



Effect of Swimming on the Induction of Vitellogenin in Conger Eel (*Conger myriaster*)

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Vitellogenin (VTG) plays a very important role in the development of oocytes. This study aims to explore the effect of swimming on the VTG synthesis of conger eel (*Conger myriaster*). Circulating water flow and hormone injection regimen were two factors set in the following trials: A1 (no injection, no water flow), A2 (no injection, water flow), B1 (injection, no water flow), and B2 (injection, water flow). The flow velocity of the flowing water was 0.4 m/s (12 h, 60 days). We examined VTG, estrogen receptor (Er) gene expression, and VTG content in the liver and serum on the 30th and 60th days. VTG gene expression in A1 and A2 gradually decreased and was not significantly different between the two groups. The expression of Er gene in A1 was significantly higher than that in A2. The expression of VTG gene in group B1 was significantly higher than that in group B2, whereas the expression level of Er gene in group B1 was slightly higher than that in group B2. The VTG content in the liver in group B1 reached 1,396.93 $\mu\text{g/L}$, which was significantly higher than that in group B2 (1,302.06 $\mu\text{g/L}$). Results showed that the flowing water factor can inhibit the expression of VTG and Er genes in the liver and reduce the synthesis of VTG in the liver. Stimulation of flowing water can inhibit the yolk accumulation during the ovarian development of conger eel.

Keywords: conger eel, swimming, ovarian development, vitellogenin, estrogen receptor

INTRODUCTION

Vitellogenin (VTG), a large-molecular-weight glycolipoprotein (Xu and Quan, 2016), is synthesized in the liver and transported to egg cells during the artificial reproduction of the Japanese eel (*Anguilla japonica*) (Wang and Lou, 2006). The gene expression and VTG synthesis are promoted by estrogen from the estrogen receptor in the liver (Williams et al., 2013; Hara et al., 2016). After being absorbed by the egg cell, VTG is converted into yolk protein (Yp), which is an important nutrient for the development of seedling embryos (Reading et al., 2017). In striped bass (*Morone saxatilis*), VTG gene is weakly expressed in organs, such as the heart and intestine, but is considerably expressed in the liver; different types of VTG are deposited in egg cells with different proportions (Williams et al., 2014). So far, two VTG genes were cloned from the

European eel (*Anguilla anguilla*), three from the Japanese eel, and only one VTG gene from the conger eel (*Conger myriaster*); conger eel VTG is the most similar to Japanese eel VTG-1 (80% identity at the amino acid level) (GenBank accession no. AY423445) (Mikawa et al., 2006). The VTG receptor in the ovary of the European eel is expressed in juvenile eels, so the VTG receptor is not a limiting factor for the uptake of VTG by the egg (Palstra et al., 2010c; Morini et al., 2020b). The synthesis of VTG has a very important effect on ovarian development.

The conger eel is a deep-sea migratory fish (Hao et al., 2020). The ovaries of the conger eel gradually mature during migration and lay eggs near the spawning grounds (Mingkun et al., 2018; Xiaolong et al., 2021). In 2008, the spawning sites of the conger eel were found in the Kyushu–Palau ridge region, 380 km west of Okinotori Island (Miller et al., 2011). The conger eel has experienced a long migration time during its gonad development, but the current artificial reproduction process is in still water and is not influenced by the external environment during migration. In 2003, female eels were first induced to mature and lay eggs by adjusting the water temperature; this study confirmed that the external environment is important for the development of ovaries (Utoh et al., 2013). A long-distance swim could promote the oocyte development of the European eel (Ginneken et al., 2007). Male European eels can mature their gonads by swimming, and female eels can promote lipid accumulation in oocytes by swimming (Palstra et al., 2007; Palstra et al., 2008). Therefore, swimming has a very important effect on the development of the eels' gonads.

