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Effects of Salinity on Five Hormones in the Hemolymph of Female *Eriocheir sinensis* during Reproduction

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ABSTRACT

Background: The Chinese mitten crab (*Eriocheir sinensis*) is widely distributed in the coastal and coastal water of China and has important economic values. Salinity is the key environmental factor affecting the mating and spawning of *E.sinensis*. During the mating and spawning of *E. sinensis*, the hormone changes in the body are usually accompanied, but there is no report about the effects of salinity on related hormones in the process of breeding of *E. sinensis*.

Methods: Mating salinity including 0‰, 2‰, 4‰, 6‰ and spawning salinity such as 3‰, 6‰, 9‰, 12‰, 15‰, 18‰, 21‰ were set up, three parallel groups are set for each salinity, 10 female and 5 male crabs are put in each tank. 5 samples were gathered to analyze the contents of 17α,20β-dihydroxy-4-pregnene-3-one (DHP), Gonadotropin (GTH), Prostaglandins (PG), Estradiol-17β (E_2) and Testosterone (T) in the hemolymph of female *E. sinensis* after mating and spawning.

Result: The contents of DHP, E_2, PG and T in female crabs hemolymph decreased first and then added with the increase of salinity before spawning. DHP, PG and E_2 added first and then declined with the increase of salinity after spawning. When salinity was 6‰, the contents of DHP, PG, E_2 and T in the hemolymph of female crabs before spawning reached the highest, the contents of DHP and PG decreased and E_2 and T increased after spawning. The comprehensive study indicated that salinity had a certain effect on five hormones in the hemolymph of female *E.sinensis* during reproduction, five hormones didn't change significantly in the process of mating, DHP, GTH and PG were involved during the spawning of *E.sinensis*.

Key words: *Eriocheir sinensis*, Hormones, Mating, Salinity, Spawning.

INTRODUCTION

The Chinese mitten crab (*Eriocheir sinensis*) is an euryhaline crustacean that is mainly distributed along the Yellow Sea region bordering China and Korea, eastern Asia (Sui *et al.*, 2009; Wang *et al.*, 2012). Adult crabs spend most of their life in fresh and brackish waters, but migrate towards the estuary or sea to reproduce (Zhang *et al.*, 2001; Bentley, 2010). After completed mating, the female crabs carrying the fertilized eggs gradually migrate further downstream for spawning and hatching in seawater (Long *et al.,* 2019). It hence can be inferred that salinity plays an important role on gonadal development, reproduction and embryo character of *E. sinensis* (Wei *et al.*, 2007; Cheng *et al.,* 2008).

The gonadal development of fish is often accompanied by a variety of sexual stimuli, the changes of hormone are closely related to gonadal development and reproduction (Lin *et al.*, 2015). Fostier *et al.,* (1975) proposed that steroid hormones include progesterone, 17α, 20β-dihydroxy-4-pregnene-3-one (DHP) and so on. DHP can effectively induce the maturation of salmon trout oocytes (Zhao *et al.*, 1985; Idler, 1960). Gonadotropin (GTH) is secreted by the pituitary gland as a factor to induce the final maturation of oocytes, early studies have shown that the ovulation activity of teleost fish is closely related to the level of GTH in the blood (Goetz, 1983).

Prostaglandins (PG) is a kind of unsaturated fatty acids with physiological activities, which have extremely extensive and complex biological effects (Zhou and Wen, 2004). Estradiol-17β (E_2) is the most important active estrogen in crustaceans, it plays an important role in ovarian development and vitellogenesis (Wang, 2012; Wei *et al.*, 2007). Burns *et al.,* (1984) found testosterone (T) in male gonads and hemolymph of American lobster (*Homarus americanus*), they thought that crustaceans also had E_2 and T, which had a certain impact on gonad development.

There are relatively few studies on the hormones of *E. sinensis* (Wei *et al.*, 2007; Yu *et al.*, 2007). The purpose of this study is to analyze the physiological mechanism of salinity affecting the mating and spawning of *E. sinensis*, so as to provide basic data and reference for the study of breeding biology of *E. sinensis*.

