Reproductive and feeding ecology of red swamp crayfish procambarus clarkii (Girard, 1852) in China

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Article 2. Shiyu Jin, Lisa Jacquin, Yan Ren, Jixin Yu, Wei Li, Sovan Lek, Jiashou Liu, Zhongjie Li, Tanglin Zhang. 2019. Growth performance and muscle composition response to reduced feeding levels in juvenile red swamp crayfish *Procambarus clarkii* (Girard, 1852). *Aquaculture Research,* 00:1-10. DOI: 10.1111/are.13968.

Article 3. Shiyu Jin, Lisa Jacquin, Feng Huang, Mantang Xiong, Ruojing Li, Sovan Lek, Wei Li, Jiashou Liu, Tanglin Zhang. 2019. Optimizing reproductive performance and embryonic development of red swamp crayfish *Procambarus clarkii* by manipulating water temperature. *Aquaculture*, 510: 32-42. DOI: 10.1016/j.aquaculture. 2019.04.066.

Article 4. Shiyu Jin, Lisa Jacquin, Jixin Yu, Sovan Lek, Wei Li, Jiashou Liu, Tanglin Zhang. Effects of reduced dietary protein levels on growth performance and muscle composition of red swamp crayfish *Procambarus clarkii*. Plan to submit to *Aquaculture International*.

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Résumé

L'aquaculture s'est développée rapidement ces dernières années et est devenue l'un des principaux contributeurs à l'approvisionnement alimentaire dans le monde. En effet, l'immense pression de pêche exercée sur les populations sauvages et d'élevage entraîne progressivement l'épuisement des stocks. Le nombre limité de larves fournies pour l'aquaculture et des stratégies d'alimentation non optimales (par exemple un apport élevé en aliments artificiels) entravent le développement d'une industrie aquacole efficace. Une gestion plus durable de l'aquaculture nécessite maintenant une amélioration de la gestion des pêches, de la reproduction artificielle et des stratégies d'alimentation.

Dans cette thèse, nous nous sommes intéressés à trois questions principales : (1) quelle est la dynamique de population et l'écologie de la reproduction des écrevisse de Louisiane en bassins articifiels? (2) Quelles sont les temperatures optimales pour permettre une reproduction artificielle et un développement embryonnaire optimal chez cette espèce? (3) Quelle est la quantité et la composition alimentation optimale en bassin pour assurer une bonne croissance des juvéniles en générant un minimum de déchets?

Cette thèse repose sur plusieurs étapes et approches expérimentales. Pour la question (1) nous avons étudié la dynamique de population et lareproduction de l'écrevisse de Louisiane (*Procambarus clarkii*) en évaluant la croissance, les taux de mortalité et le taux d'exploitation de populations cultivées en bassins commerciaux, ainsi que différents indices reproducteurs (GSI, HSI, développement ovarien et fécondité). Les résultats montrent quela ponte de *P. clarkii* se déroule en Chine de septembre à novembre, avec une fécondité moyenne de 429 ± 9 œufs par femelle, avec deux recrutements par an. Il y avait cinq cohortes de croissance at les résultats montrent que les mâles *P. clarkii* étaient surexploités. Nous suggérons donc de réduire l'intensité de la pêche sur les écrevisses immatures et d'éviter la sélection des mâles en période de reproduction afin d'améliorer la durabilité globale des populations commerciales de *P. clarkii*.

Pour la question (2), nous avons testé les effets de la température de l'eau sur les performances de reproduction et de développement embryonnaire de *P. clarkii*. Les résultats montrent que la manipulation de la température de l'eau est un moyen efficace d'induire le frai chez les femelles et d'optimiser le développement embryonnaire pour améliorer la production larvaire, avec des températures optimales de 21 — 25℃ et 25℃, respectivement. Nous avons élaboré un modèle de développement dépendant de la température pour *P. clarkii*, exprimé en D (durée du développement, jours) = 3140837 $(T-2.03)^{-3.76}$.

Enfin, pour la question (3), nous avons testé les effets de la réduction des niveaux d'alimentation et des niveaux de protéines sur les performances de croissance et la composition musculaire de *P. clarkii* juvéniles ayant accès à des aliments naturels tels que les macrophytres *Hydrilla verticillata* dans des mares commerciales Les résultats montrent que la réduction des quantités de nourriture artificielle à 60% de satiété ou à 26% de protéines n'affectait pas de manière significative les performances de croissance et la composition musculaire des écrevisses. En effet, une analyse des isotopes stables suggère que les écrevisses compensent la réduction de nourriture artificielle ou de protéines en consommant plus de macrophytes naturels *H. verticillata* facilement disponibles.

Cette thèse propose donc de nouvelles alternatives à la reproduction artificielle traditionnelle en ajustant le prélèvement d'adultes, en manipulant la température de culture et en affinant les stratégies d'alimentation, afin de réduire les coûts de production tout en améliorant la productivité et la durabilité de l'aquaculture d'écrevisses.

Mot-clé: *Procambarus clarkii*; Gestion de l'aquaculture; La reproduction; Les dynamiques de population; La température de l'eau; Développement embryonnaire; Niveau d'alimentation; Niveaux de protéines alimentaires; Performance de croissance; Analyse de la composition musculaire

Abstract

Aquaculture has developed rapidly in recent years and has become one of the primary contributors to food supply worldwide. However, the immense fishing pressure on wild and commercial-farmed populations has caused population depletion. Furthermore, limited juvenile crayfish production for aquaculture and suboptimal feeding strategies (such as high inputs of artificial diets) has hindered the development of sustainable aquaculture industry. Improving fisheries management is now necessary, based on a better scientific knowledge of population dynamics, reproductive ecology, and optimal feeding strategies, in particular by determining optimal environmental parameters for reproduction and refining artificial diets inputs.

In this thesis, we focused on three main questions. First (1) what is the population and reproduction dynamics of adult crayfish living in commercial ponds and how should we adjust the aquaculture management? Second (2) what are the optimal temperatures for artificial reproduction and embryonic development? And third (3) what are the optimal levels of feeding and protein composition of artificial food for crayfish growth?

For the first question (1), we studied the population dynamics and reproductive pattern of red swamp crayfish (*Procambarus clarkii*) by estimating growth, mortality rates, and exploitation rate of a commercial population, as well their reproductive parameters (GSI, HSI, ovarian development, and fecundity). Results showed that spawning activities took place from September to November, with a mean fecundity of 429 ± 9 eggs per female, and two recruitments yearly. There were five growth cohorts and male *P. clarkii* were overexploited. We thus suggest reducing fishing intensity on immature crayfish and avoid sex selection during the reproductive period to improve the overall sustainability of commercial *P. clarkii* populations.

For the second question (2), we experimentally tested the effects of water temperature to improve reproductive outputs and embryonic development. Results showed that manipulating water temperature was an effective way to induce spawning in females and optimize embryonic development to improve juvenile production, with

optimal temperatures of 21 — 25℃ and 25℃, respectively. We also built a temperature-dependent developmental model for *P. clarkii*, D (developmental time, days) = $3140837(T\text{-}2.03)^{-3.76}$.

Finally, for the third question (3), we experimentally tested the effects of five different feeding levels and reduced dietary protein levels (2 experiments) on growth performance and muscle composition of juvenile *P. clarkii* with natural food *Hydrilla verticillata*. Results showed that reducing the amounts of an artificial diet to 60% satiation and/or reducing the dietary protein level of the artificial diet to a level of 26% did not significantly affect the growth performance and muscle composition of *P. clarkii*. Stable isotope analysis suggested that crayfish switched diets to easily available *H. verticillata* when feeding levels or dietary protein levels decreased.

This thesis thus explored new alternatives to traditional crayfish aquaculture by adjusting fishing effort and season, manipulating crayfish culture temperature, and refining feeding strategies to reduce production costs while improving the productivity and sustainability of crayfish aquaculture.

Keywords: *Procambarus clarkii*; Aquaculture management; Reproduction; Population dynamics; Water temperature; Embryonic development; Feeding levels; Dietary protein levels; Growth performance; Muscle composition analysis

Chapter 1

General introduction

Aquaculture has undergone rapid development in recent years and has become one of the primary contributors to the nutrition supply for human demands. Its annual production was 73.8 million tons in 2014, which represented 44% of the total fishery production, and would reach 52% in 2025 (FAO, 2017). As the largest producer in the world, China has supplied more than one-third of global fish production, due to the fast developing aquaculture industry (Cao et al., 2015). In particular, freshwater aquaculture has played a dominant role and has accounted for more than 50% of the global total aquaculture production (Wang et al., 2015). However, despite the optimistic scenario of its contribution to world fish food supplies, the development of the aquaculture sector has raised many issues and concerns in recent years. First, the growing demand of market intensifies the immense fishing pressure on commercially farmed populations, which can result in population depletion and slow recovery rates (Naylor et al., 2000; Tidwell and Allan, 2001). Especially for crustacean industry, the commercial fisheries have declined worldwide for a long time, due to increasing fishing pressure and decreasing catch sizes (Nagaraju, 2011). Second, for many farmed species, larvae are obtained from spontaneous reproduction in the wild which are limited by seasonal availability and lack of scientific knowledge on the reproductive ecology of farmed species. These problems heavily hinder the development of sustainable aquaculture (Smith et al., 2002). Third, aquaculture relies highly on the input of artificial diets, which have accounted for more than 50% of the total aquaculture costs (Craig et al., 2017). The high diets inputs in culture systems can lead to depletion of natural resouces, water pollution, and low dissolved oxygen levels which can have negative effects on foraging and immunity of cultured species. Suboptimal feeding strategies can thus result in fish or crayfish disease or death and cause huge economic loss (Chávez-Crooker and Obreque-Contreras, 2010; Craig et al., 2017; Henry and Fountoulaki, 2014; Martinez-Cordova et al., 2003; VelazcoVargas et al., 2014). Exploring new feeding strategies that are efficient and less costly for farmers and for the environment is thus urgently needed. In addition, assuring a continuous and sustainable supply of larvae is now a big challenge for the whole culture industry. Therefore, there is a need to improve fisheries management, by

adjusting reproductive outputs and feeding strategies based on reliable scientific knowledge population and reproduction dynamics of cultured species, and on environmental parameters for optimal reproduction and diet (Fatemi et al., 2009; Hasan, 2000; He et al., 2011; Nentwig, 2009).

1.1 Sustainable fisheries management and population dynamics

Global total capture fisheries production was up to 90.9 million tones in 2016 (FAO, 2018). However, the proportion of fish stocks which were with sustainable levels decreased from 90% in 1974 to 66.9% in 2015 (FAO, 2018). Due to high market demands, many fish or crayfish fishing activities continued to have significant overcapacity (Coleman and Williams, 2002; Jackson et al., 2001). In 2015, it was estimated that 43% of the main fish stocks (e.g. *Thunnus alalunga, Thunnus obesus*) were exploited at unsustainable levels (FAO, 2018). Overfishing not only resulted in fish or crayfish stocks depletion, but also had subsequently negative effects on ecosystems (Coleman and Williams, 2002; Jackson et al., 2001). Considering the fact of overfishing and overcapacity of fishery resources, there is, thus, an urgent need for scientists and farmers to develop effective measures to restore the overexploited fish or crayfish stocks while maintaining global foods supplies. In this case, aquaculture has been considered as an effective solution. In 2016, global aquaculture production was 110.2 million tons and the production had been expected to continue to increase in the future (FAO, 2018). However, as the fastest growing food sector, aquaculture has gained growing concerns among scientists and farmers in fisheries management. For example, what technological and fishing solutions should be taken to develop sustainable aquaculture? What is the maximum yield of a specific fish or crayfish stock and how to adjust fishing activities to catch individuals at sustainable levels (Wilson et al., 2003)? The main challenges in achieving these goals include limited information on fish or crayfish reproductive biology, and lack of research on population dynamics and scientific fishing regulation rules (Russell et al., 2012). If accurate knowledge on spawning seasons is available to us, sustainable fishing policies such as optimal periods for fishing or fishery closures and fishing sizes limitation can be applied to improve the overall sustainability of fish or crayfish

stocks. In this situation, reducing fishing pressure on target species and during certain times (e.g. reproductive seasons) would improve the sustainability of fisheries (Zhou et al., 2010) while protecting biodiversity. Up to now, efforts towards sustainable exploitation have focused on gear restrictions, size limits, closed areas and seasons (van Overzee and Rijnsdorp, 2015). The gear restrictions and size limits provide protection for juveniles, which is important for population growth and sustainability. Fishery closures during reproductive seasons is also an effective way to reduce the fishing mortality of the larger spawners and limits adverse effects on spawning habitats (van Overzee and Rijnsdorp, 2015). Furthermore, it allows female crayfish spawning and ensures sufficient juveniles supply for exploited populations.

To address these challenges, the reproductive ecology of cultured species should be studied more in details, because reproduction is one of the most important life-history parameters and better insights into the mechanisms determining the reproductive process in natural and controlled environments are needed. Actually, species often displayed considerable plasticity and variability in their reproductive seasons in various environmental conditions. For instance, *P. clarkii* spawns once (confined to autumn) in some locations such as Germany (Chucholl, 2011), Italy (Dörr et al., 2006), and UK (Richter, 2000) while there are two or more spawning periods yearly for *P. clarkii* in Kenya (Oluoch, 1990), Portugal (Sousa et al., 2013), Italy (Scalici and Gherardi, 2007), and Spain (Gutierrez-Yurrita and Montes, 1999; Gutierrez-Yurrita et al., 1999). In China, it spawns once yearly in Poyang lake (Xiao et al., 2011), Huangjin Lake (Gong et al., 2008; Lv, 2006), and Xuyi (Xu et al., 2014) while twice a year in Wuhan (Dai et al., 2008). Therefore, accurate scientific studies on the reproduction status of species in a given location and environment are needed to improve fishery management in a specific area.

Previous studies on spawning activities of many species have focused on limited areas and seasons, such as in shrimp *Aristeus antennatus* (Sardà and Castellón, 2003), crab *Chionoecetes bairdi* (Stevens, 2003), and reef fish (De Mitcheson et al., 2008). These studies showed that high fishing pressure during the reproductive season could have negative effects on reproductive potentials, and then influence long-term stock

productivity (Van Overzee & Rijnsdorp, 2015). Furthermore, fishing may also cause the death of offspring. Thus, restricting fishing pressure on spawning crayfish is an effective measure to enhance reproductive output and promote population productivity. In addition, specific catching of one sex could have detrimental effects. For some species such as crabs and crayfish, due to the low catch rates or reproductive activities of females, more male crayfish are selectively harvested during the reproductive periods. This males-directed selectivity may also impose adverse effects on reproductive output since it causes difficulty in females finding mates (Gray & Powell, 1966; Smith & Jamieson, 1991). Such sex selection could also change the sex ratio and population sizes (Rowe and Hutchings, 2003). A better way is to selectively catch the older and low growing ones. This would also offer more access to environmental resources (e.g., food availability) for juveniles and then may increase growth rates. However, no scientific studies tested this hypothesis. Thus, more efforts to assess the reproduction and population growth dynamics is now crucial to implement better fishery management (Fatemi et al., 2009; He et al., 2011).

In population dynamics studies, population parameters such as growth (growth coefficient *K*, growth parameter index *Ø'*), and mortalities (total mortality rate *Z*, natural mortality rate *M*, and fishing mortality rate *F*) have important implications for population assessment (Rochet et al., 2000). Estimates of these parameters provide fundamental information for predicting population growth and developing sustainable exploitation strategies (Nurul AminZafar & Halim, 2008; Ochwada-Doyle et al., 2014). Usually, parameters such as *K* and *Ø'* are used for evaluation of growth performance under a variety of environmental stresses such as under aquaculture conditions (Pauly, 1991; ŽivkovTrichkova & Raikova-Petrova, 1999). Quantitative assessment of mortality is a significant step to improve our understanding of population dynamics. *M* was defined as the mortality caused by all possible causes except fishing and it could be obtained from the values of *Z* minus *F* (Pauly, 1980). *M*, *Z*, and *F* are thus crucial parameters that are commonly used in fisheries assessment and management, but they are poorly known for most commercial species, including *P. clarkii* populations (Kenchington, 2014; Nadon et al., 2015; Williams et al., 2015).

Moreover, for successful fisheries management, it is necessary to further examine the exploitation states for different populations. The previous studies suggest that a value of 0.5 for E represents the optimum exploitation condition while a value of $E > 0.5$ points toward over-fishing (Gulland, 1971; Clasing et al., 1994). There is thus an urgent need to evaluate these population dynamics parameters and optimal reproductive seasons to develop effective and sustainable management strategies of commercial populations.

1.2 Crayfish culture

1.2.1 Crayfish culture practices in China

In China, many water bodies such as rivers, lakes, and ponds are used for crayfish aquaculture. Among these, pond culture is the main aquaculture practice in China (Fisheries Department of Ministry of Agriculture, 2017). Ponds surface has expanded quickly in recent years due to the large dem and of commercial markets. In many cases of pond culture, farmers use polyculture for crayfish and other fish species such as *Siniperca chuatsi*. The polyculture systems are based on the fact that these speices have different food preferences and habitat uses, which can ensure the optimal use of the food resources and spaces in the ponds. This case can also be found in crab culture. With this culture practice, farmers earn more profits while limiting production costs. Rice-crayfish culture in ponds is also a common culture practice in the south of China. For this practice, after the rice is harvested between September and October, ponds are drained and then used for crayfish culture (Wang et al., 2015). For ponds only devoted to culture crayfish, the annual farming practices involve pond preparation, macrophyte transplanting, eradication of other aquatic organisms before stocking, crayfish stocking, and feeding (Figure 1.1).

Figure 1.1. Picture of the pond that is used for culturing crayfish.

The first step before crayfish stocking is pond preparation. The ponds are drained and fully exposed to the sunlight for two weeks. Generally, during this period, quicklime at 15-22.5 grams / m^2 is used to prevent diseases. After preparation, submerged macrophyte *Hydrilla verticillata* is planted for providing supplementary foods, refuges for crayfish and maintaining water quality. One week before cultivation, the ponds are filled with water to a depth of approximately 30 cm. Normally, *H. verticillata* is planted evenly in the ponds at an interval of 0.5 m. Based on their growth, water depth is changed before stocking crayfish. Then, eradication of other aquatic organisms is generally carried out before stocking. The common methods used for eradication (such as silver carp, rice field eel, gold fish, and loach) are the quicklime or chlorine dioxide, and the amounts varied with water depth. The time for stocking is normally from March to April, with individual sizes ranged from 2-5g. The stocking density is 10-30 individuals / m^2. For feeding management, commercial diets are the main food source for crayfish. In addition, *H. verticillata* also serves as supplementary foods for crayfish. From March to May, in order to reach commercial sizes in a short period, artificial diets with high protein levels (normally 30% level of protein) will be used. The feeding rates normally differ from time, but in general about 3% of the biomass in the pond. However, most of these practices are not based on scientific studies, and many management techniques, such as feeding rates and

protein levels could be adjusted to improve the productivity and sustainability of crayfish pond culture. There is thus a need for experipmental studies comparing the effects of different environmental factors and feeding levels on crayfish growth to improve crayfish yields and limit production costs and environmental impacts.

1.2.2 Environmental factors affecting crayfish growth

1.2.2.1 Temperature, photoperiod and salinity

One of the main environmental factors affecting crayfish growth and reproduction is temperature. It influences crayfish molting, maturation, growth, and distribution (Westhoff and Rosenberger, 2016). Temperature plays important roles in metabolic processes, which can result in animal death if the temperature is out of the optimal ranges, while within a defined temperature range, crayfish growth rates increase with temperature (Bermudes and Ritar, 1999; Camus and Koutsikopoulos, 1984). Knowledge of crayfish thermal requirements is crucial to optimize their culture conditions and predict their distribution. Previous studies have demonstrated that the optimal growth temperatures were 23 — 26℃ for *P. clarkii* (Huner and Barr, 1984), 24 — 28℃ for *Orconectes nais* (Hellman, 1992), 26 — 28℃ for *Orconectes rusticus* (Mundahl and Benton, 1990), 20 — 25℃ for *Cherax destructor* and 25 — 28℃ for *Cherax quadricarinatus* (Verhoef et al., 1998), 16℃ for *Paranephrops zealandicus* (Hammond et al., 2006), 23 — 25℃ for *Astacus leptodactulus* (Hesni et al., 2009), 20 — 26℃ *Pacifastacus leniusculus* and 20 — 26℃ for *Orconectes limosus* (Simčič et al., 2014). However, basic knowledge of how reproductive performance and embryonic development of crayfish repond to water temperature changes still remains unknown.

Besides temperature, photoperiod is also an important environmental factor that affects aquatic animals growth, cannibalism, and reproduction (Harlıoğlu and Farhadi, 2017). It has direct influences on animals growth rates, for instance in prawn *Penaeus merguiensis* (Hoang et al., 2003), and fish *Oplegnathus fasciatus* (Biswas et al., 2008). Cannibalism behaviors can be found in many crustacean species and have strong impacts on crayfish survival especially during molting periods. Higher or shorter light periods could exacerbate the cannibalism among crabs such as *Ranina ranina*

(Minagawa, 1994). Silmilar cases could also be found in the zoea stage of Australian giant crab *Pseudocarcinus gigas*. However, longer light periods improved the survival of spiny lobster *Panulirus japonics* (Matsuda et al., 2012), early phyllosoma of *Sagmariasus verreauxi* (Fitzgibbon and Battaglene, 2012), and blue swimmer crab *P. pelegicus* (Andrés et al., 2010). Some species require more light during reproductive seasons for ovarian maturation and spawning such as *P. clarkii* (optimal light-dark of 14:10h) (Daniels et al., 1994) while some species need a decrease in light such as *Astacus leptodactylus*. It's reproductive performance has been proved to be highest at constant darkness (Harlıoğlu and Barım, 2004). Similarly, fast ovarian maturation and higher spawning rates were also observed in *Procambarus llamasi* at complete darkness (Carmona-Osalde et al., 2002).

Salinity is also an important environmental factor determining survival, distribution, and reproduction of aquatic animals. Many crayfish are highly tolerant of the various environment, while they are limited in distribution because of the less tolerance to salinity. Most crayfish can survive in saline water for a short period, while long time exposure to high salinity will have adverse effects on growth. For instance, juveniles *P. clarkii* growth and reproduction were proved to be significantly affected when salinity was above 5 g/L (Meineri et al., 2014). Spinycheek crayfish *Orconectes limosus* failed to successfully reproduce and grow when salinity is above 7 ppt (Jaszczołt and Szaniawska, 2011). Similarly, for signal crayfish *Pacifastacus leniusculus* and narrow-clawed crayfish *Astacus leptodactylus*, eggs could not survive at salinity higher 14 ppt (Holdich et al., 1997).

1.2.2.2 Feed and nutrition

For crayfish intensive aquaculture, the production relies heavily on the input of artificial diets, which have accounted for more than 50% of total aquaculture costs (Keckeis and Schiemer, 1992; Wong et al., 2016). In addition, an excessive amount of artificial diet results in wastes that can induce pollution. Indeed, most aquaculture wastes were ultimately from dietary inputs, especially from high protein levels diets, containing nutrients and numerous organic compounds (e.g. ammonium, phosphorus, dissolved organic carbon, and organic matter) (Cho and Bureau, 2001; Crab et al.,

2007). This high organic and nutrient loadings result in pathogenic microorganisms occurrence, and in fish or crayfish hypoxia or even death (Chávez-Crooker and Obreque-Contreras, 2010), but can also lead to water pollution and economic loss (Chávez-Crooker and Obreque-Contreras, 2010; Craig et al., 2017; Henry and Fountoulaki, 2014; Martinez-Cordova et al., 2003; Velazco-Vargas et al., 2014).

