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The exploration of artificial incubation of *Cherax quadricarinatus* eggs

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**A R T I C L E I N F O**

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**A B S T R A C T**

The redclaw crayfish, *Cherax quadricarinatus*, is an economically valuable freshwater crayfish. However, some production obstacles, such as a low egg hatching rate and asynchronous hatching, are hindering its development in the aquaculture industry. Artificial incubation of eggs may solve these problems. This study explored the technology of artificial incubation of redclaw crayfish eggs. The following results were obtained: 1) 75% alcohol as a disinfectant for 60 s had a preferable antibacterial effect and promoted the hatching rate; 2) densities of 300 and 600 eggs/incubator resulted in significantly higher hatching and survival rates than a density of 900 eggs/incubator; 3) at a density of 600 eggs/incubator, the optimum number of net pieces for attachment of freshly hatched juveniles was 20 per incubator; 4) with a density of 600 eggs/incubator and 20 net pieces/incubator, the hatching rate was 82.05% ± 4.09%, the survival rate was 55.12% ± 7.51%, and a total of 129,200 SPF (specific pathogen-free) seedlings were cultivated. This artificial incubation system was maintained at close to an aseptic state, with an absence of white spot virus, iridovirus, *Vibrio* and ciliates; this was true for all water sources used and for incubation of both eggs and juvenile crayfish. In conclusion, if we implement reasonable methods of disinfection, SPF detection and pathogen isolation and utilize optimal egg densities and incubation systems, large-scale production of SPF seedlings of *Cherax quadricarinatus* is possible.

**1. Introduction**

*Cherax quadricarinatus*, generally known as the redclaw crayfish, is an economically valuable freshwater crayfish with outstanding advantages and broad prospects for aquaculture and development (Jones, 1990; Zheng et al., 2019). It is native to tropical northern Australia and southern New Guinea (Ghanawi and Saoud, 2012). It can reach high market prices because of its gustatory quality and high demand (Edgerton, 2005; Webster et al., 2002). Since the introduction and trial cultivation of the redclaw crayfish in the early 1990s, China has developed indoor artificial breeding technology (Gu et al., 2003). However, there are still some obstacles for industrialization of the redclaw crayfish, such as a low hatching rate and asynchronous hatching.

Artificial incubation technology can be used to solve these production problems. It also allows for closer control of ambient conditions, such as water quality and the prevalence of predators and diseases. Furthermore, it requires less space, energy and labor and allows for production of animals free from aphanomyosiosis (Jose et al., 1999; Edgerton, 1999; Evans and Edgerton, 2002). The development of this technology for the purpose of crayfish production has long attracted attention from scientists and aquaculturists in many countries (Jones and Valverde, 2020). In China, Zhu et al. (2002) first tried to hatch the eggs of *Procambarus clarkii* in vitro, but their results showed significantly lower hatchability in artificially incubated eggs compared to those attached to maternal pleopods. In the 21st century, researchers have successfully conducted a multitude of studies on artificial incubation of crayfish eggs and have achieved breakthroughs in disinfectants, incubation methods, the incubation system, and other components. In terms of disinfectants, most studies have shown that formaldehyde (1500 ppm - 4500 ppm, administered every 2–3 days for 15–25 min) can greatly control the growth of fungi, kill parasites and improve hatchability (Celada et al., 2004; Melendre et al., 2006; Melendre et al., 2007; Royuela et al., 2009; Antonín et al., 2010). However, there is concern about the user safety of formaldehyde because of its suspected carcinogenicity and its potential adverse effects on the aquatic environment (Arndt et al., 2001). The common disinfectant 75% alcohol can inactivate most bacteria and viruses (Lio-Po et al., 1982; Wang et al., 2013), including coronavirus (COVID-19).
Therefore, it is necessary to study it as a disinfectant in artificial incubation of crayfish eggs. In terms of incubation methods and systems, Brian et al. (2001) applied suspension upwellers for artificial incubation of Cherax destructor eggs and achieved survival rates averaging 87%, which was equal to or better than the survival rates observed with maternal incubation. Other researchers have evaluated artificial incubation of crayfish eggs in recirculating systems, and they found that such systems allow for successful artificial incubation of noble crayfish (Astacus astacus) eggs (Antonín et al., 2010; Antonín et al., 2011; Jones and Valverde, 2020).