During migration, the lipids and VTG in the ovaries of the conger eel gradually accumulate until maturity, but they could not spontaneously mature under cultured conditions. Based on previous experiments, the conger eel can mature under the conditions of artificial injection of hormones (Meng et al., 2021). However, the artificial breeding of the conger eel has

problems and difficulties, such as poor egg quality, low fertilization rate, and high seedling mortality (Horie et al., 2003; Horie et al., 2010). Eels cannot naturally develop to sexual maturity under artificial culture due to the insufficient secretion of gonadotropin-releasing hormone and dopamine inhibition (Burgerhout et al., 2013). In contrast to the European eel, the conger eel does not undergo the transition from freshwater to saltwater (Shimizu, 2001). We hypothesize that swimming is an important condition for the development of the eel ovary. In this study, the effects of swimming stimulation on the development of the ovary and the expression of VTG and Er genes were investigated with or without hormone injection to understand the regulatory role of swimming in artificial reproduction.

MATERIALS AND METHODS

Experimental Eels

The conger eel ($n = 86$, 303.64 ± 10.02 g) samples, whose oocytes represent the oil droplet stage and primary yolk globule stage, were obtained from Shenghang Aquatic Technology Co., Ltd. (Weihai, China). The experiment was begun in May 2021 at the company's fish farm. The eels were acclimated in a cement tank (30.0 ± 0.5 psu, $18^\circ\text{C} \pm 0.5^\circ\text{C}$) for 2 weeks before the experiment.

Swimming Tank System and Conditions

A cylindrical glass fiber tank (diameter = 0.75 m, height = 1.5 m, 1,000 L) with a built-in pump was designed to provide circulating water flow for fish swimming (Figure 1). According to a previous study on flow velocity (0.4 ± 0.05 m/s) and the findings of the eel migration route, the swimming duration was set up at 12 h per day (Hammer, 1995; Miller et al., 2011; Meng, 2020).

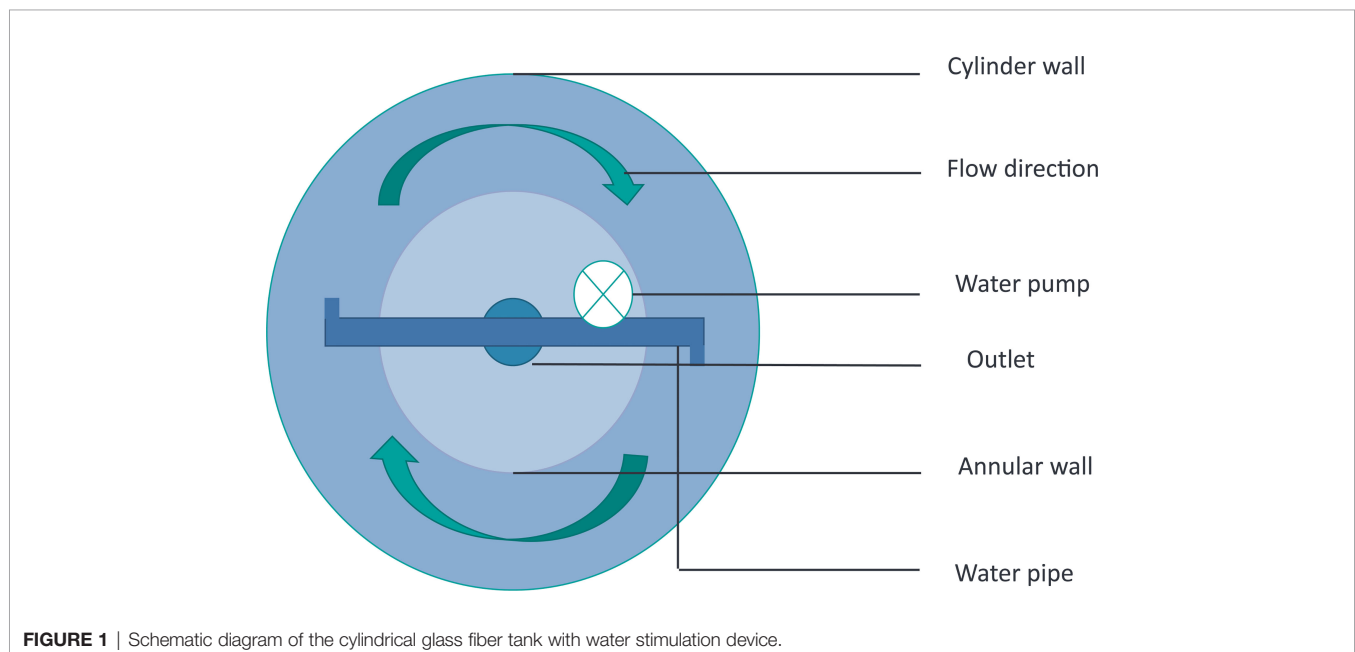


FIGURE 1 | Schematic diagram of the cylindrical glass fiber tank with water stimulation device.