Optimal dietary protein requirements were relatively well investigated for juvenile *P. clarkii* under laboratory-controlled conditions, which confirmed that optimal dietary protein levels were 24%-30% (Hai and Jie, 2012; Jover et al., 1999; Ling et al., 2012; Wu et al., 2007; Xu et al., 2013; Zhang et al., 2012) but the results from these studies could not be fully applied to pond culture conditions since many cultured organisms also derive a substantial part of nutrition from natural foods. This is particularly true for *P. clarkii*, which is capable of feeding various natural foods (e.g. macrophytes, detritus, periphyton, benthos, plankton, and microbially enriched detritus) (Alcorlo et al., 2004; Correia, 2003; Gutierrez-Yurrita et al., 1998) while little information exists concerning their dietary protein requirements under practical pond farmimg conditions where natural foods also contribute to crayfish growth. Therefore, efficiently managing the input of artificial diets and natural foods in ponds is crucial for sustainable aquaculture (Bostock et al., 2010; Bureau and Hua, 2010). This could also help to minimize feed and production costs while maintaining aquaculture production and environmental capacity to a sustainable level (Cho and Bureau, 2001).

1.3 Study species: red swamp crayfish

Among commercially farmed species, the red swamp crayfish *Procambarus clarkii* (Girard, 1852), was the second most produced species accounting for 12 % of the total crustaceans aquaculture production (FAO Yearbook, 2018). *P. clarkii*, originating from northeastern Mexico and the south-central United States, has been introduced into Nanjing, China from Japan since the late 1930s (Henttonen and Huner, 1999; Hobbs et al., 1989; Shu and Ye, 1989). It displays numerous biological traits that make it suitable for aquaculture such as short life cycles and rapid growth, and high tolerance to poor environment conditions (Cruz and Rebelo, 2007), which makes

it popular among farmers. Now it has been cultured in most provinces of China (Fisheries Department of the Chinese Ministry of Agriculture, 2017). With the fast expansion of culture areas, juveniles provided by spontaneous reproduction do not match the growing demanding of the whole aquaculture industry. Thus, it's urgent to explore effective artificial reproduction techniques to provide mass production of high quality juveniles to support sustainable aquaculture. It is thus a prerequisite to have a better understanding of the reproductive biology and growth of this species, to recommend new innovative techniques for sustainable fishery management.

The crayfish life cycle started from embryonic development and completed when crayfish spawned. Their life cycle involved three stages: (1) embryonic development; (2) grow-out of juvenile crayfish; (3) maturation and spawning of crayfish.

1.3.1 Embryonic development

After spawning, the embryos are attached to the female's pleopods and the embryonic development occurrs. However, for embryonic developmental stages, authors have different calssifications and results. For instance, it is devided into six stages: fertilized egg, cleavage and blastrula, gastrula, egg nauplius, eye pigment forming, and preparation for hatching (Dai et al., 2009). However, more specific staging scheme for *P. clarkii* were described by a previous study, dividing the embryonic development into 19 stages according to numerous morpgological characteristics such as cleavage, semi furrow, thoracic-abdominal processes, tail shapes,entennules, optix fossae, appendages, walking legs, heart, and eyes (Harper and Reiber, 2006). In China, several scholars devided the development into 9 stages: fertilized eggs, cleavage stage, blastula stage, gastrula stage, egg-nauplius stage, egg-matanauplius stage, eye pigment stage, prehatching stage and hatching stage (Feng et al., 2007) while others devided the embryonic development into twelve stages: fertilized eggs, cleavage stage, blastula stage, pregastrula stage, semi furrow stage, later gastrula stage, prenauplius stage, later nauplius stage, prezoea stage, zoea stage, and later zoea stage (Jianlin et al., 2006; Xiaoqing et al., 2009).

In this study, we synchronized the previous studies and divided embryonic development into 9 stages which was shown in Fig 1.2: I, zygote; II, cleavage; III,

blastula; IV, semicircular furrow; V, circular furrow; VI, gastrula; VII, nauplius; VIII, zoea; and IX, hatching. Within a few hours after spawing, the fertilized eggs were full of yolk and looked round in shapes. Then superficial cleavage occurred and embryos developed into the blastrula stage. The cleavage continued and blastrula invaginated into semi furrow and the furrow became circular in shape latter. Then embryos developed into the grastrula stage, the sign of this stage is the visible round hole due to invagination. We can also see the transparent area in this stage. Next, the transparent area expanded and antennae and the mandible developed, which means the embryos developed into nauplius stage. The development progressed with heart and eyes starting to develop in the zoea stage. Before hatching, the cephalothorax and abdomen were distinguished and the appendages gradually developed. After hatching, the basic shapes of crayfish were visible and the cephalothorax and abdomen were distinguishable. Although hatching from eggs, embryos still attached to females abdomen (Fig. 1.2).

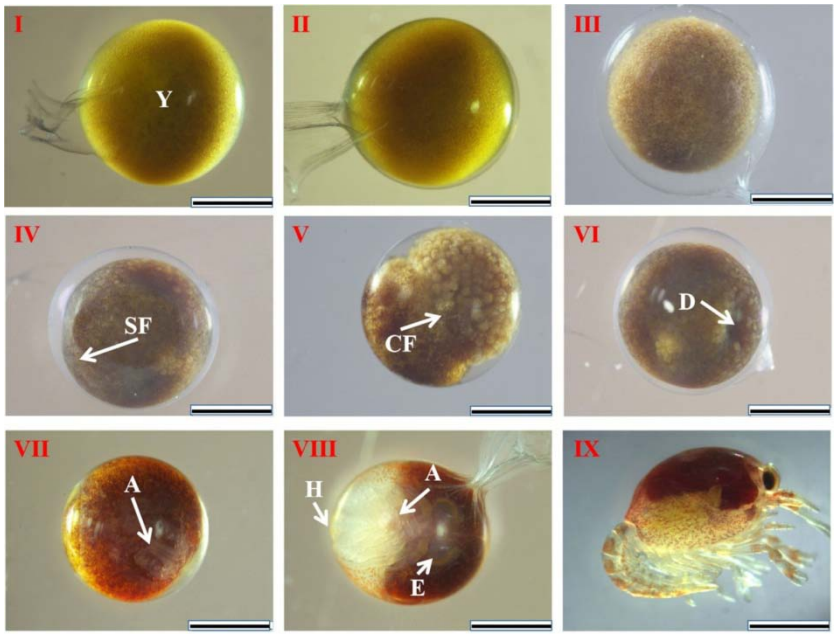

Figure 1.2. Morphological development of *Procambarus clarkii* embryos, being classified into nine stages. I, zygote with full of yolk (Y); II, cleavage; III, blastula; IV, semicircular furrow; V, circular furrow; VI, gastrula with dent visible (D); VII, nauplius with appearance of appendages (A); VIII, zoea showing the heart region (H), a pair of round eyes (E), appendages (A) and enlarged transparent area; IX, hatching.

1.3.2 Grow-out of juvenile crayfish

The juvenile crayfish growth has been devided into 13 stages. After hatching, juvenile *P. clarkii* left from the abdomen of females. For stage I, most of the body is transparent and two round eyes are visible. The cephalothorax has a black dorsal hump and has naked telson setae without uropods. For stage II, eyes have dark pigment and red dots cover the entire body. In the two stages, juveniles still remain attached to the abdomen of females. For stage III, telson and uropods are separated with bristles. For stage IV, pigmentation expands over the entire body. In stage V, embryos eyes are fully developed and the body is greenish. For stages VI to IX, body color darked and most juveniles can move independently. Juveniles in stages X start to show sexual dimorphism. The first and second pleopodes of male crayfish modified to gonopodes while females have similar five pleopodes. Suficient understanding of *P. clarkii* life cycles will allow us to optimize culture conditions ensuring better their growth and promising organisms in aquaculture.

1.3.3 Maturation and spawning of crayfish

1.3.3.1 Reproductive system of *P. clarkii*

Numerous studies have been conducted on the reproductive system of female *P. clarkii*, such as morphology, ovarian development, and vitellogenesis (Ando and Makioka, 1998; Carmona-Osalde et al., 2004; Daniels et al., 1994). The female reproductive system is composed of the ovary and oviducts (Ando and Makioka, 1998). The ovary is Y-shaped, consisting of a pair of anterior ovarian sacs with a single median posterior ovarian sac located in the cephalothorax, on the dorsal side of the stomach. The ovary of young crayfish contains several white oocytes (previtellogenic and early vitellogenic, normally less than 1 mm in diameter) while adult crayfish ovary contains yellow to dark orange oocytes, which are from 1 to 1.4 mm in diameter. Before oviposition, the ovary contains several hundred matured eggs, which are dark and 1.8 mm in diameter. According to morphological characteristics, the ovarian development of *P. clarkii* was classified into 7 stages: stageI (oogonial), stage II (immature), stage III (avitellogenic), stage IV (early vitellogenic), stage V (midvitellogenic), stageVI (late vitellogenic) , and stageVII (postvitellogenic and

resorptive) (Kulkarni et al., 1991). For stage I, oogonia are close to the ventral wall of the ovary. They are spherical and cytoplasm is narrow and weakly basophilic. For stage II, the oocyte membrane is not well defined and the oocytes are often surrounded by ovarian stromatal tissue. The nucleus is centrally located with a well-defined nuclear membrane. There are no follicle cells around the oocytes. Most oocytes in stage III are round, while only several oocytes are elliptical. The nucleus is usually central-located and oocytes often have a well-defined membrane while no yolk granules are observed. In this stage, chromatin is apparent in the nucleus and one nucleolus is next to the nuclear membrane. The follicle cells occur around the oocyte membrane. For stage IV, few yolk granules occur in oocytes and it has a centrally located nucleus. Generally, two or three nucleoli can be seen next to the nuclear membrane. Oocytes in stage V contain a large amount of yolk. There are three or four chromatin in the nucleus and nucleoli are next to the nuclear membrane. Most oocytes lose their round shape and appear squarish in stage VI. The nucleus is not always in the center. In stage VII, oocytes and nuclei appear degenerative. Follicle cells are less tightly bound to each other. Most yolk granules disappear while follicle cells are still present, decreasing in sizes.

The male *P. clarkii* reproductive system includes paired testes and sperm ducts. The process of spermiogenesis is divided into six stages: stage I (early spermatid), stage II (acrosomal granule), stage III (acrosomal cap), stage IV (biconcave nucleus), stage V (immature sperm), and stage VI (mature sperm) (Moses, 1961). For stage I, the nucleus, sausage-shaped, makes up of one-third of the cell. The most striking characteristics of this stage is the blebbing of the nuclear surface. For stage II, the nucleus shrinks and increases in density. The blebs are replaced by sheets of membrane closely associated with the nuclear surface and a large vesicle including an acrosomal granule is set off. The stage III is characterized by further elaboration of membrane sheets around the nucleus and redistribution of the material in the acrosomal granule to form a cap. Furthermore, exclusion of the remaining cytoplasmic material and delineation of a new cell periphery are observed. For stage IV, the nucleus is a biconcave disc and the membrane elaborations have consolidated

into larger sheets which are clearly extensions of the entire nuclear envelope. Slender filaments extend into the acrosomal vesicle from the dense acrosomal cap, which has begun to invaginate. The shape of the spermatid at this stage has changed slightly from the preceding one: whereas the Stage III cells tended to be a flattened sphere, with elliptical profile perpendicular to the equator, the Stage IV cells are almost circular in profile in both equatorial and polar planes. In stage V, the entire cells are surrounded by a complex, membranous integument and the nucleus has begun to extend itself radially in four directions to initiate the long processes that characterize the mature sperm and the organization of the nuclear contents has changed markedly. The invaginated acrosomal complex has undergone further structural differentiation. The nucleus is still essentially biconcave. Its contours are highly irregular, largely owing to the fact that the continuity of the nuclear envelope and the membrane sheets have become very pronounced. In stageVI, sperm is mature and most sperm appears in the testis and ducts. However, for commercially cultured crayfish, we still have limited knowledge of how their gonads develop and when they reproduce, especially in China.

1.3.3.2 Mating behavior and spawning activity

Normally, prior to mating, male and female crayfish occupy shelters for a period of time. During the mating phrase, when female crayfish approaches, their chelae contact and males arches the abdomen underneath and then turns over backward pushed by the female (Sammy, 1988). Then they kept this posture for several minutes. During this period, male *P. clarkii* deposits spermatophore into females' seminal receptacle. The mating behavior is ended by females disengaging while the male rolls over to keep an upright position (Barki and Karplus, 1999; Corotto et al., 1999).

Spawning occurs several days to months after mating. Even if female crayfish are mature, spawning cannot occur immediately until all the environmental conditions (e.g. temperature and nutrition) are optimal, which is possible because they can conserve male spermatophore for several months (Carmona-Osalde et al., 2004; Gutierrez-Yurrita and Montes, 1999). In China, the spawning activities peak from July to August (Xiao et al., 2011, Lv, 2006; Gong et al., 2008, Xu et al., 2014). In

other countries, some authors reported that the most spawning events of *P. clarkii* confined to autumn such as USA (Oluoch, 1990), Germany (Chucholl, 2011), and Italy (Dörr et al., 2006). While others argued that there existed two or more spawning periods yearly for *P. clarkii* in Portugal (Sousa et al., 2013), Italy (Scalici & Gherardi, 2007), Kenya and Spain (Gutierrez-Yurrita et al., 1999; Gutierrez-Yurrita & Montes, 1999). Thus, more data are needed on population and reproduction dynamics across years in Chinese commercial ponds to optimize catching seasons and environmental factors to induce synchronous spawning activities of female *P. clarkii*.

1.4 Current artificial reproduction techniques of crayfish

Commercial production of cultured species depends largely on larvae production, especially in crustaceans. Currently, most larvae in aquaculture are obtained from spontaneous reproduction in the wild which are limited by seasonal availability. This hinders the development of crustacean industry, especially crayfish (Smith et al., 2002). To maintain stable populations while obtaining reliable supplies of larvae for aquaculture, we need to develop innovative and sustainable artificial reproduction techniques. This would be a crucial step to meet the demands of commercial production and improve the sustainability of crayfish culture (Liu et al., 2013). One of the main obstacles for effective artificial reproduction is the difficulty or impossibility of hatching and feeding larvae, such as in the aquaculture of eel, yellowtail, and halibut (Gjerde, 1986), but also in crustaceans including crayfish.

1.4.1 Factors regulating crustacean reproduction

Normally, gonad maturation of crustacean species is regulated by two antagonistic neuropeptides: gonad inhibiting hormones (GIH) and gonad stimulating factor (GSF). GIH is secreted from the X-organ–sinus gland (XO–SG) located in the eyestalk while GSF is produced by the brain and thoracic ganglion (Eastman-Reks and Fingerman, 1984). GIH was responsible for inhibiting secondary vitellogenesis and it targeted at ovaries (LaFont, 2000). They also proposed that GIH may be a central modulator of the production or release of hormones involved in molting as well as reproduction. In crabs, GSF contents vary among different reproductive stages but there are still lacking sufficiency studies in how it affects metabolic functions in

crustacean species (Eastman-Reks and Fingerman, 1984). Crustacean hyperglycemic hormone (CHH) from the eyestalk and molt inhibiting hormone (MIH) was also proved to affect the ovarian maturation in shrimp *Penaeus semisulcatus* and *Metapenaeus ensis* (Fanjul-Moles, 2006; Gu et al., 2002). In addition to these hormones, steroids have also been proved to influence the vitellogenesis of crustacean species such as *Scylla serrata*, *Penaeus monodon*, and penaeid shrimp (Quinitio et al., 1994; Warrier et al., 2001; Yano et al., 2000). The progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, 6β-hydroxyprogesterone, 17β-estradiol, estrone and testosterone are the main steroids regulating the reproductive process. Mammalian hormones such as human chorionic gonadotrophin (HCG) and 5-HT hormones also influenced crustacean species reproduction (Nagaraju, 2011). As a consequence, hormonal manipulation has been widely used to induce spawning in fish and crustaceans. Furthermore, environmental factors could also affect crustacean reproduction.

Environmental factors such as temperature, salinity, photoperiod, and nutrition could theoretically also affect the reproductive process of crustacean species. Temperature plays a particulary central role. For instance, previous studies found that temperatures of 16 – 18 ℃ could significantly induce spawning of other species such as *Penaeus semisulcatus* (Aktaş et al., 2003), *Cherax quadricarinatus* (Tropea et al., 2010), *Astacus astacus* (Huner and Lindqvist, 1985), *Panulirus japonicus* (Matsuda et al., 2002), *Procambarus llamasi* (Carmona-Osalde et al., 2004) and *Penaeus stylirostris* (Robertson et al., 1991). For female *P. clarkii*, studies also showed that low temperature of 16-22 ℃ could also significantly induce spawning activities (Liu et al., 2013a), but very few experipmental studies are available on temperature effects in crayfish and more data are now needed.

1.4.2 Current artificial reproduction techniques

The techniques for crustacean artificial reproduction have been studied for a long time. The reproduction of crustaceans is controlled by GIH and GSF. A major source of GIH is from the XO-SG, which is located in the eyestalk. So the traditional technique in artificial reproduction to accelerate spawning activities in crustaceans is

eyestalk ablation, which has been extensively used worldwide (Aktaş and Kumlu, 1999; Browdy, 1992; Browdy and Samocha, 1985; Lumare, 1979; Muthu and Laxminarayana, 1977; Wen et al., 2015). However, this technique often leads to the death and permanent damage of females as well as the decline of larval quality (Makinouchi and Honculada-Primavera, 1987). The other method is injecting with various hormones to induce females to reproduce spontaneously under proper conditions. Lots of studies have conducted on hormonal control of reproduction in crustacean species such as crayfish, shrimp, and crab (Nagaraju, 2011). The commonly used steroids for crayfish artificial reproduction are 17 α-hydroxyprogesterone and progesterone. Other hormones used to induce spermiation and ovulation are serotonin (5-hydroxytryptamine) human chorionic gonadotropin (HCG), and domperidone (Wongprasert et al., 2006; Yano, 1985). However, this technique often causes high labor costs and endocrine problems, and potentially ethical problems and animals suffering. Numerous studies have demonstrated that hormones injection and eyestalk ablation compromised survival (from 15.56% to 51.11%). Such cases could also be found in other crustaceans, such as *Penaeus monodon*, *Penaeus vannamei*, and *Macrobrachium rosenbergii* (Vaca and Alfaro, 2000; Wei and Zhao, 1992; Wen et al., 2009). There is thus now an urgent need to find new techniques to massively produce high quality larvae in optimal artificial conditions while ensuring animal welfare.

Environmental factors such as water temperature, salinity, and nutrition play vital roles in regulating species reproductive processes such as ovarian development, mating, spawning, embryogenesis, and hatching. In particular, the temperature is the main central factor regulating these processes (Pankhurst and Munday, 2011; Planas et al., 2012). Thus, optimizing temperature in culture conditions to trigger reproduction and ensure optimal embryonic development would be an alternative to traditional artificial reproduction techniques. For other crustacean species such as *Penaeus semisulcatus* (Aktaş et al., 2003), *Cherax quadricarinatus* (Tropea et al., 2010), *Astacus astacus* (Huner and Lindqvist, 1985), *Panulirus japonicus* (Matsuda et al., 2002), *Procambarus llamasi* (Carmona-Osalde et al., 2004) and *Penaeus*

stylirostris (Robertson et al., 1991), previous studies reported that temperatures of 16 – 18 ℃ could significantly induce their reproduction. However, thermal effects on the reproduction of *P. clarkii* still remain to be determined. Such studies would be an important prerequisite for the development of effective artificial reproduction techniques for *P. clarkii*.

1.5 Questions and objectives of the thesis

There is an urgent need for better management practices to reach sustainable exploitation of *P. clarkii* commercial populations, but the information concerning reproduction and population dynamics of this species, as well as the environmental factors (especially temperature and feeding rates) affecting reproduction and adult and larvae growth is still limited. Indeed, one of the biggest challenges for crayfish aquaculture is the supply of juveniles for the *P. clarkii* aquaculture sectors. Therefore, it is necessary to optimize artificial reproduction techniques and explore more efficient feeding strategies for *P. clarkii* aquaculture. To address these questiones, we conducted a survey to determine the reproduction pattern and population dynamics for sustainable fishery management; and three experiments to (1) optimize culture conditions (water temperature) to improve reproductive performance and embryonic development; (2) evaluate optimal feeding levels for juvenile crayfish culture by maximizing the contribution of natural foods; (3) explore scientific dietary protein levels for juvenile crayfish culture by reducing the amounts and high protein inputs of artificial diet (Figure 1.3).

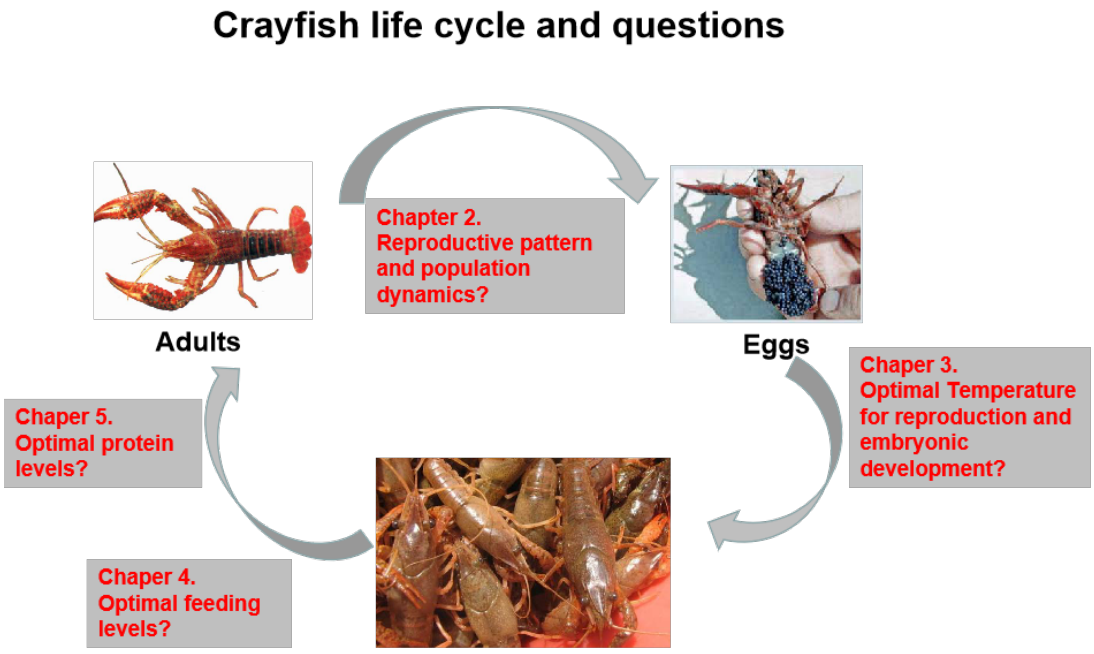

Figure 1.3. The framework of red swamp crayfish life cycle and questions of the thesis. For this thesis, I first conducted a survey on crayfish reproductive pattern and population dynamics in a commercial pond. Then based on this knowledge, I try to optimize culture conditions to induce the spawning activities of female crayfish and improve embryonic development. Next, I conducted two feeding experiments on juveniles and aimed at reducing production costs.

Chapter 2

General Methodology

2.1 Study area or culture conditions

2.1.1 Reproductive pattern and population growth dynamics

The sampling activities are carried out in the Selection and Reproduction Center of Crayfish (30.41 °N, 112.75 °E), Qianjiang, which is recognized as the land of red swamp crayfish in China by the Ministry of Agriculture of the People's Republic of China. This region extends over 200 ha and encloses many artificial ponds. The studied area has a surface area of 33,350 m^2, which is under good management and is referred as the model of crayfish culture. In this area was planted *Hydrilla verticillata*, preferred by *P. clarkii* and tolerant to high water temperatures in summer. During the sampling period, the annual mean water temperature was 19.75 ℃, ranging from 8.65 ℃ in January to 31.25 ℃ in August. The water depth was 1–1.5 m. Other water physical-chemical parameters during the sampling periods were: pH 8.61–9.30; ammonia nitrogen 0.14–0.43 mg / L; nitrite 0.15–0.25 mg / L; total nitrogen 1.06 ± 1.67 mg / L; total phosphorus 0.0445 ± 0.17 mg / L; chemical oxygen demand (to quantify the amount of oxidizable pollutants in ponds) 5.83–8.80 mg / L; chlorophyll-a 14.55–31.67 μg / L.

