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Three cDNAs encoding vitellogenin homologs from Antarctic copepod, *Tigriopus kingsejongensis*: Cloning and transcriptional analysis in different maturation stages, temperatures, and putative reproductive hormones

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ABSTRACT

Three full-length cDNAs encoding lipoprotein homologs were identified in *Tigriopus kingsejongensis*, a newly identified copepod from Antarctica. Structural and transcriptional analyses revealed homology with two vitellogenin-like proteins, Tik-Vg1 and Tik-Vg2, which were 1855 and 1795 amino acids in length, respectively, along with a third protein, Tik-MEP, which produced a 1517-residue protein with similarity to a melanin engaging protein (MEP) in insects Phylogenetic analysis showed that Vgs in Maxillopods including two Tik-Vgs belong to the arthropod vitellogenin-like clade, which includes clottable proteins (CPs) in decapod crustaceans and vitellogenins in insects. Tik-MEP clustered together with insect MEPs, which appear to have evolved before the apoB-like and arthropod Vg-like clades. Interestingly, no genes orthologous to those found in the apoB clade were identified in Maxillopoda, suggesting that functions of large lipid transfer proteins (LLTPs) in reproduction and lipid metabolism may be different from those in insect and decapod crustaceans. As suggested by phylogenetic analyses, the two Tik-Vgs belonging to the arthropod Vg-like clade appear to play major roles in oocyte maturation, while Vgs belonging to the apoB clade function primarily in the reproduction of decapod crustaceans. Transcriptional analysis of Tik-Vg expression revealed a 24-fold increase in mature and ovigerous females compared with immature female, whereas expression of Tik-MEP remained low through all reproductive stages. Acute temperature changes did not affect the transcription of Tik-Vg genes, whereas Tik-MEP appeared to be affected by temperature change. Among the three hormones thought to be involved in molting and reproduction in arthropods, only farnesoic acid (FA) induced transcription of the two Tik-Vg genes. Regardless of developmental stage and hormone treatment, Tik-Vg1 and Tik-Vg2 exhibited a strong positive correlation in expression, suggesting that expression of these genes may be regulated by the same transcriptional machinery.

1. Introduction

Copepods are a diverse group of small crustaceans, consisting of ~13,000 marine and 2800 freshwater species, divided into ten orders (Geoff and Danielle, 2008).

As these organisms are among the most numerous metazoans in the aqueous environment, and play a key role in the marine food chain, copepods have received considerable attention in marine ecological studies (Turner, 2004). Several of these species are currently being used as models for aquatic toxicology due to their short life span and extreme sensitivity to environmental toxins (LeBlanc, 2007). *Tigriopus kingsejongensis* is a newly identified copepod species originally collected from tidal pools near the King Sejong Station on King George Island, Antarctica (Park et al., 2014). Compared with more temperate latitudes, the Antarctic habitat represents a unique environment, not only in terms of temperature, but also in terms of the unique photoperiod and associated exposure to UV-radiation. Given this unique environmental niche, *T. kingsejongensis* is thought to have been exposed to considerably different evolutionary pressures relative to copepod species inhabiting more temperate latitudes. For that reason, *T. kingsejongensis* is considered a promising model for studying the processes driving adaptation to various extreme environmental stimuli in polar areas.

In order to use Antarctic copepods as a model organism, a stable culture system needs to be established with close attention paid to the reproductive physiology of the organism. Like other arthropods, copepods deposit nutrients into growing oocytes forming the yolk, with vitellogenins (Vg) serving as the major large lipid transfer proteins (LLTPs) driving this process. Compared to decapod crustaceans, fewer

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Vg genes have been identified in copepods, with only eight Vg genes from five species, *Tigriopus japonicus* (ABZ91537, ACJ12892), *Paracyclopina nana* (ADD73551, ADD73552), *Leophtheirus salmonis* (ABU41134, ABU41135), *Pseudodiaptomus annandalei* (AGT28481) and *Eurytemora affinis* (AGH68974), currently stored in GenBank (Dalvin et al., 2011; Hwang et al., 2009, 2010; Jiang et al., 2013; Lee et al., 2008). Based on these results, it is generally assumed that most copepods harbor two copies of the Vg gene. However, additional Vg-like sequences have also been identified in *L. salmonis*, suggesting that additional LLTP genes may exist.

Despite evidence of homology at the nucleotide level, little is known regarding the function of Vg or Vg-like lipoproteins during reproduction. Recent toxicological studies in copepod species have been performed under the assumption that the roles of Vg proteins in reproduction are similar to those in decapod crustaceans and insects. Currently, most of what is known regarding crustacean endocrine regulation during reproduction has come from the study of decapod crustaceans, due to their commercial importance. Since the evolutionary and functional relationship of LLTP genes in arthropods have not been clearly established, and several instances of neofunctionalization have been identified (Cheng et al., 2008b; Hall et al., 1999; Lee et al., 2000), it is likely impossible to predict the function of each LLTP gene based upon sequence homology alone.

The purpose of this study is to provide a basic understanding of the role Vg-like genes play in reproduction and lipid metabolism in copepods. The first goal was to determine how many copies of LLTP genes exist in the copepod genome and to identify the role each of these genes play in reproduction. Second, we sought to examine the role of hormones in regulating the expression of Vg genes in copepods. Among the major reproductive hormones, ecdysteroids, particularly 20-hydroxyecdysone (20E), have been shown to induce Vg expression in decapod crustaceans (Subramoniam, 2000). However, other hormones, including methyl farnesoate (MF) and farnesoic acid (FA), may also play a role in Vg production and reproduction (Mak et al., 2005; Nagaraju, 2007; Tiu et al., 2006a, 2006b, 2010). In the present study, we identified three full-length cDNAs encoding lipoproteins from *T. kingsejongensis* using a combination of next generation sequencing (NGS) and conventional molecular cloning. Transcriptional changes in response to various reproduction stages, temperatures, and hormones were also studied. Taken together, these results provide a basic understanding of the relationship between reproduction and Vg-like genes in the Antarctic copepod.

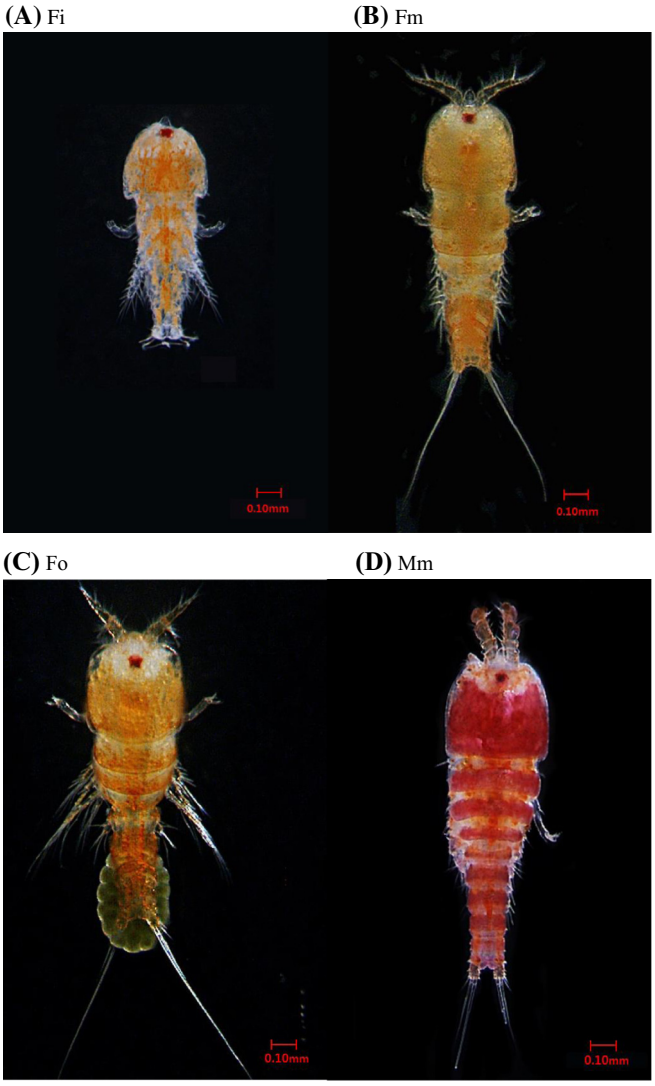

Fig. 1. Image of *Tigriopus kingsejongensis* in different developmental and maturation stages. Images were captured by a SZX10 Stereo Microscope (OLYMPUS Inc., Tokyo, Japan). Fi, immature female; Fm, mature female; Fo, ovigerous female; Mm, mature male.

