*The Israeli Journal of Aquaculture – Bamidgeh • ISSN 0792-156X • IJA.75.2023.1828192, 24 pages*
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# The involvement of GSK3β for glycogen synthesis throughout the annual cycle of Pacific oyster, *Crassostrea gigas* (*Magallana gigas*)

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(Received Nov 07, 2022; Accepted Dec 03, 2022; Published Jan 06, 2023)

Keywords: Glycogen, GSK3β, Pacific oyster, *Crassostrea gigas*, phospho GSK3β

**Abstract**

*Crassostrea gigas* is a frequently studied species in understanding physiological processes in bivalves. Similar to other animals, oysters store glucose in the body as glycogen. Glycogen is known to supply energy for germ cell development and maintenance. Glycogen is synthesized by glycogen synthase. GSK3β regulates glycogen synthase activity and plays an important role in glycogen synthesis. Therefore, this study aims to examine the effect of GSK3β on the annual cycle of oysters and the glycogen synthesis pathway and to investigate the energy pathway in comparison with seasonal variation. Oysters were sampled monthly for one year and were subjected to glycogen content, RT-PCR, FISH, and western blot analysis. The year-round glycogen content significantly differs only in the mantle edge during spring and summer of both sexes but not in labial palp, digestive gland, gonad, and adductor muscle. The expression of GSK3β mRNA level was highest in October for females and April for males. Both sexes had the lowest expression in July. In the adductor muscle, females and males showed the highest expression in April and the lowest in July and October. The pattern of GSK3β expression in gonads and adductor muscle was similarly confirmed through FISH. As a result of examining the signaling system, p-GSK3β (serine 9) increased. At the same time, glycogen synthase decreased in May when the condition index was the highest, p-GSK3β decreased in October and July when spawning occurred, and glycogen synthase increased. Overall, it is thought that p-GSK3β expression is high in *C. gigas* at ripe, which inhibits glycogen synthesis and is used as energy for growth and maturation. Glycogen synthesis occurs for energy storage during degeneration.

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## Introduction

The Pacific oyster, *Crassostrea gigas* (*Magallana gigas*), is a commercially valuable bivalve species worldwide. It is sought not only as a delicacy but also because it contains abundant nutrients such as glycogen, zinc, taurine, and amino acids (Liu et al., 2019). *C. gigas* is highly studied from an aquaculture research perspective due to its wide distribution, high economic importance, and fast growth. Like other animals, oyster stores excess glucose as glycogen through glycogenesis. This glycogen is then catabolized back to glucose during carbohydrate-limiting conditions to be used by various tissues for physiological processes (Martínez-Pita et al., 2012; Furukawa et al., 2018).

Glycogen plays an important role in energy metabolism and physiological processes in bivalves and is known to be particularly important for energy provision during gamete formation (Berthelin et al., 2000; Hamza-Chaffai et al., 2003). As such, glycogen is an important energy source for bivalves (Bacca et al., 2005). However, so far, many studies have been conducted using the whole oyster soft tissue to analyze glycogen content in bivalves (Bacca et al., 2005; Liu et al., 2019). Moreover, studies on glycogen content analysis according to tissues need to be more comprehensive. Glycogen is a storage form of glucose, and stored glycogen is a major source of glucose that can be used in various tissues (Martínez-Pita et al., 2012; Furukawa et al., 2018). The synthesis of glycogen is regulated by glycogen synthase (GS), and the activity of GS occurs by dephosphorylating GS. In contrast, GSK3β (glycogen synthase kinase 3 beta) is inactivated by phosphorylation of serine 9 residue (Patel et al., 2008). At this time, the activated GS promotes glycogen synthesis (**Figure 1**). Activated AKT induces the phosphorylation of the serine 9 residue of GSK3β and causes it to become inactive. When serine 9 phosphorylation of GSK3β occurs, it affects various reactions, including glycogen synthesis, growth and metabolic regulation of organisms, regulation of several transcription factors, and regulation of gene expression (Grimes et al., 2001; Hundsrucker et al., 2010; Souder and Anderson, 2019). The elucidation of GSK3β in glycogen metabolism has been reported in some invertebrate species, including Fujian oyster, *C. angulata* (Zeng et al., 2013), Chinese mitten crab *Eriocheir sinensis* (Wang et al., 2016), and kissing bug, *Rhodnius prolixus* (Mury et al., 2016). Previous studies have performed seasonal variation studies in glycogen content and several tissue-specific regulations of glycogen metabolism-related genes in *C. gigas* similar to the current study but failed to specify specific responses concerning sex and tissue (Liu et al., 2020). Hence, this study was conducted to specifically determine the regulation of tissue-specific GSK3β expression, seasonal glycogen content, and activity of AKT/GSK3β pathway signals about the annual reproductive cycle of both male and female Pacific oyster *C. gigas* collected off the coasts of Tongyeong, Korea.

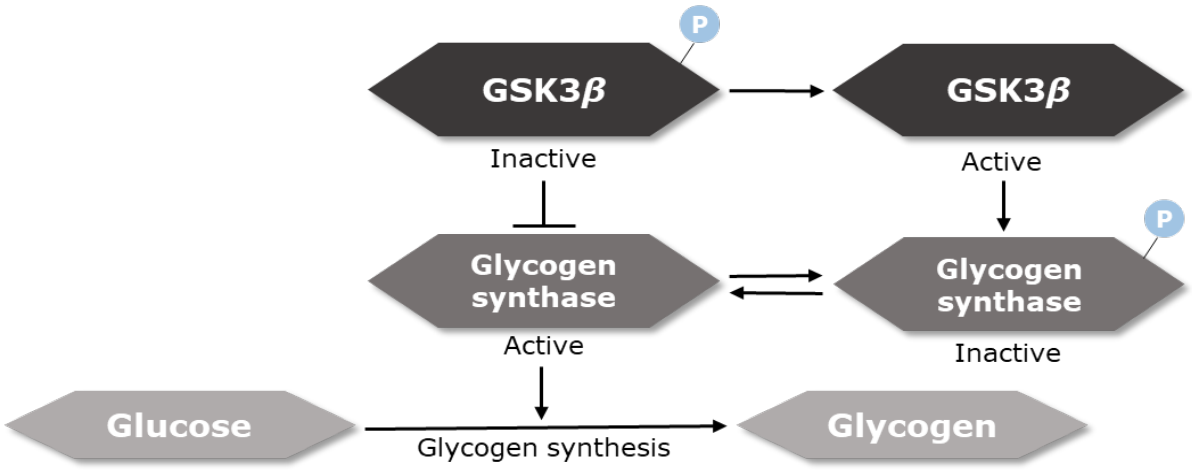


**Figure 1** GSK3β phosphorylation and activation. It inhibits GSK3β activity by phosphorylating serine 9. The inhibition of GSK3β decreases the phosphorylation of glycogen synthase, leading to an increase in the active form. As a result, glycogen synthesis is promoted.

## Materials and Methods

*Oyster sample collection and growth parameters*

Fifty oysters were randomly collected monthly for a year (August 2020 to July 2021) from the Saryangdo area of Tongyeong (34°51’32.34” N, 128°12’23.44” E), South Korea. During sampling, shell length (SL), shell height (SH), and shell width (SW) were measured using a vernier caliper capable of measuring to the nearest 0.01 mm. The total weight (TW) and soft tissue weight (STW) were measured using an electronic balance (Vibra AJ-4200E, Tokyo, Japan) to the nearest 0.01 g. Tissue weight rate (TWR) and condition index (CI) were calculated using the formula of Choi and Chang (2003) as follows.

$$TWR = STW\ (g) / TW\ (g) \times 100$$

$$CI = STW\ (g) / \{SL\ (mm) \times SH\ (mm) \times SW\ (mm)\} \times 1000$$

Thirty of fifty oyster soft tissue samples were frozen in liquid nitrogen and stored in -80ºC until analysis, and the remaining ones were fixed in 4% paraformaldehyde.

*Tissue-specific analysis of glycogen content of oyster*

The seasonal glycogen content of oysters was determined from the mantle edge, labial palp, digestive gland, gonad, and adductor muscle of three oyster samples from each sex. Samples were designated from sampling months of September, December, March, and July, coinciding with the four distinct seasons-autumn, winter, spring, and summer.

Briefly, 10 mg tissue in 100 µL Millipore-filtered distilled water was homogenized on ice and centrifuged at 13,000 x *g* for 12 mins at 4°C. The supernatant was collected and used for analysis using a commercial glycogen assay kit (Sigma-Aldrich, MO, USA) following the manufacturer’s instructions. Briefly, diluted glycogen standard (0.2 mg/mL) dispensed in a 96-well plate was added with hydrolysis buffer to adjust the total volume to 50 µL. Similarly, the tissue supernatant (25 µL) was dispensed into the sample-designated well, and 25 µL of Hydrolysis Buffer was added to adjust the final volume to 50 µL. Thereafter, 2 µL of hydrolysis enzyme mix was added and left to react for 30 minutes at room temperature. Then, 50 µL of Master Reaction Mix (Development Buffer 46 µL, Development Enzyme Mix 2 µL, and Fluorescent Peroxidase Substrate 2 µL) was added and was left again to react similarly. The

glycogen content was calculated from the absorbance readings at 570nm using a microplate reader (EZ Read 400, Biochrom, UK) against the standard curve as follows:

$$C = S_a/S_v$$

C = glycogen content in the sample

$S_a$ = amount of glycogen in the sample from the standard curve (μg)

$S_v$ = sample volume in each well (μL)

*Tissue-specific GSK3β mRNA expression of oyster*

*1. Total RNA extraction*

Female and male oyster gonad tissues were selected separately from April to October, and total RNA extraction was performed from 4 females and males each month. Also, when females and males were not distinguished from November to March, total RNA extraction was performed from 4 males and females each month. Total RNA was extracted from each tissue sample using RNAiso Plus (Takara Bio, Shiga, Japan). After homogenization, homogenates were set to react at 4°C for 1 hour. Subsequently, 200 μL of chloroform (Duksan, Seoul, Korea) was added to homogenates and reacted at room temperature for 10 minutes before centrifugation at 15,520 x g, at 4ºC. The supernatant was added with 200 μL of isopropyl alcohol (Duksan, Seoul, Korea), and reacted for 10 minutes at room temperature before centrifugation at the same conditions to obtain an RNA pellet. Then, washed with 75% and 100% ethanol and dried in a dry oven for 30 minutes at 37ºC. PCR water was used to dissolve pellets, and RNA concentrations were quantitively analyzed using a UV/VIS Nano spectrophotometer (MicroDigital, Seongnam, Korea).

