

RESEARCH ARTICLE

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# Role of the insulin-like growth factor system in gonad sexual maturation in Pacific oyster *Crassostrea gigas*



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## Abstract

**Background:** The IGF system plays important roles in controlling growth, development, reproduction, and aging of organisms.

**Methods:** To estimate maturation of the Pacific oyster *Crassostrea gigas*, we investigated the expression of insulin-like growth factor (IGF) system components and sex-specific genes. To determine the role of the IGF system in the growth and spawning period of female and male oysters, we examined mRNA expression levels of the *C. gigas* insulin receptor-related receptor (CIR), IGF binding protein complex acid labile subunit (IGFBP\_ALS), and molluscan insulin-related peptide (MIP), as well as those of vitellogenin (Vg) and receptor-type guanylate cyclase (Gyc76C) in gonads of *C. gigas* collected between April and October, when sex can be determined visually in this species.

**Results:** We found that MIP, IGFBP\_ALS, and CIR mRNA expression levels were dependent on sex and month and were greater in males than in females. CIR and Vg mRNA expression levels were very similar among females, whereas IGF system components and Gyc76C were very similarly expressed among males. The highest expression values were observed in May, when oysters are mature; CIR and Vg mRNA expression levels were highest in females, and those of MIP, IGFBP\_ALS, CIR, and Gyc76C were highest in males. Interestingly, we observed a 1:1 proportion of females to males during this period.

**Conclusion:** Our results suggest that IGF system components, as well as Vg and Gyc76C, are associated with sexual maturation in *C. gigas*.

**Keywords:** Pacific oyster, Gonad, IGF system, Vitellogenin, Gyc76C

## Background

The IGF system, which plays important roles in controlling vertebrate growth and development, comprises three components: ligands (IGF-I and -II), receptors (types I and II), and IGF-binding proteins (IGFBPs) (Duan 1997). The existence of traditional IGFs, IGF receptors, and IGFBPs has not been positively demonstrated in invertebrates (Huang et al. 2015). However, several insulin-like peptides (ILPs) have been discovered; these function in a variety of biological processes including growth, metabolism, molting, and reproduction (Nagasawa et al. 1986; Krieger et al. 2004; Wu and Brown 2006; Grönke et al. 2010; Marquez et al. 2011; Ventura et al. 2011; Chung

2014; Huang et al. 2015). Insulin receptor homologs, which exhibit structural and functional similarities to IGF receptors, have also been widely reported (Brogiolo et al. 2001; Nässel et al. 2015). In mollusks, growth and associated metabolic processes are regulated under the control of neural ganglia (Gricourt et al. 2003). Molluscan insulin-related peptides (MIPs) have been identified in many gastropods and bivalves as functional substances in neural ganglion neurosecretory cells (Roovers et al. 1995; Gricourt et al. 2003). In particular, MIPs have a beneficial effect on soft body and shell growth (Geraerts 1976, 1992; Gricourt et al. 2003). Geraerts et al. (1992) reported the various functions of MIPs according to stimulus-dependent differential patterns of MIP gene expression in the central nervous system. Recently, studies have been performed to examine the biological effects of various growth factors including

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*Crassostrea gigas* insulin receptor-related receptor (CIR) and IGFs in the mussel *Mytilus galloprovincialis* (Canesi et al. 1997, 1999, 2001), the Pacific oyster *C. gigas* (Gri-court et al. 2003; Jouaux et al. 2012), and the Yesso scallop *Patinopecten yessoensis* (Feng et al. 2014). These factors affect growth, maturation, and reproduction in a manner dependent on seasonal environmental conditions. Jouaux et al. (2012) reported a balance between growth and management of environmental stresses during reproduction and emphasized the involvement of insulin signaling in gametogenesis and reproduction in *C. gigas*. Many studies have reported intraspecific variation in growth and reproduction in a variety of invertebrates; among these, *C. gigas* has been used as a bivalve assessment model (Macdonald & Thompson 1988; Bayne 1999; Choi et al. 2018).