2.1.2 Reproductive performance and embryonic development

This study was conducted in State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. Adult crayfish (weight: 31.03 ± 1.95 g, total length: 105.41 ± 1.20 mm, mean ± SE) used in experiments were collected during the peak of ovarian maturation from the Selection and Reproduction Center of Crayfish (30.41 °N, 112.75 °E), Qianjiang, China. After transportation to the laboratory, crayfish were randomly paired and each paired crayfish (one male crayfish and one female crayfish) was kept separately in a tank (35×30×25 cm). In the beginning, all crayfish were reared under the same temperature conditions (23 ℃) in the five independent closed recirculation systems, and then water temperature was adjusted gradually at a rate of 1 ℃ per day until the experimental temperature was reached and then maintained thereafter (17 ℃, 21 ℃, 25 ℃, 29 ℃, and 33 ℃). Each tank served as an independent replicated experimental unit. In each tank, PVC pipes were provided for shelters of

crayfish (four pipes in each large tanks and one pipe in each small tank). Tap water with ultraviolet sterilization and aeration for chlorine elimination was delivered to each tank at a constant rate of 1 L/min during the study. Photoperiod was maintained at a 12:12 (light: dark) cycle. Water temperature was recorded every 2h with data loggers over the duration of the experiment. The pH, dissolved oxygen, and hardness were measured daily by a YSI probe (Yellow Springs Instruments, Yellow Springs, OH, USA). The concentration of ammonia nitrogen was determined using standard methods (APHA et al., 1989). Water quality parameters during the whole experiment were within the suitable ranges: dissolved oxygen 5.60 ± 0.9 mg/L, pH 7.12 ± 0.21, hardness 125 ± 7 mg/L, and ammonia nitrogen 0.54 ± 0.13 mg/L.

2.1.3 Effects of feeding levels on growth and muscle composition

The experiment was conducted in 15 concrete ponds (Fig. 2.1, 90 juveniles per pond of 9 m^2) at the Selection and Reproduction Center of Crayfish, Qianjiang, Hubei Province, China. The running water flow rate in ponds was approximately 7 L/min, and constant aeration was supplied to each pond. Water depth was maintained at approximately 27 cm. *H. verticillata* was planted in 35 polyethylene flowerpots (0.44 m diameter) in each pond and used as both shelters and foods for *P. clarkii*. The coverage of *H. verticillata* was 60% in each pond. The water temperature, pH, and dissolved oxygen (DO) were measured by a YSI probe (Yellow Springs Instruments, Yellow Springs, OH, USA). The concentrations of ammonia nitrogen, nitrite, chemical oxygen demand, total nitrogen, total phosphorus and chlorophyll-a were determined using standard methods (APHA, 1992). Water quality parameters for all ponds (mean ± SE) were within the ranges of crayfish growth throughout the study: temperature 27.27 ± 1.06 ℃; DO 4.33 ± 0.70 mg/L; pH 9.3 ± 0.05; ammonia nitrogen 0.1400 ± 0.005 mg/L; nitrite 0.0472 ± 0.006 mg/L; total nitrogen 1.0609 ± 0.020 mg/L; total phosphorus 0.0445 ± 0.003 mg/L; chemical oxygen demand 8.8048 ± 0.100 mg/L; and chlorophyll-a 14.5477 ± 0.340 μg/L.

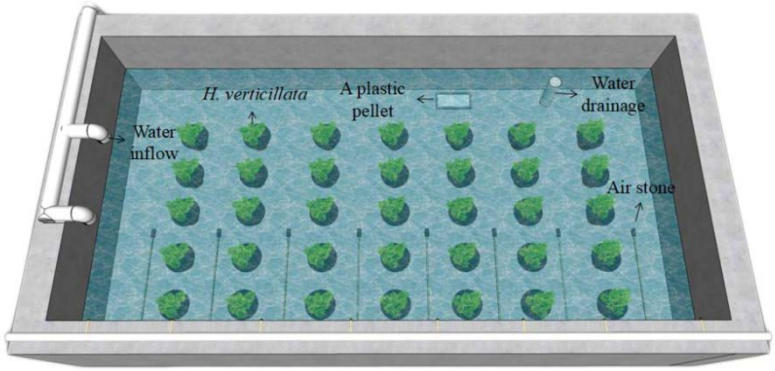

Figure 2.1. Diagram of the ponds used for culturing juvenile *Procambarus clarkii* during the experiment.

2.1.4 Effects of protein levels on growth and muscle composition

The experiment was conducted in 8 cconcrete ponds (Fig. 2.1, 90 juveniles per pond of 9 m^2) at the Selection and Reproduction Center of Crayfish, Qianjiang, Hubei Province, China. Other culture environment was same with 2.1.3.

2.2 Experimental design

2.2.1 Reproductive pattern and population growth dynamics

During the sampling period, we collected crayfish monthly from March 2016 to February 2017 with 8 cylindrical traps baited with fresh silver carp. The traps were 100 cm long with 5 mm mesh, 30 cm cross-section, and two opposing funnels 10 cm in diameter. For each sampling, trapping was performed and retrieved in the afternoon. The periods of trapping were one day from June to September; two days from March to May, and October; and three days from November to February. The same sampling site order and timetable were followed every month in order to minimize the bias in measurement. During the whole sampling period, water temperature was recorded every two hours by a HOBO data-logger (UA-002-64, HOBO Pendant temperature / light 64K data logger, Onset company, America).

For each sampling, catch per unit effort (CPUE) was calculated for each sampling as the daily number of crayfish per trap. All samples were then transported to the laboratory to dissect. After transportation to the lab, females were checked for attached eggs, if present, they were counted to determine the fecundity. The sampled crayfish were sorted by sex. Cephalothorax length (CTL, from the tip of the rostrum

to the cephalothorax posterior margin) was measured by a 0.01 mm precision caliper. Crayfish weight was determined by a 0.01g precision scale. The gonads and hepatopancreas of females were weighted to calculate the gonadosomatic index (GSI) (measuring the sexual maturity and relating to ovary development) and hepatosomatic index (HSI).

2.2.2 Reproductive performance of *P. clarkii*

The experiment was designed to evaluate the optimal temperature for the reproductive performance of *P. clarkii*. It was conducted from September to October 2017 for 50 days under five constant temperatures (17 ℃, 21 ℃, 25 ℃, 29 ℃, and 33 ℃), with 12 replicates of each treatment (total N = 480, 240 females and 240 males). Each replicate consisted of four paired female and male crayfish. The crayfish were fed twice daily with an artificial diet purchased from Charoen Pokphand Group (WHS001-2016, 30.23% protein, 10.74 % lipid, 10.18% moisture, and 8.70% ash). All crayfish were checked every day so that their mortality, accurate dates for mating and spawning could be determined. Tanks were cleaned every day.

At the beginning and the end of the experiment, crayfish weight was determined by a 0.01g precision scale. Feeding rates were measured following the methods described in a previous study (Vam Ham et al., 2003). Specifically, crayfish were fed with an excess quantity of weighted artificial diet until feeding activities stopped within one hour. Then, the remaining artificial diet was removed, dried and reweighted. Finally, we determined the given amount of artificial diet to calculate the feeding rates. The duration from mating to spawning was calculated as the number of days from mating to spawning. After spawning, all eggs were counted to determine the fecundity of female *P. clarkii*.

2.2.3 Embryonic development

This experiment was designed to determine the optimal temperature for embryonic development. It was conducted from September to December 2017 at 17 ℃, 21 ℃, 25 ℃, 29 ℃, and 33 ℃. Once females spawning, the eggs were sampled for monitoring embryonic development. There were 12 replicates in each temperature treatment (total N=60 for females). Eggs from the same ovigerous female

crayfish served as an independent replicate. Ovigerous crayfish rearing methods were identical to those for experiment 1. The experiment was terminated when all eggs in all replicates hatched.

More than 10 eggs at each treatment were collected for each sampling to determine embryos developmental stages under the dissecting microscope LEICA MVX10 (M205FA). Photographs were taken on, which was subsequently projected for calculating the various stages of development. During the 36 hours of spawning, eggs were examed every 2h and thereafter, daily until hatching. The embryonic development was classified into 9 stages according to previous studies (Dai et al., 2009; Feng et al., 2007; Harper and Reiber, 2006; Xiaoqing et al., 2009): I, zygote; II, cleavage; III, blastula; IV, semicircular furrow; V, circular furrow; VI, gastrula; VII, nauplius; VIII, zoea; and IX, hatching. The duration of development for each stage was recorded. The end of each stage was defined as the time at which 50% of the embryos sampled had passed into the next stage. This index is often chosen to compare embryonic development when different numbers of eggs are sampled in different studies (Geffen et al., 2006; Webb et al., 2007; Yang and Chen, 2005).

2.2.4 Effects of feeding levels on growth and muscle composition

Crayfish were exposed to five feeding treatments (20%, 40%, 60%, 80%, and 100% satiation) following the method described in the previous study (Vam Ham et al., 2003). Throughout the experiment, crayfish were fed twice daily (8:00 and 18:00) with a widely used artificial diet. The experimental diet (26% protein level, based on previous nutritive studies) followed a common commercial diet formulation (WHS001-2016) from Charoen Pokphand Group (Jover, et al., 1999; McClain, 1995) . Ingredients and proximate analysis of the diet are presented in Table 2.1.

Table 2.1 Ingredient composition and proximate analysis of experimental diet.

Ingredients	Diet (%)
Fish meal[a]	0.5
Rapeseed meal[b]	14
Soybean meal[c]	3
Cottonseed meal[d]	10
Wheat flour[e]	20
Rice bran[f]	8
DDGS[g]	18
Corn gluten[h]	15
Soybean oil[i]	2
Vitamin premix[j]	0.1
Mineral premix[k]	0.5
$Ca(H_2PO_4)_2$	0.5
Sodium chloride	1
Cellulose	3.9
Binder	3.5
Proximate composition	
Crude protein	26.53
Crude lipid	10.41
Ash	6.87
Moisture	13.96

[a] Fish meal was from Qingdao Great Seven Co., Ltd., Shandong, China.
[b, c, d] Rapeseed meal, soybean meal, and cottonseed meal were purchased from Jiangxi Zhengbang Tech, Jiangxi, China.
[e, f, g, h] Wheat flour, rice bran, DDGS, and corn gluten were from Wuhan Yufeng Cereals, Oils and Foodstuffs Industrial, Hubei, China.
[i] Soybean oil was from Handan Mingfu Vegetable Oil Company, Hebei, China.
[j, k] Vitamin and mineral premix were purchased from Haid Feeds Co., Ltd., Guangzhou, China.

A plastic pallet (30 × 15 cm) was placed at the bottom of each pond, and the artificial diet was placed on it (Fig. 2.1). The reference 100% satiation level was determined by feeding crayfish excess weighted artificial diet until feeding activity stopped within one hour. Then, the remaining artificial diet was removed, dried and reweighted (Vam Ham et al., 2003). We then calculated the amount of artificial diet that was consumed by crayfish under 100% satiation. *P. clarkii* in other treatments were then fed at restricted levels of 80%, 60%, 40%, and 20%, which were adjusted daily with reference to 100% satiation. At 100% satiation level, the given amount of

artificial diet was approximately 5% of the wet body weight per day (2% at 8:00 and 3% at 18:00). The experiment ended after 50 days, when the majority of the males achieved a non-growing, sexually active form.

2.2.5 Effects of protein levels on growth and muscle composition

Crayfish were exposed to two protein treatments (26% and 30% protein levels) following the same management described in 2.3. Ingredients and proximate analysis of the diet are presented in Table 2.2.

Table 2.2 Formulation and chemical composition of two artificial diets for the experiment (% dry matter)

Ingredients	Content (%)	
	26% protein level diet	30% protein level diet
Fish meal	0.5	2
Rapeseed meal	14	10
Soybean meal	3	25
Cottonseed meal	10	11
Wheat flour	20	20
Rice bran	8	
DDGS	18	20
Corn gluten	15	
Soybean oil	2	2
Vitamin premix	0.1	0.1
Mineral premix	0.5	0.4
$Ca(H_2PO_4)_2$	0.5	0.5
Sodium chloride	1	1
Cellulose	3.9	4
Binder	3.5	4
Proximate composition		
Crude protein	26.53	30.23
Crude lipid	10.41	10.74
Ash	6.87	8.7
Moisture	13.96	10.18

2.3 Parameters measurement, calculation and analyses

2.3.1 Reproductive pattern and population growth dynamics

The gonadosomatic index (GSI) (measuring the sexual maturity and relating to ovary development) and hepatosomatic index (HSI) (indice of energy status):

$GSI=100\times W_g / W_t$

$HSI = 100 \times W_h / W_t$

Where W_g, W_h, and W_t are the gonad weight, hepatopancreas weight and body weight of *P. clarkii*, respectively.

Dissected gonads were then fixed for 24 h in Bouin's solution (Wuhan Servicebio technology company) for histological analysis. Samples were dehydrated in 50%, 70%, 85%, 90%, 95%, and 100% ethanol and embedded in paraffin block. Then they were subjected to microtomy to obtain sections with 4 μm (Leica RM2016, USA). Slides were deparaffinized (2 changes of xylene, 20 min each; 3 changes of 100% ethanol, 5min each), rinsed in distilled water. Then all the slides were stained with hematoxylin and eosin (Kienan, 1999; Suvarna et al., 2012). The histopathological analyses were performed on micrographs under an Olympus BX53 microscope. The ovarian development was classified into 7 stages: stage I, stage II, stage III, stage IV, stage V, stage VI, stage VII, following the method described by the previous study (Kulkarni et al., 1991).

Furthermore, in order to estimate the population dynamics parameters (K, L_{inf}, $Ø'$, Z, M, F, and E), the cephalothorax length (CTL) data for each sex was used because it was more reliable in contrast to the flexible abdominal joint of crayfish (Ghia et al., 2015). K is referred to a relative growth rate and has dimensions of time^{-1} and $Ø'$ has a clear biological meaning (the intercept of log$K$ and logL_{inf} regression) and it is used to compare seasonal estimates of growth parameters as well as overall estimates by different fitting techniques (Al-Hosni and Siddeek, 1999). To estimate these parameters, we use the electronic length frequency analysis (ELEFAN), a system of fishery assessment procedures that is commonly employed to estimate population parameters based on length-frequency data (Pauly and David, 1980; Taylor and Mildenberger, 2017). The FISAT software has been the most frequently used for estimating population parameters. However, it is limited in importing data and performing automated analyses (Mildenberger et al., 2017). The R package "TropFishR" remedies these shortcomings and uniquely adds the further data-limited method capacity by including traditional and updated ELEFAN methods (two optimization approaches: generalized simulated annealing ELEFAN_SA, and genetic

algorithm ELEFAN_GA) for growth curves fitting and parameters estimates (Mildenberger et al., 2017; Taylor and Mildenberger, 2017). So in this study, the frequency distributions were analyzed and fitted with growth curves by the ELEFAN of R package “TropFishR”.

The parameters were calculated as follows:

$Ø'=\log K+2\log L_{inf}$ (Pauly and Munro, 1984);

The expected longevity (t_{max}): $t_{max}=3/K+t_0$ (Huang et al., 2012);

The *Z* and *M* were obtained through the Powell-Wetherall method (Wetherall, 1986). The *F* is obtained by subtracting *M* from *Z*. The *E* is defined as $E= F/Z$.

Where L_{inf} is the asymptotic CTL (calculated as $L_{max}/0.95$, where L_{max} is the maximum recorded CTL); *K* is the growth coefficient; t_0 is the initial condition parameter (when crayfish have CTL=0, although biologically meaningless, it represents an important component of curve) and can be calculated as $\ln(-t_0)=-0.3922-0.2752 \ln L_{inf} - 1.308 \ln K$.

2.3.2 Reproductive performance and embryonic development

For adult crayfish, the survival, feeding rate, and spawning rate were calculated as follows at the end of the experiment:

Survival (%) = 100 × (final crayfish number / initial crayfish number).

Feeding rate (% body weight / day) = 100 × total feed intake (dry matter, g/days) / [(initial body weight (wet weight, g) + final body weight (wet weight, g)) / 2].

Spawning rates (%) = 100 × (final spawning crayfish number / initial female crayfish number).

Based on the duration of embryonic development, we built a temperature-dependent developmental model for embryos. It was based on the law of total effective temperatures (Ikemoto and Takai, 2000): $K= D(T-\alpha)$, where *K* is the effective accumulated temperature of *P. clarkii*, *T* is the temperature (℃), α is theoretical biological zero temperature (℃), and *D* is the development time (days). Based on our data, the predictive exponential model (Bělehrádek’s equation) of the developmental time was established as follows: $D = a(T-\alpha)^b$, where *a*, *b*, and α are constants. It is commonly used to describe the relationship between temperature (℃)

and embryonic development time (Yamakawa and Matsuda, 1997). The *a* and *b* reflect the response of eggs to temperature changes, α is "theoretical biological zero temperature" (theoretical temperature below which eggs stop their development), *D* is the development time (days) and *T* is the temperature (℃) (Belehradek, 1957). Based on the relationship of embryonic development and temperature, we estimated the Bělehrádek equation parameters following the methods described by previous studies (Corkett and McLaren, 1970; Yamakawa and Matsuda, 1997).

2.3.3 Effects of feeding levels on growth and muscle composition

At the end of the experiment, all crayfish were starved for 24 h and then collected for growth performance parameters measurement. Ten males and ten females from each pond (60 crayfish for each treatment) were randomly sampled for muscle composition analysis and chill-killed using an ice-water bath. The tail muscles were removed from the shells and stored at -20 ℃ for muscle composition analysis. Samples of two individuals from each pond were also chill-killed and maintained for stable isotope analysis (six individuals for each treatment).

2.3.4 Effects of protein levels on growth and muscle composition

At the end of the experiment, all crayfish were starved for 24 h and then collected for growth performance parameters measurement. Ten males and ten females from each pond (80 crayfish for each treatment) were randomly sampled for muscle composition analysis and chill-killed using an ice-water bath. The tail muscles were removed from the shells and stored at -20 ℃ for muscle composition analysis. Samples of two individuals from each pond were also chill-killed and maintained for stable isotope analysis (eight individuals for each treatment). Three artificial diet samples and four *H. verticillata* samples were collected for stable isotope analysis.

Parameters for growth performance such as survival, final length (*L*), final weight (*W*), gonad weight, liver weight, and muscle weight were recorded and calculated as follows:

Survival (%) = $100 \times (N_t / N_0)$

Specific growth for weight (SGR_W, %, per day) = $100 \times [\ln(W_t) - \ln(W_0)] / T$

Specific growth for length (SGR_L, %, per day) = $100 \times [\ln(L_f) - \ln(L_0)] / T$

Gonadosomatic index (*GSI*, %) = 100 × W_g / W_t

Hepatosomatic index (*HSI*, %) = 100 × W_l / W_t

where N_t is the final number of *P. clarkii* per treatment, and N_0 is the initial number of *P. clarkii* per treatment; W_t is the final weight of *P. clarkii*, and W_0 is the initial weight of *P. clarkii*; L_f is the final length of *P. clarkii*, and L_0 is the initial length of *P. clarkii*; W_g is the gonad weight of *P. clarkii*, and W_l is the liver weight of *P. clarkii*; and *T* is the number of experimental days.

Crayfish muscle and diets were analysed for protein, lipid, moisture, and ash contents. Protein content was determined using the Kjeldahl method (N × 6.25) (William, 1980) with a 4800 Kjeltec Auto Analyzer (FOSS Tecator, Haganas, Sweden). Lipid content was determined by chloroform-methanol extraction. Moisture content was determined by placing a 1-g sample into a convection oven (105 ℃) for 2 h and drying it to constant weight (William, 1980). Ash content was determined by placing a 1-g sample combusting at 550 ℃ in a muffle furnace for approximately 10 h (William, 1980).

For stable isotope analysis, samples were oven dried at 60 ℃ for at least 48 h to constant weight and were very finely ground (< 200 μm). All samples were processed for $\delta^{15}N$ and $\delta^{13}C$ isotopes by the Department of Earth System Science, Tsinghua University, Beijing, China (Alfaro et al., 2006). Approximately 3-mg samples were combusted, gasses analysed by gas chromatography and continuous flow-mass spectrometry (MAT-253, Thermo Fisher Scientific, USA). Samples were referenced to pre-calibrated C_4 sucrose, which was cross-referenced to the Vienna PeeDee Belemnite standard. The reference standard of $\delta^{15}N$ was atmospheric N_2 and measured to a precision of ± 1%. The isotope values for $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) were according to the following equation:

$$\delta^{13}C\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 1000$$

$$\delta^{15}N\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 1000$$

2.4 Statistical analyses

2.4.1 Reproductive pattern and population growth dynamics

The non-parametric Kruskal-Wallis test followed by pairwise Wilcoxon Rank Sum tests (post hoc test) to detect the differences in GSI, HSI, CPUE, and the estimated population dynamics parameters. Student's t-test was used to compare the differences of CPUE between females and males. The relationships between CPUE and temperature, and GSI and HSI were analyzed by Pearson's product-moment correlation test. Chi-squared test was used to access the sex ratio balance among different months. Generalized additive model (GAM) was used to illustrate the relationships between weight, CTL, and cephalothorax width and fecundity. Statistical differences were set to 0.05 and all statistical analyses were performed in the software R version 3.3.2 (R Core Team, 2017).

2.4.2 Reproductive performance and embryonic development

We used non-parametric Kruskal-Wallis tests followed by pairwise Wilcoxon Rank Sum tests (post hoc test) to detect the differences in survival, feeding rates, spawning rates, duration from mating to the spawning, fecundity, and embryos hatching time among different temperature treatments. Independent samples *t* tests were used to analyze the differences in survival between sexes. We used non-metric multidimensional scaling analysis (NMDS) to test differences of embryos development under different temperatures. Stress (mismatch in the relationship between the distance in the original space and the reduced ordination space) is normally a factor indicating the quality of NMDS analysis, and lower values generally result in good interpretations (McCune et al., 2002; Witting and Becker, 2010). Statistical differences were set to 0.05 and all statistical analyses were performed in the software R version 3.3.2 (R Core Team, 2017).

2.4.3 Effects of feeding levels on growth and muscle composition

The pairwise permutation test was carried out to test differences of survival among treatments. Kruskal-Wallis tests were used to analyse differences of other growth parameters and muscle composition among treatments (non-parametric data) followed by Wilcox post hoc tests. Principal component analysis (PCA) was applied

to further summarize the trends in growth performance when feeding levels reduced (Næs and Risvik, 1996). For the stable isotope data, we used the Bayesian stable-isotope mixing model of the "SIAR" package in R to obtain the contributions of the artificial diet and *H. verticillata* (Parnell, 2008). This model has strong statistical power in allowing uncertainty in the sources, the consumers' isotopic signatures, and the fractionation values. We used the most appropriate fraction factor values of 3.4‰ for $\delta^{15}N$ and 0.8‰ for $\delta^{13}C$, according to the previous study (Alcorlo and Baltanas, 2013). All analyses were performed by R version 3.3.2 (R Core Team, 2017), and the significance level was set to 0.05, and the significance level was set to 0.05.

