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Effects of different conditions on the artificial incubation effect and physiological indexes of redclaw crayfish eggs

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ABSTRACT

We explored the effects of different conditions on the artificial incubation of redclaw crayfish eggs in an effort to improve this process. Samples at the egg and juvenile stages were selected. The samples at different stages were separated from the pleopods, then they were placed in incubator boxes and sterilized with different disinfectant solutions. The density was 300,400 and 500 eggs/incubator box, the vibration frequency was 11,16 and 26 vibrations/min, and the water circulation cycle was 2.1, 4.8 and 7.1 cycles/h. The results showed the eggs disinfected with 3000 ppm formaldehyde for 15 min had stronger antioxidant capacity. The hatching and survival rates of five pairs of appendage stage group were significantly lower than those of other groups. In the egg stage, acid phosphatase (ACP) level of compound eye pigmentation stage group was significantly higher than those of other groups. In the juvenile stage, malondialdehyde (MDA) content of five pairs of appendage stage group was significantly higher than those of other groups. The survival rate of 500 eggs/box group was significantly higher than that of other groups. In the egg stage, alkaline phosphatase (AKP) level of 400 eggs/box group was significantly higher than that of other groups. The survival rate of 11 vibrations/min group was significantly higher than that of other groups. In the egg stage, ACP and AKP levels of 11 vibrations/min group were significantly higher than those of 26 vibrations/min group. In the juvenile stage, superoxide dismutase (SOD), ACP and AKP levels of 11 vibrations/min group was significantly higher than those of 26 vibrations/min group. In the juvenile stage, AKP level of 4.8 cycles/h group was significantly lower than that of other groups. In conclusion, egg development at the stage after seven pairs of appendages, with a density of 400 eggs/box, vibration frequencies set at 11 vibrations/min achieved high hatching rates (93.58 %) and survival rates (75.67 %). Moreover, bronopol or hydrogen peroxide might have a better choice to replace formaldehyde if further exploration was conducted to reduce stimulation of the in vitro-grown egg. These conditions could be used on a large scale to optimize the production of redclaw crayfish.

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1. Introduction

The redclaw crayfish (*Cherax quadricarinatus*) has the advantages of fast growth rate, tender meat, high edible ratio, and broad market prospects [1–3]. However, low egg production has always restricted the development of the crawfish industry. Particularly in the areas with low water temperatures in winter, the production of juveniles has always failed to keep up with the breeding demand [4]. Presently, the method of cultivating redclaw crayfish juveniles is still based on egg holding and incubation done by the female parent, which is mainly divided into indoor and outdoor cultivation methods [5]. Between them, indoor cultivation is easier to ensure the juvenile yield [6]. Artificial incubation of redclaw crayfish eggs is a relatively new technology for cultivating juveniles. This technique is mainly used to solve problems such as a low rate of brood incubation, long brood incubation time, and inconsistent emergence time of parent crustaceans; it is also used to produce specific pathogen free juveniles [3].

Since Reichenbach first attempted to incubate the eggs of *Astacus astacus* in vitro, the technology surrounding the artificial incubation of eggs had been gradually optimized [7]. At present, this technology has proven to be effective in incubating freshwater crayfish eggs. The technology could collect the eggs incubated simultaneously to avoid notable individual differences among the eggs. Some of the more advanced incubation equipment are small in size, have a large hatching capacity, use up low volumes of hatching water, and make controlling the incubation environment easy. It was beneficial to realize the intelligent and automatic production, as well as the mass production, of high-quality juveniles. However, the artificial incubation technology could still be further optimized. As far as the current artificial incubation technology is concerned, there are still problems regarding irregular survival rates, complex operation, and lack of a complete and reliable operation method.

The disinfection methods, egg development stages, and egg densities were considered to be relatively relevant criteria for artificial incubation [3]. Royuela et al. studied the artificial incubation of *Pacifastacus leniusculushe,* and considered that the survival rate was higher when the water circulation cycle was 0.5 L/min. They speculated that the low water circulation cycle was helpful for maintaining a more stable water quality index [8]. We also observed that in the process by which the females undergo egg holding and hatching, the abdominal swimming feet of the females had different swing frequencies at different egg development stages. These swing frequencies could essentially be divided into no swinging at the beginning of egg holding, which increases when the egg develops five to seven pairs of appendages; no swinging on the day of hatching; and slow swinging after hatching. In light of this, it was necessary to explore a more appropriate vibration frequency.

SOD and MDA are significant aspects of antioxidant indexes, to a certain extent, can reflect the health of an organism. The decrease in SOD and increase in MDA contents have negative effects on normal physiological metabolism [9]. ACP and AKP are immune-related indicators, their activity levels might be used to assess the state of the body's immune system [10] Furthermore, studies have shown that the α-amylase level is closely related to nutritional metabolism [11]. Li et al. found that the digestive enzyme activities could reflect the health of the body, α-amylase in hatchable eggs and successfully developed larvae were significantly higher than those in non-hatchable eggs and stunted larvae [12]. Luo et al. believed that the normal development of shrimp and crab eggs could be monitored simply and quickly by detecting the activity of digestive enzymes during production [13]. In the artificial incubation of crayfish eggs, the eggs grown in vitro would be stimulated to different degrees by the outside world under different disinfection treatments, development stages, incubation environments, vibration frequencies, and other conditions. When the external stimulation exceeds the body's threshold, its antioxidant system would become damaged, and the antioxidant enzyme activity would decline, causing the destruction of the body's nonspecific immune defense system [14]. Therefore, detecting the changes in physiological indexes of the eggs and juveniles grown in vitro under different incubation conditions could reflect their health status under different treatment conditions.