*2. cDNA synthesis*

The cDNA was synthesized using PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). Oligo dT Primer 1 μL, dNTP Mixture 1 μL, and RNase Free $dH_2O$ 3 μL were added to RNA, reacted at 65°C for 5 minutes, and then the next step was performed at 4°C. 5X PrimeScript Buffer 4 μL, RNase Inhibitor 0.5 μL, PrimeScript RTase 1 μL, RNase Free $dH_2O$ 4.5 μL was added to the reaction mixture, reacted at 42°C for 60 minutes, and then RTase was inactivated at 95°C for 5 minutes. The synthesized cDNA was diluted by adding DNase-free $dH_2O$ 180 μL and stored at -20℃ to be used as a template when performing reverse transcription PCR (RT-PCR).

*3. Reverse transcription PCR (RT-PCR) and electrophoresis*

RT-PCR was performed using EmeraldAmp GT PCR Master Mix Kit (Takara Bio, Shiga, Japan). EmeraldAmp GT PCR Master Mix 12.5 μL, $dH_2O$ 10.5 μL, forward Primer 0.5 μL, reverse Primer 0.5 μL, and Template 1 μL were mixed to make a mixture. This experiment used EF1α (GenBank accession no. AB122066.1) and GSK3β (GenBank accession no. XM_011454458.3). The EF1α (elongation factor 1 alpha) primer was used as a housekeeping gene, and the GSK3β primer was used (**Table 1**).

The PCR reaction was repeated 30 times by initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 10 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The final extension was performed at 72°C for 7 minutes and cooled at 10°C. The PCR reaction was performed using a Takara PCR Thermal Cycler Dice Gradient (Takara Bio, Shiga, Japan) and an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems-Life Technologies, Foster City, California, USA) instrument.

The PCR product was loaded on 1.5% agarose gel containing ethidium bromide, and the band was confirmed by electrophoresis at 100 V for 20 minutes. After development with an Azure biosystem C300 imaging system (Azure Biosystems, Dublin, USA), densitometry was

confirmed using Gene Tools version 4.03 (SYNGENE, Cambridge, UK). The expression of GSK3β was analyzed by relative quantification of EF1α.

**Table 1** Primer sequence for RT-PCR

| Primer name | Forward primer | Reverse primer | Fragment length (bp) | Accession Number |
|---|---|---|---|---|
| EF1α | TGGCTTCAACATCAAGAACG | ATGTGAGCAGTGTGGCAATC | 180 | AB122066.1 |
| GSK3β | TAAGGATGGCAGCAAGAT | GATAAACAACCCCAAAAGAC | 122 | XM_011454458.3 |

*Fluorescence in situ hybridization (FISH)*

Three specimens of the Pacific Oyster (*C. gigas*) were collected on August 2020 and July 2021. Three specimens of the Pacific Oyster (*C. gigas*) were collected on August 2020 and July 2021. The gonad and adductor muscle that had been fixed were subjected to histological procedures. Tissue samples were dehydrated in increasing ethanol series (70%, 80%, 90%, and 100%), cleared with xylene, and embedded in paraffin. Then, the paraffin block was sliced to a thickness of 4-5 μm using a microtome (Tucsen, Fuzhou, China), and sectioned tissues were floated onto glass slides. Tissue slides were deparaffinized twice in xylene for 10 minutes and hydrated in decreasing ethanol series (100%, 90%, 80%, and 70%) for 10 minutes each. To block RNase activity, tissue was placed in DEPC-PBS for 10 minutes to hydrate and wash. Then, it was placed in a dry oven at 60°C and dried. GSK3β probe (5′-ACCAGCTGTGGCCAGCA–3′) was placed on the sectioned tissue and incubated overnight in an incubator at 55°C. After incubation, the slides were washed in DEPC-PBS and observed under a fluorescence microscope (Olympus IX51, Tokyo, Japan).

*Western blot*

The oyster gonad tissue was used in this experiment by selecting females and males separately from April to October. Gonad tissues homogenized using RIPA buffer (50 mM Tris, 1 mM EGTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate), containing protease inhibitor (1 μg/mL Aprotinin, 1 μg/mL Pepstatin, 1 mM Phenylmethanesulfonyl fluoride, 1 mM Sodium fluoride, 1 mM Sodium orthovanadate, 1 μg/mL Leupeptin) and centrifuged at 15,520 × g for 10 minutes to obtain supernatant. Quantitation of total protein was determined using the PierceTM BCA Protein assay kit (Thermo Scientific, Rockford, IL, USA). The total protein (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide gel and then transferred to a polyvinyl difluoride membrane. Each membrane was blocked with TBST-T (0.1% Tween 20, 10 mM Tris-HCl, 150 mM NaCl pH 7.5) containing 10% bovine serum albumin (BSA), incubated with pre-diluted (1:1,000) primary and secondary antibodies (1:10,000). The AKT/GSK3β signaling pathway was identified. Primary antibodies used were IRS-1 (Cell signaling; # 2390), PI3Kp85 (Cell signaling; # 4292), p-AKT (Santa Cruz Biotechnology; sc-7985), AKT (Cell signaling; # 9272), p-GSK3β (Santa Cruz Biotechnology; sc-373800), GSK3β (Santa Cruz Biotechnology; sc-7291), glycogen synthase 1 (Santa Cruz Biotechnology; sc-81173), β-actin (Santa Cruz Biotechnology; sc-47778) was used. As secondary antibodies, HRP-conjugated goat ant0i-rabbit IgG (Sigma-Aldrich, St Louis, MO, USA) and HRP-conjugated goat anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, USA) were used. Monoclonal mouse anti-β-actin was used as a control. Membranes were detected with an Azure biosystem C300

analyzer (Azure Biosystems, Dublin, USA), and densitometry was confirmed for activity using Gene Tools version 4.03 (SYNGENE, Cambridge, UK).

*Statistical analysis*

Data were subjected to statistical analysis using One-Way ANOVA in SPSS version 27 (IBM, USA). In case of significant difference, post-hoc Duncan's multiple range test was run. The statistical level was set at 95% confidence. Results were expressed as mean ± standard error of the mean (SEM).

## Results

*Year-round body morphometrics and condition index of oyster*

Measurement of shell length, shell height, shell width, total weight, and soft tissue weight of oysters collected for one year is shown in **Figure 2**. The shell length ranged from 27.33 - 43.25 mm, with the lowest recorded in August and the highest in April (**Figure 2A**). The shell height was 45.18 - 86.28 mm, lowest in August and highest in July (**Figure 2B**). The shell width ranged from 16.65 – 29.05 mm, with the lowest recorded in August and the highest in March and April (**Figure 2C**). The total weight of oyster samples ranged from 7.92 – 58.47 g, lowest in August and highest in May (**Figure 2D**). The soft tissue weight was recorded in lowest value during August sampling (1.98 g) and highest in May (18.44 g) (**Figure 2E**). Generally, the body morphometrics were high during late winter to late spring (February to May) and decline thereafter, before gradual increment during early autumn to mid-winter (September to January).

Meanwhile, tissue weight rate was lowest in December (14.67%) and highest in May (31.73%) (**Figure 3A**). The highest condition index was similarly recorded in May with the lowest condition index in December and July (**Figure 3B**). Generally, both tissue weight rate and CI were high in spring, decline in summer until early-winter before increasing again from mid-winter (January).

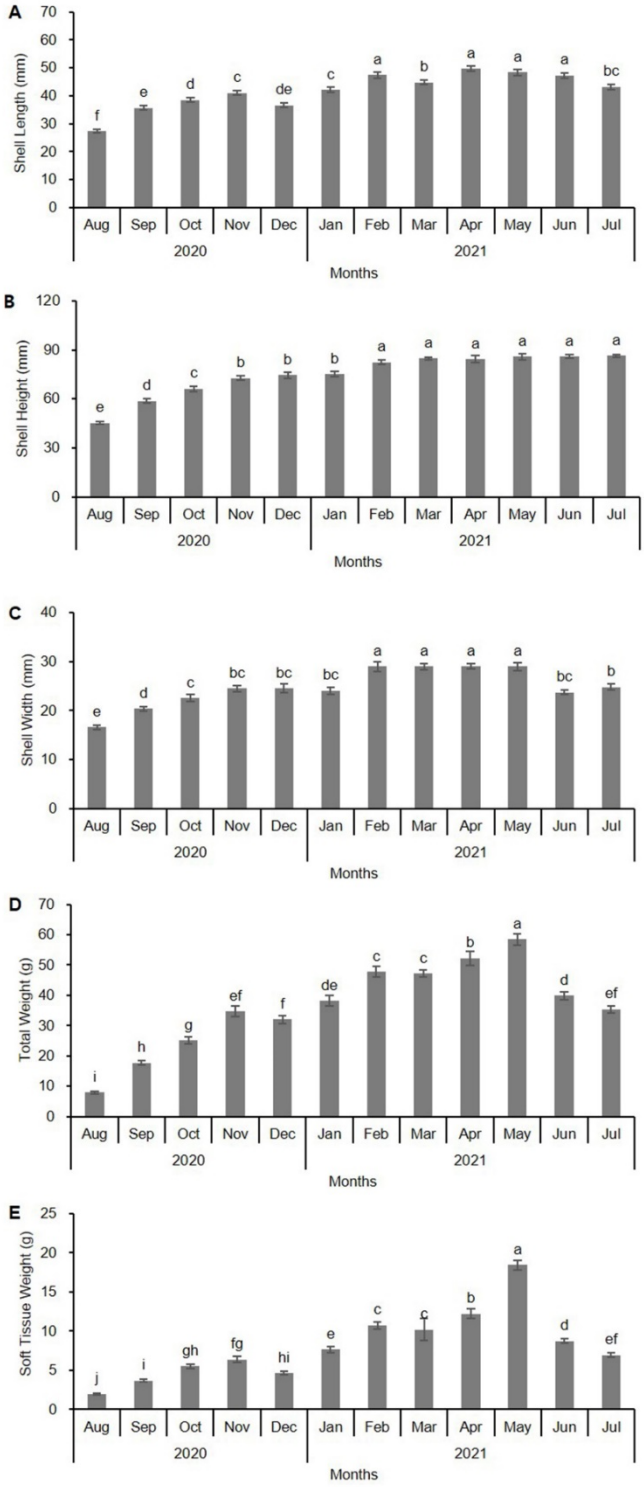


**Figure 2** Changes in Pacific oyster, *Crassostrea gigas* for (A) shell length, (B) shell height, (C) shell width, (D) total weight, and (E) soft tissue weight (mean ± SEM, n = 50). Letters indicate significant differences at $p < 0.05$.

