

Insulin-Like Growth Factors-1 Receptor (IGF-1R) Expression and the Phosphorylation of Endogenous Substrates Lead to Maturation of the Pacific oyster, *Crassostrea gigas*

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Conflict of interests

The authors declare no potential conflict of interest.

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Abstract

This study investigated the IGF-1 signal in specific tissues using Pacific oysters artificially matured via water temperature elevation. Pacific oysters were subjected to water temperature elevation from March to June, and 20 were randomly sampled each month. The condition index (CI) and tissue weight rate (TWR) were examined by measuring shell length, shell height, shell width, and soft tissue weight. The IGF-1 signal in tissues (adductor muscle, digestive glands, gills, labial palps, mantle edges, and gonads) was analyzed by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. From April to June, the TWR of females and males increased from 19.1±2.9 to 21.0±3.6 and 18.2±2.0 to 19.2±2.5, respectively, while the CI remained the same. The IGF-1 signal in each tissue differed. IGF-1 was expressed in the adductor muscle, while tyrosine was expressed in all tissues. The phosphor (p)-ERK and p-AKT activities were high in the adductor muscle, mantle edge, and gonads. IGF-1 signaling affected the growth and maturity of the Pacific oysters examined.

Keywords: Pacific oyster, Condition index, Tissue weight rate, Insulin-like growth factors-1 (IGF-1) signal

INTRODUCTION

In 2020, aquaculture in Korea yielded 325,889 tons of oysters (Korean Statistical Information Service, Ministry of Oceans and Fisheries, 2020). The main production site is the south coast, while some varieties are cultivated via various methods on the west coast (Hwang et al., 2016). However, the seedlings needed for oyster farming in Korea are dependent on natural culture. When there are few natural seedlings, it is difficult to secure a stable seed supply. Since the 1970s, the pollution of coastal waters, aging of fish farms, and deterioration of fishing conditions have led to seed shortages. In 1992, a major seed-collecting area on the southern coast was severely damaged (Park et al., 1999). Currently, artificial seed production is actively conducted to ensure a stable supply to meet the demand for oysters. The quality of the mother oysters affects seedling growth (Lee et al., 2008; Kim et al., 2013). Although

Authors' contributions

Conceptualization: Choi YH.
 Data curation: Park SJ, Choi YH.
 Formal analysis: Park SJ, Choi YH.
 Methodology: Choi YH.
 Investigation: Park SJ, Choi YH.
 Writing-original draft: Park SJ, Choi YH.
 Writing-review & editing: Park SJ, Choi YH.

Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

studies have evaluated mother oysters by examining changes in body components (Lim et al., 2011; Kim et al., 2014), a few studies have evaluated endogenous factors, such as the expression of insulin-like growth factors (IGFs), in mother oysters. The IGF system regulates somatic growth and development in fish, as in mammals (Duan, 1997; Le Bail et al., 1998; Moriyama et al., 2000). IGF-1 is an anabolic hormone that regulates development, somatic and skeletal muscle growth, and reproduction via endocrine, autocrine, and paracrine mechanisms mediated by growth hormone (Duan, 1997; Liu et al., 2017).

Here, we examined IGF-1 receptor (IGF-1R) expression and the phosphorylation of endogenous substrates in the IGF-1 pathway to evaluate the growth and maturity of artificially matured female and male Pacific oysters.

MATERIALS AND METHODS

1. Sample collection and preparation

Broodstock of artificially matured Pacific oysters, *C. gigas*, was obtained from the Southeast Sea Fisheries Institute, National Institute of Fisheries Science, Namhae, Korea. Each month from March to June 2016, 80 *C. gigas* were obtained. These were reared in running water at a flow rate of 15 L/min and fed a mixture of $10\text{--}15 \times 10^4$ cells/mL *Isochrysis galbana*, *Pheodactylum* sp., *Tetraselmis* sp., and *Chlorella* sp. The water temperature was raised slowly from 14.5°C to 22.5°C over 15 days and then kept at 22.5 ± 0.34 °C until the end of the study. The salinity was 32.2–33.8 psu.