The aim of this study is to explore the technology of artificial incubation of redclaw crayfish eggs through the selection of disinfectants and the optimization of the technological process. The results will allow for establishment of an SPF (specific pathogen-free) seedling generation technology that is suitable for production of C. quadricarinatus free of certain pathogens.

2. Materials and methods

The process of artificial incubation includes disinfection, in vitro incubation, SPF detection and pathogen isolation. Eggs were obtained from C. quadricarinatus berried females at the Zhejiang Institute of Freshwater Fisheries, Huzhou, China. 805 females were employed (weight 69.15 ± 11.04 g, body length 108.53 ± 12.44 mm, abdominal length 53.75 ± 4.15 mm). All females of their first spawning, and they’re all the same age, egg quality at the starting point was similar. The incubation system consisted of four main components: a ZISS incubator (from the company of Zissaqua in Korea, volume: 500 mL), an ozone sterilizer (power: 130 w, ozone production: 5 g/L), an incubation tank (100 × 40 × 40 cm) and a greenhouse pool (area 13 m²) with a natural gas pipeline and inflation pipe to allow the water in the pool to be heated. The ZISS incubator was the core component of the device; it was able to keep the eggs in a continuous rolling state with controllable dynamic water flow through the principle of gravity. The eggs in the ZISS incubator were in an oxygen-rich environment, and a stable circulating water flow turned them evenly and gently (Fig. 1). Seven incubators were placed in an incubation tank, and ten incubation tanks were placed in each greenhouse pool. Natural gas was used to maintain the water temperature both inside and outside the incubation tanks at 28–30 °C. One reservoir was used for incubation, and the incubation water was disinfected using the ozone sterilizer every day for 2 h. The water in the tank was exchanged every other day.

2.1. Experiment 1: study on the effect of different disinfectants

The eggs were carefully separated from the pleopods using comb to gently but firmly strip the eggs from the ovigerous setae to which they are attached and directly into a culture dish, and sterilized with different disinfectants. 18 females were employed, the eggs in each incubator from 2 females. Three treatments were tested:

- sterile water for 60 s as a control.
- 2000 ppm formaldehyde for 15 min.
- 75% alcohol for 60 s.

Then, the eggs were washed with sterile water twice, and 10 eggs from each group were ground with 200 μl of sterile water. The ground eggs were diluted 100 times and then cultured on blood agar (for detection of aerobic and facultative anaerobic bacteria) and TCBS (mainly for the detection of Vibrio). After 24 h, the number of bacteria was counted to determine the bacteriostatic effect. The remaining eggs were placed in the incubator. The water used for incubation was disinfected with ozone, and the eggs were disinfected with 75% alcohol for 60 s every other day until hatching began. The incubation water temperature was maintained at 28–30 °C. After hatching, the number of stage 1 juveniles was quantified. The hatching rate (hatching rate = number of stage 1 juveniles/number of eggs) was determined. Three parallel experiments were performed under each disinfectant condition.

2.2. Experiment 2: study on the effect of different disinfection times

The eggs were separated from the comb and sterilized with 75% alcohol for different disinfection times. 24 females were employed, the eggs in each incubator from 2 females. Four disinfection times were tested:

- 75% alcohol for 10 s, 30 s, 60 s and 90 s.

Then, the eggs were washed twice with sterile water, and 10 eggs from each group were used to determine the bacteriostatic effect following the detection steps used in experiment 1. The remaining eggs were incubated following the same steps used in experiment 1. Finally, the hatching rate was determined. Three parallel experiments were performed for each disinfection time.

2.3. Experiment 3: study on the effect of different egg densities

The eggs were separated from the comb and sterilized with 75% alcohol for 60 s. The sterilized eggs were washed with sterile oxygen-rich water and placed in the incubator. 18 females were employed, the eggs in each incubator from 1 to 3 females. Three densities were tested:

- 300, 600 and 900 eggs/incubator.

The incubation protocol was the same as that used in experiment 1. Finally, the numbers of stage 1 and stage 3 juveniles were quantified. The hatching rate and survival rate (survival rate = number of stage 3 juveniles/number of eggs) were determined. Three parallel experiments were performed for each egg density.

2.4. Experiment 4: study on the influence of net pieces on hatching

The eggs were separated from the comb and then sterilized with
75% alcohol for 60 s. The sterilized eggs were washed with sterile oxygen-rich water and placed in the incubator at a density of 600 eggs/incubator. Twelve incubators were used, and net pieces (2.0 × 2.0 cm) were added to each incubator in advance. 24 females were employed, the eggs in each incubator from 2 females. Four numbers of net pieces were tested:

- 0, 15, 20 and 25 net pieces per incubator.