MATERIALS AND METHODS

Experimental animal

The adult *E. sinensis* were collected from Qidong, Jiangsu Province, China in early October in 2021. The initial body

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weight of males and females were 125.84±8.10 g and 111.48±20.86 g, respectively. The crabs were transported to the laboratory, East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai, China. 240 female and 120 male healthy crabs were used for experiment.

Experimental design and setup

Water with salinities of 0‰, 2‰, 4‰ and 6‰ were conducted for mating experiments and three parallels were set up for each salinity. During the experiment, 10 female and 5 male crabs were taken from the temporary breeding tank and placed in the tank with different salinity, the water depth in the tank was 30 cm. After the experiment, all the remaining female crabs were dissected in all salinity groups. If the female has sperm pods in the seminal vesicles, it can be determined that the female has successfully mated. Seven salinity treatments, *i.e.* 3‰, 6‰, 9‰, 12‰, 15‰, 18‰ and 21‰ were set up and each treatment had three replicate tanks with 5 males and 10 females stocked in each tank. At the same time, a normal breeding group was conducted for each salinity, with 10 female crabs in each tank and three parallels were set up at each salinity. Approximately 40% of the bottom area of the tanks was covered with 10-20 cm sand and pieces of polyvinyl chloride (PVC) tubes (diameter 15cm) were provided as shelters for the crabs. During the experiment, all tanks were maintained under a natural photoperiod of approximately 12 h light and 12 h dark, the crabs were fed daily at 5:00 PM with a diet of *Sinonovacula constricta* at a rate of 3-5% of their live body weight and the remaining food was removed the next morning. The salinity and water temperature in each tank were measured daily at 8:00 AM and 8:00 PM, dissolved oxygen (DO), ammonia-N, nitrite and pH were measured every 2 day.

Haemolymph collection

During the mating experiment, hemolymph was collected from five mating crabs in the experimental group (salinity 2‰ and 4‰), mating but not spawning crabs (salinity 6‰) and normal breeding group (salinity 0‰). During the spawning experiment, hemolymph was collected from 5 spawning crabs in each salinity group and 5 experimental crabs in the normal breeding group. The crab was anesthetized in ice water for 15 min, the hemolymph was collected from the foot base joint in step 3 or 4 with a 2 ml disposable sterile syringe. The collected hemolymph was quickly injected into a 1.5 ml sterile centrifuge tube that had been added with EDTA-2K anticoagulant in advance. The collected hemolymph was separated with 4000 rpm at 4°C for 10 min, the supernatant was poured out, centrifuged once again and the supernatant was combined, then put into the refrigerator at -80°C for storage.

Hormone detection index and determination method

Hormone test indicators of hemolymph include DHP, GTH, PG, E_2 and T. The contents of five hormones were determined by double antibody sandwich method. 10 mg of the standard of these five substances was weighed, dissolved in methanol and fixed to 10.0 ml, prepared into 1 mg/ml standard solution and stored in 4°C refrigerator. When making the standard curve, the standard solution with a concentration of 1, 5, 25, 50, 100, 200 ng/ml was added to the sample of the control group. The main experimental steps were as follows (1) Globulin as specific antibody was diluted with carbonate buffer solution to the optimal concentration (1-10 μg/ml), 0.3 ml globulin was added to each concave hole and stayed overnight at 4°C; (2) Removed the coating solution and washed the concave hole with 0.05% PBS washing buffer for 3 times, each time for 5 minutes; (3) 0.2 ml tested sample containing antigen was added to each concave hole, then acted at 37°C for 1-2 hours; (4) Removed the coating solution and washed the concave hole with 0.05% PBS washing buffer for 3 times, each time for 5 minutes; (5) 0.2 ml enzyme labeled specific antibody solution was added to each concave hole and acted at 37°C for 1-2 hours; (6) Each concave hole was washed with 0.05% PBS buffer solution for 3 times, each time for 5 minutes; (7) 0.2 ml substrate solution was added to each concave hole and acted at room temperature for 30 minutes; (8) 0.05 ml of 2M citric acid was added to each concave hole; (9) OD value was detected at 450 nm wavelength within 30 minutes.