2. Materials and methods

2.1. Experimental animals and culture conditions

T. kingsejongensis used in this study was a generous gift from the Korean Polar Research Institute (KOPRI) where their lineage had been maintained from individuals collected from tidal pools near the King Sejong Station on King George Island, Antarctica (Park et al., 2014). *T. kingsejongensis* were cultured at a density of 40–60 individuals per dish (100 × 40 mm) containing 100 mL of 4 °C Antarctic seawater (salinity = 33 ppt). Seawater was filtered through a Whatman filter (Clifton, NJ, USA; pore size = 1.2 μm), autoclaved at 121 °C for 20 min, and stored at 4 °C until needed. The photoperiod was maintained at 16 L:8D. Copepods were fed marine alga *Tetraselmis suecica* once every three days with seawater changed before each feeding. Developmental stages and sex were determined by morphological characteristics, as described by Park et al. (2014) Fig. 1). Briefly, mature females (Fm) and mature males (Mm) can be differentiated by the morphological characteristics of their antennules (Fig. 1B and D). Immature females (Fi) were identified by their mating behavior after being caught by the antennule of a mature male (ITÔ, 1970). Ovigerous females (Fo) were easily recognized by the presence of external egg sacs.

As the transcription of three lipoprotein genes, vitellogenin 1 (Tik-Vg1), vitellogenin 2 (Tik-Vg2), and melanin-engaging protein (Tik-MEP), was below the level of detection in males, all temperature change experiments were performed using mature females. Individuals were first acclimatized at 4 °C for >1 month, after which 27 individuals were transferred to culture dishes (100 × 40 mm) containing 100 mL of filter-sterilized Antarctic seawater adjusted to the designated temperature (0 °C, 4 °C or 15 °C). Samples were then incubated for 12, 24, or 72 h, after which three individuals were selected at random and pooled together for RNA extraction and qPCR. Each experiment was replicated three times. Copepods were not fed during the temperature change experiments.

Next, we examined the effects of copepod hormones 20-hydroxyecdysone (20E) (Santa Cruz Biotechnology Inc., Texas, USA), methyl farnesoate (MF), and farnesoic acid (FA) (Echelon Biosciences Inc., Utah, USA) on lipoprotein gene expression. Stock solutions (10 mg/mL) for each hormone were prepared in 100% ethanol and stored at −80 °C until needed. Test solutions (1 μg/mL) for each hormone were then made by diluting stock solutions with filter-sterilized Antarctic seawater as described previously (Kuo and Lin, 1996; Marcial et al., 2003). Three mature females and three mature males were transferred into each well containing 1 mL of test hormone

solution or vehicle control. Samples were then incubated at 4 °C for 3 days, during which 50% of the test solution was changed every 24 h. After 12 and 72 h, test animals (Fm and Mm) were selected at random for RNA extraction. Three replicates were performed for each hormone treatment.

2.2. Construction of T. kingsejongensis RNA-seq database and identification of lipoprotein genes

Total RNA was extracted and subjected to poly-A selection. A cDNA library was then constructed using a TruSeq RNA sample prep kit (Illumina Inc., USA). RNA sequencing was performed on an Illumina Miseq platform (Illumina Inc., USA) using a 600-cycle sequencing strategy according to the manufacturer's protocol. *De novo* transcriptome assembly was performed using CLC Genomics Workbench 8.0 (CLC Bio Aarhus, Denmark). Contigs encoding the vitellogenin homologs were obtained by BLAST using two vitellogenin sequences from *T. japonicus*, Tij-Vg1 (GenBank Accession; ABZ91537) and Tij-Vg2 (GenBank Accession; ACJ12892) as queries. Full length cDNA sequences were obtained by RACE using sequence-specific primers (Table 1) as described by Lee et al. (2011, 2014) and confirmed by PCR using primers targeting the flanking regions of each open reading frame (ORF) (Table 1). Multiple amino acid sequence alignment of putative LLTPs from *T. kingsejongensis* and other homologs was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were constructed using the minimal evolution method with 1000 replications of bootstrapping using Molecular Evolutionary Genetics Analysis (MEGA5) software (Tamura et al., 2011). LPD-N domain was used to exclude potential errors originating from the different lengths of the proteins. Domains of each lipoprotein were predicted by the SMART algorithm (Letunic et al., 2012).

Table 1
Primers for Tik-Vgs and MEP study.

Primer	Sequence (5′–3′)	Description
Tik-Vg1 F1	ATGAAGGTTTTCTTCGCTTTGGCTC	Cloning for Tik-Vg1
Tik-Vg1 F2	TGCACCAACAAGAAGGATGTGGCCA	Cloning for Tik-Vg1
Tik-Vg1 F3	TGGTCAAGACTTTCGTGGACCT	Cloning for Tik-Vg1
Tik-Vg1 R1	GGAACAGCTCCTCGGCGCTCA	Cloning for Tik-Vg1
Tik-Vg1 R2	CTTGAGGAGCGACTCCAGATTCCA	Cloning for Tik-Vg1
Tik-Vg1 R3	TTAATATCTGGGAGTGGTTCGGTAG	Cloning for Tik-Vg1
Tik-Vg2 F1	ATGAAGCTTCTTGGTCTTTTCTGCCTCGT	Cloning for Tik-Vg2
Tik-Vg2 F2	CGTGGCTTCTCCCTGCTCACCAA	Cloning for Tik-Vg2
Tik-Vg2 F3	TACACCAAGATGGTGTACATGCTGG	Cloning for Tik-Vg2
Tik-Vg2 F4	AGGTTGAGAGTGGTGCTGCCATCACT	Cloning for Tik-Vg2
Tik-Vg2 R1	TGTACTTCTTGACCACCATGACGTT	Cloning for Tik-Vg2
Tik-Vg2 R2	AGGGACTCCCTCAGCTGACCATAGA	Cloning for Tik-Vg2
Tik-Vg2 R3	GTCCTTGCCTTCGAGATTGATGGACCA	Cloning for Tik-Vg2
Tik-Vg2 R4	CAATCTGCTTCTTGAGGAGAGCATCCTC	Cloning for Tik-Vg2
Tik-Vg2 3′RACE F1	CTTCACACCGCCACGAGATC	Cloning for Tik-Vg2
Tik-Vg2 3′RACE F2	TTCACACCGCCACGAGATCATC	Cloning for Tik-Vg2
Tik-MEP F1	ATGAAACTCTTTCTTGGGATCGCCAT	Cloning for Tik-MEP
Tik-MEP F2	TGGTCAAGGAATTCGAGGCCCTTTTG	Cloning for Tik-MEP
Tik-MEP F3	GTCAACGACCGCATCTACCACAAGCA	Cloning for Tik-MEP
Tik-MEP R1	CATGGCCTCGGCCGGACATGAAGGGA	Cloning for Tik-MEP
Tik-MEP R2	TGGAGGTTGGAGCCTCGGACCATGAC GCT	Cloning for Tik-MEP
Tik-MEP R3	TCAGCATTTCCACTGCCAGTTCTCAACCA	Cloning for Tik-MEP
M13F(-20)	GTAAAACGACGGCCAGT	DNA sequencing
M13R(-20)	GGAAACAGCTATGACCATG	DNA sequencing
Tik-Vg1 F	CTACATTGGTGTCCTGGAGAAG	qPCR for Tik-Vg1
Tik-Vg1 R	ACGATCTTGAGTGGCGATATTA	qPCR for Tik-Vg1
Tik-Vg2 F	CATCGTGTTTGCTGTGTTCAG	qPCR for Tik-Vg2
Tik-Vg2 R	CCTTGACAGCCTTCATCATGG	qPCR for Tik-Vg2
Tik-MEP F	GCCTCTGGAACTTCTGGATTC	qPCR for Tik-MEP
Tik-MEP R	GTTCTCGCAATCCTCAGTGTG	qPCR for Tik-MEP
Tik-18S rRNA F	ACGCTTGAATCTTCGTGCATGG	qPCR for 18S rRNA
Tik-18S rRNA R	GCCTGGTGAGATTTCCCGTG	qPCR for 18S rRNA