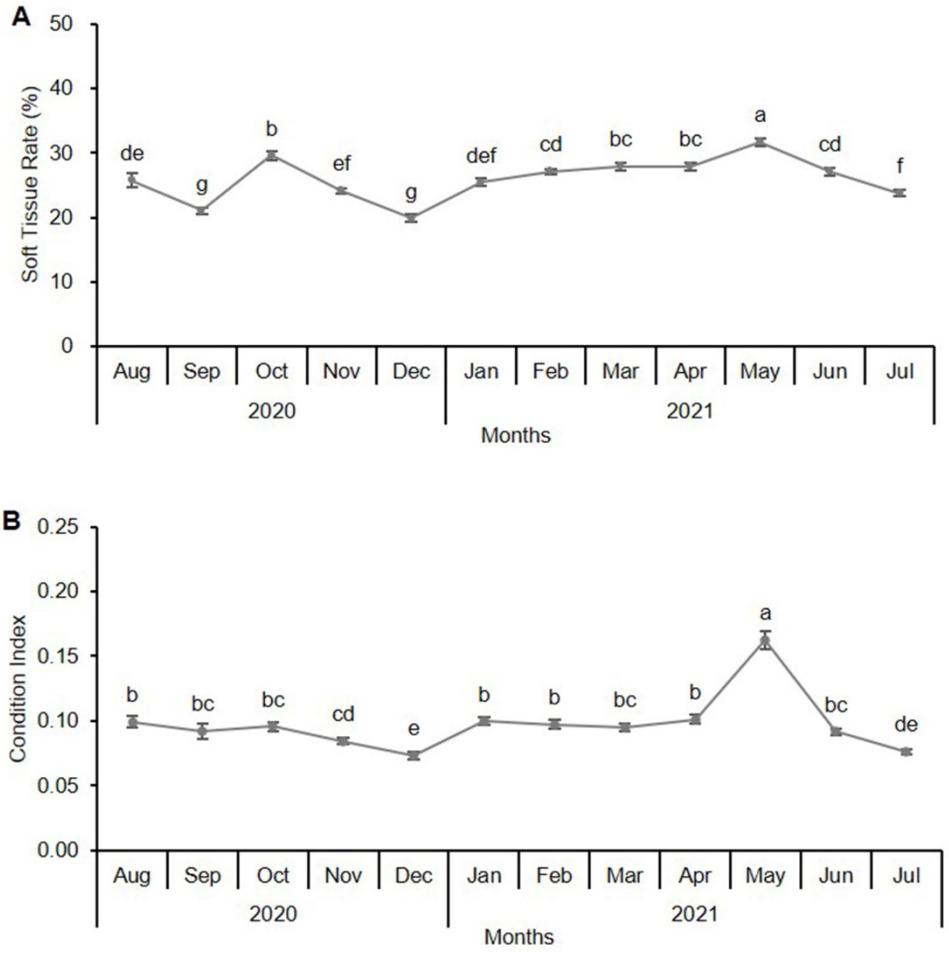


**Figure 3** Changes in Pacific oyster, *Crassostrea gigas* (A) tissue weight rate and (B) condition index (mean ± SEM, n = 50). Letters indicate significant differences at $p < 0.05$.

*Tissue-specific glycogen content in oyster*

Glycogen content was detected in all analyzed tissues (mantle edge, labial palp, digestive gland, gonad, and adductor muscle) (**Figure 4**).

The glycogen content in the mantle edge, regardless of sex, is significantly higher in spring and summer compared during autumn and winter ($p < 0.05$) (**Figure 4A**). In contrast, no significant difference was detected in the seasonal glycogen content of labial palp, digestive gland, gonad, and adductor (**Figure 4B-E**).

The glycogen content in labial palp ranged at 0.10 – 0.09 µg/µL in both sexes, and in the digestive gland at 0.12 – 0.13 µg/µL in females and 0.10 – 0.14 µg/µL in males. The female gonad recorded 0.17 – 0.14 µg/µL while the male gonad had 0.12 – 0.13 µg/µL glycogen content. The adductor muscle had glycogen content of 0.22 – 0.15 µg/µL and 0.17 – 0.18 µg/µL for male and female oysters, respectively.

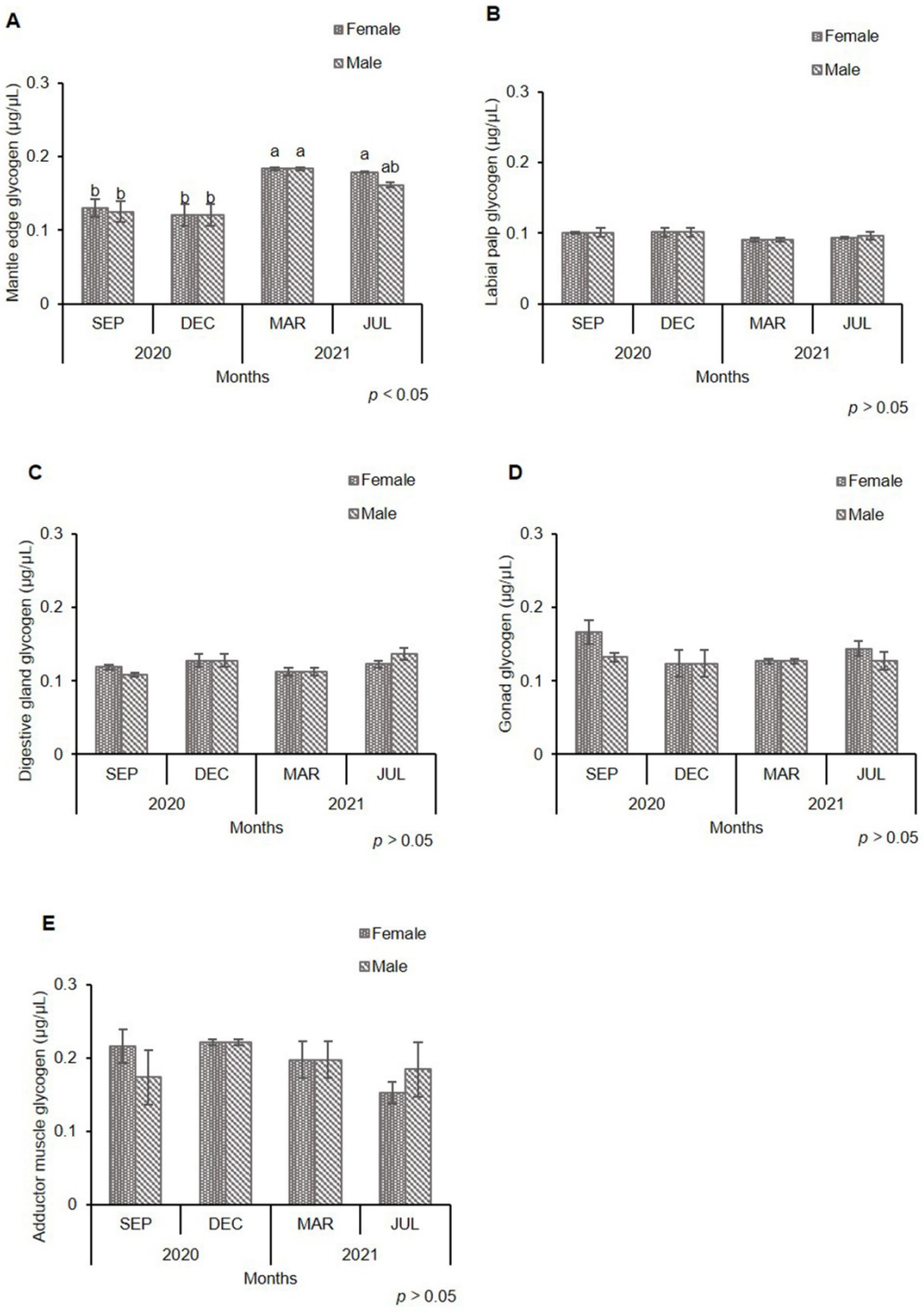


**Figure 4** Glycogen content of various tissue in Pacific oyster, *Crassostrea gigas*. (A) mantle edge, (B) labial palp, (C) digestive gland, (D) gonad, (E) adductor muscle. Values represent the mean ± SEM, n = 3. Letters indicate significant differences at $p < 0.05$. Not significantly different $p > 0.05$.

*GSK3β mRNA expression by tissue in Pacific oyster*

The expression level of GSK3β mRNA in oysters was confirmed in all analyzed tissues (mantle edge, labial palp, digestive gland, gonad, and adductor muscle).

The GSK3β mRNA expression level of in mantle edge showed a similar tendency in females and males (**Figure 5**). Both sexes were significantly higher in April, and significantly lower in November to January ($p < 0.05$). Similarly, the labial palp showed a similar pattern in males and females (**Figure 6**). Both sexes oyster showed a tendency to increase from November to May and gradually decrease thereafter. Similarly, the labial palp showed a similar pattern in males and females (**Figure 6**). Both sexes tended to increase from November to May and gradually decrease thereafter. In both sexes, the GSK3β mRNA expression level in the digestive gland was significantly higher from November to January and significantly lower from March to July ($p < 0.05$) (**Figure 7**)

In contrast, the monthly GSK3β mRNA expression level in female and male gonads differed (**Figure 8**). In the female gonad, the expression level increased from August to October, then decreased in November, plateaued until March, and then decreased thereafter (**Figure 8B**). The expression level was significantly higher in October and significantly lower in June and July ($p < 0.05$). On the other hand, the GSK3β mRNA expression level in the male gonad increased from August, gradually decreased after October, plateaued until March, then increased again in April and decreased thereafter (**Figure 8C**). Significantly higher expression levels in April and lower in June and July ($p < 0.05$). The GSK3β mRNA expression level in the adductor muscle of both female and male oysters was significantly higher in April and significantly lower from November to January ($p < 0.05$) (**Figure 9**).