Statistical analysis

The data were analyzed with SPSS statistics 22.0 software and the descriptive statistical values were expressed by mean ± standard deviation (X±SD). $P<0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

DHP

The changes of DHP in the hemolymph of female crabs before and after mating was shown in Fig 1. At salinity 0‰, the female crabs couldn't mate and DHP content in the hemolymph reached the highest, with an average of (51.31±5.47) ng/L, except for salinity 2‰, there was no significant difference among other salinity groups ($P>0.05$). The changes of DHP in hemolymph of female crab before and after spawning was shown in Fig 2. DHP content in the hemolymph after spawning at salinity 18‰ was the lowest ($P<0.05$) and the average value was (26.09±6.94) ng/L.

DHP plays a leading role in inducing salmon trout egg maturation (Lin, 1982). Zhao (1987) found that at the late stage of fish egg maturation and at the beginning of ovulation, DHP will form a peak to induce ovarian follicle maturation and egg nucleus disappearance. When the egg nucleus of rainbow trout disappears, DHP will continue to rise and then remain at a high level (Kraak *et al.*, 1984). Pu *et al.,* (2018) showed that the content of DHP in serum of *Anguilla japonica* was the highest during spawning and decreased after spawning. In this experiment, DHP content in hemolymph of female crab decreased after spawning at salinity 6‰, 12‰ and 18‰, which is basically consistent with the above research results. DHP content in other salinity

groups increased after spawning, the reason may be that the female crab is at the early stage of the second oviposition, when DHP increases to prepare for subsequent ovulation. Female crabs do not mate at salinity 0‰, they can mate but can't spawn in water below salinity 6‰, DHP in hemolymph has been kept at a low level, the reason may be that the low salinity can't stimulate *E.sinensis* to produce some nutrients, resulting in the inhibition of DHP synthesis.

GTH

The change of GTH in hemolymph of female crab before and after mating was shown in Fig 3, the results showed

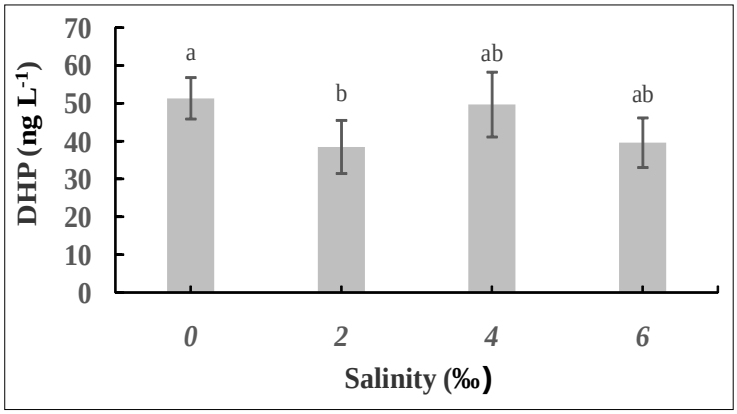

Fig 1: Changes of DHP content in the hemolymph before and after mating.

Note: Different letters represent the difference of DHP content among different salinity groups (P<0.05).

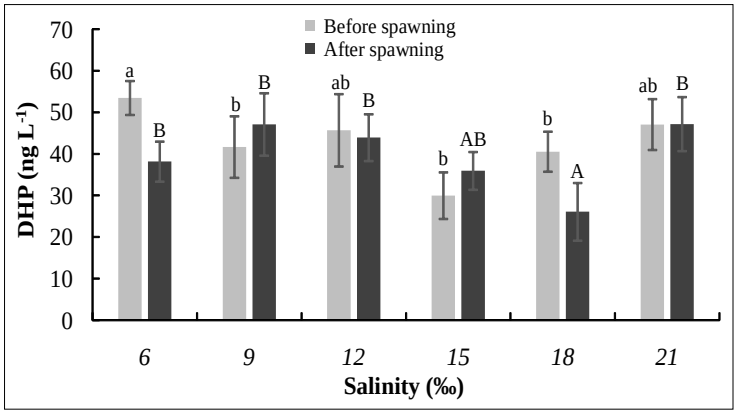

Fig 2: Effects of salinity on DHP content in hemolymph before and after spawning.