Experiment Design

Healthy eels were selected and randomly distributed into four cylindrical glass fiber tanks ($n = 20$, 30.0 ± 0.5 psu, $18^\circ\text{C} \pm 0.5^\circ\text{C}$). Six acclimated eels were sampled and measured as a control group on the first day. Circulating water flow and hormone injection regimen were two factors set in the following trials: A1 (no injection, no water flow), A2 (no injection, water flow), B1 (injection, no water flow), and B2 (injection, water flow). All experimental groups were placed in a dark environment.

The mixture of carp pituitary extract (CPE; 20 mg/kg) and human chorionic gonadotropin (HCG; 100 IU/kg) was intraperitoneally injected weekly to induce eel development until the end of the experiment.

Measurement and Sampling

On the 30th and 60th days of the trial, six eels in each group were anesthetized using MS-222 (0.05 g/L). The weight and body morphological parameters of each eel were measured. Blood samples were collected from the bulbus arteriosus using a syringe and centrifuged at 3,000 rpm for 15 min at 4°C . The serum was frozen at -80°C . The liver, digestive tract, and ovarian were fixed in Bouin's solution or stored at -80°C after weighing until analysis.

Histology

The ovarian tissue fixed in Bouin's solution was dehydrated gradient with 30%–100% ethanol, xylene transparent, paraffin-embedded, cut into 6–8- μm -thick paraffin sections (Kodi 1508A, China), and stained with H&E. The oocytes were studied visually under the microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan), and overview pictures were taken (Nikon Coolpix 4500). Per section, oocytes that were cut through the nucleus were randomly selected. For each oocyte, the developmental stage was determined.

Quantitative Real-Time PCR

After the gene sequences of VTG (GenBank accession no. AB185334.1) and Er (GenBank accession no. AB117930.1) of the conger eel were obtained at the National Center for Biotechnology Information (NCBI), specific primers were designed by primer software and used for quantitative real-time PCR to analyze gene expression. The primer for β -actin was designed from the sequences of *A. japonica* (GenBank accession no. GU001950.1). Before the experiment, the specificity and amplification efficiency of β -actin primers were verified.

Total RNA was obtained from the liver by using a kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. About 1 μg of total RNA was reverse transcribed with Hifair[®] II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (CAT: 11123ES). Diluted cDNA (1:10) was used in all qPCR experiments. The qRT-PCR experiments were carried out in triplicate on CFX96 Touch Real-time PCR Detection System (Applied Biosystems[®], Bio-Rad, Hercules, CA, USA) using 1 μl of diluted cDNA as a template for each reaction with SYBR Green PCR Master Mix

(Bio-Rad). Thermal cycling conditions included an initial heat denaturation step at 95°C for 30 s, 40 cycles at 95°C for 5 s, 60°C for 30 s, and 95°C for 15 s. Melting curves of the PCR products were determined from 60°C to 95°C to ascertain the specificity of the amplification. Relative gene expression was calculated through the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2002).

Vitellogenin Content

VTG content in the liver and serum was determined using an ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) in accordance with the instructions of the kit.

Statistical Analysis

Gonadosomatic index (GSI), digestive tract somatic index (DTSI), eye index (EI), and hepatosomatic index (HIS) were determined according to the following formulas:

- 1) $\text{DTSI} = (\text{DTW}/\text{BW}) \times 100\%$; DTW indicates digestive tract weight (g).
- 2) $\text{GSI} = (\text{GW}/\text{BW}) \times 100\%$; GW indicates gonad weight (g), and BW body weight (g).
- 3) $\text{EI} = [((\text{EDh} + \text{EDv})/4)^2 \times \pi/\text{TL}] \times 100$; TL indicates total length (cm), EDv eye diameter vertical (cm), and Edh eye diameter horizontal (cm).
- 4) $\text{HIS} = (\text{LW}/\text{BW}) \times 100\%$; LW indicates liver weight (g).