2.3. Quantitative analysis of Tik-MEP & Tik-Vgs

Functional analysis of each Tik-Vg gene was performed using end-point-RT PCR and qPCR. Primers were designed using Oligo Analyzer 3.1 software (http://sg.idtdna.com/calc/analyzer) and purchased from Macrogen Co. (Daejeon, Korea) (Table 1). In order to minimize individual differences, three individuals from each group (Fi, Fm, Fo, and Mm) were pooled together for RNA isolation. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). The integrity of isolated RNA was determined by 1% agarose gel electrophoresis and quantified by spectrophotometry (Nanodrop Technologies, Inc., USA). Qualified RNAs were aliquoted and stored at −80 °C until needed. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, USA). qPCR was performed using SYBR Green premix Ex Taq II (TakaRa Bio Inc.) with 500 ng cDNA as template, and run on a DNA Engine Chromo 4 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: denaturation at 94 °C for 1 min, followed by 40 cycles of 9 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s (Kim et al., 2005). Standard curves were constructed to confirm the efficiency of primers and to quantify copy numbers, as described previously. Sample copy numbers were normalized to 18S rRNA according to the equation: (actual copy numbers of sample/actual copy number of 18S rRNA) × 10^{10}, as described previously (Lee et al., 2011, 2014). Differences in transcription level were analyzed by independent two-sample t-tests using MINITAB software (version 12.1, Minitab Inc., USA). Correlation and regression analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (version 12.0.1, SPSS Inc., USA). The results were considered significant at $P < 0.05$.

3. Results

NGS sequencing, followed by PCR-based cloning revealed three full-length cDNAs encoding proteins homologous to vitellogenins (Fig. 2). The first cDNA encoded a 1855 amino acid protein, which exhibited the highest similarity to Vg1 proteins from *T. japonicus* (80%) and *L. salmonis* (55%). The sequence was therefore named Tik-Vg1. The second full-length cDNA was 5388 nucleotides in length, producing a protein 1795 amino acids in length. The highest sequence similarities for this protein were seen against Vg2s from *T. japonicus* (82%) and *P. nana* (58%); the sequence was therefore named Tik-Vg2. Finally, the third transcript, encoding a 4554-bp sequence 1517 amino acids in length, exhibited the highest sequence similarity to a vitellogenin-like protein from the parasitic copepod *L. salmonis* (66%). Phylogenetic analyses suggested these two copepod proteins were most closely related to the melanin-engaging proteins (MEPs) found in insect species, clustering together as an ancestral arthropod clade (Fig. 3). Given this strong sequence similarity, we named this large lipid transfer protein (LLTP) sequence Tik-MEP.

In insects, MEP is known to enhance the synthesis of melanin by activating phenoloxidase (PO) in the presence of dopamine, a major part of the immune response in insects (Lee et al., 2000). The smaller size of Tik-MEP relative to the two Tik-Vg proteins is mainly due to the short carboxyl-terminal region immediately following the von Willebrand factor type D domain (VWD) (Fig. 2). Signal peptide sequences were identified in all three Vg homologs from *T. kingsejongensis*, with cleavage occurring after residues 17, 16, and 19, respectively (Fig. 2). Sequence analyses also revealed well-conserved domains consistent across LLTP family members, including a lipoprotein N-terminal domain (LPD-N) at the amino terminus and a VWD domain at the carboxyl domain (Dalvin et al., 2011; Lee et al., 2000; Smolenaars et al., 2007). In addition to the higher amino acid sequence identity between Tik-Vg1 and Tik-Vg2 (40%), overall domain