The oyster shell length (SL), shell height (SH), and shell width (SW) were measured using Vernier calipers; total wet weight and soft tissue weight (STW) were measured using an electronic balance. The condition index (CI) and tissue weight rate (TWR) were calculated according to Choi & Chang (2003) using the following equations:

$$\text{CI} = \text{STW (g)} / \text{SL (mm)} \times \text{SH (mm)} \times \text{SW (mm)} \times 1,000$$

$$\text{TWR} = \text{STW (g)} / \text{Total wet weight (g)} \times 100$$

The adductor muscle, digestive gland, gills, labial palps, mantle edge, and gonads were isolated, and 0.5 g of each was frozen in liquid nitrogen and stored at -80 °C.

2. Western blot analysis

The tissues were homogenized in RIPA lysis buffer (50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate) containing protease inhibitors (1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 mM Na_3VO_4 , 1 mM NaF, and 1 mM PMSF). Samples were centrifuged at $14,483 \times g$ for 10 min, and the supernatant was collected. The protein concentration was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (35–50 µg) were separated on 8%–10% polyacrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in TBS-T (10 mM Tris-HCl, 150 mM NaCl pH 7.5, and 0.1% Tween 20) containing 1% BSA, incubated with the primary and secondary antibodies at room temperature for at least 1 h, and then detected using enhanced chemiluminescence Western blotting reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Between each step, the membranes were washed twice with TBST. The following primary antibodies and an anti-mouse secondary antibody, obtained from Santa Cruz Biotechnology, were diluted 1:1,000: IGF-1, IGF-1R, phosphorylated (p)-IGF-1R (PY99), AKT, p-AKT, ERK,

and p-ERK. Monoclonal mouse anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a control. The proteins were detected using enhanced chemiluminescence detection reagents (Advansta, Menlo Park, CA, USA) and visualized using the GeneSys imaging system (Syngene Synoptics, London, UK).

3. Statistical analysis

Data are presented as means \pm SD. Significant differences among means were determined by one-way analysis of variance (ANOVA) in SPSS v. 10.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

1. Growth and maturation of *C. gigas*

The growth of *C. gigas* was assessed by measuring SL, SH, SW, and STW for 4 months (Fig. 1A). Beginning in April, when it is possible to distinguish males and females, the monthly CI and TWR were assessed (Fig. 1B). SL, SH, SW, and STW were all significantly ($p < 0.05$) lower in March when maturation induction was started. Shell growth did not differ until June, but STW increased over time (Fig. 1A). In March, it was difficult to distinguish male and female oysters. While there was no difference in shell growth from April to June, females and males had the highest CI and TWR in June, when the water temperature had been elevated the longest (Fig. 1B).

Oyster maturation depends on water temperature, which affects gamete maturation, ovulation, and spawning in many fish species and oysters (Loosanoff & Davis, 1952; Anguis & Canavate, 2005; De Oliveira Ramos et al., 2014). Oyster maturation can depend on temperature, even within the genus *Crassostrea* (Loosanoff & Davis, 1952; De Oliveira Ramos et al., 2014). Mangrove oyster *C. gasar* gonadal tissue matured at 22°C and 26°C, but not at 18°C (De Oliveira Ramos et al., 2014),