The main objective of this study was to solve the issue of the irregular survival rate when the eggs are incubated using the

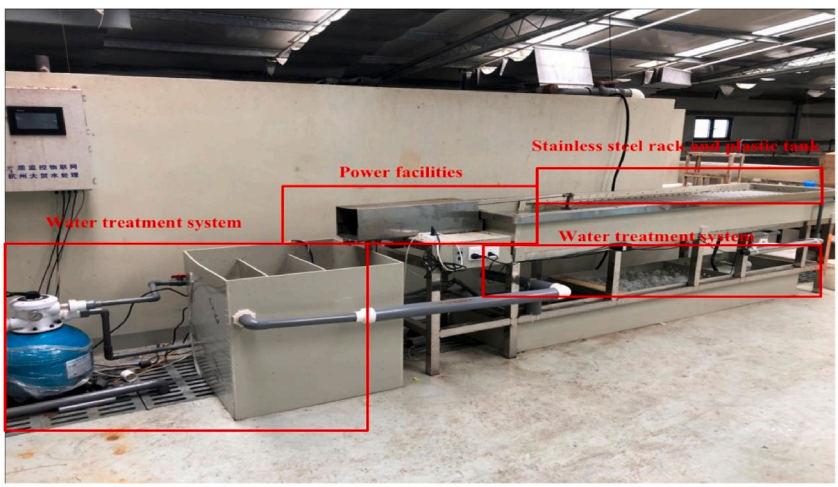

Fig. 1. Exterior of a recirculating mechanical pulling device.

recirculating mechanical pulling device [15]. Specifically, the aim was to explore methods to improve the survival rate from five perspectives: the disinfection methods, egg development stages, egg densities, vibration frequencies, and water circulation cycles. In this study we explored the effects of disinfection methods, densities, egg development stages, vibration frequencies, and water circulation cycles through the detection of physiological indexes of eggs and juveniles grown in vitro. This was done to improve the artificial incubation process for redclaw crayfish eggs.

2. Materials and methods

The experiments were conducted and eggs were obtained from *C. quadricarinatus* at the Zhejiang Institute of Freshwater Fisheries The crayfish (61.85 ± 6.52 g) used in this study were all healthy specimens. The experimental equipment used was designed and produced by the Zhejiang Institute of Freshwater Fisheries. This equipment mainly comprised incubators, incubator boxes, physical and biochemical filter areas, and motors that provided power for the incubators (Fig. 1). The volume of water used in the equipment was 800 L, 500 L water used in the physical and biochemical filtration area and 300 L used in the incubator. Each device could hold 700 incubators. A pump (300 W) was equipped for water circulation, and the switch regulating the motor speed that was equipped with the incubator could be used to control the vibration frequency of the incubator. The water could be effectively filtered, purified and controlled at a temperature of 28–29 °C by a water treatment system–composed of the water tank, sand filter tank, filter material, aerator and temperature control rod. The pH change range was 7.6–7.8. The incubator was 5.5 cm high, 6 cm long at the upper end, 2.5 cm wide at the upper end, and square at the lower end with the same side length as the upper end [16].

2.1. Experiment 1: effect of different disinfectants on vitro-grown egg

Four disinfectant groups were prepared: 1) 3000 ppm formaldehyde, 2) 3000 ppm bronopol, 3) 3000 ppm hydrogen peroxide, and 4) 75 % ethanol.

Twenty sibling females (62.70 ± 6.93 g) were selected for the experiment. The seven pairs of appendage stage eggs were carefully separated from the pleopods using a plastic comb to strip the different egg stages from the ovigerous setae. Subsequently, they were put into a culture dish (50 mm), which was then sterilized with disinfectant solutions 1–4. Disinfectant solutions 1–3 were applied to the eggs for 15 min each, and disinfectant solution 4 was applied for 30 s.

After disinfection, the eggs were washed twice with sterile water and put in incubator boxes. The eggs in each incubator box were collected from one female, the density was 400 eggs/incubator box. The eggs were disinfected every other day until hatching began. The vibration frequency was 16 vibrations/min, and the water circulation cycle was 4.8 cycles/h. The incubation water temperature was 28–29 °C. The sponge attachment was placed to the incubator boxes after egg hatching. The numbers of stage 1 juveniles and stage 3 juveniles were quantified after 8 days and 20 days, respectively. The hatching rate (hatching rate % = number of stage 1 juveniles/number of eggs × 100 %) and survival rate (survival rate % = number of stage 3 juveniles/number of eggs × 100 %) were determined [16]. Five experimental replicates were performed for each disinfectant.

When the eggs were about to hatch, or on the fourth day after hatching, egg and juvenile samples were harvested from one box in each group and stored at −80 °C. Samples were prepared by adding 9 mL of normal saline to 1 g of test sample. After grinding, the samples were transferred to 2 mL centrifuge tubes and centrifugation was performed at 3,000 rpm for 10 min at 4 °C. Thereafter, the supernatant was separated and stored at 4 °C. Next, the physiological indexes (SOD, MDA, ACP, AKP and α-amylase) were determined. In particular, enzyme activity was determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [17].

2.2. Experiment 2: effect of different egg development stages

Fifteen sibling females were randomly selected for this experiment. The eggs from groups at the five pairs of appendage, seven pairs of appendage, and compound eye pigmentation stages were collected as described above, and disinfected using 3000 ppm formaldehyde for 15 min [3]. Thereafter, the eggs were washed twice with sterile water and placed in incubator boxes. The eggs in each incubator box were collected from one female, with the density at 400 eggs/box. The vibration frequency, water circulation cycle, and incubation protocol were the same as those used in Experiment 1. After hatching, flat sponge attachments were added to the incubator boxes before testing began. Finally, the numbers of stage 1 and stage 3 juveniles were quantified, and the hatching rate and survival rate were determined. Five experimental replicates were performed for each egg development stage. When the eggs were about to hatch, or on the fourth day after hatching, egg and juvenile samples were harvested from one box in each group and stored at −80 °C.

2.3. Experiment 3: effect of different egg densities

Twenty sibling females were randomly selected for this experiment. The eggs from seven pairs of appendages were separated as described above, and then disinfected using 3000 ppm formaldehyde for 15 min. Thereafter, the eggs were washed twice with sterile water and placed in incubator boxes. The eggs in each incubator box were collected from 1 to 2 females, and had densities of 300, 400, and 500 eggs/box [3]. The vibration frequency, water circulation cycle, and incubation protocol were the same as those used in Experiment 1. After hatching, flat sponge attachments were added to the incubator boxes before testing began. Finally, the numbers of stage 1 and stage 3 juveniles were quantified, and the hatching rate and survival rate were determined. Five experimental replicates were performed for each egg density. When the eggs were about to hatch, or on the fourth day after hatching, egg and juvenile samples

were harvested from one box in each group and stored at −80 °C.