The incubation protocol was the same as that used in experiment 1. Finally, the numbers of stage 1 and stage 3 juveniles were quantified. The hatching and survival rates were determined. Three parallel experiments were performed for each number of net pieces.

2.5. Experiment 5: SPF generation (including SPF detection and pathogen isolation)

All eggs were separated from the comb and sterilized with 75% alcohol for 60 s. The sterilized eggs were washed with sterile oxygen-rich water and placed in the incubator. 721 females were employed, the eggs in each incubator from 2 females. Finally, the numbers of stage 1 and stage 3 juveniles were quantified. The hatching and survival rates were determined.

We performed SPF detection on breeding crayfish, eggs and incubation water before and after disinfection; eggs and water during incubation; and stage 1 and stage 3 juveniles. At the same time, the eggs, water, newly hatched crayfish (stage 1 juveniles) and free mobile crayfish (stage 3 juveniles) during maternal incubation were tested as the control. The detection methods were based on the World Organization for Animal Health (OIE) list of shrimp-related diseases and China entry exit inspection and quarantine industry standards. In addition to the common diseases of the redclaw crayfish, we detected white spot virus, iridescent virus, Vibrio, ciliate and other colonies; these data can help prevent and isolate the invasion of epidemic diseases. All incubation devices should be kept in a closed area. Incubation water should be sterilized regularly by an ozone sterilizer and should be isolated from other pollution sources. Possible cross-infection should be avoided, and persons should not enter the closed incubation area during the cultivation period. Strict disinfection procedures should be carried out to avoid an epidemic.

2.6. Statistical analysis

All data are presented as the mean ± SD. Statistical analysis was carried out using Statistica software for Windows. For all statistical tests, P values < .05 were considered significant.

3. Results

3.1. Selection of the best disinfectant

The results of the bacteriostatic test showed that disinfection with 75% alcohol for 60 s had the best antibacterial effect, and eggs under that condition did not carry Vibrio (Fig. 2). After 7 days of incubation, the eggs hatched with the following hatching rates: 75% alcohol for 10 s (75.53 ± 2.56)%, 30 s (81.25 ± 4.34)%, 90 s (81.78 ± 4.88)%, 60 s (84.47 ± 1.71)%. The hatching rate of the eggs disinfected with 75% alcohol for 60 s was significantly higher than that for the eggs disinfected with 75% alcohol for 10 s (P < .05), and there were no significant differences between the other groups (P > .05) (Fig. 3).

3.3. Selection of the best density of eggs

The results showed that eggs hatched after 7 days of incubation and that the eggs developed to stage 3 juveniles after 16 days. The hatching rates were as follows: 900 eggs/incubator (78.99 ± 3.15)%, 600 eggs/incubator (86.65 ± 3.09)%, 300 eggs/incubator (87.46 ± 3.63)%. The survival rates were as follows: 900 eggs/incubator (50.67 ± 2.10)%, 600 eggs/incubator (57.05 ± 2.64)%, 300 eggs/incubator (57.81 ± 1.86)%. The hatching and survival rates of eggs incubated at densities of 300 and 600 eggs/incubator were significantly higher than those for eggs incubated at 900 eggs/incubator (P < .05). There was no significant difference in hatching rate or survival rate between the densities of 300 and 600 eggs/incubator (P > .05) (Fig. 4).

3.4. The influence of different numbers of net pieces

The results showed that eggs hatched after 7 days of incubation and that the eggs developed to stage 3 juveniles after 16 days. The hatching rates were as follows: 0 net pieces (85.55 ± 3.11)%, 25 net pieces (85.57 ± 3.59)%, 15 net pieces (85.67 ± 5.01)% and 20 net pieces (86.29 ± 5.34%); there were no significant differences between the groups (P > .05). The survival rates were as follows: 0 net pieces (52.37 ± 2.30)%, 25 net pieces (60.75 ± 2.94)%, 15 net pieces (61.64 ± 5.30)%, 20 net pieces (63.19 ± 4.72)%. The survival rate was not significantly different between the groups with 15, 20 and 25 net pieces (P > .05), but the rates were significantly higher for all of those groups compared to that with 0 net pieces (P < .05). The hatching and survival rates of the group with 20 net pieces were highest when the density was 600 eggs/incubator (Fig. 5). Besides, there were moldy eggs in the core areas of clusters of freshly hatched juveniles only found in 0 net pieces groups (Fig. 5).