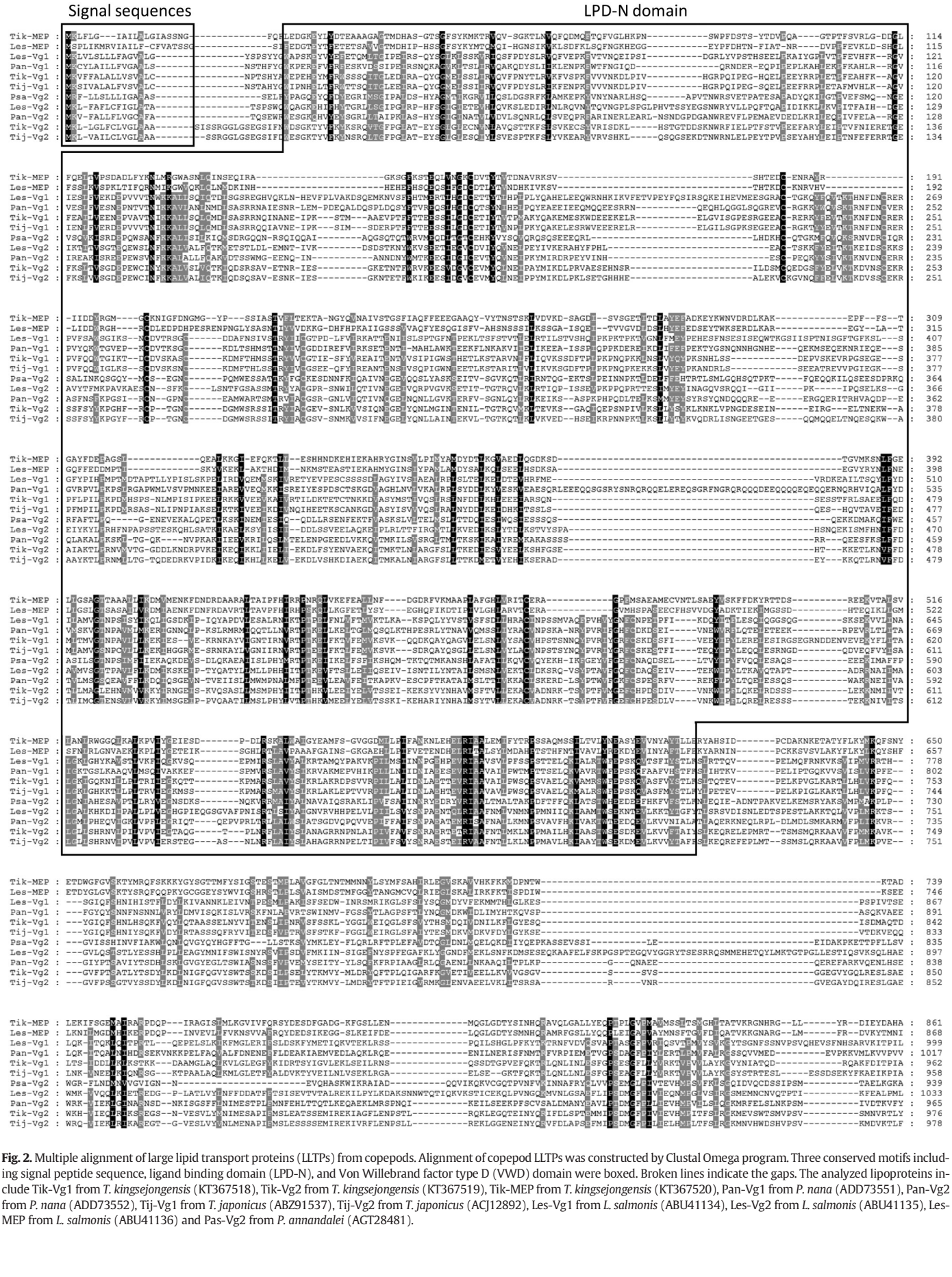

Fig. 2. Multiple alignment of large lipid transport proteins (LLTPs) from copepods. Alignment of copepod LLTPs was constructed by Clustal Omega program. Three conserved motifs including signal peptide sequence, ligand binding domain (LPD-N), and Von Willebrand factor type D (VWD) domain were boxed. Broken lines indicate the gaps. The analyzed lipoproteins include Tik-Vg1 from *T. kingsejongensis* (KT367518), Tik-Vg2 from *T. kingsejongensis* (KT367519), Tik-MEP from *T. kingsejongensis* (KT367520), Pan-Vg1 from *P. nana* (ADD73551), Pan-Vg2 from *P. nana* (ADD73552), Tij-Vg1 from *T. japonicus* (ABZ91537), Tij-Vg2 from *T. japonicus* (ACJ12892), Les-Vg1 from *L. salmonis* (ABU41134), Les-Vg2 from *L. salmonis* (ABU41135), Les-MEP from *L. salmonis* (ABU41136) and Pas-Vg2 from *P. annandalei* (AGT28481).

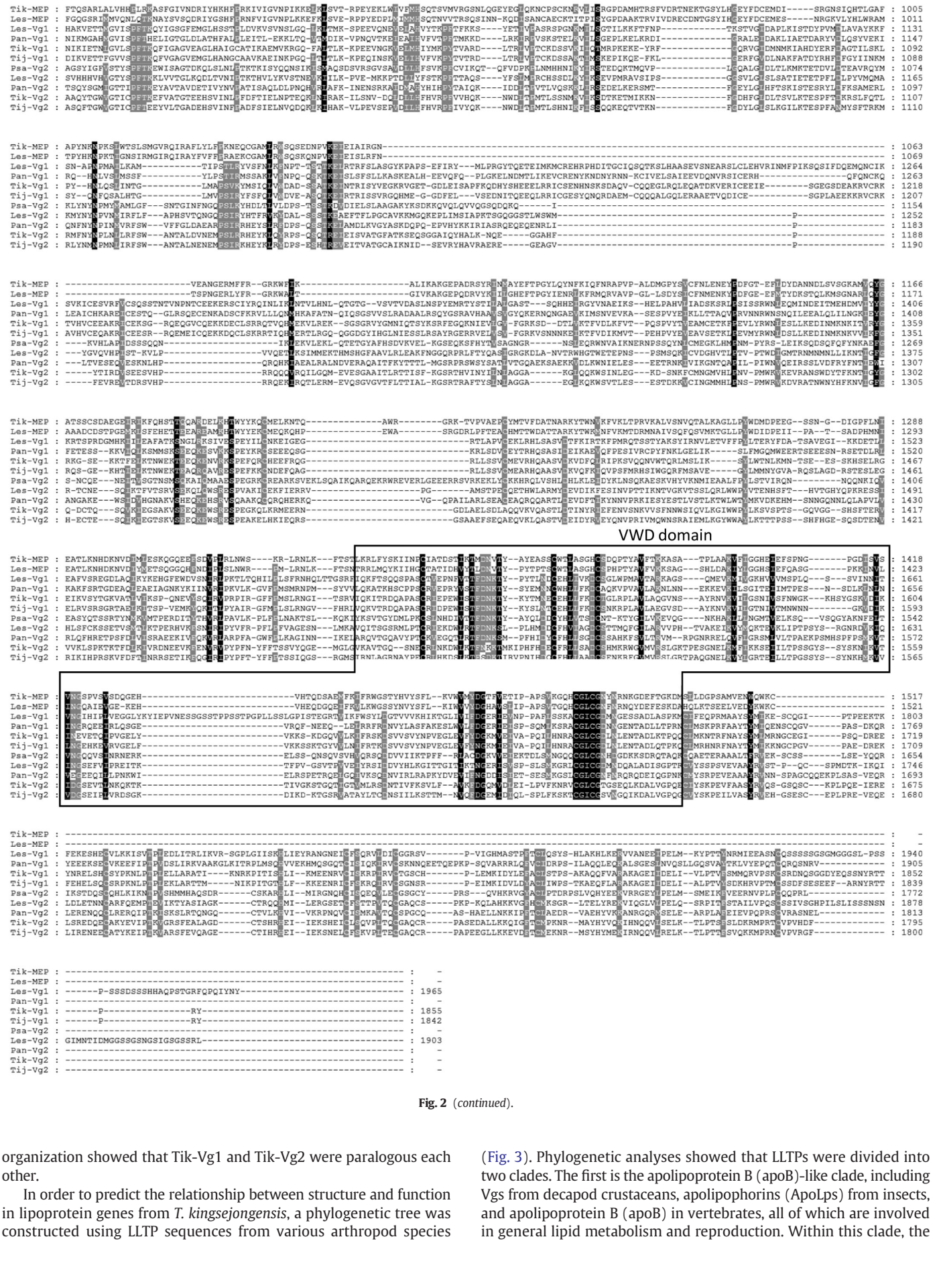

Fig. 2 (*continued*).

organization showed that Tik-Vg1 and Tik-Vg2 were paralogous each other.

In order to predict the relationship between structure and function in lipoprotein genes from *T. kingsejongensis*, a phylogenetic tree was constructed using LLTP sequences from various arthropod species (Fig. 3). Phylogenetic analyses showed that LLTPs were divided into two clades. The first is the apolipoprotein B (apoB)-like clade, including Vgs from decapod crustaceans, apolipophorins (ApoLps) from insects, and apolipoprotein B (apoB) in vertebrates, all of which are involved in general lipid metabolism and reproduction. Within this clade, the