2.4. Experiment 4: effect of different water circulation cycles

Fifteen sibling females were randomly selected for this experiment. The eggs from seven pairs of appendages were separated as described above, and disinfected using 3000 ppm formaldehyde for 15 min. The eggs were then washed twice with sterile water and placed in incubator boxes at a density of 400 eggs/box, which comprised eggs collected from one female. The eggs were disinfected every other day until hatching began. The vibration frequency of the device was set to 16 vibrations/min, and three water circulation cycles were tested (7.1, 4.8, or 2.1 cycles/h) [16]. Three identical devices were used, and five parallel groups were set up for each device. The incubation protocol was the same as that used in Experiment 1. After hatching, sponge attachments were added to each incubator box. Finally, the numbers of stage 1 and stage 3 juveniles were quantified. The hatching rate and survival rate were also determined. Five experimental replicates were performed for each water circulation cycle. When the eggs were about to hatch, or on the fourth day after hatching, egg and juvenile samples were harvested from one box in each group and stored at −80 °C.

2.5. Experiment 5: effect of different vibration frequencies

Fifteen sibling females were randomly selected for this experiment, with eggs harvested and disinfected as described above. Each incubator box contained 400 eggs harvested from one female. The water circulation cycle was 4.8 cycles/h, and three vibration frequencies of the device were tested (11, 16, or 26 vibrations/min) [16]. Three identical devices were used, and five parallel groups were set up for each device. The incubation protocol and quantification of stage 1 and stage 3 juveniles were as described for Experiment 1. The hatching rate and survival rate were also determined. When the eggs were about to hatch, or on the fourth day after hatching, egg samples were harvested from one box in each group and stored at −80 °C. At the same time, we observed the appendage swing and the status of the eggs held by the crayfish during egg holding.

2.6. Statistical analysis

All data were presented as mean ± SD. Results were examined by analysis of variance (one-way ANOVA) using the SPSS 17.0 computer program. Furthermore, Tukey's multiple comparison test was performed as a post hoc test. For all statistical tests, P values < 0.05 were considered significant [17].

3. Results

3.1. The effect of different disinfection methods

Eggs were incubated for 6–10 days and emerged after 19–23 days. There was no significant difference in hatching and survival rates among the groups ($P > 0.05$) (Fig. 2). The eggs disinfected with 3000 ppm formaldehyde for 15 min had stronger antioxidant capacity.

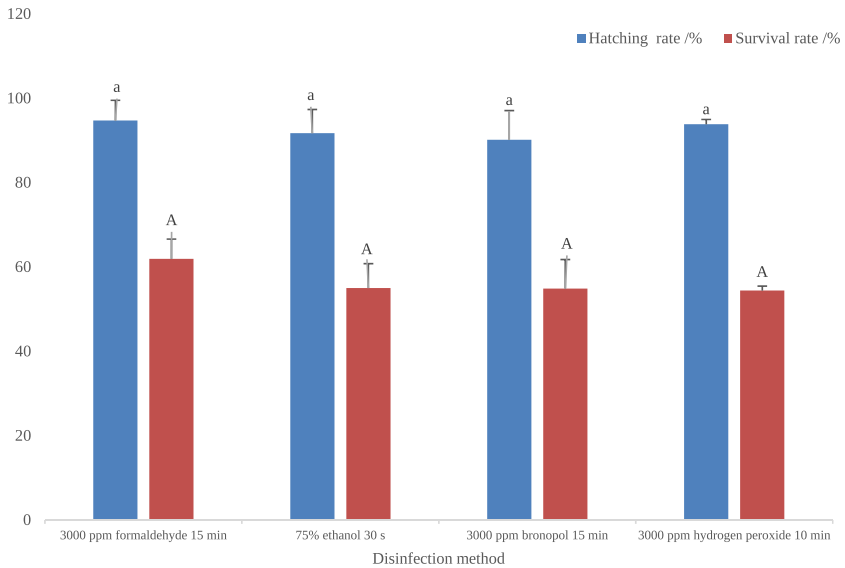

Fig. 2. Effect of different disinfection methods on the hatching and survival rates of crayfish eggs
All data are presented as the mean ± SD. The different superscript letters indicate significant differences ($P < 0.05$). This is the same in subsequent figures.

At egg stage, the SOD level in the formaldehyde group was significantly higher than that in the ethanol and hydrogen peroxide groups ($P < 0.05$), but there was no significant difference in the MDA content of each group ($P > 0.05$). At juvenile stage, there was no significant difference in SOD level among the groups ($P > 0.05$). However, the MDA content of the bronopol group was significantly higher than that of the other three groups ($P < 0.05$), and the MDA content of the ethanol group was significantly higher than that of the formaldehyde group ($P < 0.05$). At egg stage, the ACP level of the hydrogen peroxide group was significantly higher than that of the other three groups ($P < 0.05$), and the ACP level of the ethanol group was significantly lower than that of the other three groups ($P < 0.05$). The AKP levels of the formaldehyde and hydrogen peroxide groups were significantly higher than that of the ethanol group ($P < 0.05$). At juvenile stage, the ACP level of the bronopol group was significantly higher than that of the other three groups ($P < 0.05$), but the AKP level was significantly lower than that of the other three groups ($P < 0.05$). The α-amylase presented no significant difference ($P > 0.05$) (Table 1).

3.2. The effect of stripping at different development stages

Eggs at the five pairs of appendage, seven pairs of appendage, and compound eye pigmentation stages were hatched for 15–16, 11–14, and 6–7 days and emerged (became stage 3 juveniles) after 28, 23–26, and 19–20 days, respectively. There was no significant difference in hatching and survival rates between the groups in the seven pairs of appendage and compound eye pigmentation stages ($P > 0.05$). The lowest hatching rate was 80.67 %, which was observed in the group at the five pairs of appendage stage, and the highest hatching rate (96.92 %) was in the group at the compound eye pigmentation stage. The lowest survival rate (42.63 %) was in the group at the five pairs of appendage stage, and the highest survival rate (61.43 %) was in the group at the compound eye pigmentation stage. Therefore, the hatching and survival rates of the groups at the seven pairs of appendage and compound eye pigmentation stages were higher (Fig. 3).