2.4.4 Effects of protein levels on growth and musle composition

The pairwise permutation test was carried out to test differences of survival among treatments. Students' t-tests were used to analyze the differences in other growth parameters, muscle composition, and crayfish $\delta^{13}C$ and $\delta^{15}N$ values of the two treatments. Kruskal-Wallis test was used to analyze differences in $\delta^{13}C$ and $\delta^{15}N$ values of two artificial diets and *H. verticillata*. Growth performance parameters were also analyzed by principal component analysis (PCA). For the stable isotope data, we calculated the contributions of diet and *H. verticillata* to the growth of *P. clarkii* using the "SIAR" package in R. All analyses were performed by *R* version 3.3.2, and the significance level was set to 0.05.

Chapter 3

Reproductive pattern and population growth dynamics

Reproductive pattern and population dynamics of commercial red swamp crayfish (*Procambarus clarkii*) from China: implications for sustainable aquaculture management

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ABSTRACT

Background. The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is one of the most promising freshwater species for aquaculture in China. Understanding its reproductive pattern and population dynamics is crucial for sustainable management, but there is currently a lack of fundamental knowledge of commercial *P. clarkii* populations. Therefore, the purpose of this study was to investigate the reproductive pattern and population dynamics of commercial *P. clarkii* throughout the yearly cycle.
Methods. A total of 2,051 crayfish (1,012 females and 1,039 males) were collected from March 2016 to February 2017 in the area of Selection and Reproduction Center of Crayfish. The reproductive pattern was evaluated by the gonadosomatic index (GSI), hepatosomatic index (HSI), ovarian development and fecundity. Growth, mortality rates and exploitation rate were estimated by electronic length frequency analysis by R package "TropFishR" based on data of cephalothorax length (CTL).
Results. Our results demonstrated that spawning activities of *P. clarkii* took place from September to November, with a mean fecundity of 429 ± 9 eggs per female. There were two recruitments yearly, a major one from October to November and a minor one from March to May. With respect to population growth, five growth cohorts were identified for both females and males. Crayfish grew faster but attained smaller asymptotic maximum CTL as indicated by higher growth coefficient (K), growth parameter index ($\emptyset'$) and lower asymptotic CTL (L_{inf}). The estimates of total mortality rate (Z), natural mortality rate (M) and fishing mortality rate (F) were 1.93, 1.02, 0.91 year^{-1} for females and 2.32, 0.93, 1.39 year^{-1} for males, which showed that the mortality of male crayfish was mainly caused by fishing. The estimates of exploitation rate (E) indicated that male crayfish were overexploited, with the values of 0.47 and 0.60 year^{-1} for females and males, respectively.
Discussion. *P. clarkii* spawned from September to November while two recruitments were observed yearly. We inferred that some eggs, prevented from hatching by low water temperature in winter, were more likely to hatch in the next spring. Moreover, the

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fishing mortality rate was relatively high for males, which might be related to the males-directed selection during the reproductive period. The higher values of exploitation rate in our study confirmed that males *P. clarkii* were overexploited and were under high fishing pressure. We thus suggest reducing fishing intensity on immature crayfish and avoid sex selection during the reproductive period to improve the overall sustainability of commercial *P. clarkii* populations.

Subjects Aquaculture, Fisheries and Fish Science, Ecology, Zoology, Freshwater Biology
Keywords *Procambarus clarkii*, Spawning period, Population growth, Mortality and exploitation rates, Sustainable management

INTRODUCTION

Aquaculture has become a multinational industry over the last 30 years and is expected to maintain an average annual growth rate of 44% over the period 2010–2030 (*FAO, 2017*). Currently, it has been the fastest growing food-producing sector and has supplied more than 50% of global aquatic food consumption in the world (*Wang et al., 2015*). In 2016, global aquaculture production reached approximately 80 million tons, corresponding to $232 billion in sales (*FAO, 2018*). Among commercially farmed species, the red swamp crayfish *Procambarus clarkii* (Girard, 1852), was the second most produced species accounting for 12% of total crustaceans aquaculture production (*FAO Yearbook, 2018*). China, the top-ranking aquaculture country, has undergone remarkable development in its culture. *P. clarkii*, originally distributed in northeastern Mexico and the south-central United States, has been introduced into Nanjing, China from Japan since the late 1930s (*Henttonen & Huner, 1999*; *Hobbs, Jass & Huner, 1989*; *Shu & Ye, 1989*; *Li et al., 2012*). The crayfish displays an r-strategy, exhibiting short life cycles and rapid growth, and they tolerate poor environment conditions (*Cruz & Rebelo, 2007*). Now it can be found in various freshwater habitats such as rivers, ponds, rice fields and ditches of most provinces in China (*Fisheries Department of the Chinese Ministry of Agriculture, 2017*). Although its fast spread was reported to reduce the diversity of plankton, invertebrates, and tadpoles (*Zhang et al., 2003*; *Wu et al., 2008*; *Zhang et al., 2014*), the huge commercial values created great incentives for farmers to the culture of *P. clarkii*. The production has achieved 852,300 tons in 2016 and represented 41.94% of China freshwater shrimp aquaculture (*Fisheries Department of the Chinese Ministry of Agriculture, 2017*). However, the growing demand intensifies immense fishing pressure on commercial populations, which results in population depletion and slow recovery rates (*Naylor et al., 2000*; *Tidwell & Allan, 2001*). Therefore, effective fishery management efforts are now needed to alleviate fishery crises and promote commercial *P. clarkii* populations' sustainability. Correspondingly, fisheries management should be based on a better understanding of population life-history characteristics, which are supposed to induce changes in management policies.

Reproduction, growth, and mortalities are the most important life-history parameters for population maintenance, and studies on these parameters are thus crucial for fishery management (*Fatemi et al., 2009*; *He et al., 2011*). Recently, efforts have been made to assess

the status of reproduction and population dynamics of *P. clarkii* populations in wild, but little information is available on population characteristics under commercially-cultured conditions. Previous studies showed that *P. clarkii* displayed considerable plasticity and variability in reproductive patterns in different regions of the world. For example, some authors reported that the reproduction of *P. clarkii* had a clear annual periodicity, with most spawning events confined to autumn in different locations such as USA (*Oluoch, 1990*), Germany (*Chucholl, 2011*), and Italy (*Dörr et al., 2006*). While others argued that there existed two or more spawning periods yearly for *P. clarkii* in Portugal (*Sousa et al., 2013*), Italy (*Scalici & Gherardi, 2007*), Kenya and Spain (*Gutiérrez-Yurrita et al., 1999*; *Gutiérrez-Yurrita & Montes, 1999*). In China, authors also reported different results. For example, the population was proved to spawn once yearly in Poyang lake (Jiangxi province, subtropical climate with annual mean precipitation of 1996 mm and annual mean temperature of 18.9 °C, *Xiao et al., 2011*), Huangjin Lake (Wuhan, Hubei province, subtropical climate with annual mean precipitation of 1,236 mm and annual mean temperature of 17.2 °C, *Lv, 2006*; *Gong et al., 2008*), and Xuyi (Jiangsu province, transitional zone between temperate and subtropical climate with annual mean precipitation of 972 mm and annual mean temperature of 15.3 °C, *Xu et al., 2014*) while twice a year in Wuhan (Hubei province, *Dai et al., 2008*).

In fish or crayfish population dynamics studies, understanding of population parameters such as growth (growth coefficient K and growth parameter index $Ø'$), mortalities (total mortality rate Z, natural mortality rate M, and fishing mortality rate F) has important implications for population assessment (*Rochet et al., 2000*). Estimates of these parameters provide fundamental information for predicting population growth and developing sustainable exploitation strategies (*Nurul Amin, Zafar & Halim, 2008*; *Ochwada-Doyle et al., 2014*).

Usually, growth parameters such as K and $Ø'$ are used for evaluation of growth performance under a variety of environmental stresses such as under aquaculture conditions (*Pauly, 1991*; *Živkov, Trichkova & Raikova-Petrova, 1999*). Quantitative assessment of mortality is also a significant step to improve our understanding of population dynamics. M was defined as the mortality caused by all possible causes except fishing and it could be obtained from the values of Z minus F (*Pauly, 1980*). M, Z, and F are thus crucial parameters that are commonly used in fisheries assessment and management, but they are poorly known for commercial *P. clarkii* populations (*Kenchington, 2014*; *Nadon et al., 2015*; *Williams et al., 2015*). Moreover, for successful fisheries management, it will be necessary to further examine the exploitation states for different populations. The previous studies suggest that a value of 0.5 for E represents the optimum exploitation condition while a value of $E > 0.5$ points toward over-fishing (*Gulland, 1971*; *Clasing et al., 1994*).

Up to date, characteristics of those population parameters have been extensively studied on *P. clarkii* wild populations from Europe, with great emphasis on the prevention of further invasions in Italy (*Scalici & Gherardi, 2007*; *Scalici et al., 2010*; *Dörr & Scalici, 2013*; *Maccarrone et al., 2016*; *Donato et al., 2018*), France (*Coignet, Pinet & Souty-Grosset, 2012*; *Meineri et al., 2014*), Germany (*Chucholl, 2011*), Portugal (*Anastácio et al., 2009*), and Spain (*Alcorlo, Geiger & Otero, 2008*). Nevertheless, few studies have been conducted on

commercially cultured *P. clarkii* populations. Despite its high commercial importance in China, knowledge of reproduction and length-based population dynamics information, including growth, mortalities and exploitation rate of commercially cultured populations is generally limited. There is, thus, a need to target those biological characteristics of commercial populations for successful aquaculture management.

The objective of the present study was to evaluate the reproduction, growth, mortalities and exploitation rate of the commercial *P. clarkii* population in China. For this purpose, we studied: (1) reproductive pattern of females by measuring the GSI, HSI, ovarian development, and fecundity; and (2) population dynamics by estimating growth (K and $Ø'$), mortality rates (Z, M, and F) and exploitation rate (E). Our work will hopefully provide background information to develop effective and sustainable management strategies of *P. clarkii* commercial populations.

MATERIALS & METHODS

Study area

The study is carried out in the Selection and Reproduction Center of Crayfish (30.41°N, 112.75°E), Qianjiang, which is recognized as the land of red swamp crayfish in China by the Ministry of Agriculture of the People's Republic of China. This region extends over 200 ha and encloses many artificial ponds.

The studied area has a surface area of 33,350 m^2, which is under good management and is referred as the model of crayfish culture. In this area was planted *Hydrilla verticillata*, preferred by *P. clarkii* and tolerant to high water temperatures in summer. This submerged macrophyte can provide supplementary nourishment, refuge for crayfish and supports maintaining suitable water quality. Quicklime (15–22.5 grams/m^3) was used monthly to prevent diseases and eradicate other unwanted aquatic organisms (e.g., silver carp, rice field eel, gold fish, and loach). Crayfish stocking was from March to April, with individual sizes ranging from 3 to 5 g. The stocking density was 15 individuals/m^2. Two commercial diets were commonly used as a main food source for crayfish during the study period and were purchased from Charoen Pokphand Group (WHS001-2016, diet 1: 30.23% crude protein, 10.74% crude lipid, 10.18% moisture, and 8.70% ash; diet 2: 26.53% crude protein, 10.41% crude lipid, 13.96% moisture, and 6.87% ash). From March until May crayfish were fed with high protein level pellets (diet 1) in order to reach commercial sizes in a short period. In the pond feeding rates differed in time, but were in general about 3% of the crayfish biomass per day.

During the sampling period, the annual mean water temperature was 19.75 °C, ranging from 8.65 °C in January to 31.25 °C in August. The water depth was 1–1.5 m. Other water physical-chemical parameters were: pH 8.61–9.30; ammonia nitrogen 0.14–0.43 mg/L; nitrite 0.15–0.25 mg/L; total nitrogen 1.06 ± 1.67 mg/L; total phosphorus 0.0445 ± 0.17 mg/L; chemical oxygen demand (to quantify the amount of oxidizable pollutants in https://en.wikipedia.org/wiki/Water) 5.83–8.80 mg/L; and chlorophyll-a 14.55–31.67 µg/L.

Crayfish sampling

Crayfish were collected monthly from March 2016 to February 2017 with 8 cylindrical traps baited with fresh silver carp. The traps were 100 cm long with 5 mm mesh, 30 cm cross-section, and two opposing funnels 10 cm in diameter. During each sampling event, trapping was performed and retrieved in the afternoon. The periods of trapping were one day from June to September; two days from March to May, and October; and three days from November to February. The same sampling site order and timetable were followed every month in order to minimize the bias in measurement. Catch per unit effort (CPUE) was calculated for each sampling as the daily number of crayfish per trap.

Sampled crayfish were sorted by sex. Cephalothorax length (CTL, from the tip of the rostrum to the cephalothorax posterior margin) was measured by a 0.01 mm precision caliper. Crayfish weight was determined by a 0.01 g precision scale. All samples were then transported to the laboratory to dissect. During the whole sampling period, water temperature was recorded every two hours by a HOBO data-logger (UA-002-64, HOBO Pendant temperature/light 64 K data logger Onset, Bourne, MA, USA).

Reproductive pattern analysis

After transporting to the lab, females were checked for attached eggs, if present, they were counted to determine the fecundity. Then they were frozen to −20 degrees to dissect, following the European Directive 2010/63/EU for animal experiments. The gonads and hepatopancreas of females were weighted to calculate the gonadosomatic index (GSI) (measuring the sexual maturity and relating to ovary development) and hepatosomatic index (HSI) (indice of energy status):

$$\mathrm{GSI} = 100 \times W_g / W_t$$

$$\mathrm{HSI} = 100 \times W_h / W_t$$

Where W_g, W_h, and W_t are the gonad weight, hepatopancreas weight and body weight of *P. clarkii*, respectively.

Dissected gonads were fixed for 24 h in Bouin's solution (Wuhan Servicebio Technology Company, Wuhan, China) for histological analysis. Samples were dehydrated in 50%, 70%, 85%, 90%, 95%, and 100% ethanol and embedded in paraffin block. Then they were subjected to microtomy to obtain sections with 4 µm (Leica RM2016, USA). Slides were deparaffinized (2 changes of xylene, 20 min each; 3 changes of 100% ethanol, 5 min each), rinsed in distilled water. Then all the slides were stained with hematoxylin and eosin (*Kiernan, 1999*; *Suvarna, Layton & Bancroft, 2012*). The histopathological analyses were performed on micrographs under an Olympus BX53 microscope (Fig. S1). The ovarian development was classified into seven stages: stage I, stage II, stage III, stage IV, stage V, stage VI, stage VII, following the method described by the previous study (*Kulkarni, Glade & Fingerman, 1991*).

Population dynamics parameters estimates

In order to estimate the population dynamics parameters (K, L_{inf}, $Ø$, Z, M, F, and E), the CTL data for each sex was used because it was more reliable in contrast to the flexible

abdominal joint of crayfish (*Ghia et al., 2015*). K is referred to a relative growth rate and has dimensions of time^{-1} and $Ø'$ has a clear biological meaning (the intercept of log$K$ and log L_{inf} regression) and it is used to compare seasonal estimates of growth parameters as well as overall estimates by different fitting techniques (*Al-Hosni & Siddeek, 1999*).

To estimate these parameters, we used the electronic length frequency analysis (ELEFAN), a system of fishery assessment procedures that is commonly employed to estimate population parameters based on length-frequency data (*Pauly & David, 1980*; *Taylor & Mildenberger, 2017*). The FISAT software has been the most frequently used for estimating population parameters. However, it is limited in importing data and performing automated analyses (*Mildenberger, Taylor & Wolff, 2017*). The R package "TropFishR" remedies these shortcomings and uniquely adds the further data-limited method capacity by including traditional and updated ELEFAN method (two optimization approaches: generalized simulated annealing ELEFAN_SA, and genetic algorithm ELEFAN_GA) for growth curves fitting and parameters estimates (*Mildenberger, Taylor & Wolff, 2017*; *Taylor & Mildenberger, 2017*). So in this study, the frequency distributions were analyzed and fitted with growth curves by the ELEFAN of R package "TropFishR".

The parameters were calculated as follows:

$Ø' = \log K + 2\log L_{inf}$ (*Pauly & Munro, 1984*);

The expected longevity (t_{max}): $t_{max} = 3/K + t_0$ (*Huang et al., 2012*);

The Z and M were obtained through the Powell-Wetherall method (*Wetherall, 1986*). The F is obtained by subtracting M from Z. The E is defined as $E = F/Z$.

Where L_{inf} is the asymptotic CTL (calculated as $L_{max}/0.95$, where L_{max} is the maximum recorded CTL); K is the growth coefficient; t_0 is the initial condition parameter (when crayfish have CTL = 0, although biologically meaningless, it represents an important component of curve) and can be calculated as $ln(-t_0) = -0.3922 - 0.2752 lnL_{inf} - 1.308 lnK$.

Statistical analyses

Because normality and homogeneity of variance assumptions were not satisfied, we used non-parametric Kruskal–Wallis test followed by pairwise Wilcoxon Rank Sum test (post hoc test) to detect the differences in GSI, HSI, CPUE, and the estimated population dynamics parameters. Student's t-test was used to compare the differences of CPUE between females and males. The relationships between CPUE and temperature, and GSI and HSI were analyzed by Pearson's product-moment correlation test. Chi-squared test was used to access the sex ratio balance among different months. Generalized additive model (GAM) was used to illustrate the relationships between weight, CTL, and cephalothorax width and fecundity. Statistical differences were set to 0.05 and all statistical analyses were performed in the software R version 3.3.2 (*R Core Team, 2017*).

RESULTS

Sampling features

A total of 2,051 individuals (1,012 females and 1,039 males) were captured in the studied area from March 2016 to February 2017. During the entire sampling period, the sex ratio (females/males, Fig. 1) did not differ significantly from expected 1:1 sex ratio (chi-square

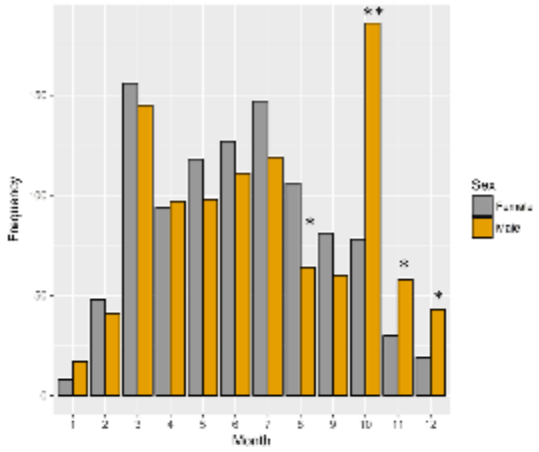

Figure 1 Frequency histogram for females and males of *Procambarus clarkii* throughout the year. Significant differences from expected 1:1 ratio between sexes are shown by asterisks (* $P < 0.05$ and ** $P < 0.001$).

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test: $\chi^2 = 0.36$, $P = 0.55$). However, they showed significant differences from 1:1 from August to December except September, with females abundant in August, and the situation reversed from October to December (chi-square test: August, $\chi^2 = 10.38$, $P = 0.001$; September, $\chi^2 = 3.13$, $P = 0.080$; October, $\chi^2 = 44.18$, $P < 0.001$; November, $\chi^2 = 8.91$, $P = 0.003$; December, $\chi^2 = 9.29$, $P = 0.002$).

Catch per unit effort (CPUE)

The temperature and CPUE of female and male crayfish are shown in Fig. 2. The CPUE were significantly different across months both for females and males (Kruskal–Wallis test, females: $\chi^2 = 91.34$, $P < 0.001$; males: $\chi^2 = 89.04$, $P < 0.001$). Post hoc analyses showed that the CPUE of females in June, July, and August were significantly higher than other months, while for males, July was significantly higher than other months except for June (pairwise Wilcoxon Rank Sum test, females: June-March: $P = 0.002$, June–September: $P = 0.004$, others: $P < 0.001$; males: July–June: $P = 0.395$, others: $P < 0.05$). There were no significant differences observed between the CPUE of females and males (Students' t-test, $t = 1.97$, $P = 0.052$). We further found that there were strong correlations between temperature and CPUE for females and males (Pearson correlation test, females: $r = 0.93$, $t = 8.25$, $P < 0.001$; males: $r = 0.81$, $t = 4.41$, $P = 0.001$).

Reproductive pattern analysis

The monthly variations of GSI and HSI for females are shown in Fig. 3. There were significant differences in GSI throughout months. The GSI in August, September, and October were significantly higher than other months by Kruskal-Wallis and pairwise

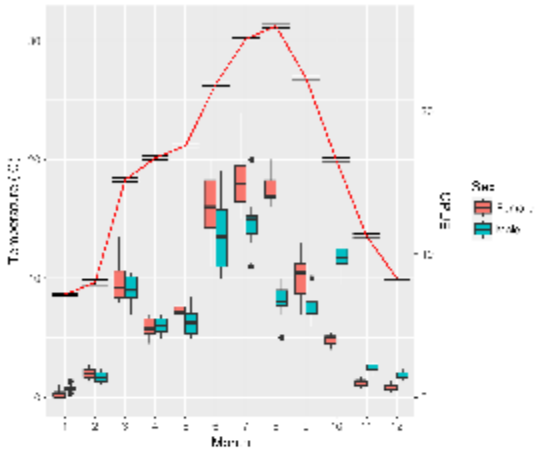

Figure 2 Variation of temperature and catch per unit effort (CPUE) for females and males of *Procambarus clarkii* throughout the year. Box-plot representation: the horizontal line inside the box represents the median, and the lower and upper borders of the box represent the 25th and 75th percentiles, respectively. The upper and lower whiskers indicate the maximum and minimum range of the data excluding outliers. Temperature values are shown as mean ± SE.

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Wilcoxon Rank Sum test ($\chi^2 = 369.84$, September–January: $P = 0.003$, October–January: $P = 0.02$, others: $P < 0.001$). It increased remarkably from August to September. Although there was slight increase in February, no significant differences were observed, when compared with January, March, November and December ($\chi^2 = 369.84$, January: $P = 0.70$; March: $P = 0.06$; November: $P = 0.78$; December: $P = 0.80$).

The HSI decreased progressively from September to October. Comparisons among different months by Kruskal-Wallis and pairwise Wilcoxon Rank Sum test showed that April, May, June, and July had significantly higher HSI values than that of other months ($\chi^2 = 266.99$, all $P < 0.05$). Furthermore, we found that GSI was negatively correlated with HSI (Pearson correlation test $r = -0.38$, $t = -10.79$, $P < 0.001$).

The proportions of different ovarian stages across the year are shown in Fig. 4. In February, ovaries with stage I were present at maximum abundance, and ovaries with stage II increased until May. In June, the percentage of ovaries with stage III was the highest. In July, although ovaries with stage IV increased, the proportion showed only a slight increase due to more juveniles with stage I occurrence. The proportion of ovaries with stage V peaked in August. Most female crayfish ovaries developed to stage VI from August to October, with a peak in September. In November, the proportion decreased dramatically and ovaries with stage II dominated. From December to January, most ovaries developed to stage VII after spawning.

Fecundity was only assessed from September to December. The relationships of weight, CTL, cephalothorax width and fecundity explained by the GAM model are shown in

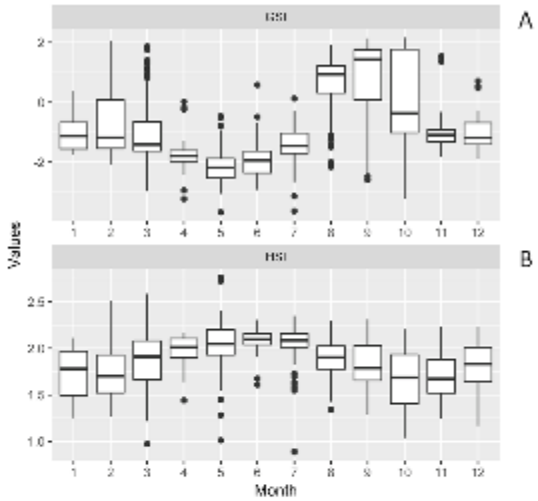

Figure 3 Box-plot of gonadosomatic index (GSI) and hepatosomatic index (HSI) for females of *Procambarus clarkii* during the sampling period. Box-plot representation: the horizontal line inside the box represents the median, and the lower and upper borders of the box represent the 25th and 75th percentiles, respectively. The upper and lower whiskers indicate the maximum and minimum range of the data excluding outliers.