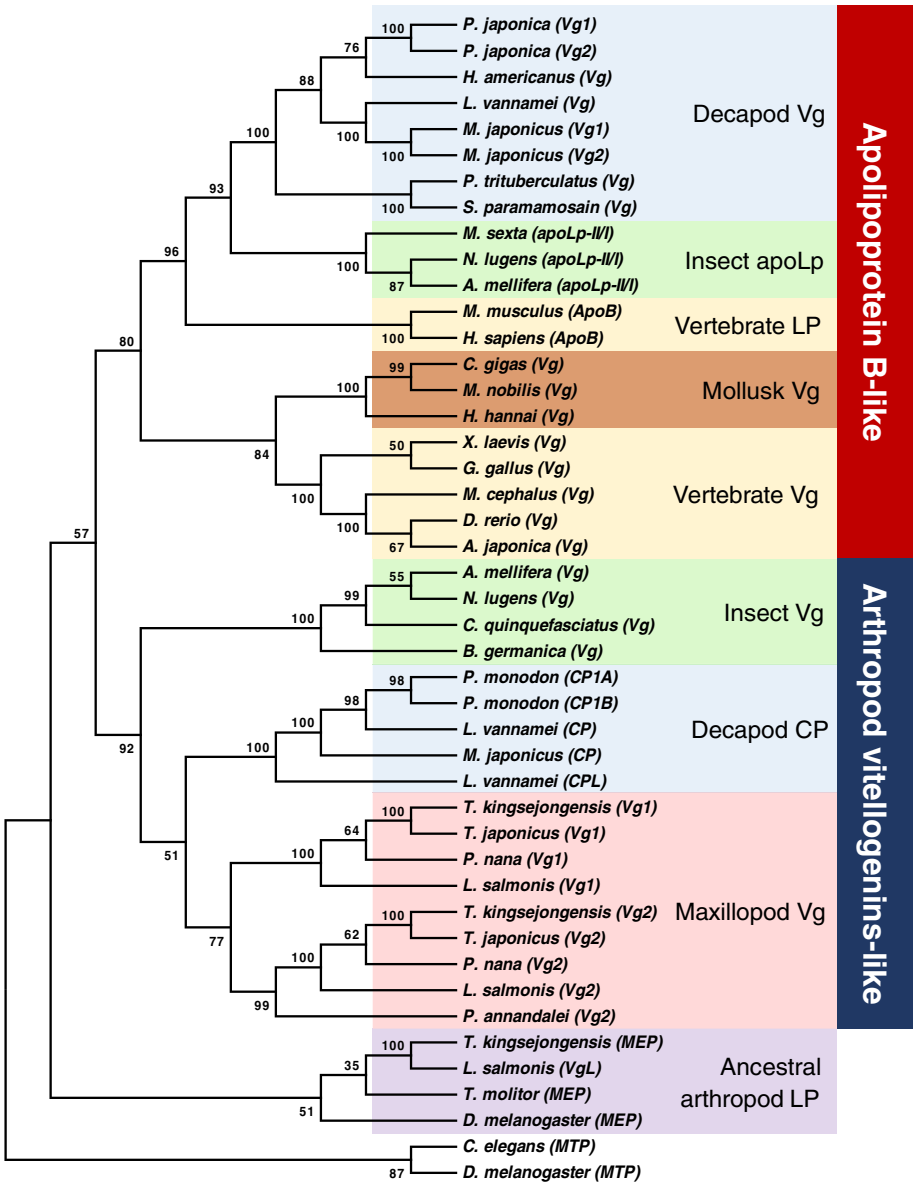

Fig. 3. Phylogenetic tree of LLTPs. Ligand binding domain (LPD-N) of each protein was used for the analysis. The phylogenetic trees were constructed by the minimum evolution method with 1000 bootstrap replications using MEGA5 software. Note two clades with different colors, Apolipoproteins (red) and Arthropod Vg-like (blue). Two microsomal triglyceride transfer proteins (MTPs) from *Caenorhabditis elegans* (AAR27937) and *D. melanogaster* 2 (AAF53946) were used as out group members. Analyzed cDNA sequences included the Decapod Vgs of *Pandalopsis japonica* 1 (ACU51164), *P. japonica* 2 (AHD26978), *Homarus americanus* (ABO09863.1), *Litopenaeus vannamei* (AAP76571), *Marsupenaeus japonicas* 1 (BAB01568), *M. japonicas* 2 (BAD98732), *Portunus trituberculatus* (AAX94762) and *Scylla paramamosain* (ACO36035); the insect apoLp-II/I of *Apis mellifera* (XP_006561555), *Manduca sexta* (Q25490) and *Nilaparvata lugens* (BAG75121); the vertebrate apoLp of *Homo sapiens* (CAA28420) and *Mus musculus* (NP_033823); the mollusk Vg of *Crassostrea gigas* (BAC22716), *Mimachlamys nobilis* (AFO66775) and *Haliotis discus* (BAF98238); the vertebrate Vg of *Gallus gallus* (AAA49139), *Xenopus laevis* (NP_001152753), *Danio rerio* (NP_001038759), *Anguilla japonica* (AAV48826) and *Mugil cephalus* (BAF64835); the decapod CP of *Litopenaeus vannamei* (ABI95361), *Marsupenaeus japonicas* (ABK59925), *Penaeus monodon* 1 (AAF19002), *P. monodon* 2 (ABW77320) *L. vannamei* (KT336921) and *Pacifastacus leniusculus* (AAD16454); the insect Vg of *Blattella germanica* (CAA06379), *Nilaparvata lugens* (AEL22916), *Apis mellifera* (NP_001011578) and *Culex quinquefasciatus* (XP_001843135); the maxillopod Vgs of *Tigriopus kingsejongensis* 1 (KT367518), *T. kingsejongensis* 2 (KT367519), *Tigriopus japonicas* 1 (ABZ91537), *T. japonicas* 2 (ACJ12892), *P. nana* 1 (ADD73551), *P. nana* 2 (ADD73552), *L. salmonis* 1 (ABU41134), *L. salmonis* 2 (ABU41135) and *P. annandalei* (AGT28481); the ancestral arthropod LP of *L. salmonis* (ABU41136), *T. kingsejongensis* 3 (KT367520), *Drosophila melanogaster* 1 (AAM50129) and *Tenebrio molitor* (BAB03250).

Vgs of mollusks and vertebrates have evolved to the point where they form their own distinct subclade (Fig. 3), while Vgs in insects, clottable proteins (CPs) in decapod crustaceans, and Vgs in copepods, including Tik-Vg1 and Tik-Vg2, formed an arthropod Vg-like clade. This result indicates that Tik-Vgs are orthologous with Vgs in insects and CPs in decapod crustaceans within the arthropod Vg-like clade. In contrast, Tik-MEP clustered with MEPs from insects, which appear to be the ancestral genes of the two clades. Interestingly, no ortholog belonging to the apoB-like clade was identified in copepod lipoprotein genes (Fig. 3). Both Vg1 and Vg2 in copepods, including Tik-Vg1 and Tik-Vg2, clustered together in each group, suggesting that a Vg duplication event may have occurred before the emergence of the copepod species (Fig. 3).

Relative expression levels of each of the three lipoprotein sequences were measured across different maturation stages by qPCR (Fig. 4). Tik-Vg1 and Tik-Vg2 transcript levels were 25- and 24-fold higher in mature and ovigerous females, respectively, relative to those in immature females (Fig. 4A). In contrast, no statistically significant differences were observed between the transcription levels of Tik-Vg1 and Tik-Vg2 in mature and ovigerous females (Fig. 4A). This strong co-regulation is particularly striking given the dynamic expression patterns of Vg genes, which are upregulated during vitellogenic states and decrease

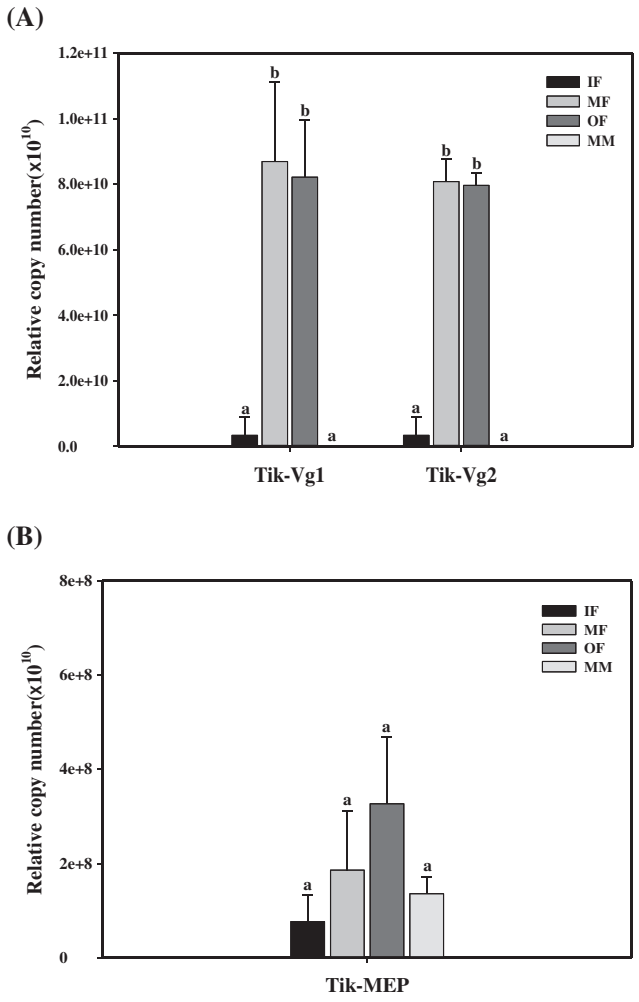

Fig. 4. Relative copy numbers of three LLTP genes from *T. kingsejongensis*. The relative copy numbers were normalized by the number of 18S rRNA. Bars show the standard error and dissimilar letters indicate the significant difference ($P < 0.05$) after Student's t-test. Fi, immature female; Fm, mature female; Fo, ovigerous female; Mm, mature male.

in the ripe stage (Okumura et al., 2004; Santhoshi et al., 2009). Expression of both Tik-Vg1 and Tik-Vg2 in immature females was 45-fold higher than that of males, showing that regardless of maturation stage, these two Vg genes can be used as potential sex markers in *T. kingsejongensis*. In contrast, Tik-MEP expression was reduced ~450-fold relative to Tik-Vgs in mature females with no statistically significant differences in Tik-MEP transcription levels among different sex and maturation stages (Fig. 4B).