All statistical analyses were performed using SPSS Statistics 12.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as average \pm SE (av \pm se). Differences at $p \leq 0.05$ were considered statistically significant.

RESULTS

Swimming Behavior and Distance

The eels from groups A2 and B2 swam around 518.4 and 1,036.8 km over 30 and 60 days, respectively. Few eels were observed resting at the bottom of the tanks during water flow.

Oocyte Developmental Status

The ovaries of the control group, group A1, and group A2 did not develop. However, the ovaries of groups B1 and B2 were further developed. Eels of the control group (**Figure 2A**), group A1 (**Figures 2B, C**), and group A2 (**Figures 2D, E**) contained oocytes representing the oil droplet stage and primary yolk globule stage according to Tomoko et al. (Tomoko et al., 2003). The ovaries of eels in groups B1 (**Figure 2F**) and B2 (**Figure 2H**) were at the secondary pharmaceutical globule stage and tertiary pharmaceutical globule stage on the 30th day. The ovaries of eels in groups B1 (**Figure 2G**) and B2 (**Figure 2I**) are in the tertiary globule stage and migratory nucleus stage on the 60th day.

Effect of Swimming on Ovarian Development

Two groups (A1 and A2) without injection presented no significant difference in HIS and GSI and had no ovaries

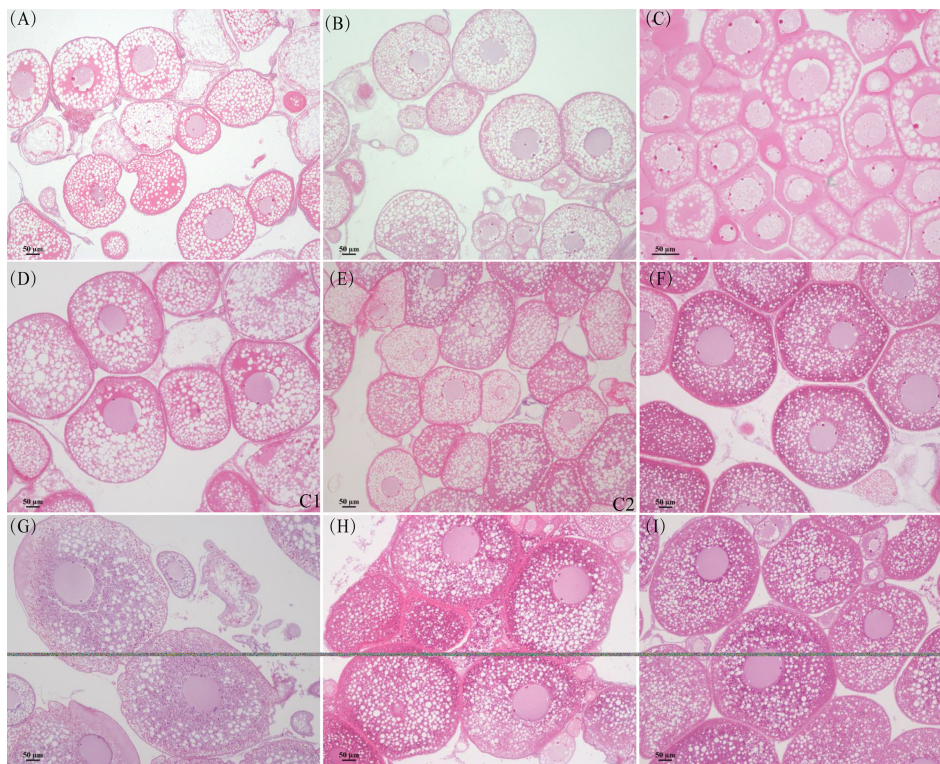


FIGURE 2 | Histology section of ovary in different groups. **(A)** Control group. **(B)** Group A1 (30 days). **(C)** Group A1 (60 days). **(D)** Group A2 (30 days). **(E)** Group A2 (60 days). **(F)** Group B1 (30 days). **(G)** Group B1 (60 days). **(H)** Group B2 (30 days). **(I)** Group B2 (60 days).