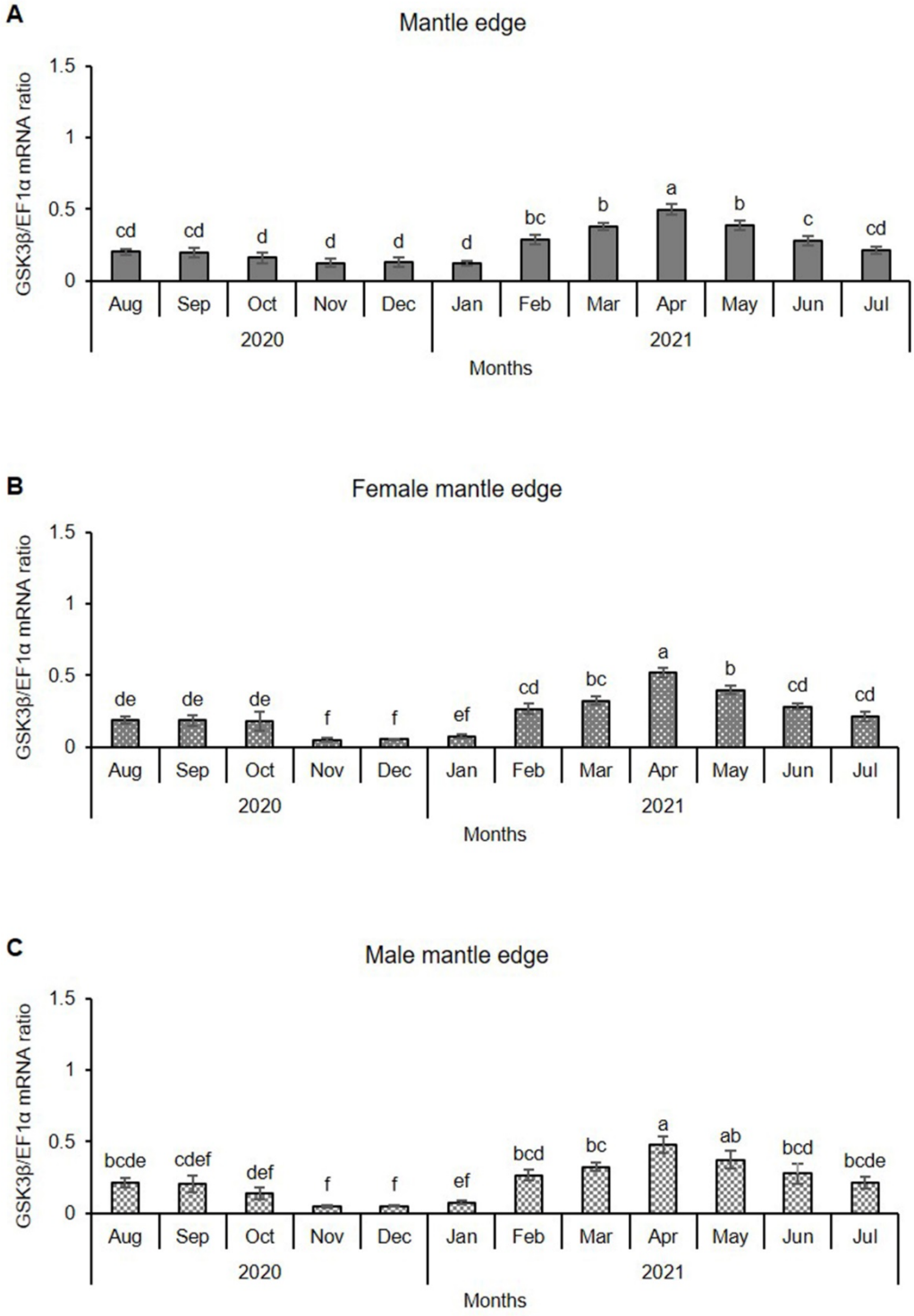


**Figure 5** GSK3β mRNA expression of mantle edge in Pacific oyster, *Crassostrea gigas*. (A) total mantle edge (n = 8), (B) female mantle edge (n = 4) and (C) male mantle edge (n = 4). Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

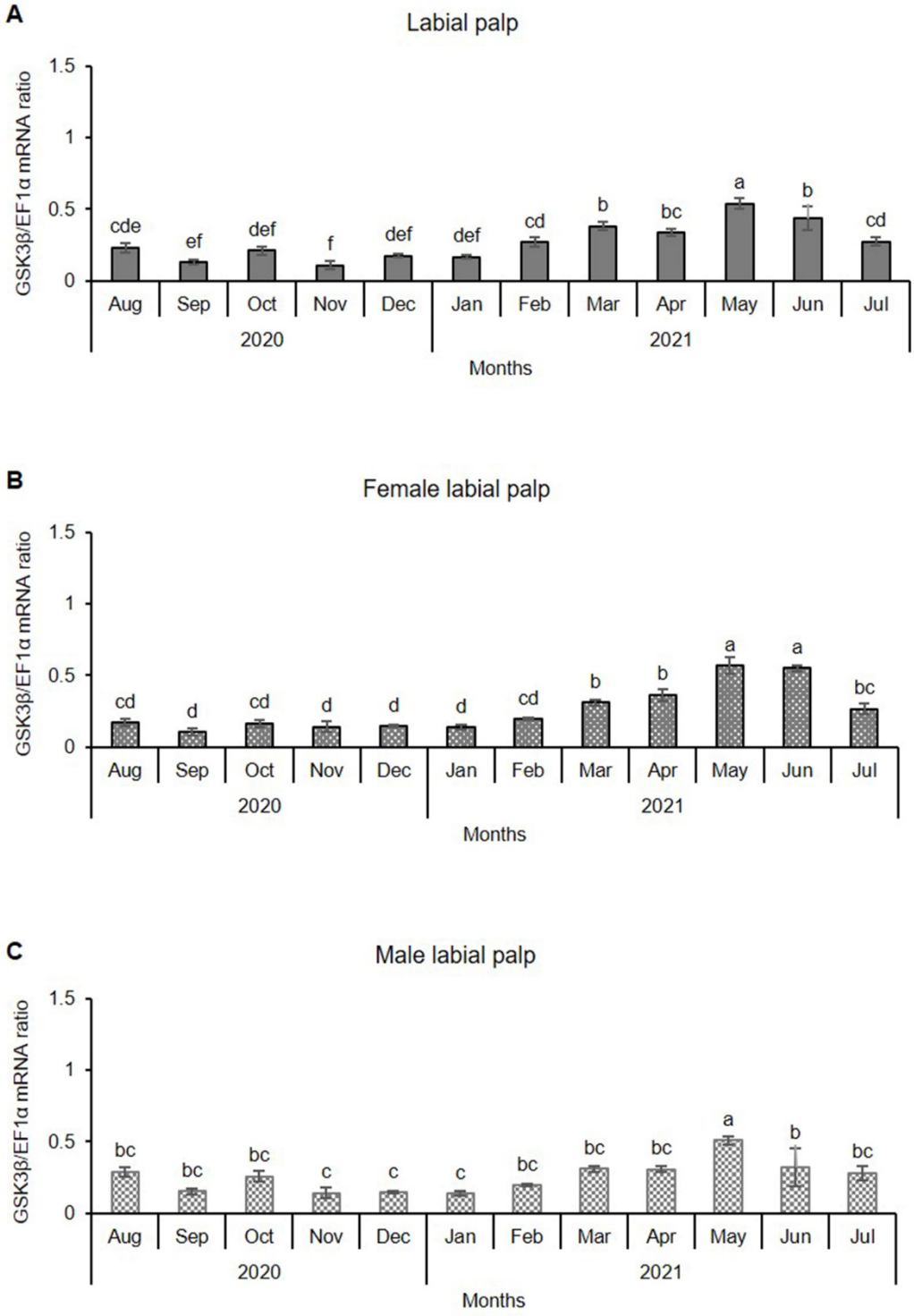


**Figure 6** GSK3β mRNA expression of labial palp in Pacific oyster, *Crassostrea gigas*. (A) total labial palp (n = 8), (B) female labial palp (n = 4) and (C) male labial palp (n = 4). Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