Next, we examined the effect of temperature on Tik-Vgs/MEP gene expression. Expression levels were assessed at both increased (15 °C) and decreased (0 °C) incubation temperatures relative to baseline (4 °C). Acute temperature changes for up to 72 h did not affect transcriptional levels of Tik-Vg genes (Fig. 5A and B). In contrast, incubation at 15 °C did increase the expression of Tik-MEP 3-fold at 72 h (Fig. 5C), though no effects were seen at lower temperature (0 °C; Fig. 5C). Despite this clear distinction in transcriptional regulation between Tik-Vgs and Tik-MEP, the relatively small change in gene expression was too little to determine the biological function of Tik-MEP in response to temperature changes.

Since neither the evolutionary nor functional relationships between crustacean Vg homologs are clearly understood, we assessed the effects of three putative copepod hormones on Tik-Vgs/MEP gene expression. Transcription of Tik-Vg1 was upregulated in mature females at both 12 and 72 h in response to 1 μg/mL FA (Fig. 6A). Transcription of Tik-Vg2 was also significantly upregulated 72 h after FA incubation

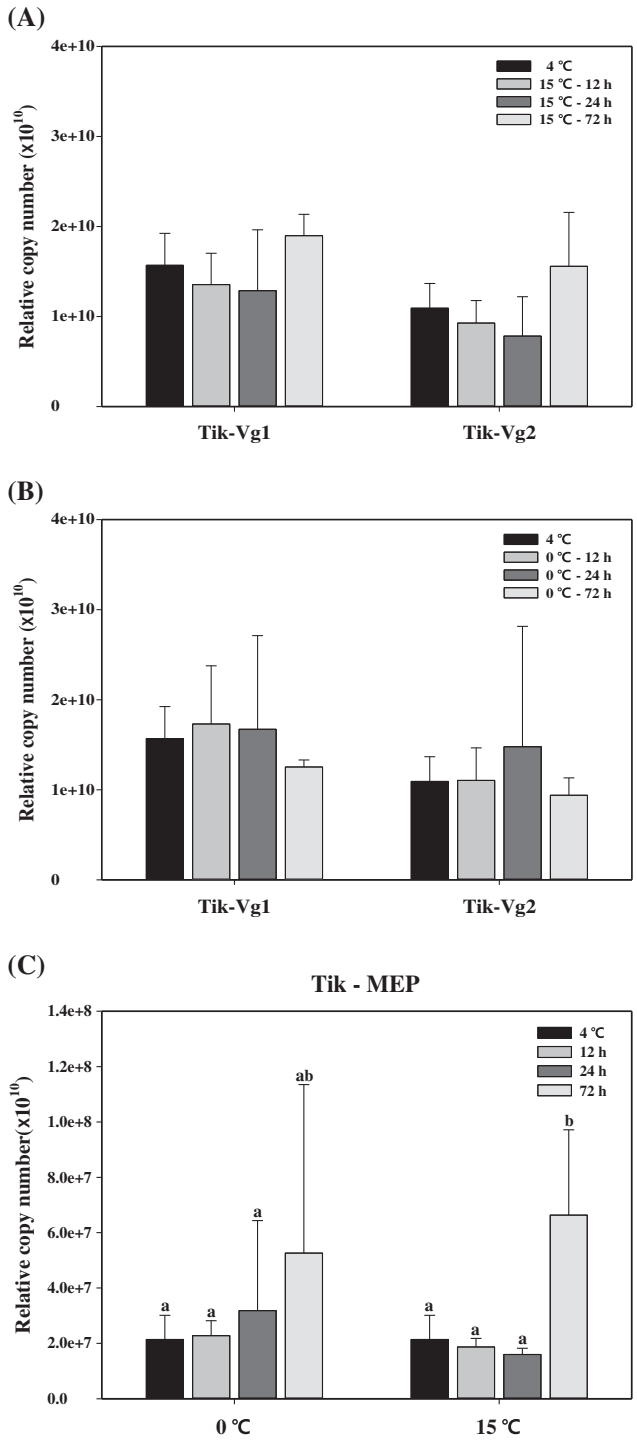

Fig. 5. Effects of acute temperature changes on the transcription of three LLTP genes in *T. kingsejongensis*. (A) Effects of increased temperature from 4 °C to 15 °C on two Tik-Vg genes. (B) Effects of decreased temperature from 4 °C to 0 °C on two Tik-Vg genes. (C) Effects of acute temperature changes on Tik-Lp gene. The relative copy numbers were normalized by the number of 18S rRNA. Bars show the standard error and dissimilar letters indicate the significant difference.

(Fig. 6B). Interestingly, MF, a relative of FA, did not affect the expression of Tik-Vgs genes in mature females (Fig. 6). A third hormone, 20E, also failed to affect Tik-Vg transcription. Expression of both Tik-Vg1 and Tik-Vg2 was below the detection limit in mature males before and after hormone incubation (data not shown) with the overall expression of Tik-MEP extremely low regardless of hormone treatment (Fig. 7). We also failed to identify any notable transcriptional changes in Tik-MEP

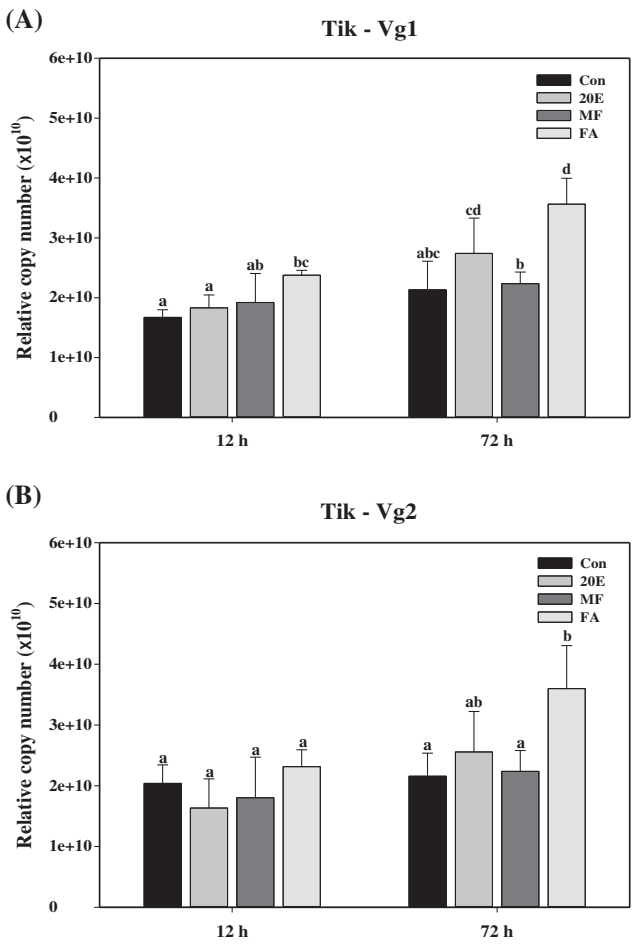

Fig. 6. Effects of three hormones on the transcription of two Tik-Vg genes. Transcriptional changes of two Tik-Vg genes after exposure to 1 μg/mL of 20-hydroxyecdysone (20e), methyl farnesoate (mf) and farnesoic acid (fa), respectively. Only mature females were used for the experiment. The relative copy numbers were normalized by the number of 18S rRNA. The relative copy numbers were normalized by the number of 18S rRNA. Bars show the standard error and dissimilar letters indicate the significant difference.