Note: Different small letters represent the difference of DHP content before spawning of female crabs in different salinity groups and different capital letters represent the significant difference of DHP content after spawning of female crabs in different salinity groups (P<0.05).

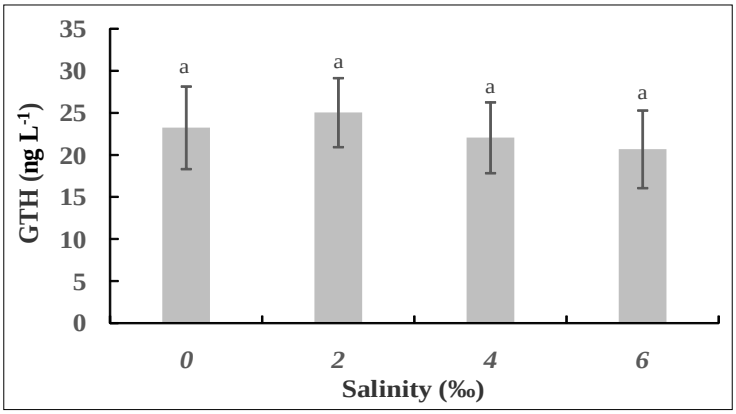

Fig 3: Changes of GTH content in female crab before and after mating.

Note: Different letters represent the difference of GTH content among different salinity groups (P<0.05).

that there was no significant difference in GTH content under different salinity (P>0.05). Changes of GTH in female crabs hemolymph before and after spawning was shown in Fig 4. At salinity 6‰ and 15‰, GTH in hemolymph of female crab increased after spawning, however, when salinity was 9‰, 12‰, 18‰ and 21‰, GTH decreased after spawning, but there were no significant differences among the salinity groups after spawning (P>0.05).

GTH is a hormone secreted by the pituitary gland of teleost fish and plays an important role in the regulation of its reproductive cycle (Li *et al.*, 2013). When the final physiological maturity was reached, the oocytes could ovulate and fertilize normally. During this process, GTH must be used to stimulate the growth, development, maturation and ovulation of germ cells (Otsu, 1963; Yano *et al.*, 1988). When rainbow trout was approaching egg maturation, the content of GTH in hemolymph continued to rise and then stabilized at a high level until all eggs were produced (Fostier *et al.*, 1978). GTH could also induce the ovarian follicles of Atlantic salmon and rainbow trout to produce DHP until the egg nucleus disappeared (Fostjer *et al.*, 1978). In this experiment, the trend of DHP and GTH in the hemolymph of female crab after spawning was basically the same, which showed that DHP also needed the participation of GTH to jointly play an important role in the ovulation process of female *E. sinensis*.

PG

The change of PG in hemolymph of female crab before and after mating was shown in Fig 5, the results showed that there was no significant difference in the hemolymph under different salinity (P>0.05). The changes of PG in female crab hemolymph before and after spawning was shown in Fig 6. When the salinity was 6‰ and 18‰, PG content in the hemolymph after spawning decreased and when the salinity was 6‰, PG content was significantly different from that before spawning (P<0.05). After spawning, PG content reached the highest at salinity 12‰, with an average of (274.30±37.58) ng/mL.