There was no significant difference in the SOD level among groups ($P > 0.05$). However, at juvenile stage, the MDA content of the group at the five pairs of appendage stage was significantly higher than that of the groups at the seven pairs of appendage and compound eye pigmentation stages on the fourth day after egg hatching ($P < 0.05$). There was no significant difference in the AKP level among the groups ($P > 0.05$). However, at egg stage, the ACP level of the group in the compound eye pigmentation stage was significantly higher than that of the groups at the five and seven pairs of appendage stages ($P < 0.05$). At egg stage, the α-amylase level of the group in the compound eye pigmentation stage had significantly higher values than those of the groups at the five and seven pairs of appendage stages ($P < 0.05$) (Table 2).

3.3. The effect of different egg densities

Eggs were incubated for 6–8 days and emerged after 19–21 days. There was no significant difference in hatching rate among groups ($P > 0.05$). The lowest hatching rate was observed in the 500 eggs/box group (88.47 %), and the highest hatching rate was seen in the 400 eggs/box group (94.73 %). The survival rate in the 500 eggs/box group was significantly higher than that in the 300 eggs/box group ($P < 0.05$). The lowest survival rate was seen in the 300 eggs/box group (56.22 %), whereas the highest survival rate was observed in the 500 eggs/box group (62.47 %) (Fig. 4).

There was no significant difference in the contents of SOD and MDA among groups ($P > 0.05$). At egg stage, the AKP level of the 400 eggs/box group was significantly higher than that of the other two groups ($P < 0.05$), but there was no significant difference in ACP levels among the three groups ($P > 0.05$). Furthermore, At juvenile stage, there was no significant difference in ACP or AKP levels among the groups ($P > 0.05$). The α-amylase yielded no significant difference ($P > 0.05$) (Table 3).

3.4. The effect of different vibration frequencies

We observed the appendage swing and the status of eggs held by crayfish during egg holding. It was found that the egg-holding

Table 1
Effect of different disinfection methods on the physiological indexes of the eggs and juveniles.

Group		3000 ppm formaldehyde, 15 min	75 % ethanol, 30 s	3000 ppm bronopol, 15 min	3000 ppm hydrogen peroxide, 10 min
SOD (*U/mgprot*)	Egg	77.28 ± 1.32^{a}	68.10 ± 1.62^{b}	74.25 ± 4.07^{ab}	64.72 ± 7.58^{b}
	Juvenile	82.21 ± 0.45^{a}	86.79 ± 7.70^{a}	81.27 ± 0.99^{a}	81.58 ± 7.49^{a}
MDA (*nmol/mgprot*)	Egg	0.52 ± 0.11^{a}	0.52 ± 0.25^{a}	0.53 ± 0.21^{a}	0.48 ± 0.04^{a}
	Juvenile	0.50 ± 0.04^{a}	0.77 ± 0.13^{b}	1.04 ± 0.25^{c}	0.59 ± 0.16^{ab}
ACP (*U/mgprot*)	Egg	152.73 ± 11.41^{a}	116.52 ± 6.04^{b}	168.66 ± 1.65^{a}	180.66 ± 4.64^{c}
	Juvenile	179.01 ± 12.51^{a}	170.33 ± 22.94^{a}	192.23 ± 5.79^{b}	145.10 ± 17.89^{a}
AKP (King unit/ *gprot*)	Egg	14.74 ± 2.33^{a}	9.97 ± 0.47^{b}	12.10 ± 0.97^{ab}	14.08 ± 0.65^{a}
	Juvenile	11.87 ± 0.57^{a}	11.64 ± 1.04^{a}	7.59 ± 0.34^{b}	12.87 ± 1.03^{a}
α-amylase (*U/ mgprot*)	Egg	1.28 ± 0.24^{a}	1.51 ± 0.42^{a}	0.98 ± 0.04^{a}	1.02 ± 0.06^{a}
	Juvenile	2.25 ± 0.04^{a}	2.32 ± 0.19^{a}	2.53 ± 0.12^{a}	2.13 ± 0.51^{a}

All data are presented as the mean ± SD. The different superscript letters indicate significant differences ($P < 0.05$). The same superscript in the same line. This is the same for subsequent tables.

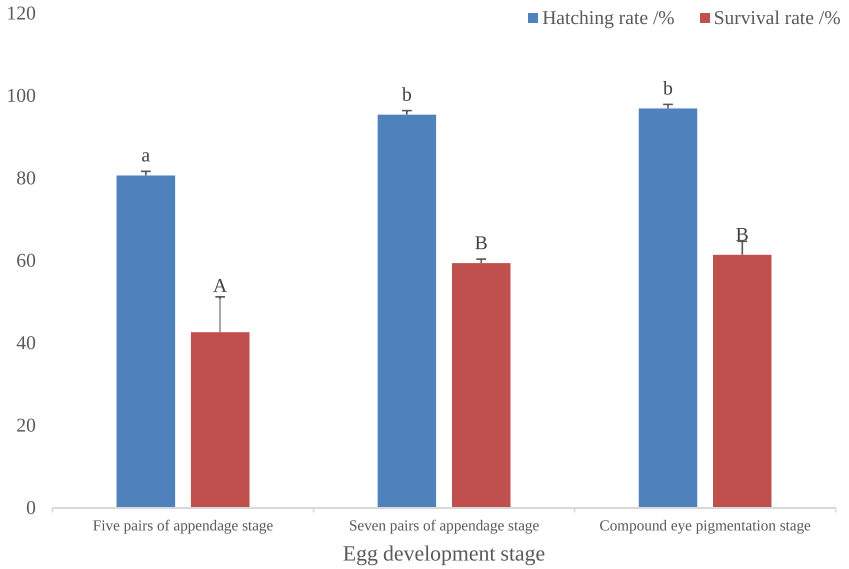

Fig. 3. The hatching and survival rates of crayfish eggs at different development stages.