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Fig. 5. The model explained the 76.6% of total deviance, with a high value of $R^2 = 0.745$. The relationship between weight and fecundity was approximately linear, indicating the fecundity increased with increasing weight ($F = 36.72$, $P < 0.001$). Although increased with cephalothorax width at the beginning, the fecundity was at the onset of decrease after 23 mm of the cephalothorax width ($F = 3.80$, $P = 0.006$). The fitted curve for CTL was slightly concave based on the interpretation of the GAM plots, however, there was no evidence of interactions observed ($F = 1.97$, $P = 0.16$). The average number of eggs berried per female crayfish was 429 ± 9, with the minimum and maximum value of 290 and 610, respectively.

Population dynamics parameters estimates

The frequency distributions of monthly CTL (distinguished by sexes) and the growth curves, fitted by ELEFAN using "TropFishR" package, are presented in Figs. 6A, 6B and 7A, 7B. The CTL data of collected crayfish was classified into 17 size classes of 4 mm interval size classes. From the analysis of the CTL frequency distributions, five growth cohorts were observed for both females and males, each cohort corresponding to 1 size class.

For females, the growth curves highlighted five cohorts (Fig. 6B). In the first cohort, offspring released from May 2016 had about 25 mm CTL in February 2017. For the second and third cohorts, individuals had about 28 mm and 43 mm CTL in March 2016 and reached the CTL of about 42 mm and 49 mm in February 2017. For the fourth and fifth cohorts, crayfish did not show obvious growth during the whole sampling period.

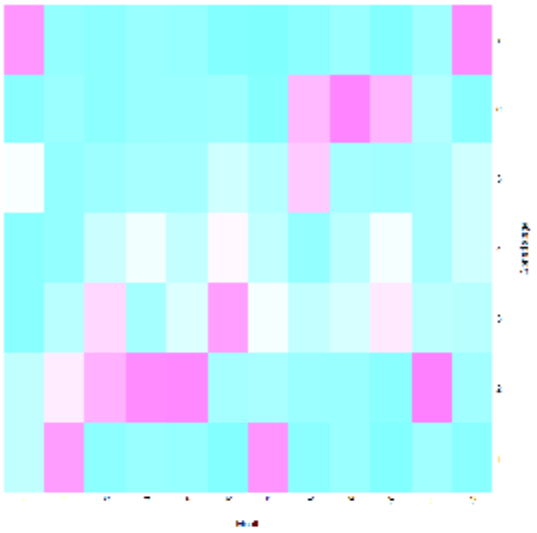

Figure 4 Heatmap of gonad stages of female *Procambarus clarkii* in different months. As shown in color key, white color represents absent and purple represents the highest occurrences. Samples were eight for January, 48 for February, 156 for March, 94 for April, 118 for May, 127 for June, 147 for July, 106 for August, 81 for September, 78 for October, 30 for November, and 19 for December, respectively.
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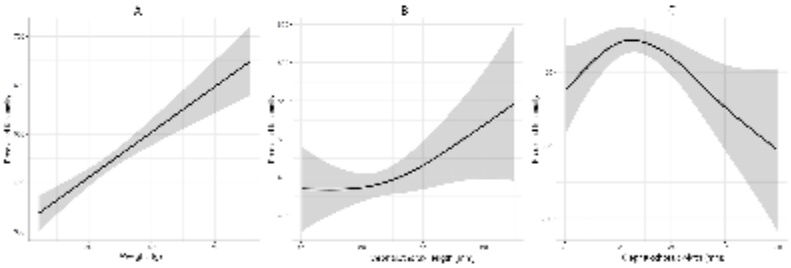

Figure 5 Generalized additive model (GAM) explaining the relationship between weight, cephalothorax length, cephalothorax width and fecundity of *Procambarus clarkii*. Solid lines represent the estimated smooth function and the grey areas represent the 95% confidence interval.
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For males, there were also five growing cohorts and showed similar growth patterns with females (Fig. 7B). In the first cohort, offspring from May 2016 had about 28 mm CTL in February 2017. For the second and third cohort, individuals had about 29 mm and 45 mm CTL in March 2016 and reached the CTL of about 42 mm and 52 mm in February 2017. Crayfish composed of the fourth and fifth cohorts also did not show obvious growth.

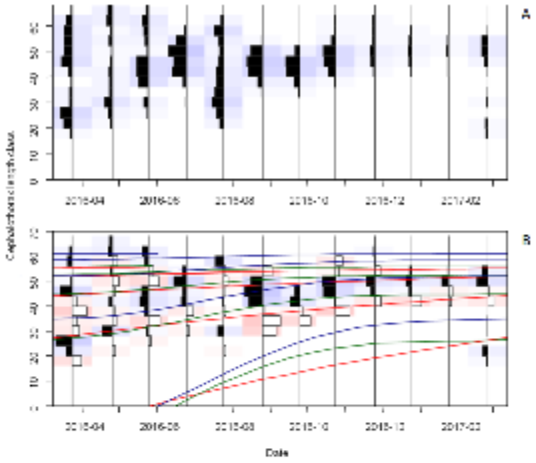

Figure 6 Cephalothorax length (CTL) frequency distribution and growth curves of female *Procambarus clarkii* during sampling time. CTL frequency data visualized in terms of catches (A) and restructured data (B) with a moving average setting of MA = 5. Graphical fit of estimated and true growth curves plotted through the CTL frequency data. The growth curves with the true values are displayed in red, while the blue and green curves represent the curves of ELEFAN_SA (Electronical length frequency analysis-simulated annealing, estimate growth paramaters with simulated annealing) and ELEFAN_GA (estimate growth paramaters with genetic algorithm), respectively. Positive (black) and negative (white) scored bins are indicated by the histogram direction.

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The estimated population dynamics parameters (L_{inf}, K, t_0, and t_{max}, Z, M, F, and E) for both females and males during the sampling period are shown in Table 1. Although females had higher values of K and M, results of pairwise Wilcox test showed no significant differences in those parameters between sexes ($P = 0.19$).

DISCUSSION

The present study was based on a large sample size (2051 crayfish), aiming to improve the knowledge of reproduction and population dynamics of commercial *P. clarkii* population.

Sex ratio

The overall sex ratio was near 1:1, but it varied throughout sampling months. In August and September, females were abundant and the situation was reversed from October to December, as reported by previous studies (*Dörr et al., 2006*; *Mueller, 2007*; *Alcorlo, Geiger & Otero, 2008*; *Peruzza et al., 2015*). This discrepancy in the sex ratio observed in our study was probably due to the reproductive activities of females, which tended to stay in burrows for parental care to their offspring and could be less easily trapped (*Gherardi & Barbaresi, 2000*; *Thiel, 2000*; *Dörr et al., 2006*; *Donato et al., 2018*). In addition, we also recorded an interesting phenomenon regarding the increase in proportion of males being observed

Table 1 Von Bertalanffys parameters of the studied *Procambarus clarkii* population and others from Europe reported in previous studies.

Sex	K (year^{-1})	L_{inf} (mm)	t_0 (year)	t_{max} (year)	Ø′ (year^{-1})	Z (year^{-1})	M (year^{-1})	F (year^{-1})	E	References
Male	0.81	60.93	−0.29	3.41	8.01	2.32	0.93	1.39	0.60	Present study
Female	0.86	58.12	−0.27	3.22	7.97	1.93	1.02	0.91	0.47	
Male	0.340	68.25	−0.110	8.71	3.19	3.43	1.14	2.29	0.67	*Maccarrone et al. (2016)*
Female	0.350	67.20	−0.260	8.31	3.19	3.83	1.16	2.67	0.70	
Male	0.59	69.35	−0.09	5.08		5.50	2.83	2.67	0.49	*Dörr & Scalici (2013)*
Female	0.58	73.71	−0.14	5.17		5.10	2.77	2.33	0.46	
Male	0.49	74.60	−0.022	6.1	3.44	2.26	2.26	0.00	0.00	*Chucholl (2011)*
Female	0.45	79.80	−0.027	6.6	3.46	2.79	2.55	0.24	0.09	
Male	0.33	68.3	−0.37	8.73		2.88	1.63	1.25	0.43	*Scalici et al. (2010)*
Female	0.32	74.6	−0.43	8.95		3.11	1.77	1.34	0.43	
Male	0.69	62.71	−0.1	4.25	3.43	2.99				*Scalici & Gherardi (2007)*
Female	0.68	65.52	−0.1	4.31	3.47	4.71				

Notes.

L_{inf}, asymptotic cephalothorax length (CTL); K, growth coefficient; t_0, initial condition parameter (when crayfish have CTL = 0, although biologically meaningless, it represents an important comp
of curve); t_{max}, expected longevity; Ø′, growth parameter index; Z, total mortality rate; M, natural mortality rate; F, fishing mortality rate; E, exploitation rate.

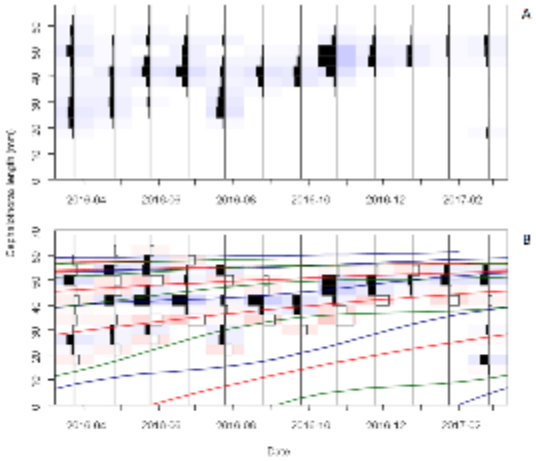

Figure 7 Cephalothorax length (CTL) frequency distribution and growth curves of male *Procambarus clarkii* during sampling time. CTL frequency data visualized in terms of catches (A) and restructured data (B) with a moving average setting of MA = 5. The graphical fit of estimated and true growth curves plotted through the CTL frequency data. The growth curves with the true values are displayed in red, while the blue and green curves represent the curves of ELEFAN_SA (Electronical length frequency analysis-simulated annealing, estimate growth parameters with simulated annealing) and ELEFAN_GA (estimate growth parameters with genetic algorithm), respectively. Positive (black) and negative (white) scored bins are indicated by the histogram direction.

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during the reproductive period (September to December), likely due to the search for a mate (*Peruzza et al., 2015*).

In order to maximize short-term catch rates and profitability, farmers intentionally target particular sizes or sex of crayfish during catching periods (*Zhou et al., 2010*). For example, due to the low catch rates and reproductive activities of females, more male crayfish are selectively harvested during the reproductive period. This males-directed selectivity may impose adverse effects on reproductive output since it causes difficulties in females finding mates. Similar cases were also found in crabs (*Gray & Powell, 1966*; *Smith & Jamieson, 1991*). Thus, in fishery management, the possible side effects of sex selection on reproductive success of the population should be considered (*Zhou et al., 2010*).

Reproductive pattern analysis

In the present study, spawning activities of female *P. clarkii* mostly took place from September to November. However, several ovigerous females were also caught from March to May, which suggested the possibility of two recruitment phases yearly (March to May and October to November). This was also confirmed by the characteristics of the samples collected in spring, where the release of larvae frequently took place. We inferred that those females had most likely laid eggs at the end of the previous autumn. This was because eggs development was strongly linked to water temperature and previous studies showed that

it would take up to 130 days until eggs hatching at the temperature below 10 °C (*Suko, 1954*; *Suko, 1956*). During our study, the mean temperature was 13.58 °C and 10.03 °C for November and December, which suggested that eggs in late autumn were probably prevented from hatching by low water temperature. Those eggs, having survived the harsh winter conditions, would be more likely to hatch in the next spring when the environment is favorable. Accordingly, we found crayfish larvae in spring. Thus, delaying hatching could be an adaptive strategy of *P. clarkii* for unfavorable environmental conditions such as low water temperature in winter (*Lass et al., 2005*).

In this study GSI increased rapidly from July reaching its peak in September and then diminished from October, and was near to the lowest value in November. This indicated that crayfish spawning initiated in September and was achieved in November. Although February encountered a slight increase in GSI values, we speculated that it might be attributed to the small sampling sizes since most crayfish slowed down their activities and were hard to catch due to low water temperature (*Rodríguez, Bécares & Fernández-Aláez, 2003*).

In some places, different recruitment events were found per year. For instance, there were two-yearly distinct recruitments in Italy (*Scalici & Gherardi, 2007*; *Maccarrone et al., 2016*), southern Portugal (*Adao & Marques, 1993*), Spain (*Cano & Ocete, 1997*; *Alcorlo, Geiger & Otero, 2008*), America (*Sommer, 1984*) and Japan (*Suko, 1958*), while one main recruitment occurred in central Portugal (*Anastácio & Marques, 1995*) and Germany (*Chucholl, 2011*). The differences in plastic recruitment patterns were difficult to explain, because gonad development and eggs incubation depended on different environmental features, such as water temperature, habitat uses, and food resources (*Sastry, 1983*; *Harlioğlu & Farhadi, 2017*). In our study, the single spawning peak with two recruitment patterns is most likely driven by the low water temperature, but further studies are still needed to test it.

Generally, the fecundity of crustaceans is correlated with females' body sizes or weight, and it shows variability in different populations (*Harlioğlu et al., 2004*; *Nakata & Goshima, 2004*). Our study accords with those findings. The strong linear relationship between weight and fecundity indicated that heavier females tended to produce more eggs. Similar results have been reported for other crustaceans such as *Cherax quadricarinatus* (*Ondes, Kaiser & Murray, 2017*) and *Oziothelphusa senex senex* (*Swetha, Girish & Reddy, 2015*). Moreover, it was noteworthy that fecundity started to decrease when cephalothorax width was over 23 mm. Actually, this result was in contrast from what were reported in several previous studies, which showed fecundity always increased with the increasing cephalothorax width (*Lizarraga-Cubedo et al., 2003*; *Hamasaki, Fukunaga & Kitada, 2006*; *González-Pisani & Greco, 2014*). We inferred that the declined fecundity was mostly due to the onset of senescence of larger females, and thus resulting in lower relative reproductive output (*Sudha & Anilkumar, 1996*). Furthermore, the average fecundity of *P. clarkii* in the current study was much higher than those in Germany (*Chucholl, 2011*) and its native range (*Penn, 1943*), but similar to that in Kenya (*Oluoch, 1990*). Such differences in fecundity could be explained by the different female sizes or the temporal variations in food availability for different populations (*Beyers & Goosen, 1987*). In our study, favorable environment such

as abundant food resources (e.g., artificial diet) could result in large young females and thus higher reproductive output (*Alcorlo, Geiger & Otero, 2008*).

Population dynamics parameters estimates

Length-frequency analysis showed the structure of commercial *P. clarkii* population constituted of five cohorts for both females and males. The second and third cohorts were constituted of abundant younger crayfish, which were fast-growing individuals. Actually, cohorts of *P. clarkii* varied considerably in numbers across populations. For example, there were five cohorts in Portugal (*Anastácio et al., 2009*), six in Italy (*Dörr & Scalici, 2013*), seven in China (*Huang et al., 2012*), and eight and nine for males and females in Germany (*Chucholl, 2011*). It was easy to observe differences in CTL sizes of *P. clarkii* among those studies. We inferred that the differences were mainly attributed to trapping activities. In our study, only crayfish with a CTL higher than 15.20 mm were captured, which could be caused by the selectivity of sampling traps used in studies. Therefore, it was possible that the CTL frequency analysis only partially described the real population structure.

Comparing with previous studies on Von Bertalanffy's growth parameters of *P. clarkii* showed that the L_{inf} in our study was smaller than others (Table 1, *Scalici et al., 2010*; *Chucholl, 2011*; *Dörr & Scalici, 2013*; *Maccarrone et al., 2016*). We speculated that it could be related to density-dependent growth. Generally, higher density leads to a decline in resources availability, which consequently could result in a decrease in L_{inf} (*Svedäng & Hornborg, 2014*). The K and $Ø'$ obtained for females and males in our study were higher, which suggested that the *P. clarkii* in our studied area maintained a relatively high growth rate. The variability in growth rates of *P. clarkii* may relate to several ecological factors, especially temperature and nutrition (*Dörr & Scalici, 2013*). The optimal temperature for *P. clarkii* growth is approximately 23 °C and low temperatures at higher latitudes in the previous studies probably lead to slow growth rates (*Espina & Herrera, 1993*). Crayfish growth is highly correlated with nutrition, and the high food availability and nutrition-sufficiency of artificial diet in our study could guarantee *P. clarkii* better growing conditions than the wild ones.

Our findings showed that fishing mortality rate F of male *P. clarkii* was higher than females, which indicated that males were under high fishing pressure (0.91 and 1.39 $year^{-1}$ for females and males, accounting for 47% and 60% of Z, respectively). We inferred that this was related to the males-directed fishing selection during the reproductive period. In our study, high proportions of males were captured during reproduction. This fishing selection generally causes damage and stress to males, which has negative effects on their growth and survival (*Chopin & Arimoto, 1995*). Even though some crayfish escape from fishing, they may be injured and die later due to physical damage, which might account for the elevated mortality in our study. M is related to many factors except fishing, such as predation and starvation. For the aquaculture practice, sufficient nutrition supplies and farmers' efforts to eradication other unwanted fish or crayfish guarantee crayfish under very low starvation and predation pressure in our study, which could explain why M is lower than that of wild populations (Table 1). Generally, M has been widely used as the upper limit of F for sustainable fishing, which suggests that E should be less than 0.5 to

prevent populations from overfishing (*Gulland, 1971*; *Gulland, 1983*; *MacCall, 2009*; *Froese et al., 2016*). The estimated E of 0.60 for males in our study was higher, indicating that the male *P. clarkii* was overexploited and under high fishing pressure. In such a situation, the fishing activities should be well monitored to protect the commercial *P. clarkii* population from further depletion.

Implications for aquaculture management

In recent years, *P. clarkii* has become one of the most important freshwater products in China, and the market demands greatly exceed aquaculture supplies. As the males *P. clarkii* have been overexploited, efforts to improve productivity and sustainability of the crayfish population are crucial for the aquaculture management. Therefore, balanced exploitation should be encouraged to alleviate fishing mortality arising from unsustainably fishing activities and increase the overall sustainability of *P. clarkii* populations (*Svedäng & Hornborg, 2014*). Based on the results of our study, we suggest reducing fishing pressure for commercial *P. clarkii* population through the following two parts.

First, we suggest reducing the fishing intensity on immature crayfish before they reach maximum sizes. In our study, the second and third growth cohorts were made of abundant fast-growing individuals while crayfish of the fourth and fifth growth cohorts showed extremely slow growth. For sustainable exploitation scenario, reducing fishing on younger crayfish and selectively catching old crayfish with slow growth or small sizes will help to promote large-sized individuals and render crayfish culture more profitable. These old crayfish can be distinguished by CTL sizes (more than 50 mm, Figs. 6B and 7B) and maturity (*Penn, 1943*; *Cang, Miltner & Avault, 1982*; *Jarboe & Romaire, 1995*; *Taketomi, Murata & Miyawaki, 1990*; *Taketomi, Nishikawa & Koga, 1996*). This would also offer more access to environmental resources (e.g., food availability) for juveniles and then may increase growth rates.

We also suggest reducing the fishing intensity and avoiding sex selection during the reproductive phase of *P. clarkii*. Trapping is a widespread method for management and is considered to be highly efficient especially for higher crayfish sizes. However, this high efficiency is achieved only when the trapping activity is conducted for a proper period of time. The high fishing pressure during the reproductive season could have negative effects on reproductive potentials, and then influence long-term stock productivity (*Van Overzee & Rijnsdorp, 2015*). Furthermore, fishing may also cause the death of offspring. Thus, restricting fishing pressure on spawning crayfish would be an effective measure to enhance reproductive output and promote population productivity. In our study, more male crayfish were harvested during the reproductive period, which may cause difficulties in females finding mates, and thus affect reproductive success. Based on our findings that the spawning activities and the main recruitment occurred from October to November, we suggest reducing fishing pressure and avoid male selection during this period.

CONCLUSIONS

This study was conducted to determine the reproductive pattern and population dynamics of commercial *P. clarkii* population. The spawning activities of female *P. clarkii* took place

from September to November. There were two recruitments yearly, with a major one from October to November and the minor one from March to May. There were five growth cohorts for females and males, with higher growth rates than previous studies. Males *P. clarkii* were overexploited and under high fishing pressure, as evidenced by a high exploitation rate of 0.60 for males. Our findings thus suggest reducing fishing intensity on immature crayfish and avoid male selection during the reproductive phase to improve aquaculture sustainability. With this study, we hope to encourage further works on commercial crayfish stock assessment and management to promote population productivity and sustainable fisheries.

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ADDITIONAL INFORMATION AND DECLARATIONS

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The authors declare there are no competing interests.

Author Contributions

- Shiyu Jin conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Lisa Jacquin and Wei Li authored or reviewed drafts of the paper, approved the final draft.

- Mantang Xiong and Ruojing Li prepared figures and/or tables, approved the final draft.
- Sovan Lek conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, approved the final draft.
- Tanglin Zhang conceived and designed the experiments, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data and code are provided in the Supplemental Files 2 and 3.

Supplemental Information

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Chapter 4

Thermal effects on reproduction and embryonic development

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Optimizing reproductive performance and embryonic development of red swamp crayfish *Procambarus clarkii* by manipulating water temperature

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ABSTRACT

Aquaculture of red swamp crayfish, *Procambarus clarkii* (Girard, 1852), has developed rapidly worldwide in recent years with promising prospects. However, limited knowledge about temperature effects on reproductive performance and embryonic development has hindered the development of crayfish aquaculture. The two present studies were conducted to identify optimal water temperatures (17 °C, 21 °C, 25 °C, 29 °C and 33 °C) for reproductive performance (experiment 1) and embryonic development (experiment 2) of *P. clarkii*. Totally, there were 12 replicates, with 480 adults and embryos from 60 ovigerous crayfish selected for experiment 1 and 2, respectively. In the first experiment, the survival of adult crayfish was not significantly affected by the temperatures tested. However, significantly higher feeding rates, spawning rates, and fecundity were obtained at 21 °C and 25 °C when compared to those at 29 °C and 33 °C. Polynomial models and loess regression fitted to the experimental data showed that highest spawning rates and fecundity occurred at 21 °C while shortest duration from mating to spawning was found at 33 °C. In the second experiment, we found that optimal embryonic development was at 25 °C with shorter hatching time and no abnormalities observed. However, while embryos showed abnormalities and subsequently died at 29 °C and 33 °C. We further built a temperature-dependent developmental model for *P. clarkii* embryos: D (developmental time, days) = $3{,}140{,}837(T\text{-}2.03)^{-3.76}$. Based on these results, the temperature range 21 °C – 25 °C was recommended for adult crayfish reproduction and 25 °C was recommended for embryonic development. This study indicates that manipulating water temperature is an effective alternative to current artificial reproduction techniques (e.g. eyestalk ablation and injection hormones) to induce spawning and embryonic development and thus provides mass production of juvenile *P. clarkii* for aquaculture.