grown during the experiment (**Table 1**). The BW of eels in A2 declined fast (BW, -21.78%) and was significantly lower than that in A1 when sampled on the 30th day (250.67 ± 23.05 vs. 284 ± 34.98 ; **Table 1**). The average value of TL in A2 was significantly

lower than that in A1 on the 60th day (54.67 ± 2.4 vs. 59.02 ± 2.9 ; **Table 1**). The DTSI in group A2 showed a downward trend (DTSI, -48.94%) and was significantly lower than that in A1 on the 30th day (0.0021 ± 0.0003 vs. 0.0028 ± 0.0006 ; **Table 1**).

TABLE 1 | Effects of swimming and hormone injection on body indexes of conger eel.

	1 day	A1		A2	
		30 days	60 days	30 days	60 days
BW (g)	303.64 ± 10.02^a	284 ± 34.98^{Aa}	269.17 ± 33.11^{Aa}	250.67 ± 23.05^{Ba}	237.5 ± 19.30^{Ba}
TL (cm)	57.65 ± 3.92^a	58.27 ± 1.61^{Aa}	59.02 ± 2.93^{Aa}	57.35 ± 3.92^{Ba}	54.67 ± 2.40^{Ba}
EI	1.19 ± 0.09^a	1.23 ± 0.13^{Aa}	1.06 ± 0.13^{Aa}	0.98 ± 0.12^{Aa}	1.01 ± 0.14^{Aa}
DTSI	0.0047 ± 0.0024^a	0.0028 ± 0.0006^{Aa}	0.0029 ± 0.0008^{Aa}	0.0021 ± 0.0003^{Bb}	0.0024 ± 0.0004^{Bb}
GSI	0.0266 ± 0.0218^b	0.0639 ± 0.0251^{Aa}	0.0436 ± 0.0264^{Aab}	0.0413 ± 0.0356^{Aa}	0.0403 ± 0.0535^{Aa}
HIS	0.0138 ± 0.0021^a	0.0114 ± 0.0019^{Aa}	0.0105 ± 0.0010^{Aa}	0.0120 ± 0.0009^{Aa}	0.0114 ± 0.0029^{Aa}
		B1		B2	
BW (g)	303.64 ± 10.02^b	368.33 ± 46.63^{Aa}	358.33 ± 95.84^{Aa}	358.5 ± 43.07^{Aa}	340.33 ± 71.39^{Aa}
TL (cm)	57.65 ± 3.92^a	59.48 ± 1.52^{Aa}	58.37 ± 4.10^{Aa}	61.73 ± 2.33^{Aa}	59.37 ± 2.67^{Aa}
EI	1.19 ± 0.09^a	0.93 ± 0.17^{Aa}	0.99 ± 0.07^{Aa}	0.86 ± 0.09^{Aa}	0.98 ± 0.08^{Aa}
DTSI	0.0047 ± 0.0024^a	0.0011 ± 0.0002^{Bb}	0.0011 ± 0.0005^{Ab}	0.0015 ± 0.0002^{Ab}	0.0012 ± 0.0015^{Ab}
GSI	0.0266 ± 0.0218^b	0.1762 ± 0.0420^{Aa}	0.2636 ± 0.1709^{Aa}	0.1141 ± 0.0437^{Ba}	0.23748 ± 0.1221^{Aa}
HIS	0.0138 ± 0.0021^b	0.0172 ± 0.0044^{Aa}	0.0141 ± 0.0019^{Ab}	0.0168 ± 0.0028^{Aa}	0.0149 ± 0.0032^{Aa}

The capital letters marked in the chart indicate significant differences between the swimming group and the resting group ($p < 0.05$). Lowercase letters indicate differences within the same group ($p < 0.05$).

BW, body weight; A1, hormone-free still water group; A2, hormone-free flowing water group; B1, hormone still water group; B2, hormonal flowing water group; TL, total length; EI, eye index; DTSI, digestive tract somatic index; GSI, gonadosomatic index; HIS, hepatosomatic index.