*C. gigas* is an important aquaculture product that is mainly produced off the southern coast of Korea. However, the aquaculture production of oysters in Korea declined from 321,276 tons in 2007 to 303,183 tons in 2018 (MOF 2020). There was a similar tendency in the worldwide aquaculture production (FAO 2020). Oyster value is dependent on condition; however, fishing environments are becoming increasingly polluted due to high-density rearing, contaminated runoff from the coast, and climate change. As a result of physical activity by the parent oyster in such farming environments, oyster larval health is reduced, resulting in mass destruction and repeated seedling shortages. Oysters produced in coastal areas that have become clearer and cleaner through nitrogen and phosphorus regulation fail to gain weight due to a lack of food suitable for shellfish. These phenomena have unfavorable effects on oyster growth and maturity.

In this study, to estimate maturation of *C. gigas* oysters, we investigated the expression of various IGF system components. To determine the role of the IGF system in the growth and spawning of male and female oysters, we determined CIR, IGFBP\_ALS, and MIP mRNA expression levels and those of sex-specific genes such as vitellogenin in *C. gigas* gonads.

## Methods

### Sample preparation

We collected 30–40 *C. gigas* individuals every month from April to October in 2017 (when gonads can be discriminated visually) at an oyster farm in **Tongyeong**, Gyeongsangnam-do, Korea (34° 51' 32.34" N, 128° 12' 23.44" E). We measured shell length (SL), shell height (SH), shell width (SW), total wet weight (TW), and soft tissue weight (STW) using a Vernier caliper (Mitutoyo, Kawasaki,

Japan) and a digital balance (AJ Vibra, Shinko Denshi, Japan). Male and female gonad pieces were dissected, immediately frozen in liquid nitrogen, and stored at – 75 °C until use.

### Protein identification

We investigated protein expression in gonads using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Specific proteins were confirmed using tandem mass spectrometry (MS/MS) and electrospray ionization quadrupole time-of-flight MS/MS (ESI-Q-TOF MS/MS, ABI, USA) as previously described (Choi et al. 2015). Proteins were identified via the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov>) and UniProt Knowledgebase (<http://www.uniprot.org/uniprot>) databases using the MASCOT program (Matrixscience, London, UK).

### cDNA synthesis and reverse-transcription polymerase chain reaction (RT-PCR)

Male and female gonads were pulverized by adding 1 mL Trans-Zol UP (TransGen Biotech, Beijing, China), and total RNA was extracted using Trans-Zol UP according to the manufacturer's instructions. cDNA was synthesized from 2 µg total RNA using the PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. RT-PCR was performed using Emerald Amp GT PCR Master Mix (TaKaRa Bio, Otsu, Japan). Each primer set was designed according to sequences of CIR (accession no. AJ 535669.1), IGFBP\_ALS (XM\_011417921.2), MIP (NM\_001308866.1), Gyc76C (XM\_011452292.2), Vg (AB084783.1), and EF1α (AB122066.1) (Table 1). PCR amplification was performed using *C. gigas* template cDNA with the following parameters: 1 cycle at 95 °C for 5 min, 25 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, followed by 1 cycle at 72 °C for 5 min. PCR products were analyzed by gel electrophoresis on 1% agarose gels. Densitometry was conducted using the GeneTools v. 4.03 software (Syngene, Cambridge, UK).

### Statistical analyses

Data are presented as means ± standard deviations. Significant differences among means were tested using one-way analysis of variance (ANOVA) in the SPSS v. 10.0 software environment (SPSS, Inc., Chicago, IL).