Table 2
Effects of different egg peeling stages on the physiological indexes.

Group		Five pairs of appendage stage	Seven pairs of appendage stage	Compound eye pigmentation stage
SOD (*U/mgprot*)	Egg	69.54 ± 0.40[a]	76.92 ± 3.78[a]	76.44 ± 6.17[a]
	Juvenile	86.98 ± 5.32[a]	87.28 ± 6.63[a]	92.65 ± 3.24[a]
MDA (*nmol/mgprot*)	Egg	0.48 ± 0.12[a]	0.49 ± 0.13[a]	0.49 ± 0.26[a]
	Juvenile	1.01 ± 0.39[a]	0.55 ± 0.02[b]	0.55 ± 0.28[b]
ACP (*U/mgprot*)	Egg	160.83 ± 13.32[a]	165.39 ± 28.95[a]	229.07 ± 18.71[b]
	Juvenile	165.64 ± 17.33[a]	159.85 ± 1.93[a]	187.49 ± 10.88[a]
AKP (King unit/*gprot*)	Egg	10.10 ± 6.33[a]	8.35 ± 4.82[a]	10.08 ± 1.47[a]
	Juvenile	15.45 ± 8.88[a]	15.52 ± 0.06[a]	22.39 ± 5.95[a]
α-amylase (*U/mgprot*)	Egg	1.45 ± 0.09[a]	1.52 ± 0.03[a]	2.34 ± 0.12[b]
	Juvenile	1.94 ± 0.47[a]	1.69 ± 0.01[a]	1.91 ± 0.63[a]

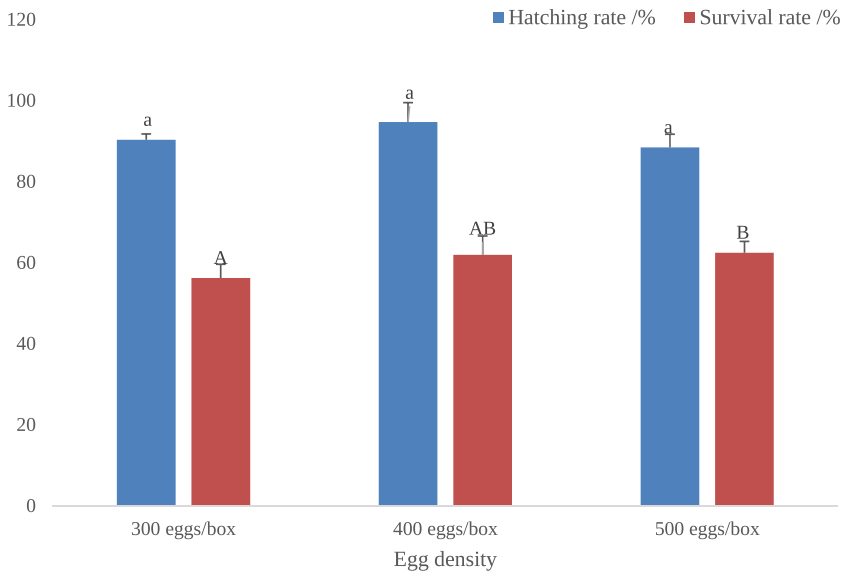

Fig. 4. The hatching and survival rates of crayfish eggs at different egg densities.

crayfish kept their tummy rolled up for the first 3 days; thereafter, the abdomen began to swing slightly. When the eggs were in the early development stage, the frequency of appendage swinging was low, about 0.5–0.8 times/s, and most of them were continuously swung. However, after the eggs reached the seven pairs of appendage stage and the egg membrane thickens, the frequency of swinging increased to 1.7 times/s, but continuous swinging was not obvious. This was accompanied by tummy rolling, the removal of dead eggs, and other behaviors. After the eggs hatched, the swing frequency decreased and the amplitude became low, the action became soft, and the swing amplitude of the appendages became lower than that during the egg stage, which was mainly characterized by a low amplitude and a slow swing.

Eggs were incubated for 10 days and emerged after 23 days. The hatching rates of all three groups exceeded 90 %. The lowest and highest hatching rates were observed in the 16 vibrations/min group (90.17 %) and the 11 vibrations/min group (93.58 %), respectively, but differences among the three groups were not significant ($P > 0.05$). The survival rate of each group ranged from 59.00 % to 75.67 %. The lowest and highest survival rates were observed in the 26 vibrations/min group (59.00 %) and the 11 vibrations/min group (75.67 %), respectively. The survival rate of the 11 vibrations/min group was significantly higher than that of the other two groups ($P < 0.05$) (Fig. 5).

At egg stage, there was no significant difference in the levels of SOD or MDA among groups ($P > 0.05$). At juvenile stage, there was no significant difference in MDA content among the groups ($P > 0.05$), but the SOD level in the vibration frequency group of 11 vibrations/min was significantly higher than that in the vibration frequency group of 26 vibrations/min ($P < 0.05$). At egg stage, the ACP and AKP levels in the 11 vibrations/min group were significantly higher than those in the 26 vibrations/min group ($P < 0.05$). At juvenile stage, the ACP and AKP levels in the 11 vibrations/min group were significantly higher than those in the other two groups ($P > 0.05$). The α-amylase yielded no significant difference ($P > 0.05$) (Table 4).

3.5. The effect of different water circulation cycles

Eggs were incubated for 6–8 days and emerged after 19–21 days. The results showed that the hatching and survival rates of each group all exceeded 90.00 % and 65.00 %, respectively, with no significant differences among groups ($P > 0.05$). The lowest hatching rate in the groups under the water circulation cycles was 2.1 cycles/h (92.88 %) and the highest hatching rate in the groups under the water circulation cycles was 7.1 cycles/h (95.92 %), respectively. The lowest survival rates in the groups under water circulation cycles was 4.8 cycles/h (65.67 %) and the highestsurvival rates in the groups under water circulation cycles was 2.1 cycles/h (66.58 %), respectively (Fig. 6).