PG is widely distributed in aquatic animals, they can promote gonadal development and ovulation and are closely related to reproductive ability (Xing *et al.*, 2019). Nagaraju

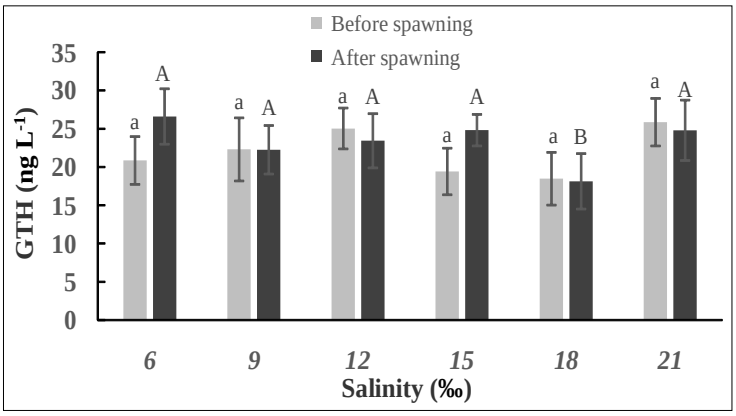

Fig 4: Effects of salinity on GTH content of female crab before and after spawning.

Note: Different small letters represent the difference of GTH content before spawning of female crabs in different salinity groups and different capital letters represent the significant difference of GTH content after spawning of female crabs in different salinity groups (P<0.05).

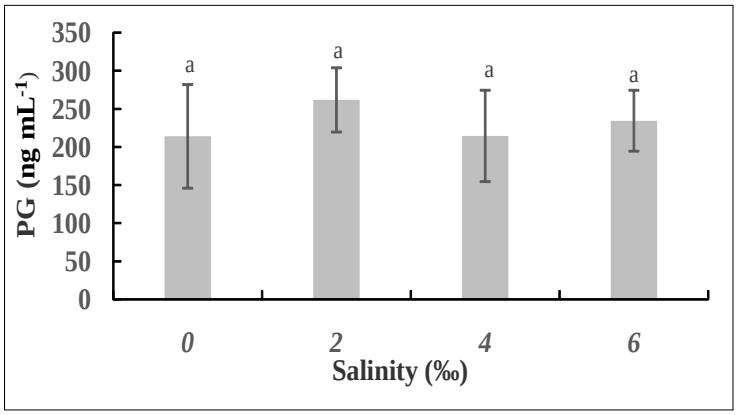

Fig 5: Changes of PG content in female crab before and after mating.

Note: Different letters represent the difference of PG content among different salinity groups (P<0.05).

et al., (2004) confirmed that PG existed in the tissues of *Oziotelphusa Senex Senex* and can promote the ovarian development of crabs, which was related to the reproductive ability of organisms. Meunpol *et al.,* (2010) found that PG levels in ovaries and hemolymph of female shrimp fluctuated with different stages of ovarian development and PG could significantly accelerate the growth of oocytes. In this experiment, there was no significant change in PG content after mating under different salinity, indicating that PG had no significant effects on the mating of female crab. The follicles of *Perca flavescens* produce a large number of PG under DHP stimulation and result in ovulation effect (Berndtson, 1989). In this experiment, when the salinity was 18‰, GTH, DHP and PG contents in the hemolymph after spawning significantly reduced, the reason may be that the reproductive performance parameters of female crabs, such as spawning volume, fecundity and reproductive index, reached the maximum at salinity 18‰ (Huang *et al.,* 2022). The trends of above three hormones are basically the same, indicating that three hormones may interact during the reproduction of *E. sinensis*.

E_2 and T

Change of E_2 content in hemolymph of female crab before and after mating was shown in Fig 7. The results indicated that there was no significant difference in E_2 content before and after mating (P>0.05). Change of E_2 in female crab hemolymph before and after spawning was shown in Fig 8. When salinity was 9‰ and 18‰, E_2 content decreased after spawning. Change of T content in hemolymph of female crab before and after mating was showed in Fig 9. When the salinity was 0‰, T content was the lowest, with an average of (16.82±1.02) nmol/L; with the increase of salinity, T content also increased gradually, but there was no significant difference between each group (P >0.05). The changes of T content in hemolymph before and after spawning was showed in Fig 10. When the salinity was 6‰, T content in hemolymph before and after spawning was the highest, with an average of (21.68±3.25) nmol/L and (23.21±0.86) nmol/L, respectively, but there was no significant difference between them (P>0.05).