## Results

### Growth parameters

We collected 40 *C. gigas* individuals monthly from April to October 2017, except in June. The ratio of females to males differed each month, being about 1:1 in May and September and 2:1 in June and July (Fig. 1). One hermaphrodite was found in May and one in

**Table 1** Nucleotide sequences of primers used in reverse-transcription polymerase chain reaction (RT-PCR) analysis of adductor muscles of the Pacific oyster *Crassostrea gigas*

Gene	Accession no.	Sequence (5'-3')	Amplicon (bp)
<i>CIR</i>	AJ535669	(F)-TGAGGAGGGTGATGAGGATA (R)-ATTGCACTGTAGGGATTGGA	375
<i>IGFBP-ALS</i>	XP_011442465.1	(F)-CTTGCCCTGAAAACCCTACA (R)-GGGCAAGTAGCAGAATTTGG	500
<i>MIP</i>	XM_011417420.2	(F)-TAAATACAAGCGGTCGGGTG (R)-AGATCCTTTCTTCTTGGCGC	214
<i>Gyc76C</i>	XM_011452292.2	(F)-ATGAATGCAGTGAATCGCTTGA (R)-TGATACTGTGCAGCTCTTGG	274
<i>Vg</i>	AB084783.1	(F)-ATGCTGGAGATGAACCTTGAG (R)-TGCTTCGTGGTGGATATCCT	273
<i>EF1a</i>	AB122066.1	(F)-CCACTGGCCATCTCATTAC (R)-TGTTGACACCAATGATGAGC	393

bp, base pairs; F, forward; R, reverse; *CIR*, *C. gigas* insulin receptor-related receptor; *IGFBP-ALS*, insulin-like growth factor binding protein complex acid labile subunit; *MIP*, molluscan insulin-related peptide; *Gyc76C*, receptor-type guanylate cyclase; *Vg*, vitellogenin

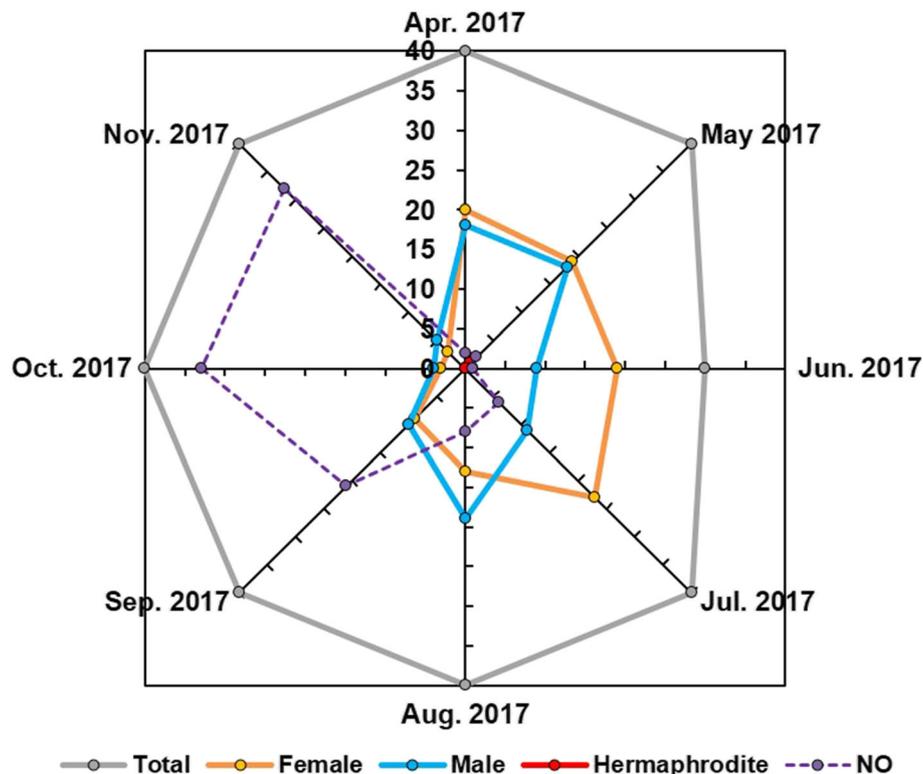
June; the proportion of males was higher than that of females in August (Fig. 1).