1. Introduction

Crustacean aquaculture has developed rapidly and global production has reached 7.9 million tons in 2016 (FAO, 2018). Among commercially farmed species, red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is the second most produced species and accounts for 12% of total crustacean aquaculture production (FAO, 2018). As the top-ranking aquaculture country, China has witnessed the rapid development of *P. clarkii* culture industry, with the production increasing from 0.26 million tons in 2007 to 0.85 million tons in 2016 (USD 8.14 billion, Fisheries Department of Ministry of Agriculture, 2017). This encourages the development of optimal artificial reproduction techniques, which are the key steps for improving reproductive outputs and juvenile crayfish production in aquaculture (Rakaj et al., 2019). However, we still lack basic knowledge on how crayfish reproductive performance and embryonic development respond to different culture conditions (especially water temperature). Such knowledge will be helpful to facilitate the management of crayfish broodstock and embryos in aquaculture. In addition, this species is one of most invasive species in the world and has been listed as "100 of the worst" invasive alien species in Europe (DAISIE, 2010; Nentwig, 2009). More information on the reproduction and embryonic development will help to

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understand populations status and predict juveniles recruitment time. Thus popualtion control methods (e.g. fishing) with emphasis on young crayfish and exact time (e.g. reproductive seasons) will be therefore of the high priority and more effective to control population sizes (Rogowski et al., 2013). Furthermore, the related information is also helpful to predict the potential distribution areas and thus management efforts can be made to prevent their further introduction, establishment, and spread (Egly et al., 2019).

Currently, the biggest challenge in *P. clarkii* aquaculture is the limited supplies of juveniles (Song et al., 2015). Up to now, most juvenile *P. clarkii* in aquaculture are obtained from spontaneous reproduction which is limited by seasonal availability (Xu et al., 2011). This hinders the development of the crayfish industry (Smith et al., 2002). It is, thus, of great importance to develop artificial reproduction techniques to trigger a mass production of juveniles. This would be important to meet the demands of commercial production and improve crayfish culture sustainability (Liu et al., 2013a). The crayfish gonad maturation is controlled by two antagonistic neuropeptides: gonad inhibiting hormone (GIH) and gonad stimulating factor (GSF) (Eastman-Reks and Fingerman, 1984; Chaves, 2000). GIH is secreted from the X-organ sinus gland complex, located in the eyestalk of crustaceans. GSF is produced by brains and thoracic ganglion, which has significant effects on stimulating gonads development. Based on this reproductive rationale, there are currently two techniques used to induce the reproduction of *P. clarkii*: eyestalk ablation and hormones injection. The eyestalk ablation, by elimination of GIH to accelerate gonad development, is widely used to induce crayfish reproduction (Chaves, 2000). Besides *P. clarkii*, it has been also extensively used on various crustacean species such as *Cherax quadricarinatus*, *Penaeus monodon*, and *Penaeus semisulcatus* (Aktaş and Kumlu, 1999; Browdy, 1992; Browdy and Samocha, 1985; Liu et al., 2014; Lumare, 1979; Muthu and Laxminarayana, 1977; Sagi et al., 1997; Wen et al., 2015). Hormones injection is through injecting hormones involved in the control of crayfish reproduction to stimulate gonad maturation and spawning. Various hormones such as serotonin (also called 5-hydroxytryptamine), progesterone, 17 α-hydroxyprogesterone, human chorionic gonadotropin (HCG), and domperidone have been proved to significantly induce gonad development and spawning of crayfish (Yano, 1985; Wongprasert et al., 2006; Zhang, 2011; Liu et al., 2014; Liu et al., 2013b). However, eyestalk ablation and hormones injection the two techniques led to death and permanent damage of adult females and also had negative effects on offspring quality (Makinouchi and Honculada-Primavera, 1987; Liu et al., 2013b; Liu et al., 2014; Zhang, 2011). Furthermore, they might cause endocrine and potentially ethical problems. There is thus now an urgent need to find new techniques to produce a mass of high quality juveniles while ensuring animal welfare.

One potential means would be to optimize culture conditions especially water temperature to induce reproduction and ensure optimal embryonic development. It was proved that reproductive processes such as ovarian development, mating and spawning activities of *P. clarkii* were highly related to temperature, with variability in spawning events in different locations. For instance, *P. clarkii* had only one spawning event yearly in Germany (Chucholl, 2011), Italy (Dörr et al., 2006), and UK (Richter, 2000), while two or more spawning events occurred in USA (Huner, 2002; Penn, 1943), Portugal (Anastácio and Marques, 1995; Sousa et al., 2013), Kenya and Spain (Cano and Ocete, 1997; Gutierrez-Yurrita and Montes, 1999; Gutierrez-Yurrita et al., 1999; Lowery and Mendes, 1977; Oluoch, 1990). Furthermore, embryogenesis and hatching also highly depended on temperature, which could be accelerated or delayed under different water temperature conditions (Planas et al., 2012; Tong et al., 2000). Typically, increasing temperature in a certain range shortened embryos hatching time while temperature below or above a specific threshold delayed hatching and even caused abnormalities and/or mortality (Das et al., 2006; Folkvord et al., 2015; Lin et al., 2006; Pandian and Katre, 1972; Seuffert et al., 2012; Sfakianakis et al., 2004). This was particularly true in China. For instance, it took three months for *P. clarkii* embryos hatching in Poyang lake (annual min-max and mean water temperature: 3.52 °C—31.18 °C, 18.37 °C, Hu, 2009; Li et al., 2012; Xiao et al., 2011), while it took four months in Huangjin Lake (annual min-max and mean water temperature 7.0–34.1 °C, 17.4 °C, unpublished data provided by Qidong Wang and Kai Feng, Gong et al., 2008; Lv, 2006). These studies suggest the potential for improving *P. clarkii* reproductive performance and embryonic development by manipulating water temperature in controlled conditions. However, only few studies have attemped to determine temperature effects on hatching time of *P. clarkii* embryos (Lv et al., 2004, 2006; Suko, 1956; Wang, 2012). More detailed information would be an important prerequisite for the development of artificial reproduction techniques. We thus hypothesized that the manipulation of water temperature would be an effective alternative to improve reproductive performance and embryonic development of *P. clarkii*.

The objectives of the present study were to: (1) evaluate the effects of water temperature on the reproductive performance of adult *P. clarkii* (survival, feeding rates, spawning rates, duration from mating to spawning and fecundity); (2) determine the effects of water temperature on embryonic development (morphological abnormalities, relationship between embryonic development and temperature); (3) build a temperature-dependent developmental model for *P. clarkii* embryos to better predict their hatching time.

To tackle these questions, we reared *P. clarkii* under five different temperatures (17 °C, 21 °C, 25 °C, 29 °C, and 33 °C), which were the typical temperature range during reproductive seasons in the wild (Qianjiang, China, see supplementary material 1). The present study will hopefully provide theoretical basic knowledge for optimizing crayfish reproductive performance and embryos culture conditions in crayfish aquaculture.

2. Material and methods

2.1. Broodstock and embryos collection and holding

Adult crayfish (weight: 31.03 ± 1.95 g, total length: 105.41 ± 1.20 mm, mean ± SE) used in experiment 1 and experiment 2 were collected during the peak of ovarian maturation from the Selection and Reproduction Center of Crayfish (30.41°N, 112.75°E), Qianjiang, China. After transportation to the laboratory, crayfish were randomly paired and each paired crayfish (one male crayfish and one female crayfish) was kept separately in small tanks (35 × 30 × 25 cm). They were then acclimated to the experimental conditions for two weeks in five recirculation systems with constant aeration, during which they were fed a 30% protein commercial crayfish diet twice daily. In the beginning, all crayfish were reared under the same temperature conditions (23 °C) in the five recirculation systems, and then water temperatures were adjusted gradually at a rate of 1 °C per day until the experimental temperatures were reached and then maintained thereafter. All paired crayfish were checked every hour so that the accurate dates for mating and spawning could be determined. Embryos used in experiment 2 were obtained from 60 ovigerous females. They were incubated attached to the pleopods of females (thereby under the same temperature conditions as females). Eggs from each female were used as an independent replicated experimental unit. At the beginning of the experiment, we randomly dissected 20 ovaries from female crayfish to determine ovarian developmental stages through histological analyses. They were first weighted to calculate the gonadosomatic index (GSI, 3.18 ± 0.15, mean ± SE) and then fixed in Bouin's solution (Wuhan Servicebio Technology Company, China). The samples were dehydrated in 50%, 70%, 85%, 90%, 95%, and 100% ethanol and embedded in paraffin blocks. Finally, the slides were stained with hematoxylin and eosin (Kiernan, 1999; Suvarna et al., 2012). The histological analyses were conducted on micrographs under an Olympus BX53 microscope. The ovarian development of *P. clarkii* was divided

into seven stages (Kulkarni et al., 1991): stage I, stage II, stage III, stage IV, stage V, stage VI, and stage VII. The results are shown in supplementary material 2, with 3 crayfish ovaries developing to stage IV and 17 crayfish ovaries developing to stage V or VI (considered as mature ovaries).

2.2. Culture conditions

In the laboratory, adult crayfish were reared in five independent closed recirculation systems, operating at a fixed temperature of 17 °C, 21 °C, 25 °C, 29 °C, and 33 °C. This temperature range was chosen to represent the average water temperature during the reproductive seasons of *P. clarkii* in Qianjiang, China (mean temperature of 31.25 °C in August, 26.56 °C in September, and 19.94 °C in October, see supplementary material 1), which was recorded every two hours by data HOBO loggers (UA-002-64, HOBO Pendant temperature/light 64 K data logger Onset, Bourne, MA, USA).

Each system consisted of 16 large tanks (35 × 120 × 25 cm, experiment 1) and 64 small tanks (35 × 30 × 25 cm, experiment 2). Each tank served as an independent replicated experimental unit. In each tank, PVC pipes were provided for shelters of crayfish (four pipes in each large tanks and one pipe in each small tank). Tap water with ultraviolet sterilization and aeration for chlorine elimination was delivered to each tank at a constant rate of 1 L/min during the study. Tanks were cleaned every day. Photoperiod was maintained at a 12:12 (light: dark) cycle. Water temperatures during the whole experimental period were controlled with high precision, which were recorded every two hours with data loggers and shown in supplementary material 3. The pH, dissolved oxygen, and hardness were measured daily by a YSI probe (Yellow Springs Instruments, Yellow Springs, OH, USA). The concentration of ammonia nitrogen was determined using the standard method (APHA et al., 1989). Water quality variables during the whole experiment were within the suitable ranges: dissolved oxygen 5.60 ± 0.9 mg/L, pH 7.12 ± 0.21, hardness 125 ± 7 mg/L, and ammonia nitrogen 0.54 ± 0.13 mg/L.

2.3. Experiment 1

2.3.1. Experimental design

Experiment 1 was designed to evaluate the effects of water temperature on the reproductive performance of *P. clarkii*. It was conducted from September to October 2017 for 50 days under five constant temperatures (17 °C, 21 °C, 25 °C, 29 °C, and 33 °C), with 12 replicates of each treatment (total N = 480, 240 females and 240 males). Each replicate consisted of four paired female and male crayfish. The crayfish were fed twice daily with an artificial diet purchased from Charoen Pokphand Group (WHS001-2016, 30.23% protein, 10.74% lipid, 10.18% moisture, and 8.70% ash). All crayfish were checked every day so that their mortality, accurate dates for mating and spawning could be determined. Tanks were cleaned every day.

2.3.2. Data collection and measurement

At the beginning and the end of the experiment, crayfish weight was determined by a 0.01 g precision scale. Feeding rates were measured following the methods described in a previous study (Van Ham et al., 2003). Specifically, crayfish were fed with an excess quantity of weighted artificial diet until feeding activities stopped within one hour. Then, the remaining artificial diet was removed, dried and reweighted. Finally, we determined the given amount of artificial diet to calculate feeding rates. The duration from mating to spawning was calculated as the number of days from mating to spawning. After spawning, all eggs were counted to determine the fecundity of female *P. clarkii*. Other parameters were calculated as follows:

Survival (%) = 100 × (final crayfish number/initial crayfish number)

Feeding rate (%body weight/day) = 100 × total feed intake (dry matter, g /days)
/[(initial body weight (wet weight, g)
+ final body weight (wet weight, g))/2]

Spawning rate (%) = 100 × (final spawning crayfish number
/initial female crayfish number)

2.4. Experiment 2

2.4.1. Experimental design

Experiment 2 was designed to determine the optimal temperature for embryonic development. It was conducted from September to December 2017 (90 days) at 17 °C, 21 °C, 25 °C, 29 °C, and 33 °C. There were 12 replicates for each temperature treatment, and each replicate included one ovigerous crayfish (a total of 60 females for experiment 2). Eggs from the same ovigerous female crayfish served as an independent replicate. Once females spawning, the eggs were sampled for monitoring embryonic development. Ovigerous crayfish rearing methods were identical to those for experiment 1.

2.4.2. Data collection and measurement

For the 21 °C and 25 °C treatments, 30 eggs were collected for each sampling to determine developmental stages under the dissecting microscope LEICA MVX10 (M205FA). Because embryos at 17 °C developed slowly with insufficient embryos to be sampled later, sometimes only 10 eggs were sampled. Photographs of eggs were taken to determine their developmental stages. During the 36 h of spawning, eggs were examined every two hours and thereafter, daily until hatching.

In order to provide accurate time for each stage of embryos development, we first classified embryonic development into 9 stages according to previous studies (Dai et al., 2009; Feng et al., 2007; Harper and Reiber, 2006; Lei et al., 2009): I, zygote; II, cleavage; III, blastula; IV, semicircular furrow; V, circular furrow; VI, gastrula; VII, nauplius; VIII, zoea; and IX, hatching. Characteristics of each developmental stage are shown in Fig. 1. Then we recorded the duration of development for each stage. The end of each stage was defined as the time at which 50% of the embryos sampled had passed into the next stage. This index is often chosen to compare embryonic development when different numbers of eggs are sampled in different studies (Geffen et al., 2006; Webb et al., 2007; Yang and Chen, 2005).

Based on our data, the predictive exponential model (Bělehrádek's equation) of the developmental time was established as follows: $D = a(T-\alpha)^b$, where a, b, and α were constants, D was the development time (days) and T was the temperature (°C) (Belehradek, 1957). It was commonly used to describe the relationship between temperature (°C) and embryonic development time (Yamakawa and Matsuda, 1997). Based on the relationship of embryonic development and temperature, we estimated the Bělehrádek equation parameters following the methods described by previous studies (Corkett and McLaren, 1970; Ozaki and Ikeda, 1997; Yamakawa and Matsuda, 1997). Specifically, a represented for the differences in mean slope (shifts on the development scale) and b depicted the degree of curvilinearity over the vital temperature range. The α was "theoretical biological zero temperature" (theoretical temperature below which eggs stop their development). The equation was first converted to logarithm: $D = \log_{10}a + b\log_{10}(T-\alpha)$. Then, it was fitted to get constants by successive approximation to that value of α having the smallest sums of squares of deviations of observed hatching times.

2.5. Statistical analysis

We used non-parametric Kruskal-Wallis tests followed by pairwise Wilcoxon Rank Sum tests (post hoc test) to detect the differences in

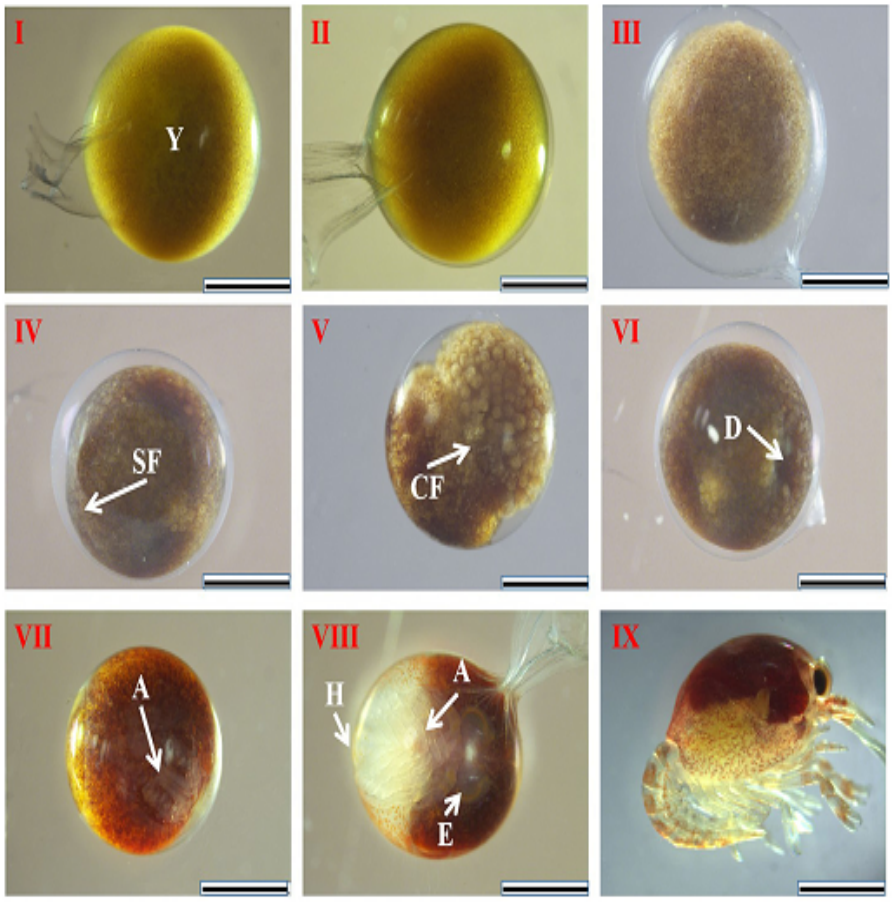

Fig. 1. Morphological characteristics of *Procambarus clarkii* embryos in nine different developmental stages. I, zygote, opaque and full of yolk (Y); II, cleavage; III, blastula; IV, semicircular furrow (SF, due to blastula invagination); V, circular furrow (CF); VI, gastrula with dent (D) visible (the sign of gastrula); VII, nauplius with appearance of appendages (A); VII, zoea showing the heart (H) region, a pair of round eyes (E), appendages (A) and enlarged transparent area; IX, hatching. Scale bars are 1 mm.

survival, feeding rates, spawning rates, duration from mating to the spawning, fecundity, and embryos hatching time among different temperature treatments. Independent samples *t*-tests were used to analyze the differences in survival between sexes. In order to estimate optimal temperature for *P. clarkii* reproductive performance, polynomial models were fitted to the data of spawning rates and fecundity, and loess regression was fitted to the data of duration from mating to spawning. We used non-metric multidimensional scaling analysis (NMDS) to ordinate samples of embryos developmental stages under different temperatures. Results were presented according to five levels of experimental temperatures. Stress (mismatch in the relationship between the distance in the original space and the reduced ordination space) is normally a factor indicating the quality of NMDS analysis, and lower values (< 0.2) generally result in good interpretations (McCune et al., 2002; Wittig and Becker, 2010). Statistical differences were set to 0.05 and all statistical analyses were performed in the software R version 3.3.2 (R Core Team, 2017).

3. Results

3.1. Experiment 1

3.1.1. Survival

Adult crayfish survival was not significantly affected by temperature for both females and males (Kruskal-wallis test, females: $\chi^2 = 4.27$, $P = 0.37$; males: $\chi^2 = 3.01$, $P = 0.56$). Furthermore, no significant differences between sexes were observed (Independent samples *t*-tests, 17 °C: $t = 1.68$, $P = 0.11$; 21 °C: $t = 1.26$, $P = 0.22$; 25 °C: $t = 1.15$, $P = 0.26$; 29 °C: $t = 1.70$, $P = 0.11$; 33 °C: $t = 1.17$, $P = 0.25$. Original data see supplementary material 4).

3.1.2. Feeding rates

The feeding rates of adult crayfish were significantly affected by temperature (Kruskal-wallis test, $\chi^2 = 43.51$, $P < 0.001$). Specifically, at 21 °C and 25 °C, feeding rates of adults were significantly higher than those at other temperatures (pairwise Wilcoxon test, all $P < 0.001$) (Supplementary material 5).

3.1.3. Spawning rates

Spawning events occurred from 17 °C to 33 °C, but spawning rates were significantly affected by temperature (Kruskal-wallis test,

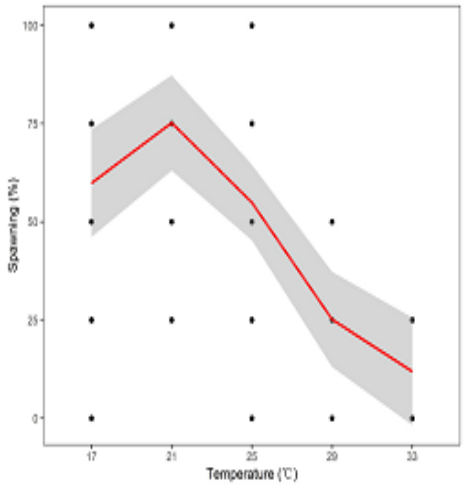

Fig. 2. Scatterplot of spawning rates of female *Procambarus clarkii* exposed to five different temperatures. Total sample sizes were 109, with 29 samples for 17 °C (2, 3, 3, and 3 samples overlapped in the spawning rates values of 25%, 50%, 75%, and 100%, respectively), 35 for 21 °C (3, 4, and 4 samples overlapped in 50%, 75%, and 100%, respectively), 28 for 25 °C(4 and 5 samples overlapped in 50% and 75%, respectively), 11 for 29 °C (3, 7, and 2 samples overlapped in 0%, 25%, and 50% respectively), and 6 for 33 °C(6 samples overlapped in 0% and 25%, respectively). Red line denotes the fitted values from the polynomial model and the grey area denotes 95% confidence interval. The relationship between spawning rates and temperature is shown as: $Y = 45.42 - 159.75X - 61.72X^2 + 57.05X^3$ ($F = 19.14$, $P < 0.001$, $r^2 = 0.48$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

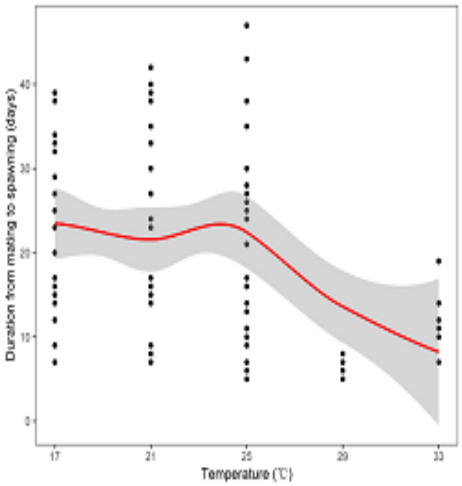

Fig. 3. Scatterplot of duration from mating to spawning of female *Procambarus clarkii* at five different temperatures. Total sample sizes were 109, with 29, 35, 28, 11, and 6 samples for 17 °C, 21 °C, 25 °C, 29 °C, 33 °C, respectively. Red line denotes the fitted values from the loess regression and the grey area denotes 95% confidence interval (*span* = 0.75). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$\chi^2 = 31.04$, $P < 0.001$). More specifically, the spawning rates of *P. clarkii* at 29 °C and 33 °Cwere significantly lower than those at 17 °C, 21 °C, and 25 °C (pairwise Wilcoxon test, 17–29 °C: $P = 0.002$; 17–33 °C: $P < 0.001$; 21–29 °C: $P < 0.001$; 21–33 °C: $P < 0.001$; 25–29 °C: $P = 0.001$; 25–33 °C: $P < 0.001$). The spawning rates were also fitted by a polynomial model. Based on the model, the optimal spawning rate occurred at 21 °C. The relationship between spawning rates and temperature is shown as: $Y = 45.42 - 159.75X - 61.72X^2 + 57.05X^3$ ($F = 19.14$, $P < 0.001$, $r^2 = 0.48$, Fig. 2).