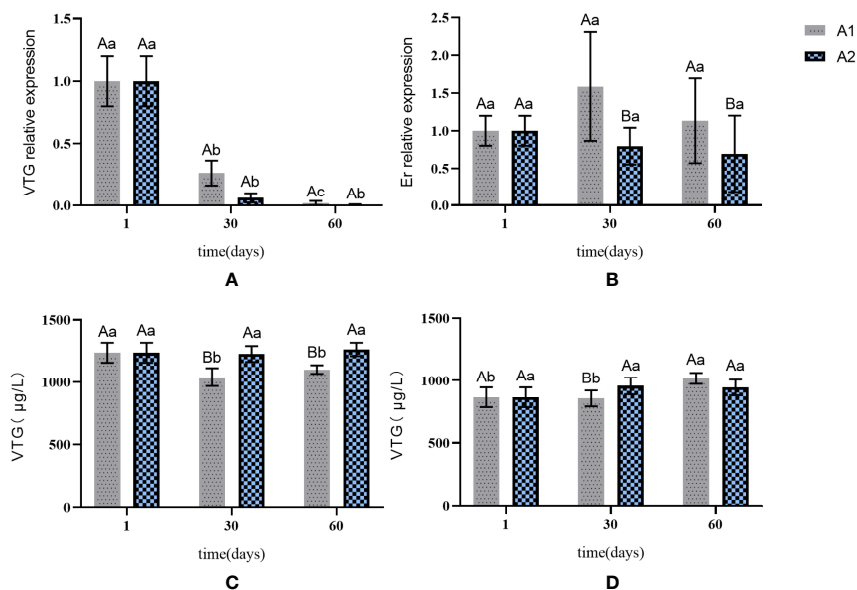


FIGURE 3 | The expression of VTG and Er genes in liver and the content of VTG in liver and serum in A1 and A2. **(A)** VTG gene expression in the liver. **(B)** Er gene expression in the liver. **(C)** changes in the content of VTG in the liver. **(D)** changes in the content of VTG in the serum. The capital letters marked in the chart indicate significant differences between the swimming group and the resting group ($p < 0.05$). Lowercase letters indicate differences within the same group ($p < 0.05$). VTG, vitellogenin; Er, estrogen receptor.

The expression of VTG in A1 and A2 was gradually reduced compared with that in the control group (**Figure 3A**). Although the expression in A1 was slightly higher than that in A2 on the 30th day, the difference between the two groups was not significant. The expression of Er gene in both groups did not change significantly compared with that in the control group (**Figure 3B**), but the expression in group A1 was significantly higher than that in A2 through the 60-day treatment ($p < 0.05$).

The VTG concentration in serum was not significantly different between A1 and A2 (**Figure 3D**). The VTG content in the liver in group A1 showed a downward trend, while that in A2 remained stable (**Figure 3C**). No obvious change in the VTG content was observed in the liver and serum in the two groups.

Effect of Swimming Under Hormone Injection on Ovarian Development

The GSI of eels in groups B1 and B2 increased gradually during the experiment (**Table 1**). The GSI in B1 was significantly higher than that in B2 on the 30th day (0.1762 ± 0.042 vs. 0.1141 ± 0.0437), but no significant difference was observed on the 60th day. The HIS in groups B1 and B2 increased on the 30th day (HIS, +24.64% and +21.74%, respectively). On the 60th day, the HIS in B1 decreased and that in B2 remained unchanged, but the difference between the two groups was not significant (**Table 1**). The body weight in groups B1 and B2 increased gradually (weight, +21.30% and +18.07%, respectively) and had no significant difference between the two groups (**Table 1**). The DTSI of eels in B1 was significantly lower than that in B2 on the 30th day ($p < 0.05$, **Table 1**).

The expression levels of VTG and Er increased gradually in B1 and B2 compared with those in the control group (**Figures 4A, B**). The VTG expression was significantly higher in B1 than in B2 on the 30th day but was not significantly different on the 60th day. The Er gene expression showed no significant difference between B1 and B2 during the entire experiment (**Figure 4B**).