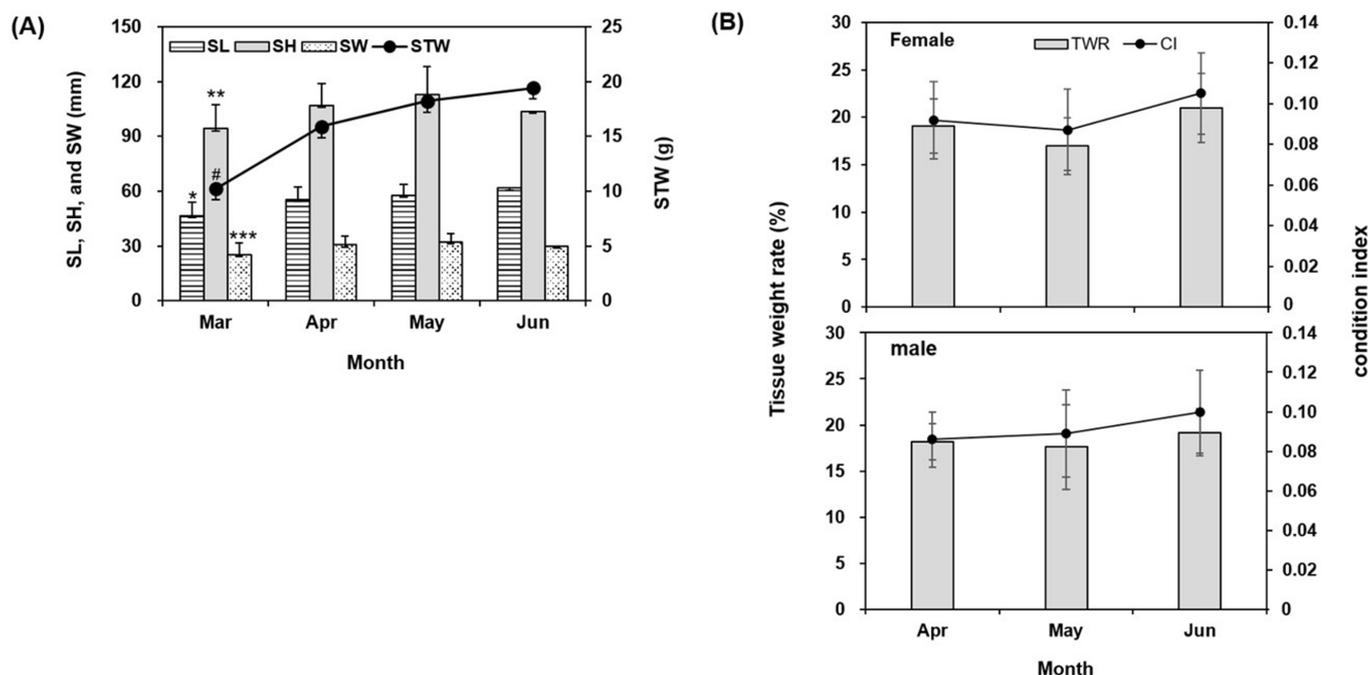


Fig. 1. The shell length (SL), shell height (SH), and shell width (SW), soft tissue weight (STW), tissue weight rate (TWR), and condition index (CI) of the Pacific oyster, *Crassostrea gigas*. Values are means \pm SD. *, **, *** and # indicate significant differences at $p < 0.05$.

and the CI did not differ among the temperature treatments at the beginning of the experiment.

In oysters of the genus *Crassostrea*, an individual can be male or female depending on the environmental conditions (De Oliveira Ramos et al., 2014). In this study, the mean ratio of *C. gigas* females to males from April to June was 1:0.875.

2. IGF-1R expression and the phosphorylation of endogenous substrates

IGF-1R has two extracellular α -subunits with ligand-binding sites. Each α -subunit couples to one of two membrane-spanning β -subunits, which contain an intracellular domain with intrinsic tyrosine kinase activity (Adams et al., 2000). IGF-1 binding to its receptor initiates a complex signaling cascade in the cell (Foncea et al., 1997). Activation is initiated by triggering the β -subunit kinase domains, leading to receptor autophosphorylation and tyrosine phosphorylation of multiple substrates (Adams et al., 2000). IGF-1R activation may be involved in the survival of surf clam (*Spisula sachalinensis*) larvae (Choi & Nam, 2014) and the growth of *C. gigas* (Choi et al., 2018). We investigated the expression of IGF-1R and the phosphorylation of its endogenous substrates in various tissues, including the gonads of oysters artificially matured via water temperature elevation. IGF-1R β was detected in all female and male tissues, whereas tyrosine phosphorylation (PY99) of the intracellular β -subunits was clearly detected in females, especially the gonads ($p < 0.05$, Fig. 2A).