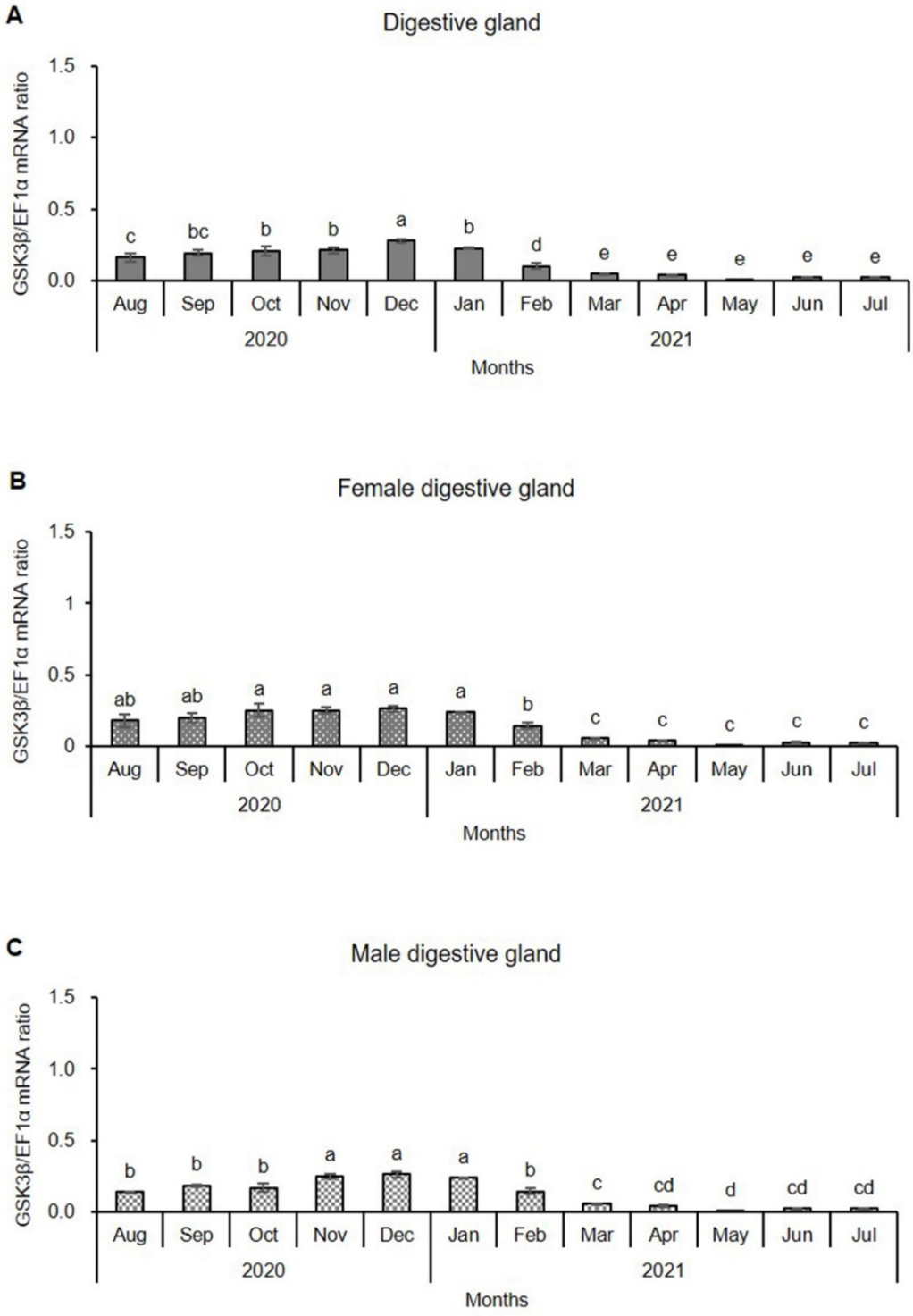


**Figure 7** GSK3β mRNA expression of the digestive gland in Pacific oyster, *Crassostrea gigas*. (A) total digestive gland (n = 8), (B) female digestive gland (n = 4) and (C) male digestive gland (n = 4). Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

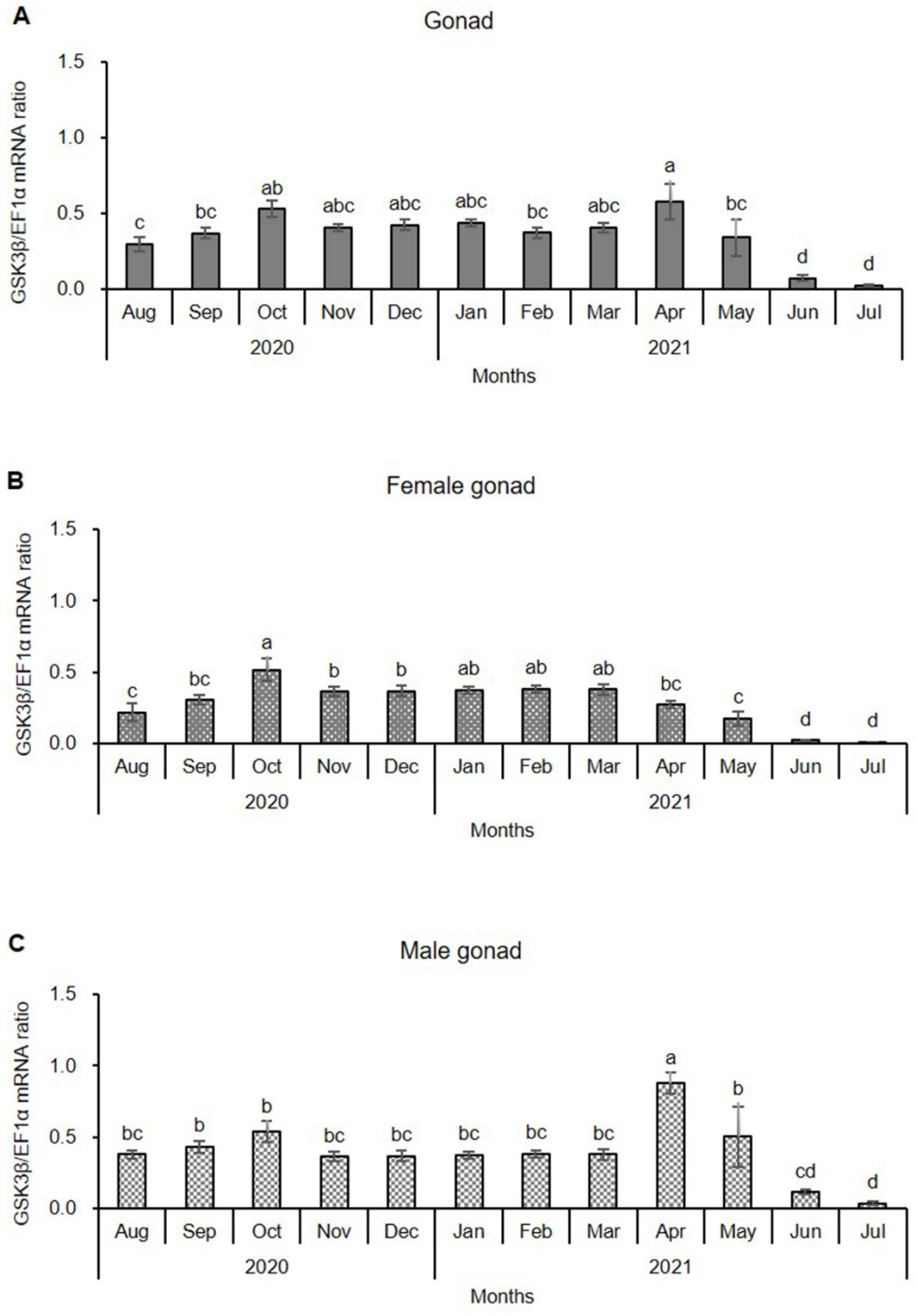


**Figure 8** GSK3β mRNA expression of the gonad in Pacific oyster, *Crassostrea gigas*. (A) total gonad (n = 8), (B) female gonad (n = 4) and (C) male gonad (n = 4). Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

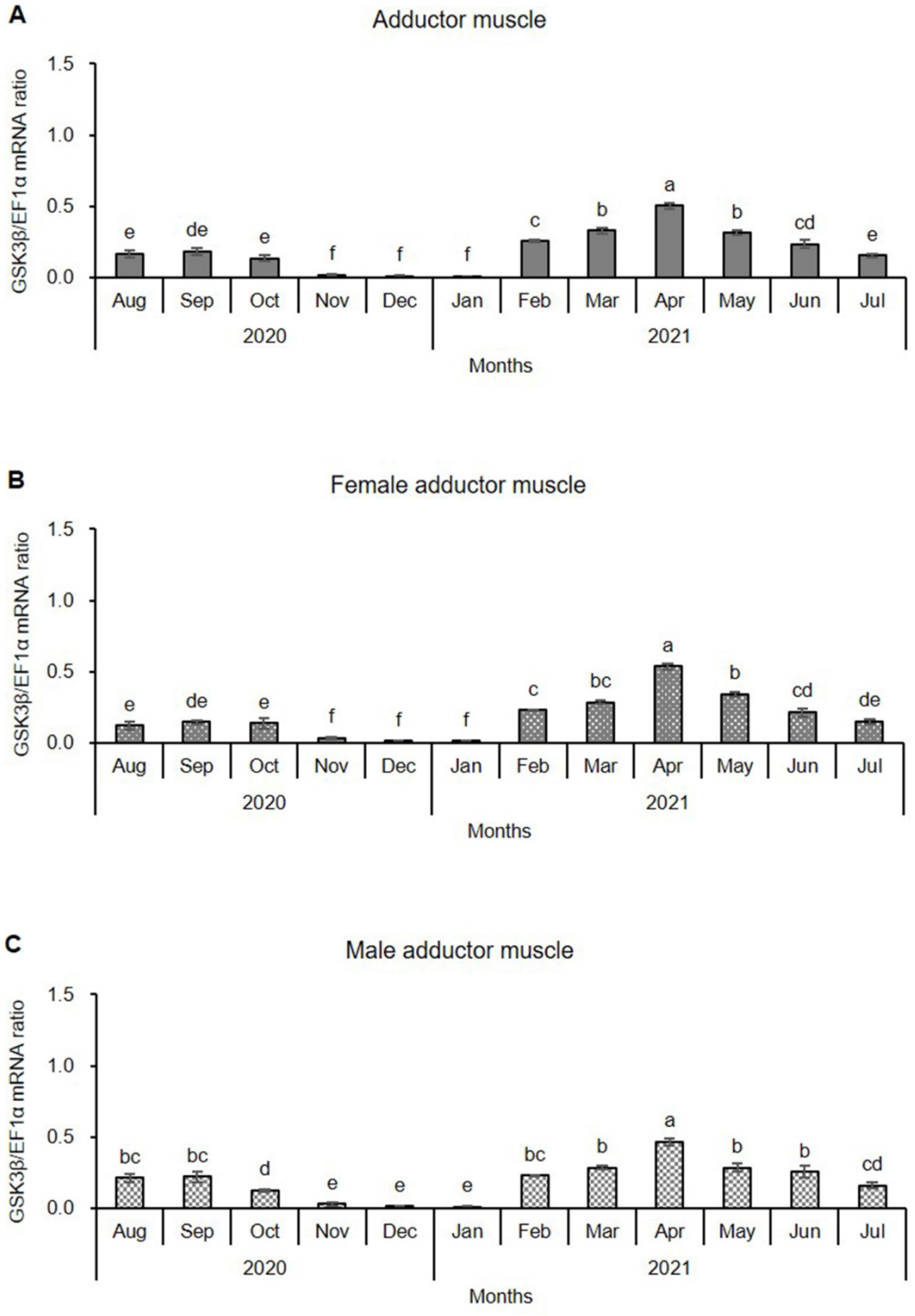


**Figure 9** GSK3β mRNA expression of the adductor muscle in Pacific oyster, *Crassostrea gigas*. (A) total adductor muscle (n = 8), (B) female adductor muscle (n = 4) and (C) male adductor muscle (n = 4). Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

*Distribution of GSK3β mRNA expression through fluorescence in situ hybridization (FISH)*

The FISH method was performed to confirm the distribution of GSK3β mRNA expression based on year-round in the gonads and adductor muscle of oysters. GSK3β mRNA expression was detected throughout seasonal variation of gonad and adductor muscle.