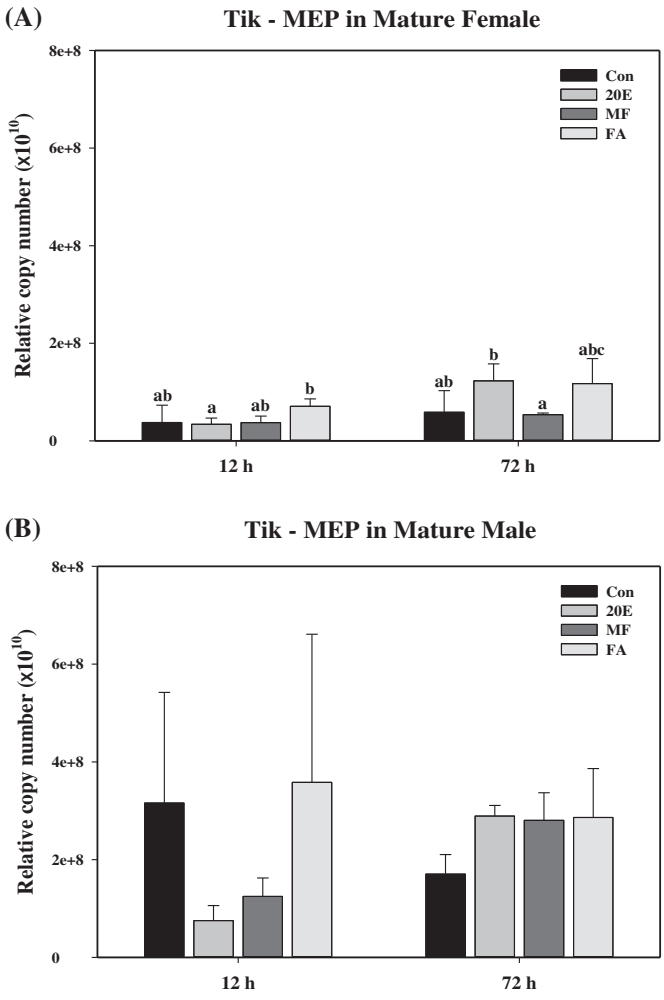

Fig. 7. Effects of three hormones on the transcription of Tik-MEP. Transcriptional changes of Tik-MEP in mature male (A) and in mature female (B) after exposure to 1 μg/mL of 20E, MF and FA, respectively. The relative copy numbers were normalized by the number of 18S rRNA. Student's t-test was used to determine significant effects. Bars show the standard error and dissimilar letters indicate the significant difference.

when incubated in the presence of three putative molt and reproductive hormones (Fig. 7A and B).

Since there were no statistically significant differences in transcription levels between Tik-Vg1 and Tik-Vg2, a simple linear regression analysis was performed (Fig. 8). This analysis revealed a strong positive correlation between Tik-Vg1 and Tik-Vg2 ($R^2 = 0.8682$), with a strong similarity in transcription levels (slope = 1.010). Although a positive correlation was evident between the two Tik-Vg genes and Tik-MEP, this relationship was not statistically significant ($R^2 = 0.4511$ and 0.2949 for Tik-Vg1 and Tik-Vg2, respectively; Fig. 8).

4. Discussion

T. kingsejongensis is a recently identified species of copepod, originally collected from tidal pools near the King Sejong Station on King George Island, Antarctica. Since the establishment of a stable culture system, this species has been considered a promising model for studying the physiology of crustacean species inhabiting polar areas. Here, we describe three genes homologous to Vgs in an Antarctic copepod species. These genes exhibit unique characteristics in terms of evolutionary phylogeny and their roles in species maturation. Furthermore, the transcriptional regulation of these genes in response to temperature and reproductive hormones differed from that of other crustaceans, particularly the more well-studied decapod crustaceans. To our knowledge, this is the first report examining the relationship between Vg transcription and reproductive characteristics in copepod species. Future studies will be necessary to determine if these characteristics are unique to *T. kingsejongensis* or can be applied to copepods inhabiting other environmental conditions. For example, we identified consistent coexpression of Tik-Vg1 and Tik-Vg2 regardless of maturation stage or hormone treatment, suggesting that their expression is subject to the same control mechanism (Fig. 8). While two Vg genes are also found in decapod crustaceans, there is usually only one gene that plays a major role in reproduction while the second copy is expressed at low levels (Jeon et al., 2011). Further studies will be necessary to determine if these two Vg paralogs have different functions.

Three cDNAs predicted to encode LLTPs were identified from a transcriptomic database constructed from a whole organism lysate of *T. kingsejongensis*. The presence of three Vg homologs appears to be a common characteristic of copepod genomes, with orthologs to Tik-Vg1, Tik-Vg2, and Tik-MEP also present in *L. salmonis* (order Siphonostomatoida; Fig. 2) (Dalvin et al., 2011; Eichner et al., 2008). Furthermore, we failed to identify any genes with homology to other currently known copepod Vg-like lipoprotein genes, including two from order Harpacticoida, two from order Cyclopoida, and one from order Calanoida, supporting the notion of three Vg homologs as the standard in copepod genomes. Phylogenetic analyses failed to identify any LLTP gene belonging to the apoB clade, which includes apoLps in insects and Vgs in decapod crustaceans (Fig. 3). Considering the fact

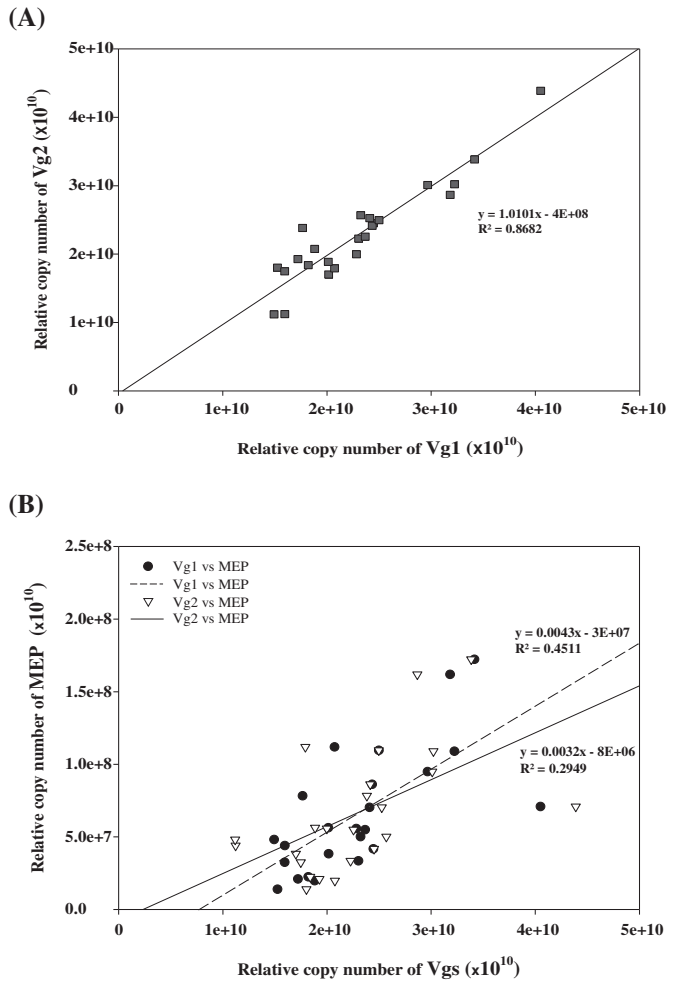

Fig. 8. Transcriptional correlation of two Tik-Vgs and Tik-MEP. The correlation in transcriptional levels between Tik-Vg1 and Tik-Vg2 (A) and between Tik-Vgs and Tik-MEP (B) were analyzed with a simple and multiple linear and regression analysis with logarithmic transformation of relative copy number data. R^2 value and regression equation are shown on each regression line.