3.5. SPF generation

On the basis of previous experiments, we carried out large-scale experiments. The eggs were sterilized with 75% alcohol for 60 s, washed with sterile oxygen-rich water and placed in the incubator. During incubation, the water was disinfected with ozone, and the eggs were disinfected with 75% alcohol for 60 s every other day. The results showed that the eggs developed to be stage 3 juveniles after 12–16 days. The hatching and survival rates were 82.05 ± 4.09% and 55.12 ± 7.51%, respectively, and a total of 129,200 SPF seedlings were cultivated (Fig. 6).

The results of SPF detection showed that in the process of hatching in vitro, the water, eggs and juvenile crayfish were all close to an aseptic state and did not carry white spot virus, iridovirus, Vibrio and ciliates, indicating that the measures of disinfection and isolation of external bacteria were appropriate.

4. Discussion

There are four key components to the process of artificial incubation: the disinfectant, egg density, incubator, and incubation management.

First, disinfectants have a great influence on hatching. Fungal infections caused by Saprolegnia and other related oomycetes are serious problems for incubated crayfish eggs and can result in 100% mortality.
Fig. 3. The antibacterial effect and hatching rate effect of different disinfectants. (A) The antibacterial effect of different disinfectants. (a) Sterile water for 60 s as a control. (b) 2000 ppm formaldehyde for 15 min. (c) 75% alcohol for 60 s. The upper row was detected by blood agar; the lower row was detected by TCBS. (B) Hatching rates with different disinfectants. Data are means of triplicates. Error bars represent S.E.M. Columns marked with different letters are significantly different (P < .05).

Fig. 2. The antibacterial effect and hatching rate effect of different disinfectants. (A) The antibacterial effect and hatching rate effect of different disinfectants. (a) Sterile water for 60 s as a control. (b) 2000 ppm formaldehyde for 15 min. (c) 75% alcohol for 60 s. The upper row was detected by blood agar; the lower row was detected by TCBS. (B) Hatching rates with different disinfectants. Data are means of triplicates. Error bars represent S.E.M. Columns marked with different letters are significantly different (P < .05).

Fig. 4. The hatching rate and survival rate at different egg densities. Data are means of triplicates. Error bars represent S.E.M. Columns marked with different letters are significantly different (P < .05).

(edgerton et al., 2002). The principle means of addressing this problem in previous reports were chemical antifungal baths or removal of dead and infected eggs (antonín et al., 2010). Although the periodic removal of dead eggs had a positive effect on the hatching rate by reducing fungal growth, it was laborious, and manipulation could cause damage to healthy eggs (celada et al., 2004; gómez et al., 2011). Thus, the antifungal treatments used in this study were combinations of chemical antifungal baths, disinfection of incubation water and periodic removal of dead eggs. We used 75% alcohol for 60 s as the chemical antifungal bath every other day up to the beginning of hatching, and the water for incubation was disinfected with ozone. We took advantage of the small size, light weight and easy operation of the incubator to regularly gently pour off the dead eggs floating on the upper layer of the water surface of the incubator. In contrast, most prior studies used formaldehyde for chemical antifungal baths (celada et al., 2004; melendre et al., 2006; melendre et al., 2007; royuela et al., 2009; antonín et al., 2010). Furthermore, lio-po et al. (1982) reported that alcohol was mycostatic at very high concentrations. The results of this study showed that 75% alcohol for 60 s is an effective alternative chemical antifungal bath to formaldehyde.

Second, we examined egg density. celada et al. (2004) reported that the efficiency rates obtained in their experiments were greatly affected by egg density. royuela et al. (2009) reported that dead crayfish eggs were a good substrate for Saprolegnia spp. and other oomycetes. Hyphae were capable of spreading from infected eggs to surrounding healthy ones, and this was a greater issue at higher egg densities. In agreement with the results of celada et al. (2004) and royuela et al. (2009), this study showed that the hatching and survival rates using egg densities of 300 and 600 embryos/incubator were significantly higher than those at a density of 900 embryos/incubator (P < .05).

brian et al. (2001) suggested that the incubator should imitate the pleopods of female crayfish. When females incubate eggs on their pleopods, they constantly move their pleopods to "fan" the eggs; this provides a constant source of freshwater and associated oxygen to the eggs. Therefore, we used a suspension system to suspend the eggs within a column of moving water. We considered this system to be effective because it allowed for good provisioning of freshwater and oxygen to the eggs and separated the eggs from surfaces that could encourage bacterial or parasitic infections. Additionally, the incubation system used in this study kept the eggs in a continuous rolling state with controllable dynamic water flow through the principle of gravity.