The female gonads showed the highest expression GSK3β in October and lowest in July (**Figure 10**). Meanwhile, GSK3β in male gonads were highly expressed in October, April, and May, and low in June and July (**Figure11**). The GSK3β mRNA expression in adductor muscle showed the highest expression in April in both sexes, and the lowest expression in November and December (**Figure12,13**).

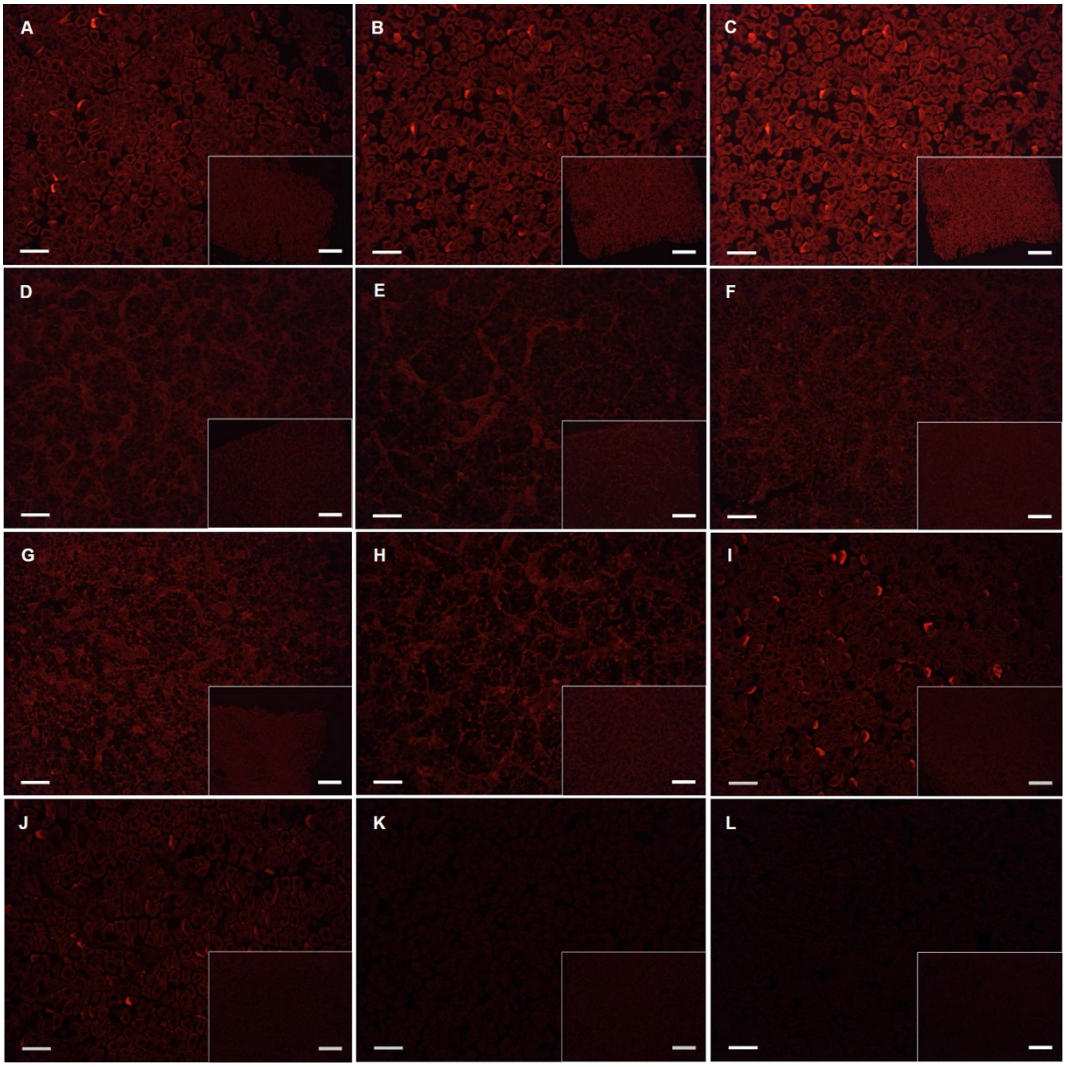


**Figure 10** Expression of GSK3β mRNA fluorescence in situ hybridization female gonad of Pacific oyster, *Crassostrea gigas*. (A) August; (B) September; (C) October; (D) November; (E) December; (F) January; (G) February; (H) March; (I) April; (J) May; (K) June; (L) July. The large image is × 100 (scale bar, 100 μm); the small image is × 40 (scale bar, 500 μm).

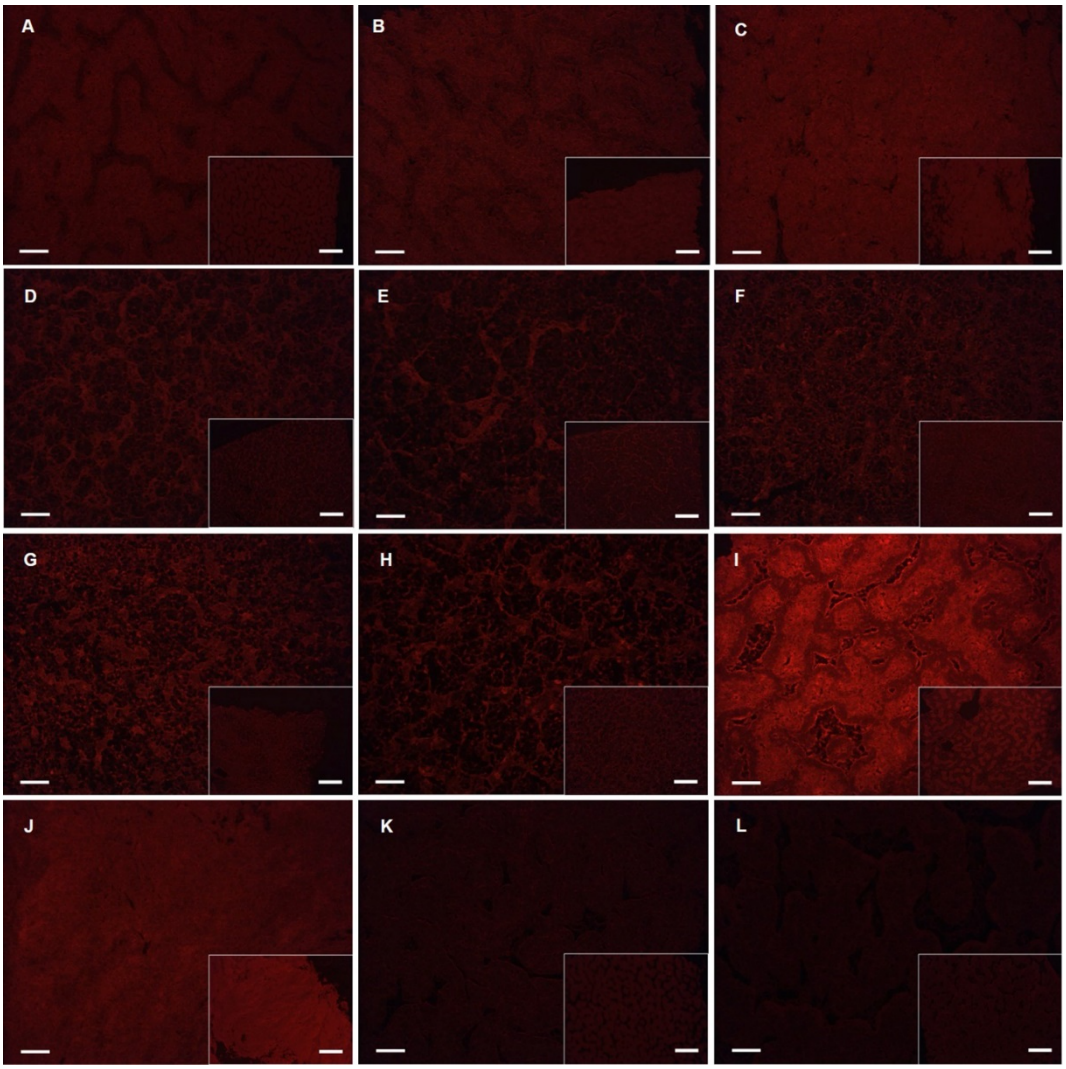


**Figure 11** Expression of GSK3β mRNA fluorescence in situ hybridization male gonad of Pacific oyster, *Crassostrea gigas*. (A) August; (B) September; (C) October; (D) November; (E) December; (F) January; (G) February; (H) March; (I) April; (J) May; (K) June; (L) July. The large image is × 100 (scale bar, 100 μm); the small image is × 40 (scale bar, 500 μm).