The ratios of STW to TW ranged from 0.07 to 0.29 (mean,  $0.15 \pm 0.04$ ) and 0.08 to 0.34 (mean,  $0.16 \pm 0.04$ ) in females and males, respectively (Fig. 2a, c). The ratio of SH to SL ranged from 1.35 to 3.58 (mean,  $2.07 \pm 0.37$ ) and from 1.02 to 3.36 (mean,  $2.07 \pm 0.36$ ) in females and males, respectively (Fig. 2b, d). The ratio of SW to SL ranged from 0.07 to 0.29 (mean,  $0.15 \pm 0.04$ ) and from

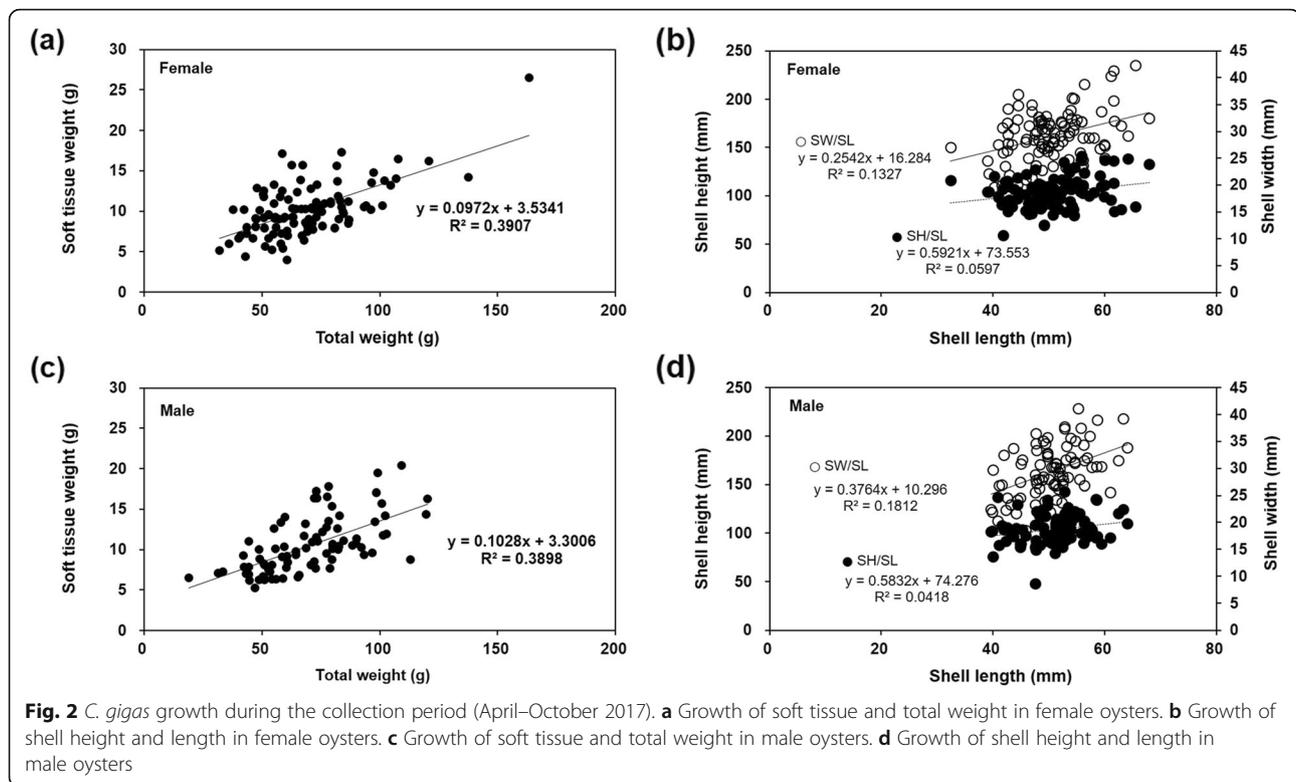
0.08 to 0.34 (mean,  $0.16 \pm 0.04$ ) in females and males, respectively (Fig. 2b, d). Thus, growth rates of STW/TW, SH/SL, and SW/SL were positive in both females and males in this study (Fig. 2).