There was no significant difference in the levels of SOD or MDA among groups ($P > 0.05$). At egg stage, there was no significant difference in the ACP or AKP level among the groups ($P > 0.05$), while at juvenile stage, there was no significant difference in the ACP level among the groups ($P > 0.05$). However, the AKP level of the 4.8 cycles/h group was significantly lower than that of the other two groups ($P < 0.05$). The α-amylase yielded no significant difference ($P > 0.05$) (Table 5).

4. Discussion

4.1. Effect of different disinfection methods on physiological indexes

Royuela et al. believed that disinfection was the key factor in the artificial incubation of shrimp eggs [8]. Different disinfectants had different toxic and side effects [18]. Among the disinfectants, formaldehyde, ethanol, bronopol, and hydrogen peroxidewere often used and yielded good results [3,19–21]. Formaldehyde at 3000 ppm was the most widely used disinfectant, however, formaldehyde might carcinogenic or have disadvantageous influences on the aquatic environment [21]. Therefore, bronopol, hydrogen peroxide, and ethanol maybe the alternate disinfectants.

The research on the effect of disinfectants on crustacean eggs hatching in vitro mostly focused on the apparent indicators of hatching rate, survival rate, and fungal infection [16,19]. Less focus was placed on the effect of the physiological indexes of the disinfectants on crustacean eggs. Indicators such as SOD, MDA, ACP, AKP, and digestive enzymes can be used to evaluate the degree of damage that disinfectants deal to the body [22]. In this study, the eggs disinfected with 3000 ppm formaldehyde for 15 min had stronger antioxidant capacity, we believe that formaldehyde had less of a stimulating effect on the in vitro-grown eggs. However, the

Table 3
Effects of different incubation densities on the physiological indexes.

Group		300 eggs/box	400 eggs/box	500 eggs/box
SOD (*U/mgprot*)	Egg	76.68 ± 0.64^{a}	72.00 ± 13.37^{a}	67.15 ± 4.34^{a}
	Juvenile	85.20 ± 14.57^{a}	76.03 ± 0.14^{a}	78.31 ± 3.71^{a}
MDA (*nmol/mgprot*)	Egg	0.36 ± 0.09^{a}	0.51 ± 0.10^{a}	0.47 ± 0.06^{a}
	Juvenile	0.60 ± 0.02^{a}	0.56 ± 0.21^{a}	0.61 ± 0.20^{a}
ACP (*U/mgprot*)	Egg	168.01 ± 25.45^{a}	140.33 ± 20.93^{a}	141.81 ± 23.42^{a}
	Juvenile	152.97 ± 28.74^{a}	157.80 ± 7.20^{a}	167.46 ± 16.04^{a}
AKP (King unit/*gprot*)	Egg	4.84 ± 2.20^{a}	8.34 ± 1.22^{b}	5.94 ± 0.37^{a}
	Juvenile	9.48 ± 2.40^{a}	9.07 ± 0.72^{a}	7.36 ± 1.50^{a}
α-amylase (*U/mgprot*)	Egg	1.91 ± 0.19^{a}	1.74 ± 0.19^{a}	1.65 ± 0.49^{a}
	Juvenile	2.16 ± 0.05^{a}	1.96 ± 0.51^{a}	1.69 ± 0.02^{a}

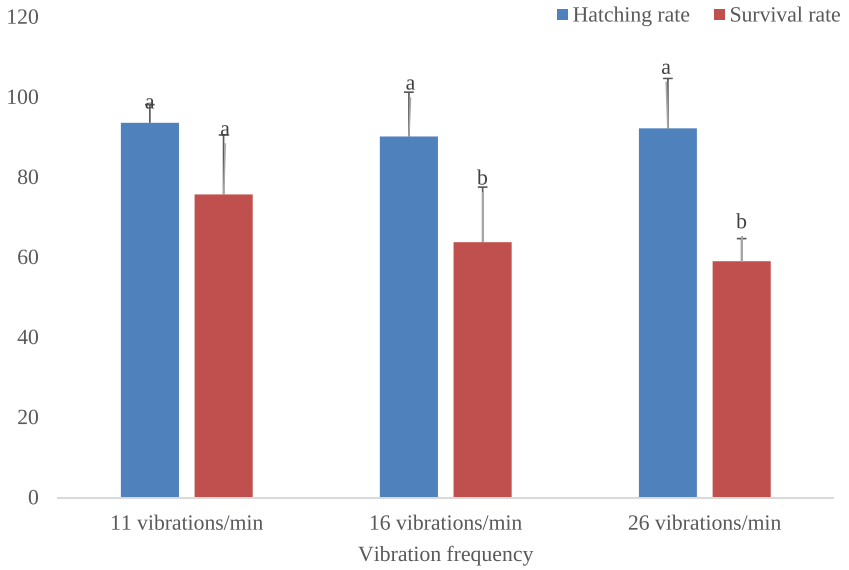

Fig. 5. Effect of different vibration frequencies on the hatching and survival rates of crayfish eggs.

Table 4
Effects of different vibration frequencies on the physiological indexes.

Group		11 vibrations/min	16 vibrations/min	26 vibrations/min
SOD (*U/mgprot*)	Egg	75.00 ± 0.43^{a}	70.94 ± 4.54^{a}	72.71 ± 12.22^{a}
	Juvenile	81.29 ± 4.46^{a}	75.52 ± 6.10^{ab}	65.93 ± 0.12^{b}
MDA (*nmol/mgprot*)	Egg	0.35 ± 0.16^{a}	0.40 ± 0.02^{a}	0.38 ± 0.03^{a}
	Juvenile	0.51 ± 0.17^{a}	0.64 ± 0.13^{a}	0.58 ± 0.11^{a}
ACP (*U/mgprot*)	Egg	163.31 ± 18.29^{a}	146.52 ± 23.25^{ab}	127.75 ± 21.29^{b}
	Juvenile	194.41 ± 81.48^{a}	135.10 ± 3.31^{b}	136.84 ± 6.24^{b}
AKP (King unit/*gprot*)	Egg	14.80 ± 1.39^{a}	10.80 ± 2.29^{ab}	7.86 ± 0.12^{b}
	Juvenile	7.77 ± 0.98^{a}	5.59 ± 0.24^{a}	7.86 ± 0.62^{a}
α-amylase (*U/mgprot*)	Egg	1.92 ± 0.20^{a}	1.62 ± 0.04^{a}	1.45 ± 0.10^{a}
	Juvenile	1.75 ± 0.74^{a}	1.66 ± 0.43^{a}	1.70 ± 0.44^{a}