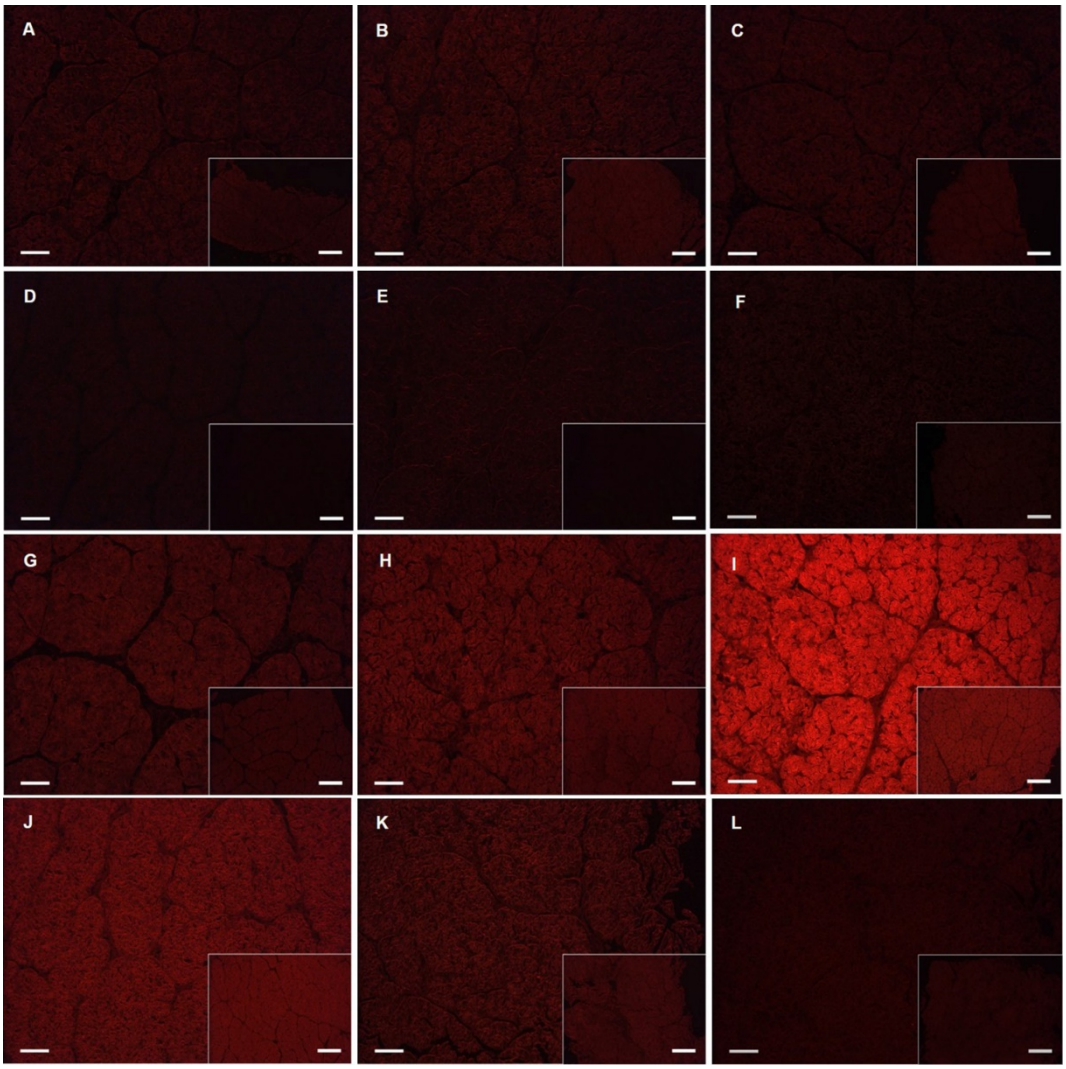


**Figure 12** Expression of GSK3β mRNA fluorescence in situ hybridization female adductor muscle of Pacific oyster, *Crassostrea gigas*. (A) August; (B) September; (C) October; (D) November; (E) December; (F) January; (G) February; (H) March; (I) April; (J) May; (K) June; (L) July. The large image is × 100 (scale bar, 100 μm); the small image is × 40 (scale bar, 500 μm).

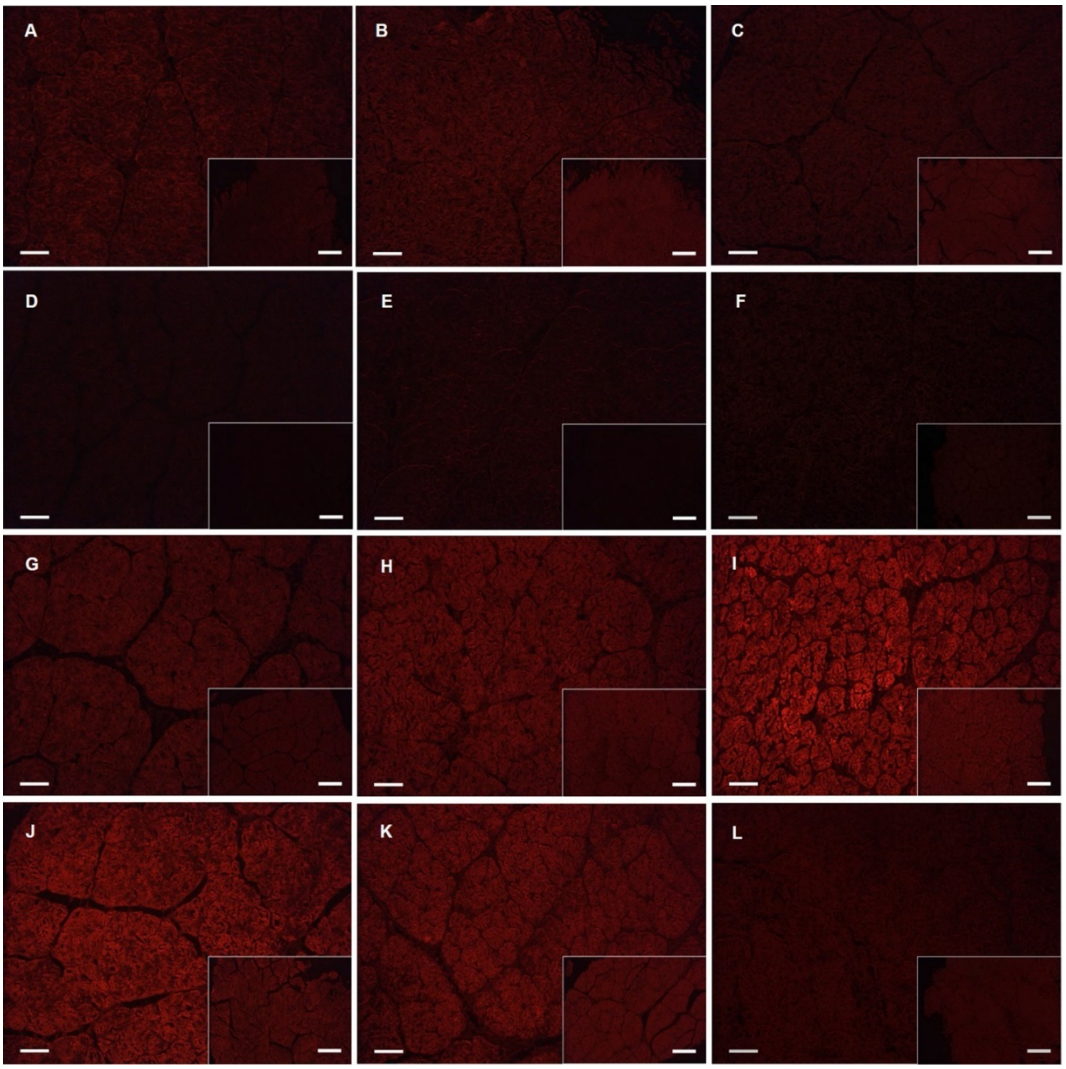


**Figure 13** Expression of GSK3β mRNA fluorescence in situ hybridization male adductor muscle of Pacific oyster, *Crassostrea gigas*. (A) August; (B) September; (C) October; (D) November; (E) December; (F) January; (G) February; (H) March; (I) April; (J) May; (K) June; (L) July. A large image is × 100 (scale bar, 100 μm); a small image is × 40 (scale bar, 500 μm).

*Western blot*

The activity of AKT/GSK3β pathway signals was confirmed in all sampled months through western blot analysis (**Figure 14A**). IRS-1 showed low activity throughout the year, and there no significant difference ($p > 0.05$) was detected (**Figure 14B**). PI3Kp85 showed a significantly highest activity in September and lowest activity in April and July ($p < 0.05$) (**Figure 14B**). Activated AKT showed significantly high activity in August to September and May to June and significantly low activity in October and July ($p < 0.05$) (**Figure 14B**). p-GSK3β (serine 9) increased from August to September, decreased in October, increased significantly until May, and decreased again after June ($p < 0.05$). GSK3β showed no significant difference throughout the year ($p > 0.05$). Glycogen synthase 1 (GS 1) increased from August to October, then decreased until June, and again increased significantly in July, showing the highest activity ($p < 0.05$). In particular, p-GSK3β (serine 9) increased while glycogen synthase 1 decreased in May when oysters were ripened, and p-GSK3β decreased

in October and July, when spawning and degeneration occurred, whereas glycogen synthase 1 increase was observed (**Figure 14C**).