#### Protein identification

Protein expression differed between males and females in this study. Proteins were identified as vitellogenin-6 C-term fragments of 180 kDa and N-



**Fig. 1** The ratio of females to males in the Pacific oyster *Crassostrea gigas* from April to October. NO, not observed



term fragments of 110 kDa (accession no. tr|K1QNA2|K1QNA2\_CRAGI, <https://www.uniprot.org/uniprot/K1QNA2>) in females and guanlylate cyclase (Gyc76C) protein of 96 kDa (accession no. tr|K1QS46|K1QS46\_CRAGI, <https://www.uniprot.org/uniprot/K1QS46>) mixed with 143 kDa of uncharacterized protein in males (Fig. 3).

#### MIP, IGFBP\_ALS, CIR, Vg, and Gyc76C expression analysis

Among components of the IGF system (MIP, IGFBP\_ALS, and CIR), mRNA expression was greater in males than in females (Fig. 4a, b). CIR mRNA expression was significantly higher in both females and males in May and June and was 1.75–2.02 times higher in males than in females ( $p < 0.05$ ; Fig. 4a, b). MIP and IGFBP\_ALS mRNA expression levels were negligible in females except in September, whereas those of males were significantly higher in May and June, the maturation season ( $p < 0.05$ ; Fig. 4a, b). MIP, IGFBP\_ALS, and CIR mRNA expression were relatively constant throughout the collection period (Fig. 4b) but decreased in spawning season and then increased slowly after August in both females and males (Fig. 4a, b).

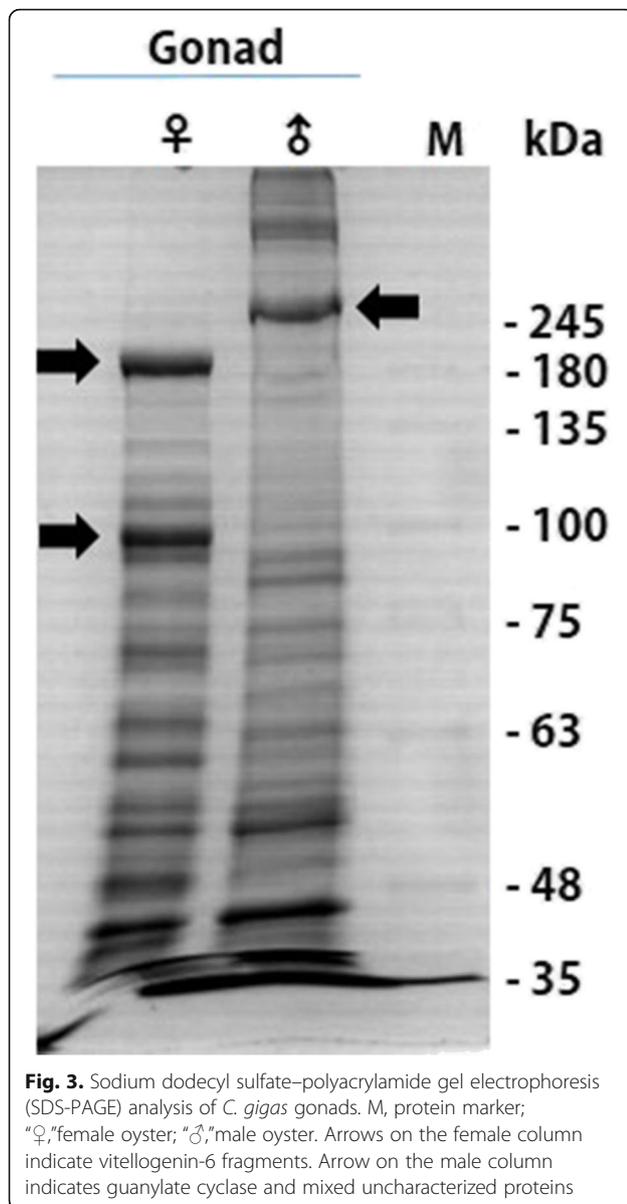
Vg and receptor Gyc76C expression levels were examined based on their identification among male and female gonad proteins. Expression levels were high in May and then decreased until August, as observed in IGF system component expression (Fig. 5a, b). Vg showed high

mRNA expression in September; however, the difference between this and other months was not significant ( $p > 0.05$ ; Fig. 5a).