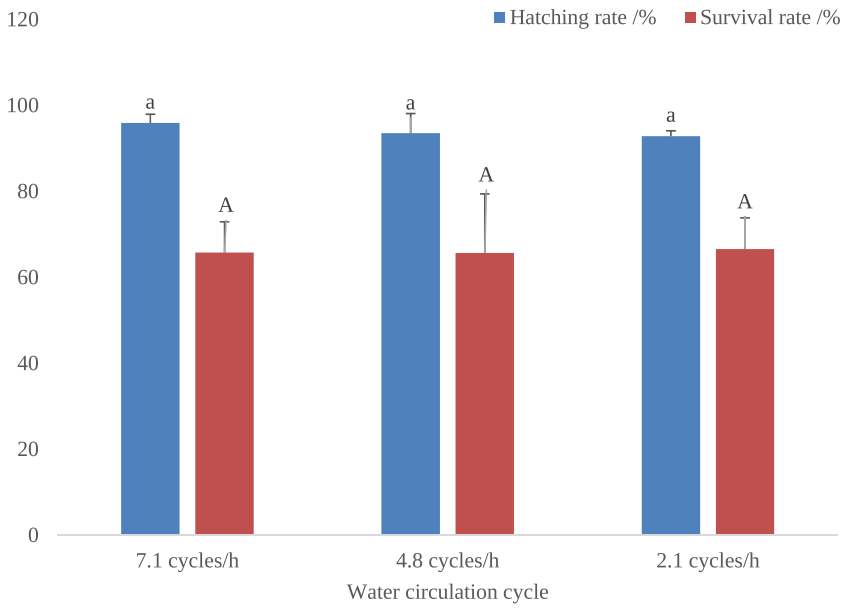

Fig. 6. Effect of different water circulation cycles on the hatching and survival rates of crayfish eggs.

Table 5
Effects of different water circulation cycles on the physiological indexes.

Group		7.1 cycles/h	4.8 cycles/h	2.1 cycles/h
SOD (*U/mgprot*)	Egg	77.28 ± 1.32[a]	75.00 ± 0.43[a]	71.19 ± 0.39[a]
	Juvenile	82.74 ± 1.21[a]	81.29 ± 4.46[a]	76.03 ± 0.14[a]
MDA (*nmol/mgprot*)	Egg	0.52 ± 0.11[a]	0.35 ± 0.16[a]	0.43 ± 0.04[a]
	Juvenile	0.68 ± 0.09[a]	0.51 ± 0.17[a]	0.56 ± 0.21[a]
ACP (*U/mgprot*)	Egg	152.73 ± 11.41[a]	163.31 ± 18.29[a]	155.03 ± 10.83[a]
	Juvenile	128.10 ± 31.90[a]	113.41 ± 38.14[a]	157.80 ± 7.20[a]
AKP (King unit/*gprot*)	Egg	14.74 ± 2.33[a]	14.80 ± 1.39[a]	12.49 ± 2.64[a]
	Juvenile	10.33 ± 0.73[b]	6.84 ± 0.88[a]	10.42 ± 1.20[b]
α-amylase (*U/mgprot*)	Egg	1.28 ± 0.24[a]	1.92 ± 0.20[a]	1.72 ± 0.18[a]
	Juvenile	1.55 ± 0.02[a]	1.75 ± 0.74[a]	1.96 ± 0.51[a]

eggs disinfected with 3000 ppm bronopol for 15 min or hydrogen peroxide for 10 min had less of a stimulating effect on the egg stage or juvenile stage, respectively. Therefore, we speculate that if further exploration was conducted to reduce stimulation of the in vitro-grown egg, bronopol or hydrogen peroxide might have a better choice to replace formaldehyde.

4.2. Effect of different egg densities

It had been shown that different egg densities have varied effects on the artificial incubation of crayfish eggs [3,8]. Royuela et al. reported that dead eggs act as a medium for the growth of fungi and other bacteria [8]. The mycelium could spread from the infected eggs to the surrounding healthy eggs, especially when the density is high.

In this study, the egg density had no significant effect on the hatching and survival rates, but the survival rate of the 300 eggs/box group was generally lower than that of the other two groups. This might be because of the relatively large swing amplitude of the sponge attached to the juveniles, which led to an increase in the probability of the juveniles being hit and rubbed by the outside world, and in the probability of the sponge falling off during molting or in the probability of a relatively large mutual impact amplitude of the eggs and the newly hatched juveniles without attachments. In addition, when the density reached or exceeded 500 eggs/box, those at the bottom of the incubator easily died from hypoxia due to the accumulation of juveniles, and the dead eggs or fungi bred by the dead juveniles were easily spread to healthy eggs and juveniles. Royuela et al. reported that the dead eggs or juveniles were the medium facilitating the growth of fungi and other bacteria [8]. The mycelium could spread from the infected ones to the surrounding healthy ones. Therefore, the density of 400–500 eggs/box was generally regarded as appropriate, which was conducive to enhance the attachment effect of juveniles and protected them.

Nevertheless, some studies have shown that with the increase in density, the antioxidant capacity of the body decreases [23]. In this study, the egg density of 300, 400, and 500 eggs/box would not affect the physiological indexes of the eggs and juveniles. Only the AKP value of eggs in the 400 eggs/box group significantly higher than that of the other two groups. Therefore, the egg density of the 400 eggs/box might make the eggs have a stronger immune capacity.