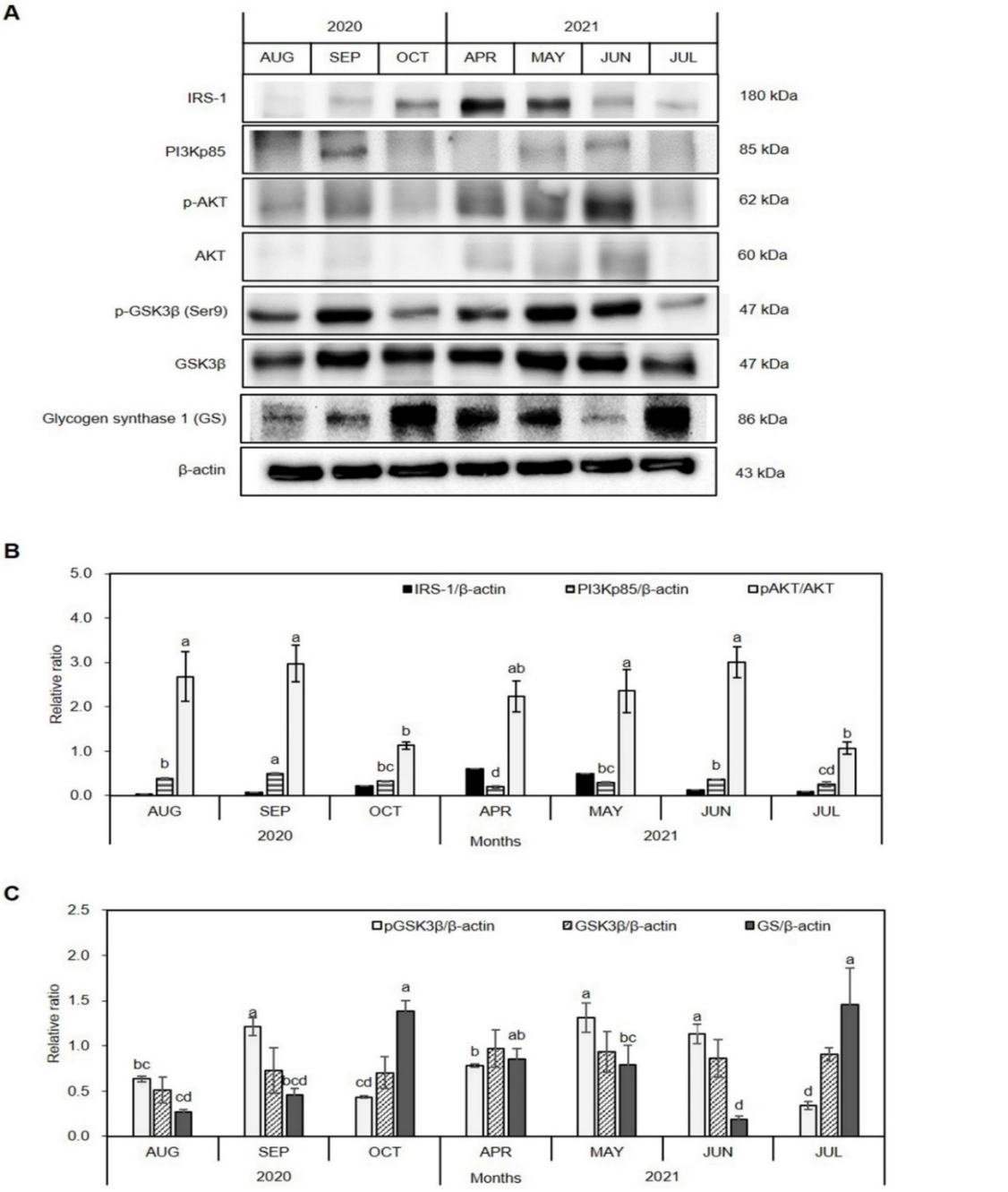


**Figure 14** Western blot analysis. (A) Image of western blot, (B) Activity of IRS-1, PI3Kp85, p-AKT, and AKT in Pacific oyster, Crassostrea gigas. (C) The activity of p-GSK3β, GSK3β, and Glycogen synthase 1 (GS) in Pacific oyster, *Crassostrea gigas*. Values represent the mean ± SEM. Letters indicate significant differences at $p < 0.05$.

## Discussion

Condition index has been widely reflected in the physiological condition of reproduction, growth, and health of organisms, including bivalves (Clark et al., 2013). In this study, the condition index increased in May, coinciding with maturation, and decreased in June and July during spawning seasons. This result is consistent with the high condition index in May in oysters cultivated in Namhae in 2000 (Choi et al., 2003). Similarly, the tissue weight rate steadily increased from January to May and decreased after June, the spawning season. This tendency depends on the area where the oysters are grown (Min et al., 2004). This presents that tissue weight and the condition index is highly dependent on the annual reproductive cycle (gonadal maturation cycle) of oysters.

Glucose, important for providing energy for metabolism in the body, is mainly stored in glycogen (Chang et al., 2007). Glycogen is important for metabolic and physiological processes and is a major energy source in various organisms, from vertebrates to invertebrates (Furukawa et al., 2018). In oysters, glycogen is a major energy source for gametogenesis and has been used as a physiological indicator to evaluate health status (Berthelin et al., 2000; Vodáková et al., 2019). In this study, glycogen content was confirmed in several tissues of oysters. The Glycogen content of the mantle edge was high in spring and summer and low during autumn and winter, regardless of sex. This result confirmed with previous studies in some bivalve species and thought glycogen stored in the mantle edge is utilized in autumn and winter to provide energy during gamete formation and gonad development (Li et al., 2009; Liu et al., 2020). Glycogen content of labial palp and digestive gland in both sexes increased from autumn to winter, decreased in spring, and increased again in summer. Glycogen storage cells are present in the labial palp, and glycogen stored is used for gamete formation (Liu et al., 2020). The digestive gland is the site for energy accumulation and nutrient availability and plays a role in delivering energy and nutrients to other tissues (De la Parra et al., 2005; Mayrand et al., 2017). In addition, nutrients stored in the digestive gland are known to play a role in replenishing nutrients required for gonad growth (Sastry et al., 1971).

The glycogen content of female gonads and adductor muscles was higher than that of male gonads. This is consistent with the glycogen content of the gonads and adductors of Fujian oyster, *Crassostrea angulata* (Zeng et al., 2013). Glycogen is used to produce gametes in male oysters and for oocyte maturation in female oysters with an accumulation of yolk and nutrient substances (Qin et al., 2018). And the glycogen content of adductor muscle increased from autumn to winter and decreased from winter to summer of the following year. This is similar to the tendency of adductor muscle glycogen content according to seasonal changes in the Pacific oyster *Crassostrea gigas* (Berthelin et al., 2000). In particular, when compared with other tissues, the glycogen content of the adductor muscle was high, which is thought to be the major energy storage tissue in oysters, consistent with previous studies (Zeng et al., 2013). As reported by the previous study, glycogen in oysters appears to be used and stored selectively regulated for each tissue (Li et al., 2009).

GSK3β, which regulates glycogen synthase activity, plays an important role in glycogen metabolism (Souder and Anderson, 2019). The expression of GSK3β related to glycogen metabolism was previously confirmed in oysters such as the Pacific oyster and Fujian oyster (Bacca et al., 2005; Zeng et al., 2013). GSK3β expression patterns in female and male oysters were similar in the mantle edge, labial palp, digestive gland, and adductor muscle tissues except in the gonads. The GSK3β mRNA expression level in female gonads is similar to the pattern in previous studies, which increases from early development to maturity and decreases after maturity (Zeng et al., 2013). The GSK3β mRNA expression level in female gonads was highest in October when spawning occurred after ripe, and the lowest in July, when spawning and regression occurred. In addition, GSK3β mRNA expression level in the digestive gland was low in spring and summer and high in autumn and winter and showed a similar tendency to

GSK3β expression in female gonads. Meanwhile, the GSK3β mRNA expression level in male gonads increases during ripe, similar to Zeng et al., 2013. The GSK3β mRNA expression level in the male gonads was highest in April when ripening occurred, and the lowest expression was shown in July when spawning and regression occurred in the same way as in the female gonads. This difference in the GSK3β expression level is because male gametes tend to ripe faster than their female counterparts (Min et al., 2004; Antonio et al., 2019). GSK3β expression in male gonads showed a similar tendency to GSK3β expression in mantle edge, labial palp, and adductor muscle. GSK3β expression was high in spring and summer and low in autumn and winter in the mantle edge, labial palp, and adductor muscle, and females and males showed similar expression changes in each tissue. GSK3β is known to be closely related to the reproductive cycle of the bivalve (Zeng et al., 2013). Likewise, in this study, GSK3β expression in the gonads differed according to the reproductive cycle. In particular, the GSK3β expression in the male gonads was high in April, which is thought to be the result of male oysters ripening faster than females, as in previous studies (Min et al., 2004; Antonio et al., 2019). As such, GSK3β expression was confirmed in all oyster tissues, there was a difference in the expression level for each tissue, and it is thought to be selectively regulated depending on the tissue.

As a result of fluorescence in situ hybridization (FISH) observation of GSK3β mRNA expression in gonad and adductor muscle of oyster, an expression pattern similar to the GSK3β mRNA expression result confirmed by RT-PCR was detected. When the expression of GSK3β was confirmed in the oyster gonad, the expression was predominantly in the cytoplasm of eggs and sperm. This was similar to confirm the expression of GSK3β in the female gonad and adductor muscle of the Fujian oyster, *Crassostrea angulata* in Zeng et al., 2013 study.

Insulin is known to affect energy storage by promoting glycogen synthesis, and the action of insulin mainly occurs through the PI3K (phosphoinositide 3-kinase) signaling pathway (Friedrichsen et al., 2013). Phosphorylated IRS-1 (insulin receptor substrate-1) activates PI3Kp85 to regulate the activation of AKT (protein kinase B) (Villalobos-Labra et al., 2017). As such, AKT is influenced by the IRS-1/PI3K pathway, inducing phosphorylation of GSK3β and stimulating glycogen synthesis in each tissue (Friedrichsen et al., 2013). Activated AKT showed a pattern similar to p-GSK3β (serine 9), with high expression in May when the tissue weight rate and condition index were a high and low expression in July when the tissue weight rate and condition index were low. As was known from previous studies, AKT influences the regulation of the activity of GSK3β (Shi et al., 2012).

Glycogen synthase plays an important role in glycogen synthesis. There are two forms of glycogen synthase. Glycogen synthase 1 is expressed in various tissues except for the liver, and glycogen synthase 2 is specifically expressed in the liver (Marr et al., 2022). Concerning glycogen metabolism, GSK3β is inactivated and inhibited by serine 9, which promotes phosphorylation and activation of glycogen synthase, thereby affecting glycogen synthesis (Wang et al., 2017). In the previous study, when the role of GSK3β in glycogen synthesis was confirmed in mice, the expression of p-GSK3β (serine 9) and glycogen synthase 1 was found to be complementary (Patel et al., 2008). This appeared the same as the pattern in the results of this study. The activity of p-GSK3β (serine 9) was increased. In contrast, glycogen synthase 1 decreased in May, when the tissue weight ratio and condition index of the bivalve was high, and ripe occurred. In addition, it was confirmed that p-GSK3β (serine 9) decreased while glycogen synthase 1 increased in October and July when the tissue weight ratio and condition index of the bivalve was low, and spawning and degeneration occurred. It is thought that p-GSK3β (serine 9) and glycogen synthase also affect glycogen synthesis in oysters.

In conclusion, glycogen is thought to play a central role in providing energy during the development and growth of oysters. Glycogen synthesis is regulated by glycogen synthase, and GSK3β regulates glycogen synthase. GSK3β expression in oysters showed seasonal differences and was associated with gonad maturation, which appears to lead to glycogen

synthesis. Therefore, this study confirmed the effect of GSK3β on the growth and maturation of oysters and is expected to be used as basic data to understand the energy pathway of oysters.

## Acknowledgments

This work was supported by a Research Grant from Pukyong National University (2021).

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