#### Discussion

It is necessary that salinity, food resources, and water flow for oyster growth (Williamson et al. 2015). The growth difference depends on the oyster farm regions affected by environmental factors such as water temperature, salinity, dissolved oxygen, nutrients, water flow and so on (Min et al. 2004). In the present study, the growth difference was not found between females and males within the same farm. In addition, it was showed positive relationship among STW/TW, SH/SL, and SW/SL.

The IGF system plays numerous roles in oyster growth and development; its components have been detected as 150-kDa ternary complexes comprising one molecule each of IGF, IGFBP-3/IGFBP-5, and an 85 kDa acid labile subunit (ALS) in circulation (Baxter 1994; Brogiolo et al. 2001). ALS is a glycosylated protein that binds to IGFBP-3:IGF and IGFBP-5:IGF binary complexes but does not bind to IGFBP except in complex with IGF (Brogiolo et al. 2001). The main function of ALS is to increase the half-life of IGFs in circulation (Forbes et al. 2012). The IGF insulin receptor (IGF-1R) and insulin receptor-related receptor (IRR) form subclass II of the receptor tyrosine kinase superfamily, sharing covalently

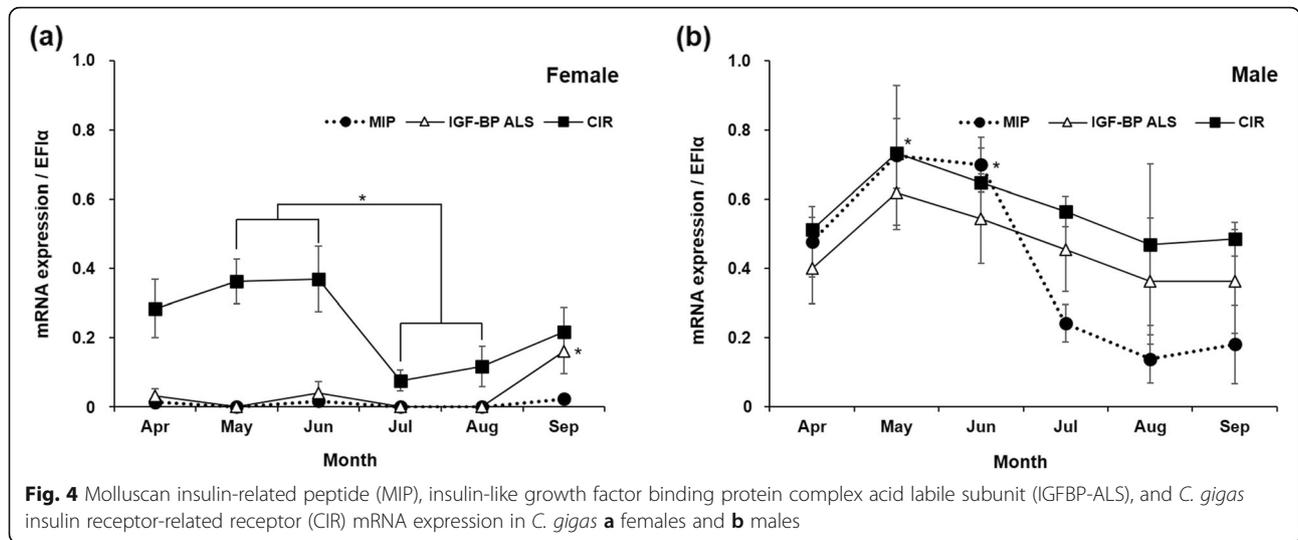


linked homodimers and several structural domains (Rentería et al. 2008). Invertebrates have only a single IRR, which regulates growth and metabolism (Leevers 2001); there is no clear evidence of the existence of traditional IGFs, IGF receptors, or IGFBP1-6 in invertebrates (Huang et al. 2015). However, several insulin-related peptides (IRPs) have been identified based on biochemical purification or cDNA identification (Cherf-Feildel et al. 2019). Various biological functions and members of the insulin family observed in vertebrates are generally conserved in invertebrates. This functional conservation has been clearly demonstrated in *Drosophila melanogaster* and *Caenorhabditis elegans* (Cherf-Feildel et al. 2019). IRPs play key roles in controlling growth, development, energy storage, stress resistance, response

to diet restriction, lifespan, and fecundity in these species. Some research has also been conducted on the involvement of IRPs (molluscan IRP, MIPs) in controlling growth, reproduction, and nutritional status in *C. gigas* (Gricourt et al. 2003; Jouaux et al. 2012).