The VTG content in the liver increased first and then decreased in B1 and B2 (**Figure 4C**). The VTG content in B1 was significantly higher than that in B2 on the 30th day (**Figure 4C**). However, changes in the VTG content in the serum of the two groups had no obvious regularity (**Figure 4D**).

DISCUSSION

In this experiment, the conger eel presented a very high swimming efficiency, similar to the European eel, which swam a maximum of 1,000 km without eating and adverse reactions, such as illness or death (Burgerhout et al., 2013). Swimming consumed much energy mainly from fat and protein, leading to a more significant reduction in the BW of the conger eel in A2 than in A1 (Nowosad et al., 2014). The European eel consumes 32% fat for its migration to spawning grounds (Clevestam et al., 2011). The body weight and GSI increased gradually in B1 and B2. The abdomen expanded during ovarian development, and no weight loss was found in eels in group B2. Hydration may be the main reason for counteracting the depleting effects of swimming (Qi, 2010). During gonadal development,

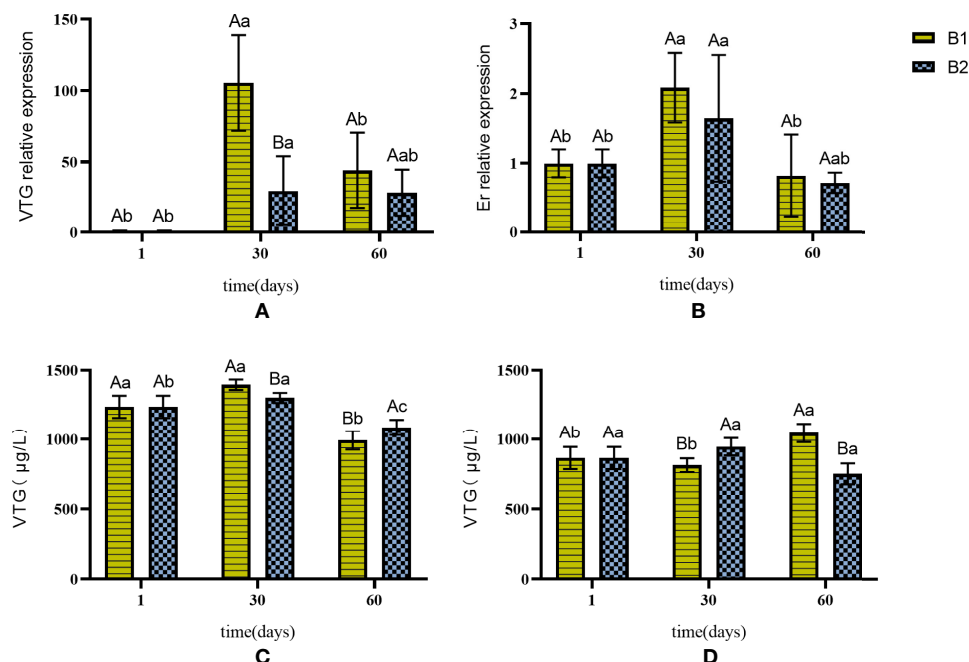


FIGURE 4 | The expression of VTG and Er genes in liver and the content of VTG in liver and serum in B1 and B2. **(A)** VTG gene expression in the liver. **(B)** Er gene expression in the liver. **(C)** changes in the content of VTG in the liver. **(D)** changes in the content of VTG in the serum. The capital letters marked in the chart indicate significant differences between the swimming group and the resting group ($p < 0.05$). Lowercase letters indicate differences within the same group ($p < 0.05$). VTG, vitellogenin; Er, estrogen receptor.

various nutrients, such as fatty acids and proteins, are transferred from the muscle to the gonad (Nowosad et al., 2014). In the European eel, swimming is believed to be key to the activation of lipid metabolism (Palstra and van den Thillart, 2010). Therefore, sufficient nutrient reserve is one of the necessary conditions for eel migration.