3.1.4. Duration from mating to spawning

The duration from mating to spawning of crayfish was significantly affected by temperature (Kruskal-wallis test, $\chi^2 = 27.77$, $P < 0.001$). At 29 °C, it was significantly shorter than other treatments (pairwise Wilcoxon test, 17 °C: $P < 0.001$; 21 °C: $P < 0.001$; 25 °C: $P < 0.001$; 33 °C: $P = 0.003$) while at 33 °C, it was significantly shorter than those at 17 °C (pairwise Wilcoxon test, $P = 0.006$). We used loess regression to fit the observed data, which showed that crayfish at 17 °C had the longest duration while crayfish at 33 °C had the shortest duration (*span* = 0.75, Fig. 3).

3.1.5. Fecundity

Fecundity of female *P. clarkii* ranged from 163 to 624 in the present study. They were significantly higher at 21 °C and 25 °C when compared to other treatments, and *P. clarkii* at 17 °C also had significantly higher fecundity compared to 29 °C and 33 °C (Kruskal-wallis test and pairwise Wilcoxon test, $\chi^2 = 60.64$, 29 °C — 33 °C: $P = 0.01$, others: $P < 0.001$). The polynomial model showed that the optimal temperature for improving fecundity was 21 °C. The relationship between fecundity and temperature is shown as: $Y = 442.38 - 536.62X - 745.43X^2 + 197.35X^3$ ($F = 75.48$, $P < 0.001$, $r^2 = 0.67$, Fig. 4).

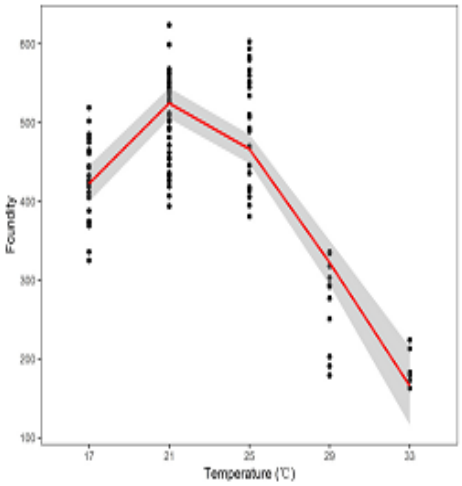

Fig. 4. Scatterplot of the fecundity of female *Procambarus clarkii* exposed to five different temperatures. Total sample sizes were 109, with 29 for 17 °C, 35 for 21 °C, 28 for 25 °C, 11 for 29 °C, and 6 for 33 °C, respectively. Red line denotes the fitted values from the polynomial model and the grey area denotes 95% confidence interval. The relationship between fecundity and temperature is shown as: $Y = 442.38 - 536.62X - 745.43X^2 + 197.35X^3$ ($F = 75.48$, $P < 0.001$, $r^2 = 0.67$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Experiment 2

3.2.1. Morphological abnormalities

During the early stages of embryo development (< 72 h), we observed abnormalities and death of all eggs at 29 °C and 33 °C. These abnormalities included abnormal cleavage (Fig. 5A), blastula lesions (Fig. 5B), punctured membranes (Fig. 5C), abnormal invagination of blastula (Fig. 5D), and gastrulation lesions (Fig. 5E). However, no abnormalities were observed in embryos at 17 °C, 21 °C, and 25 °C.

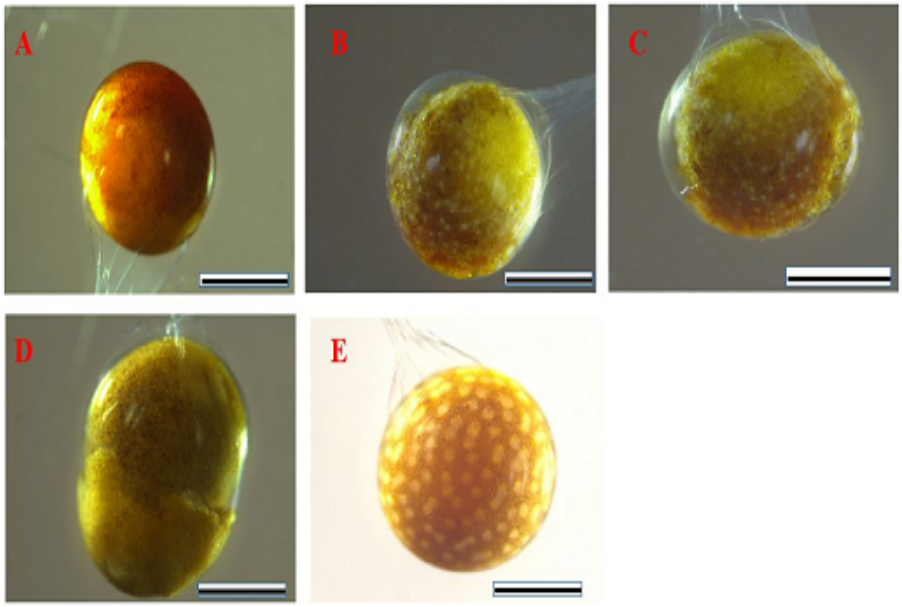

Fig. 5. Morphological abnormalities of *Procambarus clarkii* embryos exposed to 29 °C and 33 °C. (A) abnormal cleavage, (B) blastula lesions, (C) punctured membranes, (D) abnormal invagination of blastula, (E) gastrulation lesions. Scale bars are 1 mm.

3.2.2. Relationship between embryonic development and temperature

The NMDS analysis on embryos developmental stages at five different temperatures revealed that successful hatching of *P. clarkii* only occurred when they were exposed to 17 °C, 21 °C, and 25 °C (*stress* = 0.04, Fig. 6). The development of embryos at 29 °C and 33 °C was aborted and they were dead before hatching. Embryos hatching time was significantly shortened when *P. clarkii* exposed to 25 °C (Kruskal-Wallis and pairwise Wilcoxon Rank Sum tests, $\chi^2 = 31.07$, 17 °C — 25 °C: $P < 0.001$; 21 °C — 25 °C: $P < 0.001$).

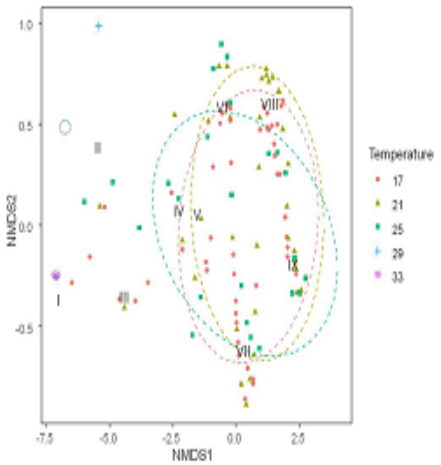

Fig. 6. Non-metric multidimensional scaling (NMDS) plot based on embryos developmental stages for female *Procambarus clarkii* exposed to five different temperatures (17 °C, 21 °C, 25 °C, 29 °C, and 33 °C). Each point (different shapes and colors representing that they were exposed to different water temperatures) in the two-dimensional space represents an individual embryo developmental stage. Ellipses indicate 68% confidence intervals of embryos developmental stages exposed to different water temperature. I ~ IX represents *P. clarkii* embryos developmental stages. Specifically, I, zygote; II, cleavage; III, blastula; IV, semicircular furrow; V, circular furrow; VI, gastrula; VII, nauplius; VII, zoea; and IX, hatching. *Stress* is 0.04 for NMDS analysis, indicating good interpretations of the results.

3.2.3. Temperature-dependent developmental model

The 50% developmental time of the sampled eggs is an important index to compare embryonic development. When exposed to 17 °C, the embryonic development took significantly more time compared to 21 °C and 25 °C. The average time taken for 50% of embryos hatching at 17 °C was 85 days, while they were 29 days for 21 °C and 21 days for 25 °C (Fig. 7).

Based on the relationship of embryos developmental time and temperature, we built a predictive exponential model as follows: $D = 3{,}140{,}837(T\text{-}2.03)^{-3.76}$ ($r^2 = 0.96$, $F_{1,\ 34} = 765.8$, $P < 0.001$, Fig. 7), where D was the embryonic development duration from spawning to hatching in days and T was the hatching temperature in °C. The model described the curvilinear relationship between the duration of embryonic development and temperature, and it fitted the experimental data very well, with a very high value of $r^2 = 0.96$. In addition, it indicated that the theoretical biological zero temperature of 2.03 °C for embryonic development of *P. clarkii*. This meant that under this temperature, embryonic development would be aborted.

4. Discussion

4.1. Optimal temperature for female reproductive performance

The results of experiment 1 showed that female *P. clarkii* at 21 °C and 25 °C had significantly higher reproductive outputs than other treatments. According to the polynomial models and loess regression, crayfish at 17 °C had a long duration from mating to spawning while they had significantly lower spawning rates and fecundity at 29 °C and 33 °C. Taken together, we thus suggest the optimal temperature range of 21 °C – 25 °C for improving reproductive performance of female *P. clarkii*.

Temperature is generally a crucial factor influencing the spawning activities of crayfish (Carmona-Osalde et al., 2004; Liu et al., 2013a;

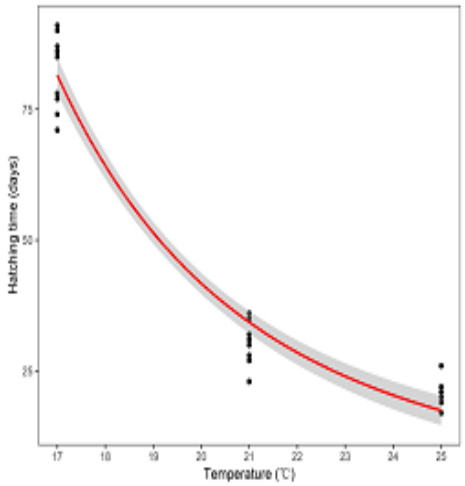

Fig. 7. Scatterplot of hatching time of *Procambarus clarkii* embryos across temperatures fitted by the predictive exponential model (r^2 = 0.96). Each black point represents 50% sampled eggs hatching time from each replicate in different temperatures of the current study (N = 36). The solid red curve represents the estimated results from the model and the grey areas represent the 95% confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Tropea et al., 2010). In the current study, the spawning rates of *P. clarkii* exposed to 21 °C and 25 °C were significantly higher than those at other temperatures. This was different from a previous study reporting that 16–18 °C could significantly induce spawning of *P. clarkii* (Liu et al., 2013a). Similar results could also be found on other species such as crayfish *Cherax quadricarinatus* (Tropea et al., 2010), *Procambarus llamasi* (Carmona-Osalde et al., 2004), crabs *Callinectes sapidus* (Bembe et al., 2017), *Genus menippe* (Bert et al., 2016), shrimps *Penaeus semisulcatus* (Aktaş et al., 2003), *Exopalaemon carinicauda* (Liang et al., 2017), and lobster *Panulirus japonicus* (Matsuda et al., 2002). We inferred that the discrepancy among studies might be due to the fact that spawning activities were not only dependent on temperature, but that other environmental factors (e.g. photoperiod, foods, and salinity) not tested in this study could also interact with temperature and then influence spawning activities (Gutierrez-Yurrita and Montes, 1999; Harlioğlu and Duran, 2010; Liu et al., 2013a; Meineri et al., 2014). Furthermore, differences between populations in different studies could also be responsible for the discrepancy as each population might be adapted to different environmental conditions, which thus might result in variability in life history traits such as spawning activities (Alcorlo et al., 2008; Chucholl, 2011; Peruzza et al., 2015). In this case, it appears that different *P. clarkii* populations may have different optimal temperatures for spawning, thus resulting in the discrepancy between our study and previous ones.

Our study also showed that the duration from mating to spawning were significantly shortened at 29 °C and 33 °C (mean duration were 6 and 12 days, respectively). However, in the wild, the mean duration from mating to spawning for *P. clarkii* in China was much longer (approximately two months in Xuyu and one month in Wuhan, Gong et al., 2008; Xu et al., 2014). This could be due to the fact that temperature was continuously decreasing in the wild while it was kept constant all the time in our study. Furthermore, protein and lipid-rich artificial diet used in our study may shorten the duration (30.23% and 10.74% for crude protein and lipid contents). However, in the wild, *P. clarkii* fed on natural foods such as macrophytes, which contained lower protein and lipid contents (Carreira et al., 2014; Carvalho et al., 2016). For instance, a previous study demonstrated that protein and lipid contents were 2.57% and 0.44% for *Hydrilla verticillata*, 2.40% and 0.54% for *Ceratophyllum demersum*, 3.77% and 0.79% for *Elodea Canadensis*, 1.39% and 0.45% for *Vallisneria spiralis* (Zhang et al., 2016). For experiment 1, we also found an interesting phenomenon regarding no significant differences observed in survival among treatments. These results were different from what were reported by several previous studies (Gherardi and Paglianti, 2004; Han et al., 2011; Mazlum and Eversole, 2005; Zhang et al., 2015). We inferred that it may be due to the different experimental designs and culture conditions (e.g. constant or ambient temperature variations, different temperature ranges tested, experimental *P. clarkii* sizes, and even whether other environmental factors synchronically action with temperature). For instance, some authors set the experimental minimum temperature (10 °C) lower than ours (17 °C), which thus might more or less relate to different survival results (Han et al., 2011; Mazlum and Eversole, 2005). Furthermore, in our study, the temperature tested (17 °C, 21 °C, 25 °C, 29 °C, and 33 °C) were within the temperature range that the experimental *P. clarkii* experienced during their reproductive seasons in the wild. In this case, the *P. clarkii* in the current study might have tolerance and resistance to these temperatures, which in turn resulted in the no differences in survival results.

The ability to induce spawning spontaneously is a key step for large-scale production of juvenile crayfish in aquaculture. Currently, eyestalk ablation and hormones injection are widely used to induce spawning of crustaceans including *P. clarkii*. The current study demonstrated that manipulating water temperature could achieve similar effects on inducing spawning of *P. clarkii* (mean spawning rates: 72% and 58.33% for 21 °C and 25 °C) when compared with eyestalk ablation and hormones injection (mean spawning rates with eyestalk ablation: 63.33%, HCG injection: 77.5%, LD injection: 55%, domperidone injection: 66.67%, domperidone and serotonin injection: 59.26% and 17 α-hydroxyprogesterone injection: 20%) (Liu et al., 2014; Liu et al., 2013b; Zhang, 2011). In addition, hormones injection and eyestalk ablation compromised with lower adults survival (mean survival range from 15.56% to 51.11% in previous studies while 83.9% in our study) and even decreased fertilization rates and offspring sizes (especially for nauplii) (Fornies et al., 2001; Magana-Gallegos et al., 2018; Mylonas et al., 1992). Such cases could also be found in other crustacean species (Pillai et al., 2011; Weng et al., 2012; Vaca and Alfaro, 2000; Wen et al., 2009). All these results show that temperature manipulation could be a more efficient and ethical alternative technique for *P. clarkii* reproduction. Although the present study convinced that 21 °C – 25 °C was optimal for improving female *P. clarkii* reproductive performance, we could not exclude the point that different developmental stages of female crayfish ovaries (3 crayfish ovaries developing to stage IV and 17 crayfish ovaries developing to stage IV or stage V) at the beginning of the experiment might influence the results of spawning rates and duration from mating to spawning to a certain degree during a 50-day experimental period. More studies are encouraged to address this question and confirm whether asynchronous ovaries development has potential effects on crayfish spawning activities and the duration from mating to spawning. Anyway, these results support our hypothesis that water manipulation is an efficient alternative technique of reproduction, and 21 °C– 25 °C is suggested for improving the the reproductive performance of female *P. clarkii*.

4.2. Optimal temperature on embryonic development

Results from experiment 2 and temperature-dependent developmental model showed that the optimal temperature for embryonic development was 25 °C. The model was described as $D = 3{,}140{,}837(T-2.03)^{-3.76}$.

In the present study, embryos hatching time was shortened as temperature increased within the defined temperature range (17–25 °C). Optimal embryonic development was observed at 25 °C (mean duration of 21 days) while it was delayed (85 days) at 17 °C. This suggests that the embryonic development of *P. clarkii* could be

accelerated by manipulating water temperature. The results were consistent with previous studies reporting faster development with increasing temperatures within a suitable range: 23–30 days at 21 °C and 15–20 days at 25.8 °C for *P. clarkii* (Lv et al., 2006; Suko, 1956; Wang, 2012). Hatching temperature was previously shown to have similar effects on embryonic development of other crustacean species (Branford, 1978; Brillon et al., 2005; Perkins, 1972; Sachlikidis et al., 2010; Stevens et al., 2008; Tong et al., 2000; Webb et al., 2007) and fish (Brown et al., 2011; Morehead and Hart, 2003; Peña et al., 2014; Wen et al., 2013; Yang and Chen, 2005). All these findings further indicate that manipulating water temperature to induce spawning and embryos hatching is a potentially effective way to provide mass production of juveniles in a limited time.

4.3. Temperature-induced abnormality and death of embryos

Abnormality of embryos is one of the most serious problems in aquaculture, which is mainly due to suboptimal culture conditions (Cobcroft et al., 2001; Fraser and De Nys, 2005). For instance, high temperature could induce abnormalities of embryos especially during cleavage, blastomere and gastrulation stages of many hatchery-reared species (Aritaki and Seikai, 2004; Huang et al., 2010; Sfakianakis et al., 2004; Wang and Tsai, 2000). In the current study, *P. clarkii* exposed to the high temperatures (29 °C and 33 °C) during embryonic development also showed abnormalities and ceased to develop while no abnormalities were detected at lower temperatures (17 °C, 21 °C, and 25 °C). Similar phenomena have also been reported in many fish species such as *Solea senegalensis* (Dionísio et al., 2012), *Danio rerio* (Casper et al., 2015), *Vimba vimba* (Lugowska and Kondera, 2018), *Sparus aurata* (Georgakopoulou et al., 2010), and *Dicentrarchus labrax* (Georgakopoulou et al., 2007).

High mortalities of embryos also occurred when hatching temperatures were out of the suitable ranges (Lahnsteiner et al., 2012; Lugowska and Witeska, 2018). In our study, all the embryos failed to hatch above 29 °C while a previous study showed that 40% of the embryos of *P. clarkii* died at 30 °C, and 100% died at 41 °C (Lv et al., 2004). This discrepancy and reduced thermal tolerance in our study might be attributed to different maternal thermal history, which has been considered as the most important factor influencing thermal tolerance, thus resulting in the different results of embryos thermal tolerance between studies (Lutterschmidt and Hutchison, 1997; Soundarapandian et al., 2014). Previous studies also found that animals exposed to dynamic temperature changes would have faster acclimation rates and thus increase their thermal tolerance (Beitinger et al., 2000; Heath, 1963; Hutchison and Ferrance, 1970; Mora and Maya, 2006). The different duration that crayfish embryos exposed to suboptimal temperature conditions could also affect their survival and abnormalities. In the present study, we did not observe abnormalities when embryos were exposed to 17 °C, 21 °C, and 25 °C, which suggested that embryos at these temperatures displayed better ontogeny. Based on these results, we thus recommend performing embryos hatching at 25 °C and avoiding hatching temperatures higher than 29 °C to perform balanced embryonic development.

4.4. Embryo developmental model

The relationship between temperature and developmental time was best described by a nonlinear model that fitted the experimental data very well. This model is useful to predict embryos hatching time based on temperatures and would hopefully help farmers to predict juveniles recruitment time and optimize their culture conditions. According to the Match/Mismatch theory, a mismatch of hatching time and food availability would subsequently lead to low survival and poor growth (Cushing, 1990). In the wild, the diets of juvenile *P. clarkii* include a variety of zooplankton (e.g. *Daphnia magna*), and macrophytes (e.g. *Myriophyllum spicatus*) (Carreira et al., 2014; Carvalho et al., 2016; Dan et al., 2007). The abundance of these food sources has pronounced influences on survival of juveniles (Fiksen and Jørgensen, 2011; González-Ortegón et al., 2015). With this model, farmers and scientists will be able to predict the occurrence time of juveniles in farmed and natural ponds. This could help match food resources and optimize feeding strategies in aquaculture to make multiple hatchery productions thus increasing potential production.

5. Conclusions

A sustainable and continuous supply of juvenile crayfish is now needed for the development of *P. clarkii* culture industry. Our study suggests that manipulating water temperature is an effective way to induce spawning and optimize embryonic development to improve juvenile crayfish production, which is of high interest for sustainable aquaculture. In the present study, the optimal temperatures for improving *P. clarkii* reproductive performance were 21 °C – 25 °C. Furthermore, the optimal temperature for embryonic development was 25 °C to shorten embryonic development time while avoiding embryos abnormalities and death. We also built a temperature-dependent developmental model, which could help farmers to predict juveniles recruitment time depending on their culture conditions. Other factors such as photoperiod, food quality, and salinity will also affect the reproductive performance and embryonic development of *P. clarkii*, and further experimental studies aiming at optimizing the culture conditions for *P. clarkii* aquaculture should be encouraged.

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Declarations of interest

None. The funding sponsors had no roles in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, nor in the decision to publish the results.

Declarations of submission

All authors approved the authorship and submission of the manuscript for peer review. The authors confirm that this manuscript has not been published and is not currently under consideration by any other journals.

Authorship

Shiyu Jin and Tanglin Zhang conceived and designed the investigation; Shiyu Jin, Feng Huang, Mantang Xiong, and Ruojing Li conducted the test; Shiyu Jin and Lisa Jacquin drafted the initial manuscript; Sovan Lek contributed to the data analysis; Wei Li, Sovan Lek, Jiashou Liu, and Tanglin Zhang provided guidance for data analysis, provided critical feedback on the manuscript and approved the final manuscript.

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Chapter 5

Effects of feeding levels on crayfish growth and muscle composition

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ORIGINAL ARTICLE

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Growth performance and muscle composition response to reduced feeding levels in juvenile red swamp crayfish *Procambarus clarkii* (Girard, 1852)

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Abstract

Overfeeding in aquaculture can lead to suboptimal growth and increased production costs. Red swamp crayfish, *Procambarus clarkii*, is one of the most noteworthy species cultured in China, but little information is available on the optimal feeding levels of this species, especially in ponds cultured with macrophytes. In this study, we tested the effects of five different feeding levels (20%, 40%, 60%, 80% and 100% satiation) of an artificial diet on growth performance and muscle composition of juvenile *P. clarkii* in 15 concrete ponds cultured with the macrophyte *Hydrilla verticillata* (three replicates for each treatment). The results showed that growth performance decreased only when feeding levels were below 60% satiation. Muscle composition analysis revealed that the moisture and ash contents of muscle did not vary significantly with feeding levels but that the lipid and protein contents of muscle significantly decreased when *P. clarkii* was fed to 40% satiation. Stable isotope analysis suggested a shift in crayfish diets to easily available *H. verticillata* when feeding levels decreased. With this study, we hope to encourage crayfish farmers to reduce feeding levels and increase natural food items such as macrophytes in cultured ponds to maximize crayfish yields and reduce production costs.

KEYWORDS
growth performance, muscle composition analysis, principal component analysis, *Procambarus clarkii*, stable isotope analysis

1 | INTRODUCTION

Aquaculture has undergone rapid development in recent years and has become a primary source of nutrition for human. Its annual production in 2014 was as high as 73.8 million tons, which represented 44% of the total fishery production, and the proportion would reach 52% in 2025 (FAO, 2017). However, aquaculture production relies heavily on the input of artificial diets, and overfeeding increases organic waste and pollution (Bureau & Hua, 2010; Grigorakis & Rigos, 2011; Sapkota et al., 2008). In addition, overfeeding and underfeeding can lead to suboptimal growth of cultured species as demonstrated in *Litopenaeus vannamei* (Peter, 1999), *Megalobrama amblycephala* (Xu, Li, Tian, Jiang, & Liu, 2016), *Tinca tinca* (Kamler, Myszkowski, Kamiński, Korwin-kossakowski, & Wolnicki, 2006) and *Paralichthys olivaceus* (Lee et al., 2018). Therefore, efficiently managing the input of artificial diets and their feeding levels is crucial for sustainable aquaculture (Bostock et al., 2010; Bureau & Hua, 2010).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), has high commercial values because of its short life cycles, rapid growth, high fecundity and environmental tolerance (Cruz & Rebelo,

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2007). Originating from northeastern Mexico and south-central United States, *P. clarkii* has been introduced to aquaculture worldwide (Barbaresi & Gherardi, 2000; Geiger, Alcorlo, Baltanas, & Montes, 2005; Hobbs, Jass, & Huner, 1989; Vedia & Miranda, 2013). According to the *China Fishery Statistical Yearbook*, the production of *P. clarkii* was 852,300 tons in 2016 and represented 41.94% of China's freshwater shrimp aquaculture production, thereby constituting a very important part of the national fishery output (Fisheries Department of Ministry of Agriculture, 2017).