In the present study, expression levels of IGF-related genes (*MIP*, *CIR*, and *IGFBP-ALS*) and sex-specific genes (*Vg* and *Gyc76C*) were significantly higher in May, when oysters have matured and are about to undergo spawning. The observed changes in gene expression levels may therefore be used as indicators of annual growth, maturity, and spawning period in cultured *C. gigas*. The application of IGF system (*MIP*, *IGFBP-ALS*, and *CIR*) expression as a growth indicator is an attractive alternative to physiological methods, which are prone to processing errors (Tran et al. 2007).

IGF system component mRNA expression is associated with changes in *Vg* and *Gyc76C* expression. *Vg* is a major precursor of vitellins, which are egg yolk proteins that provide energy reserves for embryonic development in oviparous organisms (Matozzo et al. 2008). To date, six *Vg* genes (1 to 6) have been identified in the nematode *Caenorhabditis elegans* (Boag et al. 2001), and a female-specific *Vg-6* has been identified in *Haemonchus contortus* (Hartman et al. 2001), *Trichostrongylus vitrinus* (Nisbet and Gasser 2004), and *Toxocara canis* (Zhu et al. 2017). These *Vg* genes play roles in reproduction and may be involved in adult biological processes (Zhu et al. 2017). In the present study, we identified *Vg-6* C-term and N-term fragments in female gonads. *Vg* mRNA expression was highest during the maturation stage and decreased thereafter. Our results are consistent with those reported for the scallop *Chlamys farreri* (Qin et al. 2012) and the Fujian oyster *Crassostrea angulata* (Ni et al. 2014). Ni et al. (2014) described the stages of oyster ovarian development in detail as follows: (1) maturation stage, when ovaries accumulate nutrients for oogenesis, and mRNA is abundant due to active *Vg* gene expression to promote *Vg* protein synthesis, yolk intake, and nutrient accumulation in oocytes; (2) ripeness stage, when oocytes grow and accumulate yolk, decreasing the demand for yolk protein and *Vg* gene expression; and (3) partially spent stage, when ovaries stop developing and before new oogonium inception so that *Vg* expression levels are very low (Boutet et al. 2008; Zheng et al. 2012). Davis et al. (2008) reported that endogenous in females and exogenous *Vg* in 17 $\beta$ -estradiol ( $E_2$ )-treated males downregulate GH/IGF-1 axis support of vitellogenesis in the liver.  $E_2$  induces the shift of energy away from somatic growth by suppressing the haptic GH/IGF axis and toward vitellogenesis by estrogen receptor  $\alpha$ -mediated upregulation of multiple *Vg* genes, which is