Swimming reduced the DTSI in the conger eel but had no significant effect on EI. Scholars believe that EI and other external morphological changes are important markers of ovarian development in fish. Changes in DTSI and EI were found during the ovarian development of the Japanese eel and the European eel. In particular, in the Japanese eel, the EI increased and the DTSI decreased with the gradual development of the ovary (Fontaine et al., 1995; Meng et al., 2021). In the present study, swimming reduced the DTSI in eels without hormone injection; however, no significant difference in DTSI was found between swimming and resting eels with injection. Exogenous hormones play a key role in reducing variations in DTSI caused by swimming. As another important marker, the EI in the European eel would increase through silver plating, which can be promoted by swimming until it reaches the silver eel stage (Ginneken et al., 2007; Palstra et al., 2007). In the present experiment, swimming did not accelerate the growth of EI in the conger eel, but long-term swimming decreased the DTSI. Fish coping with environmental pressure during long-term swimming among different species is a complicated system (Rousseau K et al., 2020).

Swimming inhibits the gene expression of VTG and Er in the liver. The ovary of eels in B1 and B2 developed gradually compared with that in the control group; meanwhile, swimming inhibited the VTG expression in B2 compared with that in B1. Similar to the present experiment, previous work reported that swimming inhibits the expression of two VTGs in the European eel (Palstra et al., 2010b). However, the VTG expression was not inhibited by swimming in A1 and A2 when the ovary did not develop. During the ovarian development of eels, the Er in the liver combines with estradiol and promotes the synthesis of VTG (Morini et al., 2020a). Swimming inhibited Er expression in group A without hormone injection. The expression was slightly higher in B1 than in B2, but the difference was not significant. The inhibition of VTG expression could be due to exogenous hormones and the suppression of Er expression. In our previous experiment, swimming increased the serum cortisol level (unpublished results). In the European eel, swimming and salinity changes can increase the cortisol content (Cutler et al., 2007; Palstra et al., 2009). In response to environmental stress, the ACTH axis is activated and regulated by secreting corresponding hormones, such as cortisol (Ganesh, 2020). Cortisol inhibited hepatic Er expression in rainbow trout (Lethimonier, 2000). The inhibitory effect of cortisol could be related to the inhibition of VTG and Er genes. Changes in salinity during swimming are similar to migration conditions for eels, so the migration process may be accompanied by increased cortisol levels. The inhibition of VTG

expression by swimming delayed the accumulation of yolk. The GSI was higher in B1 than in B2, suggesting that fluid stimulation slowed down the rate of pre-ovarian development. Prolonged swimming can delay ovulation in rainbow trout (*Oncorhynchus mykiss*) (Contreras, 1998). Meanwhile, in the European eel, swimming promoted lipid deposition in egg cells (Palstra et al., 2008). In the conger eel, the inhibition of VTG expression by swimming may promote lipid accumulation.

The VTG content in the liver and serum was also detected. The change in the serum VTG content is similar to that in the European eel under the two conditions with and without hormone injection, and no obvious change rule existed. The same phenomenon was observed in swimming experiments in rainbow trout (Palstra et al., 2007; Palstra et al., 2010a). The inhibition of VTG by swimming may be responsible for the difference in the serum VTG content (Palstra et al., 2010a). Although the amount of VTG in the liver in group B1 was significantly higher than that in group B2 for the first 30 days, the exogenous hormone plays an important factor than swimming through the continuous treatment. A higher VTG content was observed in the liver of group B2 than B1 on the 60th day. The impact of both swimming and exogenous hormone in conger eel liver remains to be further studied.

The results showed that swimming could significantly reduce DTSI in the conger eel without affecting EI. During artificial reproduction, swimming inhibited the expression of VTG gene and slowed down the early development of the conger eel.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

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ETHICS STATEMENT

All fish samples were handled in accordance with the Animal Ethics Committee of Shanghai Ocean University (2016NO.4) and the Regulations for the Administration of Affairs Concerning Experimental Animals approved and authorized by the State Council of the People's Republic of China.

AUTHOR CONTRIBUTIONS

RL carried out the experiment, analyzed the data, and wrote the manuscript. KL and LL were responsible for guiding the experiment and modifying the article. GW, XB, and ZJ helped RL to collect and analyze the experimental samples. All authors contributed to the article and approved the submitted version.

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