that LLTPs belonging to apoB clades are involved in general lipid metabolism and reproduction in both vertebrates and invertebrates, it is interesting to see that no such orthologous genes exist in Maxillopoda. In fact, Avarre et al. (2007) suggested that Vgs in decapod crustaceans were an inappropriate classification of these proteins from an evolutionary standpoint, instead suggesting the name apolipocrustacein. This distinction is not without merit, given that Vgs in decapod crustaceans were originally named based upon their functions in reproduction, not their evolutionary relationship with Vgs from other species (Kung et al., 2004; Mak et al., 2005; Tiu et al., 2006a). ApoLps in insects and Vgs in decapod crustaceans are orthologous, with no notable functional differences having been reported in terms of their roles in reproduction.

Despite their similarity to genes found in the apoB clade, Tik-Vg genes appear to form an evolutionally distinct clade of LLTPs, playing a major role in copepod reproduction. The paralogs of Tik-Vgs include Vgs in insects and CPs in decapod crustaceans, which instead belong to the arthropod Vg-like clade (Fig. 3). Although Vg is involved in oocyte maturation in insects, additional functions have been reported, including important roles in innate immunity and the regulatory control of somatic maintenance functions (Amdam et al., 2004; Zhang et al., 2011). Similarly, in addition to their role in oocyte maturation, CPs in decapod crustaceans appear to play a role in innate immunity by clotting hemolymph (Cheng et al., 2008a; Maningas et al., 2013). Consistent with these observations, Tik-MEP was shown to cluster with immune-related genes. However, its role in copepod immunity has yet to be identified. Similarly, we failed to find any relationship between Tik-MEP and reproduction in *T. kingsejongensis*, suggesting further analyses will be necessary to understand the relationship between evolution and function in arthropod Vg-like proteins in copepods. This will likely involve developing strategies for controlling gene expression, such as RNAi, in copepod species. While targeted gene inhibition has been successful in both insects and decapod crustaceans (Pamuru et al., 2012; Treerattrakool et al., 2011; Zhang et al., 2013), its application in copepods is still largely unknown. Injection of long dsRNA is the most widely used strategy (Lee et al., 2015; Sagi et al., 2013) but difficult to implement in tiny copepods. Oral administration of dsRNA has been successful in both insect and decapod crustaceans (Coy et al., 2012; Treerattrakool et al., 2013), Despite several tests, we were unable to achieve detect any considerable gene suppression in *T. kingsejongensis* (data not shown).

Since temperature is one of the major physical factors affecting lipid composition and physical properties in crustaceans (Chapelle, 1978; Lahdes et al., 2000), the reproductive physiology of *T. kingsejongensis* represents a good model for understanding cold adaptation. The response to increased temperature has generally been an increase in Vg synthesis (Berg et al., 2004), with hepatic Vg mRNA levels accumulating more rapidly at 15 °C than at 9 °C in rainbow trout *Oncorhynchus mykiss* (Mackay and Lazier, 1993). Conversely, a 4- to 7-fold upregulation of Vg expression at lower temperature (10 °C) was observed in the branchiopod crustacean *Daphnia pulex* (Schwerin et al., 2009), highlighting the potential for significant variation depending on the species in question. In the present study, we observed no effect in terms of the transcription levels of two Tik-Vg genes in response to acute temperature changes (Fig. 5). However, we cannot exclude the possibility of indirect effects due to temperature changes, as temperature and salinity are among the most important physical factors affecting growth and reproduction in aquatic animals. For example, maturation of shrimp has been induced by changing water temperature (Cripe, 1994) with Vg synthesis mediated by an estrogen receptor and heat shock protein 90 (Hsp90) (Wu and Chu, 2008).

No significant differences in Tik-Vg expression were observed between mature and ovigerous females (Fig. 4). This result may have been due to the relatively long spawning period in *T. kingsejongensis*, during which maturation and spawning occur simultaneously. Transcription of Vg genes usually increases during the early maturation stages in single spawners and decrease during the ripe stage once the egg yolk has fully accumulated (Okumura et al., 2004; Santhoshi et al., 2009). Further studies will be necessary to address the relationship between Vg levels in ovigerous and spent females, and to determine whether a common maturation strategy exists in copepod species.

It is noteworthy that only FA upregulated Tik-Vg expression, with only minimal expression changes seen in response to MF or 20E (Fig. 6). 20E is one of the most well-known ecdysteroid hormones, controlling ecdysis (molting) and reproduction in arthropods (Subramoniam, 2000), while MF and FA are sesquiterpenoids and derivatives of insect juvenile hormone (JH III). Since crustaceans lack epoxidase and juvenile hormone acid methyltransferase (JHAMT) (Daimon and Shinoda, 2013; Hui et al., 2010), JH III is absent in decapod crustaceans. FA is the substrate of farnesoic acid O-methyltransferase, which produces MF. It is noteworthy that MF did not induce the transcription of Tik-Vgs, despite its obvious structural similarity to FA.

MF is responsible for enhancing reproductive maturation, maintaining juvenile morphology, and influencing male sex determination (Homola and Chang, 1997; Laufer et al., 1993; Rotllant et al., 2000). Additionally, injection of MF accelerated molting in both females and males of the crab *Oziotelphusa senex senex* (Reddy et al., 2004). Feeding *Cherax quadricarinatus* females with MF also induced molting frequency (Abdu et al., 2001) in addition to stimulating the gonads in *Macrobrachium rosenbergii* (Wilder et al., 1995), *Macrobrachium malcolmsonii* (Nagaraju et al., 2004), *O. senex senex* (Reddy et al.,

2004), and *Penaeus indicus* (Nagaraju et al., 2002). In a separate study, feed containing MF induced maturation in *Procambarus clarkii* (Laufer et al., 1998).

The first step in determining the mechanism of hormone activity in *T. kingsejongensis* would be to perform a comparative analysis of putative steroid and sesquiterpenoid receptors, the majority of which are nuclear receptors. Ecdysone receptors and ultraspiracles (USPs) have already been identified in both insects and decapod crustaceans (Asazuma et al., 2007; Chan, 1998; Maestro et al., 2005; Ogura et al., 2005). Analysis of these nuclear receptors in copepods would help us understand the relationship between hydrophobic hormones, such as MF, and reproduction in copepods.

Taken together, the results presented here provide strong evidence regarding the presence of three functional lipoprotein genes in the Antarctic copepod *T. kingsejongensis*. Based on high expression levels during maturation and the transcriptional upregulation induced by the putative reproductive hormone, two of these proteins, Tik-Vg1 and Tik-Vg2, belong to an arthropod Vg-like clade. These proteins play a major role in yolk production during maturation, a clear divergence from that of other arthropods, including insects and decapod crustaceans. Further studies will be necessary to clarify the evolutionary and functional relationships among the lipoproteins in arthropod species. Such a study would not only provide important details regarding the roles of lipoproteins in *T. kingsejongensis* reproduction, but would also help extend our knowledge of reproductive physiology in model copepod systems.

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