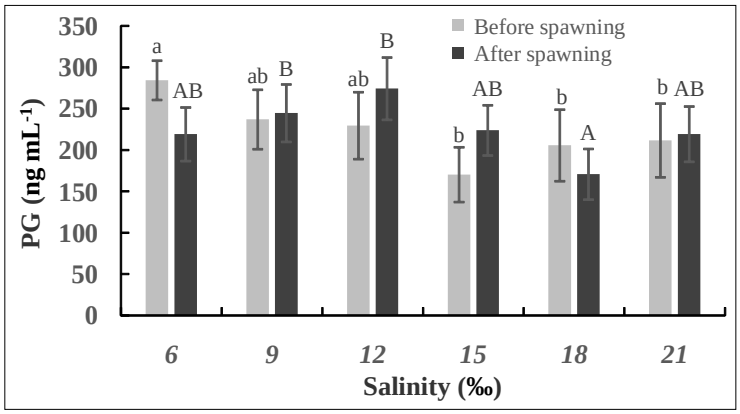

Fig 6: Effects of salinity on PG content of female crab before and after spawning.

Note: Different small letters represent the difference of PG content before spawning of female crabs in different salinity groups and different capital letters represent the significant difference of PG content after spawning of female crabs in different salinity groups (P<0.05).

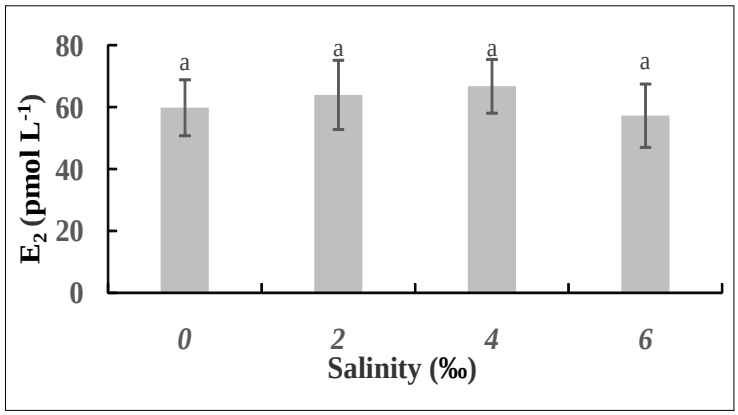

Fig 7: Changes of E_2 content in female crab before and after mating.

Note: Different letters represent the difference of E_2 content among different salinity groups (P<0.05).

This experiment found that when the salinity was 6‰, T content in the hemolymph of female crabs before and after spawning all reached the maximum. The reason may be that when the salinity was 6‰, the reproductive performance of female crabs was the lowest (Huang *et al.*, 2022). At this time, the female crab still retains a lot of nutrients, which may be in the early stage of the second spawning. Teleost fish ovaries can synthesize T, which is then converted into

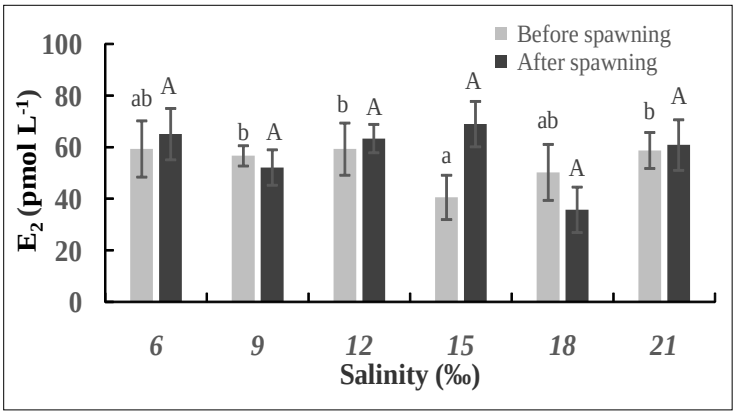

Fig 8: Effects of salinity on E_2 content of female crab before and after spawning.

Note: Different small letters represent the difference of E_2 content before spawning of female crabs in different salinity groups and different capital letters represent the significant difference of E_2 content after spawning of female crabs in different salinity groups (P<0.05).