Last, incubation management was another important factor for the success of incubation. We focused on the two following points. First, disinfection of eggs and incubation water and periodic removal of dead eggs guaranteed successful incubation. Second, infected eggs consistently became core areas of clusters of freshly hatched juveniles, which is the most likely explanation for their consequent deaths. Crayfish hatchlings naturally tend to attach to the setae of maternal pleopods (vogt and toley, 2004), but this substrate is not available under artificial incubation. Therefore, utilizing an appropriate number
of net pieces is critical. In this study, there were moldy eggs in the core areas of clusters of freshly hatched juveniles only found in 0 net pieces groups. Besides, the survival rates of 15, 20 and 25 net pieces groups were significantly higher than 0 net pieces group ($P < .05$). We speculate that this was because the aggregation of stage 1 juveniles after hatching, resulting in the anoxic death of other eggs or juveniles, or the hatching was not synchronous, the first hatched juveniles attached to the non-hatched eggs, resulting in the death and mildew of the eggs. Therefore, in order to improve the survival rate and hatching rate, it was necessary to put the net pieces into the incubator as the attachment. Moreover, the hatching and survival rates of the group with 20 net pieces were highest when the density was 600 eggs/incubator, so 20 net pieces per incubator was the most suitable number for attachment of 600 freshly hatched juveniles.

Fungal (i.e., *Psorospermum*) and bacterial infections along with protozoan (i.e., *Epistylis*) and metazoan parasites are restricted to the outside of the egg. Therefore, if eggs are stripped from the female and their outer surfaces are sterilized, they can be incubated separately from the mother to produce juveniles that are specific-pathogen-free (SPF) (Rudge and O’Sullivan, 2007). Rudge and O’Sullivan (2007) reported that female crayfish did not pass viruses to their eggs; rather, the viruses were transferred after the eggs hatched or, more specifically, once the juveniles began to feed. Therefore, the production of SPF seedlings can control the spread of disease from the seedling source, which can improve the safety of germplasm conservation and improve seedling quality. In view of this, we examined artificial incubation technology for *C. quadricarinatus* eggs and applied this technology in the large-scale production of SPF seedlings with positive results. We also discovered that the incubation synchronization of SPF seedlings cultured with the incubation technology was high. This is beneficial for production because all the juveniles can easily be released into the ponds at the same weight and age, resulting in less cannibalism and precise knowledge of the number of juveniles being stocked in each pond.

The market for redclaw is growing, so better hatchery management are required (Ghanawi and Saoud, 2012). Future research needs to scale up the approach to a commercial level. We should combine aquatic knowledge with physical and mechanical knowledge, Besides, it is better to effectively simulate the wafting of the egg-bearing pleopods of the female crayfish. The AquaVerde Incubator described in the Jones and Valverde (2020) paper may be a good choice.

### 5. Conclusion

In this study, we determined a reasonable disinfection method (75% alcohol for 60 s every other day up to the beginning of hatching), egg density (600 eggs/incubator), incubation method (disinfecting the water for incubation with ozone for 2 h every other day; exchanging the water in the tank every other day; and using 20 net pieces/incubator for attachment) and incubation system (with a ZISS incubator as the core component). We also achieved SPF detection and pathogen isolation (close to an aseptic state and free of white spot virus, iridovirus, *Vibrio*...
and the first application of this artificial incubation system in the breeding of the redclaw crayfish and the preservation of new germplasm.

Compliance with ethical standards

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

Author statement

CHENG shun, JIA Yong-yi, CHI Mei-li, LIU Shi-li, ZHENH Jian-bo, WANG Dan-li, GU Zhi-min conceived and designed the experiments. WANG Dan-li, GU Zhi-min supervised the project. CHENG shun, JIA Yong-yi, CHI Mei-li performed the experiments. CHENG shun, JIA Yong-yi, CHI Mei-li analyzed the data. WANG Dan-li, GU Zhi-min supervised the project. CHENG shun, JIA Yong-yi, WANG Dan-li, GU Zhi-min wrote the paper. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors declare that there are no competing interests.

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