In the recirculating mechanical pulling device, the main consideration was whether it was more susceptible to the breeding of bacteria and fungi when the density was increased, and whether it was more likely to cause damage during the incubation process, such as via mechanical movement. According to our analysis, the eggs had relatively better protection. In addition, after the eggs were hatched, sponges were added to the incubator, which did not squeeze the juveniles in the space. The possibility of the mutual infection of fungi was small, and the probability of local hypoxia was low. This density test only considered the test effect in a small incubator. The whole set of equipment incubated fewer eggs at the same time, produced fewer organic substances during the test, and had little impact on water quality. It did not consider the egg capacity tolerance test in the entire incubator. Therefore, the three incubation densities had little impact on the antioxidant performance, immune function, and digestive capacity of eggs. The equipment egg carrying capacity test should be added in the future to provide evaluation data for the actual incubation effect of equipment.

4.3. Effect of different egg development stages

Studies have shown that the hatching rate of crayfish eggs in the early stage was lower than that in the late stage [3]. In this experiment, the different egg development stages had a greater impact on the artificial incubation of redclaw crayfish eggs. The hatching and survival rates of eggs in the seven pairs of appendage and compound eye pigmentation stages were higher, but these were lower in the eggs in the five pairs of appendage stage. Therefore, it is appropriate to the choose compound eye pigment formation or seven pairs of appendage stage for artificial incubation. The membrane of eggs in this stage is thicker, which facilitates optimal development and the eggs can effectively resist external physical damage during stripping.

Moreover, the eggs at the seven pairs of appendage and compound eye pigmentation stages were considered to be better for artificial incubation as they also had a stronger antioxidant and immune capacity. Simultaneously, eggs at the compound eye pigmentation stage had stronger immune capacities and higher digestive enzyme activities. Based on the incubation effect and enzyme activity test results, it was speculated that the reason why the incubation effect of late-stage eggs was better than that of early-stage eggs was that the membrane of early-stage eggs was thin and easily damaged during egg stripping, which might lead to infection. The membrane damage might also lead to the overflow of enzymes from the egg, which might cause the decrease in the enzyme activity

that maintains physiological function; furthermore, the antioxidant capacity or immune capacity might also be reduced. Additionally, compared with the late-stage eggs, early-stage eggs incubate for a longer time in the recirculating mechanical pulling device. During this period, changes in the water environment, and in physiological and biochemical indicators, would have an impact on hatching. Therefore, it is more appropriate to select eggs in the compound eye pigment formation or seven pairs of appendages stage for artificial incubation.

4.4. Effects of different vibration frequencies

Clive et al. used similar equipment to incubate redclaw crayfish, but the survival rate was unstable [15]. This study further explored the vibration frequency to improve the stability of the survival rate. The results showed that the group with a vibration frequency of 11 vibrations/min had a higher survival rate, indicating that the reduction in vibration frequency was helpful for improving the survival rate. However, Wang et al. and Zeng discovered that the rhythmic fanning of the abdominal limbs could sustain the flow of water, thereby reducing the percentage of egg death due to local hypoxia [24,25]. We also observed that the rate of appendage swaying gradually increased from low frequency to high frequency after hatching. Therefore, we believe that low vibration frequencies reduced the probability of external physical damage to eggs and juveniles. In particular, the juveniles without egg membrane protection require reduced vibration frequencies because of their weak resistance to external impact and friction. In addition, in light of the analysis of the egg-holding behavior of female crayfish, when eggs were in the incubation box during artificial incubation we controlled the vibration frequency to cause vibrations around all the eggs, but this frequency was reduced when juveniles were in the incubation box.

The results of this study also showed that the group with the lowest vibration frequency had higher antioxidant and immune capacities. The reduction in vibration frequency also contributed to the improvement of the survival rate. It was speculated that the low vibration frequency reduced the probability of eggs and juveniles suffering external physical damage. This is especially so for juveniles without egg membrane protection. Intact eggs and juveniles had relatively high antioxidant and immune-related indicators, and the antioxidant system and immune defense system were not greatly damaged. This resulted in higher survival rates than in other groups with high vibration frequencies.

4.5. Effect of different water circulation cycles

In studying the effect of water cycles on the artificial incubation of crayfish eggs, we examined whether it was easier to breed fungi if the water circulation cycle was too slow and whether it had a negative impact on the hatching and survival rates if the cycle was too fast. Royuela et al. compared the effect of water circulation cycles of 0.5 L/min and 1 L/min on the hatching of *Pacifastacus leniusculus* eggs [8]. The results showed that the survival rate was higher when the cycle was 0.5 L/min. It was believed that the water quality was more stable under this cycle. However, their research also showed that, unlike disinfection treatment and egg density, the influence of the water circulation cycle was not a key factor. Antonín et al. also believed that reductions in the water circulation cycle would help to maintain the state of excellent water quality [19]. In this experiment, the cycles of 7.1, 4.8, and 2.1 times/h had no significant effect on the hatching and survival rates.

At the same time, the results of this study also showed that different water circulation cycles had no significant effect on the enzyme activities of eggs and juveniles. However, the water circulation cycle of 4.8 times/h may reduce the immune capacity of juveniles. Considering the conclusion obtained by Antonín et al., and Cao et al. (2020) which stated that reducing the water circulation cycle was beneficial to the stability of water quality [19,26], this cycle was conducive to the stability of physiological and biochemical indicators, such as immune-related enzymes, during egg incubation. Although the cycle was 2.1–7.1 times/h, which had little impact on eggs and juveniles, and had no obvious impact on the hatching effect, it is recommended to set the water circulation cycle between 2.1 and 4.8 times/h. In the process of equipment design and optimization in the future, whether or not the organics can be timely removed from the filtration system should also be considered.

5. Conclusion

In this study, we selected the best egg development stage (after the formation of seven pairs of appendages), egg density (400–500 eggs/box), and vibration frequency (11 vibrations/min) for egg rearing under artificial incubation. Under these conditions, eggs and juveniles had higher hatching and survival rates, and higher antioxidant and immune capacities. Moreover, bronopol or hydrogen peroxide might have a better choice to replace formaldehyde if further exploration was conducted to reduce stimulation of the in vitro-grown egg. These conditions could be used on a large scale to optimize the production of redclaw crayfish.

Compliance with ethical standards

This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

Ethics approval

This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

Availability of data and material

The data and material were available.

Code availability

Software application.

Declaration of competing interest

The authors declare that there are no competing interests.

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