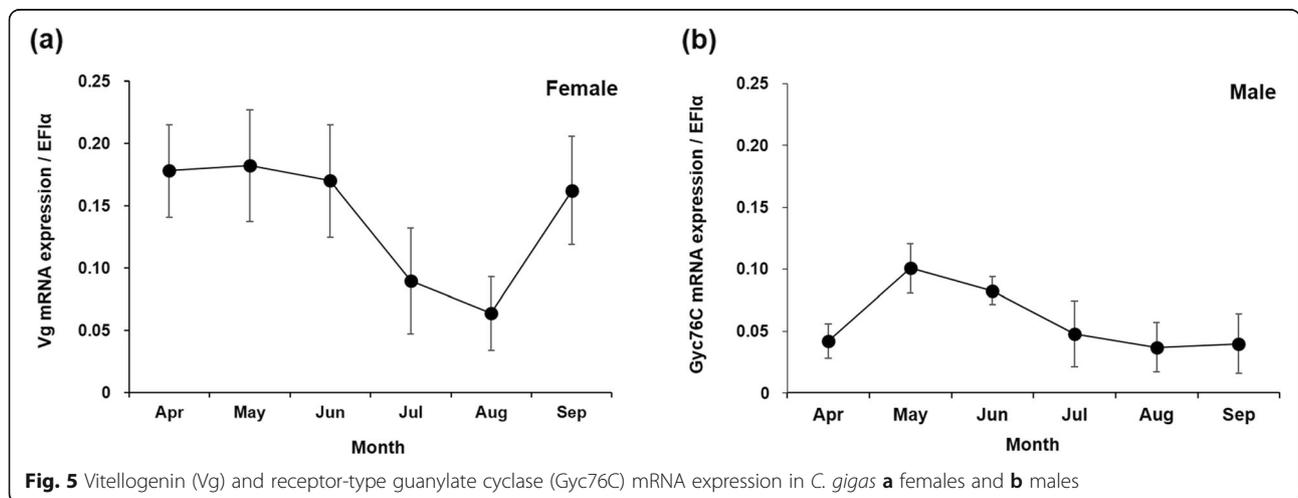
a potential endocrine mechanism contributing to sexual dimorphism (Davis et al. 2008).

Guanylate cyclase (Gyc) is a family of soluble receptor-type enzymes that catalyze the conversion of GTP to cGMP in both vertebrates and invertebrates (Patel et al. 2012). The expression of Gyc mRNA is involved in oogenesis, egg chamber development (Gigliotti et al. 1993; Ayoob et al. 2004), retinal development (Patel et al. 2012), wing development (Schleede and Blair 2015), and lumen formation (Myat and Patel 2016) in *Drosophyla* and in embryonic and adult retinal development in *Oryzias latipes* (Harumi et al. 2003). In the present study, Gyc76C mRNA expression was high in males during the maturation season, and its trends were similar to those of the IGF system. Therefore, we cautiously suggest that Gyc76C plays a sex-specific role in male oysters. IGF-1 inhibits adenylate cyclase and stimulates Gyc activity, thereby lowering cyclic AMP concentrations and elevating cycle GMP levels in a manner similar to the action of insulin

(Hadley 1988; Deeming 1991). Further studies focusing on the association between the function of cleaved Vg genes and the GH/IGF axis in ovaries and on Gyc and the IGF system in each developmental stage are necessary.

**Conclusion**

The expression of IGF system components including MIP, IGFBP-ALS, and CIR was associated with sex and developmental stage in Pacific oyster gonads. CIR and Vg expression levels were very similar among females, whereas those of MIP, IGFBP\_ ALS, CIR, and Gyc7C were very similar among males. The highest expression levels occurred in May, which is the maturation season. CIR and Vg are female-specific genes in Pacific oysters, whereas MIP, IGFBP-ALS, CIR, and Gyc76C are male specific. The results of the present study suggest that IGF system components, as well as Vg and Gyc76C, are associated with sexual maturation in *C. gigas*.



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### Authors' contributions

YHC contributed to the conceptualization, investigation, writing, supervision, project administration, and funding acquisition. JSM contributed to the investigation and data analysis. Both authors read and approved the final manuscript.

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### Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interest.

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