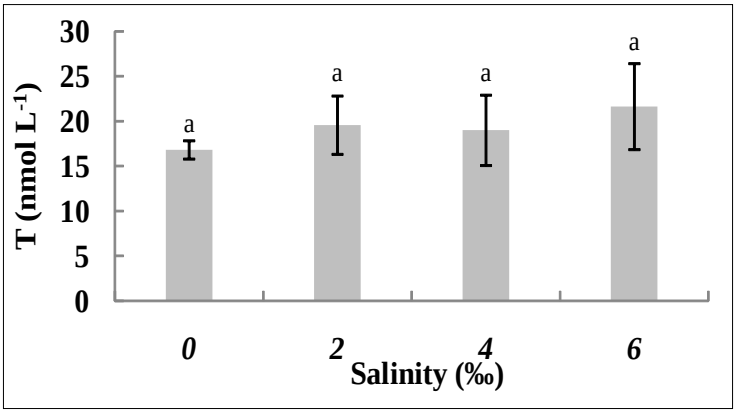

Fig 9: Changes of T content in female crab after mating before and after mating.

Note: Different letters represent the difference of T content among different salinity groups (P<0.05).

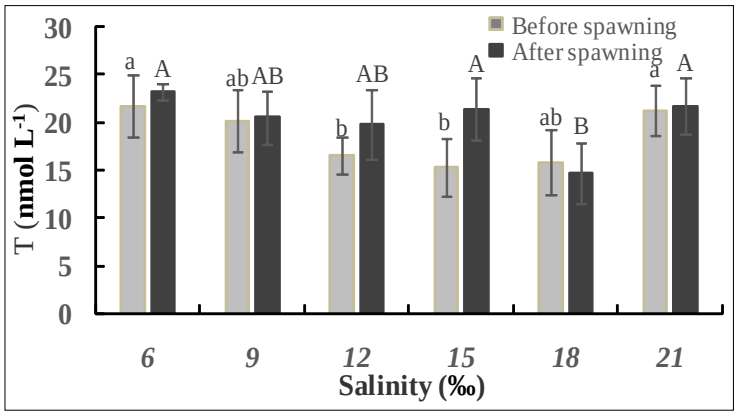

Fig 10: Effects of salinity on T content of female crab before and after spawning.

Note: Different small letters represent the difference of T content before spawning of female crabs in different salinity groups and different capital letters represent the significant difference of T content after spawning of female crabs in different salinity groups (P<0.05).

E_2 under the action of aromatase (Zhao *et al.*, 1985). At the end of the vitellogenesis of rainbow trout, the contents of E_2 reached the maximum, but the concentration of E_2 decreased rapidly at the final maturation of oocytes and ovulation and then gradually increased until stable (Kraak *et al.*, 1984). The concentration of E_2 in hemolymph of rainbow trout decreased 30 days before ovulation, while GTH increased and GTH induced the production of T in ovary (Scott *et al.*, 1983). In this experiment, except for salinity 18‰, the content of T in the hemolymph of female crabs in other salinity groups increased after spawning, which may be due to the difference in the mechanism of T on female crabs and other fish during the spawning stage of *E.sinensis*. Zhao *et al.,* (1985) found that T can promote GTH induced DHP synthesis, E_2 inhibits the synthesis of DHP by GTH, meanwhile T can be converted to E_2. In this experiment, the content of T decreased after spawning, while the content of E_2 increased, which was basically consistent with the above results. There was no obvious correlation between E_2 and T contents and salinity, which may be because the effects of two hormones on mature crabs were mainly concentrated at the stage of gonadal development, they probably played a small role during the mating and spawning.

CONCLUSION

At salinity 0‰, female crabs didn't mate, when the salinity was lower than 6‰, female crabs only mated but didn't spawn. Salinity had a certain effect on five hormones in the hemolymph of female *E. sinensis* during reproduction, five hormones didn't change significantly in the process of mating, DHP, GTH and PG were involved during the spawning of *E. sinensis*.

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Conflict of interest: None.

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