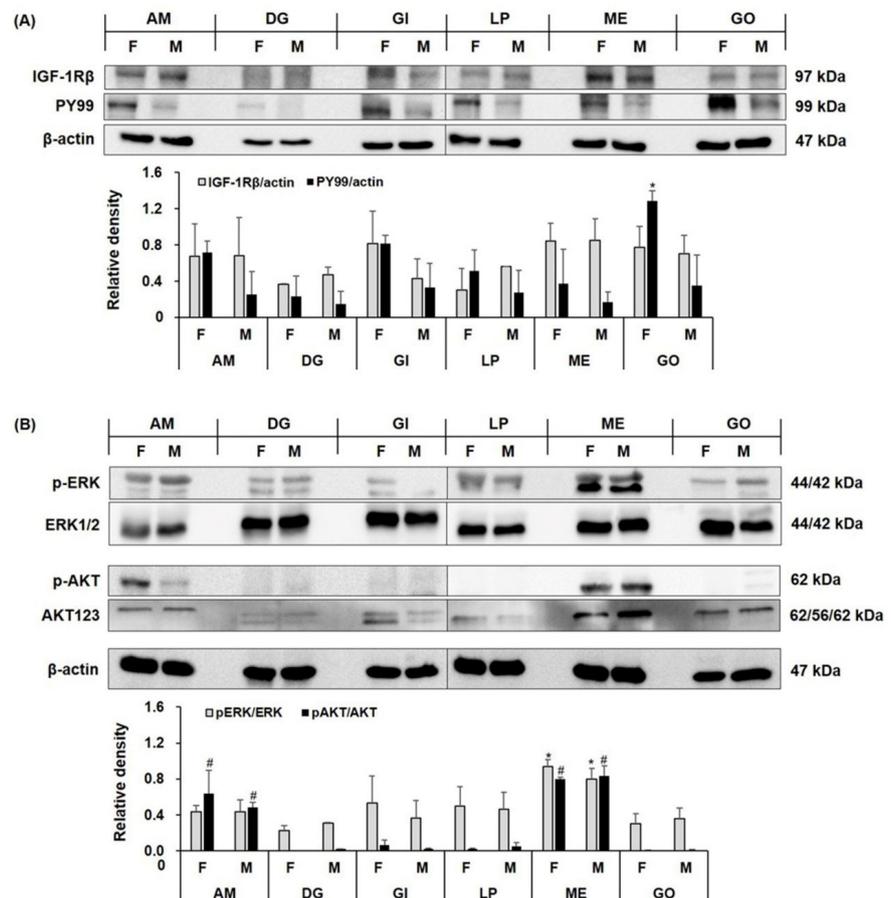


Fig. 2. Expression of the IGF-1R β -subunit, phosphorylated tyrosine residue 99 (PY99), ERK1/2, p-ERK, AKT123, and p-AKT in Pacific oyster tissues. Cell lysates were resolved by SDS-PAGE and analyzed by Western blot analysis. Values are means \pm SEM. * and # indicate significant differences at $p < 0.05$. AM, adductor muscle; DG, digestive gland; GI, gill; LP, labial palp; ME, mantle edge; GO, gonad; F, female; M, male; IGF, Insulin-like growth factors; SDS-PAGE, sulfate-polyacrylamide gel electrophoresis.

There was significant p-ERK and p-AKT expression in the adductor muscle and mantle edge ($p < 0.05$, Fig. 2B). While there were differences in the expression of endogenous substrates, the expression of downstream factors is believed to be involved in IGF signaling in artificially matured oysters. Further studies are needed to examine the association between the IGF-1 signaling pathway (PI3K and Ras/MAPK pathways) and maturation mechanisms in shellfish.

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