In intensive crayfish aquaculture, artificial diets account for more than 50% of total aquaculture costs (Keckeis & Schiemer, 1992; Wong, Mo, Choi, Cheng, & Man, 2016). Many studies have confirmed that natural food items (e.g. benthic organisms, aquatic plants, plankton and detritus) are an important part of crayfish diets, but the effects of these food items on crayfish growth in commercial ponds are not well characterized (Anderson, Parker, & Lawrence, 1987; Gherardi & Barbaresi, 2008; Nunes & Parsons, 1999; Soares, Peixoto, Wasielesky, & D'Incao, 2005). Although many studies have investigated the optimal feeding levels of different cultured fish or crayfish species, most have been based on the results from a laboratory-controlled environment without considering the dietary contributions of natural food items (Baloi et al., 2017; El-Dahhar, Fayed, Sallam, El-Zaeem, & El-Greisy, 2015; Liu, Wen, & Luo, 2018; Luo et al., 2015; Sun et al., 2016). To date, very little information on the optimal feeding levels of *P. clarkii* is available, especially considering natural food items contributions such as *Hydrilla verticillata*, which are widely planted in many crayfish cultured ponds in China. Thus, improving the feeding strategies of crayfish is an important step towards cost-effective aquaculture (Hasan, 2000; Martinez-Córdova, Emerenciano, Miranda-Baeza, & Martinez-Porchas, 2015; Yuan et al., 2010).

In this study, we hypothesized that reducing the amounts of an artificial diet to an appropriate level would not negatively affect the growth or muscle composition of *P. clarkii*, and that the crayfish diets would shift from an artificial diet to one of the natural food items such as *H. verticillata*. To test this hypothesis, we placed 1,350 juvenile *P. clarkii* in 15 concrete ponds cultured with *H. verticillata* and fed them at five different levels. Thus, we (1) investigated the effects of feeding levels on the growth performance and muscle composition of juvenile *P. clarkii* and (2) determined the optimal feeding level for *P. clarkii* culture to maintain crayfish growth and minimize the amounts of artificial diet provided. In addition, we explored by using stable isotope analysis whether juvenile *P. clarkii* adjusted their diets and consumed more *H. verticillata* from the ponds when feeding levels decreased.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Juvenile *P. clarkii* (4.82 ± 0.15 g, 60.03 ± 0.52 mm, mean ± *SE*, no significant differences among treatments for crayfish sizes at the beginning of experiment) were obtained from ponds at the Selection and Reproduction Center of Crayfish, Qianjiang, Hubei Province, China. A 50-day feeding experiment was conducted in 15 experimental concrete ponds (90 juveniles per pond of 9 m^2), following the European Directive 2010/63/EU for animal experiments. Three replicate ponds were randomly assigned to each of the five feeding treatments (20%, 40%, 60%, 80% and 100% satiation). Before the experiment, crayfish were acclimated to the culture conditions for 1 week. At the beginning of the experiment, healthy juveniles were collected and randomly allocated to 15 concrete ponds. The running water flow rate in ponds was approximately 7 L/min, and constant aeration was supplied to each pond. Water depth was maintained at approximately 27 cm. *H. verticillata* was planted in 35 polyethylene flowerpots (0.44 m diameter) in each pond and used as both shelter and foods for *P. clarkii*. The coverage of *H. verticillata* was 60% in each pond. The water temperature, pH and dissolved oxygen (DO) were measured by a YSI probe (Yellow Springs Instruments, Yellow Springs, OH, USA). The concentrations of ammonia nitrogen, nitrite, chemical oxygen demand, total nitrogen, total phosphorus and chlorophyll *a* were determined using standard methods (APHA, 1992). Water quality parameters for all ponds (mean ± *SE*) were within the ranges of crayfish growth throughout the study: temperature 27.27 ± 1.06°C; DO 4.33 ± 0.70 mg/L; pH 9.3 ± 0.05; ammonia nitrogen 0.1400 ± 0.005 mg/L; nitrite 0.0472 ± 0.006 mg/L; total nitrogen 1.0609 ± 0.020 mg/L; total phosphorus 0.0445 ± 0.003 mg/L; chemical oxygen demand 8.8048 ± 0.100 mg/L; and chlorophyll *a* 14.5477 ± 0.340 μg/L.

2.2 | Feeding treatments

Throughout the experiment, crayfish were fed twice daily (8:00 and 18:00) with a widely used artificial diet. The experimental diet (26% protein level, based on previous nutritive studies) followed a common commercial diet formulation from Charoen Pokphand Group (WHS001-2016, Jover, Fernandez-Carmona, Rio, & Soler, 1999; Wu et al., 2007; Zhang et al., 2012; McClain, 1995; Xu et al., 2011). Ingredients and proximate analysis of the diet are presented in Table 1.

Crayfish were exposed to five feeding treatments (20%, 40%, 60%, 80% and 100% satiation) following the method described in the previous study (Van Ham et al., 2003). A plastic pallet (30 × 15 cm) was placed at the bottom of each pond, and the artificial diet was placed on it (Figure 1). The reference 100% satiation level was determined by feeding crayfish excess weighted artificial diet until feeding activity stopped within 1 hr. Then, the remaining artificial diet was removed, dried and reweighted (Van Ham et al., 2003). We then calculated the amount of artificial diet that was consumed by crayfish under 100% satiation. *P. clarkii* in other treatments were then fed at restricted levels of 80%, 60%, 40% and 20%, which were adjusted daily with reference to 100% satiation. At 100% satiation level, the given amount of artificial diet was approximately 5% of the wet body weight per day (2% at 8:00 and 3% at 18:00). The experiment ended after 50 days, when the majority of the males achieved a non-growing, sexually active form.

TABLE 1 Ingredient composition and proximate analysis of experimental diet

Ingredients	Diet (%)
Fish meal[a]	0.5
Rapeseed meal[b]	14
Soybean meal[c]	3
Cottonseed meal[d]	10
Wheat flour[e]	20
Rice bran[f]	8
DDGS[g]	18
Corn gluten[h]	15
Soybean oil[b]	2
Vitamin premix[i]	0.1
Mineral premix[k]	0.5
$Ca(H_2PO_4)_2$	0.5
Sodium chloride	1
Cellulose	3.9
Binder	3.5
Proximate composition	
Crude protein	26.53
Crude lipid	10.41
Ash	6.87
Moisture	13.96

[a]Fish meal was from Qingdao Great Seven Co., Ltd., Shandong, China. [b]Soybean oil was from Handan Mingfu Vegetable Oil Company, Hebei, China. [b,c,d]Rapeseed meal, soybean meal and cottonseed meal were purchased from Jiangxi Zhengbang Tech, Jiangxi, China. [e,f,g,h]Wheat flour, rice bran, DDGS and corn gluten were from Wuhan Yufeng Cereals, Oils and Foodstuffs Industrial, Hubei, China. [i,k]Vitamin and mineral premix were purchased from Haid Feeds Co., Ltd., Guangzhou, China.

2.3 | Sample collection

At the end of the experiment, all crayfish were starved for 24 hr and then collected for growth parameters measurement. Ten males and 10 females from each pond (60 crayfish for each treatment) were randomly sampled for muscle composition analysis and chill-killed using an ice-water bath. The tail muscles were removed from the shells and stored at −20°C for muscle composition analysis. Samples of two individuals from each pond were also chill-killed and maintained for stable isotope analysis.

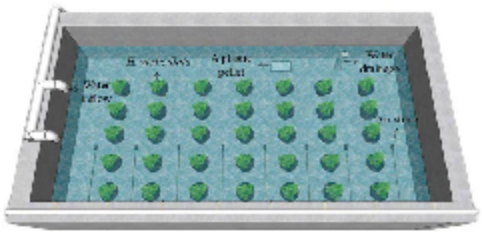

FIGURE 1 Diagram of the ponds used for culturing juvenile *Procambarus clarkii* during the experiment

2.4 | Growth performance

Parameters for growth performance such as survival, final length (*L*), final weight (*W*), gonad weight, liver weight and muscle weight were recorded and calculated as follows:

$$\text{Survival}\,(\%) = 100\times\left(\frac{N_t}{N_0}\right)$$

$$\text{Specific growth for weight}\,(SGR_W,\%,\text{per day}) = 100\times\left|\ln(W_t)-\ln(W_0)/T\right|$$

$$\text{Specific growth for length}\,(SGR_L,\%,\text{per day}) = 100\times\left|\ln(L_T)-\ln(L_0)/T\right|$$

$$\text{Gonadosomatic index}\,(GSI,\%) = 100\times\frac{W_g}{W_t}$$

$$\text{Hepatosomatic index}\,(HSI,\%) = 100\times\frac{W_l}{W_t}$$

where N_t is the final number of *P. clarkii* per treatment, and N_0 is the initial number of *P. clarkii* per treatment; W_t is the final weight of *P. clarkii*, and W_0 is the initial weight of *P. clarkii*; L_T is the final length of *P. clarkii*, and L_0 is the initial length of *P. clarkii*; W_g is the gonad weight of *P. clarkii*, and W_l is the liver weight of *P. clarkii*; and T is the number of experimental days.

2.5 | Muscle composition analysis

Crayfish muscle and diets were analysed for protein, lipid, moisture and ash contents. Protein content was determined using the Kjeldahl method (N × 6.25) (William, 1980) with a 4800 Kjeltec Auto Analyzer (FOSS Tecator, Haganas, Sweden). Lipid content was determined using chloroform-methanol extraction (Folch, Lees, & Sloane Stanley, 1957). Moisture content was determined by placing a 1-g sample into a convection oven (105°C) for 2 hr and drying it to constant weight (William, 1980). Ash content was determined by placing a 1-g sample combusting at 550°C in a muffle furnace for approximately 10 hr (William, 1980).

2.6 | Stable isotope analysis

In total, 30 crayfish (three males and three females from each treatment), three artificial diet samples and four *H. verticillata* samples were collected at the end of the experiment and were analysed for their carbon and nitrogen isotope ratios respectively. Muscle samples of crayfish (one male and one female randomly chosen from each pond, six individuals for each treatment) were oven dried at 60°C for at least 48 hr to constant weight and were very finely

ground (<200 μm). All samples were processed for $\delta^{15}N$ and $\delta^{13}C$ isotopes by the Department of Earth System Science, Tsinghua University, Beijing, China (Alfaro, Thomas, Sergent, & Duxbury, 2006). Approximately 3-mg samples were combusted, gasses were analysed using gas chromatography and continuous flow-mass spectrometry (MAT-253, Thermo Fisher Scientific, USA). Samples were referenced to pre-calibrated C_6 sucrose, which was cross-referenced to the Vienna PeeDee Belemnite standard. The reference standard of $\delta^{15}N$ was atmospheric N_2 and measured to a precision of ±1%. The isotope values for $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) were according to the following equation:

$$\delta^{13}C\,(‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000$$

$$\delta^{15}N\,(‰) = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000.$$

2.7 | Statistical analyses

The pairwise permutation test was carried out to test the differences of survival among treatments. Kruskal–Wallis tests were used to analyse the differences of other growth parameters and muscle composition among treatments (non-parametric data) followed by Wilcoxon post hoc tests. Principal component analysis (PCA) was applied to further summarize the trends in growth performance when feeding levels reduced (Næs & Risvik, 1996). For the stable isotope data, we used the Bayesian stable isotope mixing model of the 'SIAR' package in R to obtain the contributions of the artificial diet and *H. verticillata* (Parnell, 2008). This model has strong statistical power in allowing uncertainty in the sources, the consumers' isotopic signatures and the fractionation values. We used the most appropriate fraction factor values of 3.4‰ for $\delta^{15}N$ and 0.8‰ for $\delta^{13}C$, according to the previous study (Alcorlo & Baltanas, 2013). All analyses were performed by R version 3.3.2 (R Core Team, 2017), and the significance level was set to 0.05.

3 | RESULTS

3.1 | Growth performance

The growth parameters such as survival, *W*, *L*, *GSI*, *HSI*, SGR_W, SGR_L and muscle weight of males and females are shown in Table 2. Most parameters did not differ among the 60%, 80% and 100% satiation treatments, showing that feeding crayfish to 60% satiation did not decrease the growth of crayfish. Crayfish survival ranged from 73.33% to 87.04%, and there were no significant differences among the treatments (Kruskal–Wallis test, $\chi^2 = 6.57$, $p = 0.16$).

Most specifically, for males, no significant differences in any parameters were observed among the 60%, 80% and 100% satiation treatments (Kruskal–Wallis tests, *W*: $\chi^2 = 1.16$, $p = 0.56$; *L*: $\chi^2 = 0.14$, $p = 0.93$; SGR_W: $\chi^2 = 1.21$, $p = 0.55$; SGR_L: $\chi^2 = 0.13$,

TABLE 2 The growth parameters of female and male *Procambarus clarkii* fed with five feeding levels (mean ± *SE*)

	Treatments				
	100% Satiation	80% Satiation	60% Satiation	40% Satiation	20% Satiation
Survival (%)	77.41 ± 3.21	87.04 ± 3.53	79.63 ± 2.53	78.89 ± 1.11	73.33 ± 1.1
Males					
W (g)[a]	19.70 ± 0.45[a]	19.66 ± 0.52[a]	20.15 ± 0.59[a]	19.19 ± 0.92[a]	14.09 ± 0.40[b]
L (mm)[b]	83.21 ± 0.70[a]	83.39 ± 0.67[a]	83.50 ± 0.78[a]	82.23 ± 1.03[a]	77.00 ± 0.58[b]
GSI (%)[c]	0.080 ± 0.004	0.064 ± 0.003	0.053 ± 0.002	0.063 ± 0.003	0.059 ± 0.002
HSI (%)[d]	7.72 ± 0.05[a]	7.59 ± 0.10[a]	7.93 ± 0.11[a]	6.67 ± 0.10[c]	6.95 ± 0.10[c]
SGR_W (%, day^{-1})[e]	2.75 ± 0.05[a]	2.74 ± 0.05[a]	2.78 ± 0.06[a]	2.62 ± 0.08[a]	2.07 ± 0.06[b]
SGR_L (%, day^{-1})[f]	0.65 ± 0.02[a]	0.65 ± 0.02[a]	0.65 ± 0.02[a]	0.62 ± 0.02[a]	0.49 ± 0.02[b]
Muscle weight (g)	1.70 ± 0.04[b]	1.91 ± 0.05[a]	1.9 ± 0.04[a]	2.06 ± 0.07[a]	1.55 ± 0.03[b]
Females					
W (g)	18.26 ± 0.39[a]	18.72 ± 0.41[a]	18.15 ± 0.33[a]	17.09 ± 0.62[b]	12.81 ± 0.32[c]
L (mm)	85.39 ± 0.57[a]	92.27 ± 0.66[a]	86.14 ± 0.77[a]	85.01 ± 0.91[a]	77.11 ± 0.49[b]
GSI (%)	0.46 ± 0.014[a]	0.30 ± 0.012[a]	0.29 ± 0.010[a]	0.29 ± 0.029[b]	0.27 ± 0.017[b]
HSI (%)	9.82 ± 0.12[a]	10.46 ± 0.10[a]	10.32 ± 0.08[a]	8.92 ± 0.09[b]	9.67 ± 0.09[b]
SGR_W (%, day^{-1})	2.62 ± 0.04[a]	2.65 ± 0.04[a]	2.61 ± 0.04[a]	2.43 ± 0.06[b]	1.89 ± 0.05[c]
SGR_L (%, day^{-1})	0.70 ± 0.01[a]	0.73 ± 0.01[a]	0.71 ± 0.03[a]	0.68 ± 0.02[a]	0.50 ± 0.01[b]
Muscle weight (g)	2.10 ± 0.04[b]	2.66 ± 0.06[a]	2.45 ± 0.04[a]	2.54 ± 0.07[a]	1.91 ± 0.03[c]

Note. Values in the same row sharing the same superscript are not significantly different ($p > 0.05$).

[a]*W*: final weight (g). [b]*L*: final length (mm). [c]*GSI*: gonadosomatic index (%) = 100 × (gonad weight, g)/(final weight, g). [d]*HSI*: hepatosomatic index (%) = 100 × (liver weight, g)/(final weight, g). [e]SGR_W: specific growth for weight (%/day) = 100 × [ln(final weight) − ln(initial weight)]/experimental days. [f]SGR_L: specific growth for length (%/day) = 100 × [ln(final length) − ln(initial length)]/experimental days.

$p = 0.94$; *GSI*: $\chi^2 = 5.55$, $p = 0.06$; *HSI*: $\chi^2 = 2.14$, $p = 0.34$; muscle weight: $\chi^2 = 3.80$, $p = 0.15$). The *W*, *L*, SGR_W and SGR_L in the 20% satiation treatment were significantly lower than the other treatments (Kruskal–Wallis tests, *W*: $\chi^2 = 87.33$, $p < 0.001$; *L*: $\chi^2 = 59.75$, $p < 0.001$; SGR_W: $\chi^2 = 87.47$, $p < 0.001$; SGR_L: $\chi^2 = 59.89$, $p < 0.001$). No significant differences were observed in *GSI* among all treatments (Kruskal–Wallis test, $\chi^2 = 5.70$, $p = 0.22$), while *P. clarkii* fed to 40% satiation had significantly lower *HSI* values (Kruskal–Wallis test, $\chi^2 = 34.69$, $p < 0.001$). No significant differences were observed in muscle weight among all treatments, except 20% satiation treatment (Kruskal–Wallis test, $\chi^2 = 19.15$, $p < 0.001$).

Female *P. clarkii* fed to 60% satiation showed no significant differences from those fed to 80% or 100% satiation in any parameters except muscle weight (Kruskal–Wallis tests, *W*: $\chi^2 = 0.68$, $p = 0.71$; *L*: $\chi^2 = 3.42$, $p = 0.18$; SGR_W: $\chi^2 = 0.68$, $p = 0.71$; SGR_L: $\chi^2 = 3.42$, $p = 0.18$; *GSI*: $\chi^2 = 11.74$, $p = 0.06$; *HSI*: $\chi^2 = 5.37$, $p = 0.07$). *P. clarkii* fed to 20% and 100% satiation had significantly lower muscle weight than did those in the other treatments (Kruskal–Wallis test, $\chi^2 = 33.704$, $p < 0.001$).

Principal component analysis was performed to summarize the main trends in growth performance of both males and females (Figure 2). PC1 included *W*, *L*, SGR_W, SGR_L and muscle weight, explaining 66.8% of the variance among samples. PC2 mainly separated females and males into two groups by *GSI* and *HSI*, explaining 22.25% of the variance. The two components explained 89.05% of the total variance. Considering both males and females, Figure 2 illustrated that crayfish fed to 20% satiation had the lowest *W*, *L*, SGR_W, SGR_L and muscle weight among all treatments. When fed to 40% and to 20% satiation, crayfish had lower *GSI* and *HSI* values. However, *P. clarkii* exhibited similar growing properties when feeding levels were to 60% satiation or above.

3.2 | Muscle composition

The ash, lipid, moisture and protein contents of *P. clarkii* among the five different feeding levels are shown in Figure 3. Neither the ash nor moisture content in the muscle of *P. clarkii* was significantly different among treatments (Kruskal–Wallis tests, ash: $\chi^2 = 1.04$, $p = 0.90$; moisture: $\chi^2 = 5.32$, $p = 0.26$). However, *P. clarkii* fed to 40% satiation had a significantly lower lipid content than that in the other treatments and a lower protein content than that in the 80% satiation treatment (Kruskal–Wallis tests, lipid: $\chi^2 = 12.20$, $p = 0.02$; protein: $\chi^2 = 8.57$, $p = 0.04$).

The ash, lipid, moisture and protein contents in the muscle of juvenile *P. clarkii* within five different treatments are presented in Figure S1. The moisture content accounted for the largest share of muscle composition, ranging from 69.86% to 75.67%, followed by protein content, which ranged from 21.91% to 27.02%. The contents of ash and lipid were low, at 1.51%–1.68% and 2.71%–3.24% respectively.

3.3 | Stable isotope analysis

The $\delta^{13}C$ and $\delta^{15}N$ values of *P. clarkii* from the different treatments were not significantly different (Kruskal–Wallis test, $\delta^{13}C$: $\chi^2 = 2.19$, $p = 0.70$; $\delta^{15}N$: $\chi^2 = 2.98$, $p = 0.56$). Individuals exhibited variability in their isotopic signatures, which ranged from −24.36‰ to −19.12‰ for $\delta^{13}C$ and from 2.14‰ to 5.66‰ for $\delta^{15}N$. The mean $\delta^{13}C$ and $\delta^{15}N$ values for the artificial diet were −21.49‰ and 4.59‰ respectively. *H. verticillata* had lower $\delta^{13}C$ and $\delta^{15}N$ values than did the artificial diet, at −25.19‰ and 2.88‰ respectively. The $\delta^{13}C$ and $\delta^{15}N$ values of the artificial diet and *H. verticillata* were significantly different (Kruskal–Wallis test, $\delta^{13}C$: $\chi^2 = 4.50$, $p = 0.03$; $\delta^{15}N$: $\chi^2 = 4.50$, $p = 0.03$) (Figure 4).

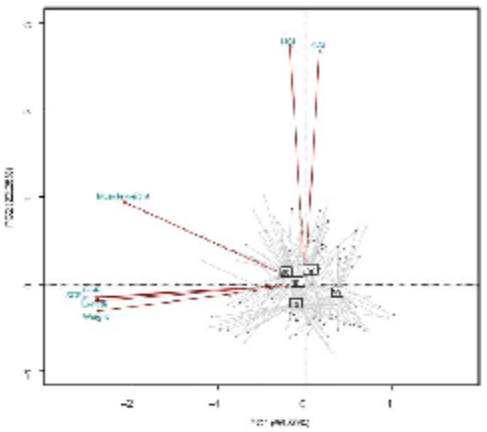

FIGURE 2 Principal component analysis (PCA) of growth parameters for both male and female *Procambarus clarkii* fed with five different feeding levels. Each point represents a specific crayfish. Abbreviations are as follows: 20, 20% satiation levels ($n = 33$); 40, 60, 80 and 100 are 40% ($n = 33$), 60% ($n = 35$), 80% ($n = 34$) and 100% satiation levels ($n = 28$) respectively. *GSI*, gonadosomatic index; *HSI*, hepatosomatic index; *SGRW*, specific growth rate for weight; SGRL, specific growth rate for length

The Bayesian mixing model results revealed that *H. verticillata* was an important component of the crayfish diets especially when crayfish were fed to 40% and 20% satiation (mean contribution: 50.26% and 49.31%; 95% confidence interval: 9.96%–92.98% and 10.67%–93.70%). The mean contribution of *H. verticillata* increased from 27.84% to 50.26% when feeding levels decreased from 100% to 20% satiation, although the 95% confidence intervals overlapped (Table 3).

4 | DISCUSSION

4.1 | Effects of feeding levels on growth performance of *P. clarkii*

This study demonstrated that reducing the amounts of artificial diet to a feeding level of 60% satiation did not significantly affect the growth performance and muscle composition of both male and female *P. clarkii*. This suggested that feeding crayfish at a reduced feeding level of 60% satiation could ensure crayfish production

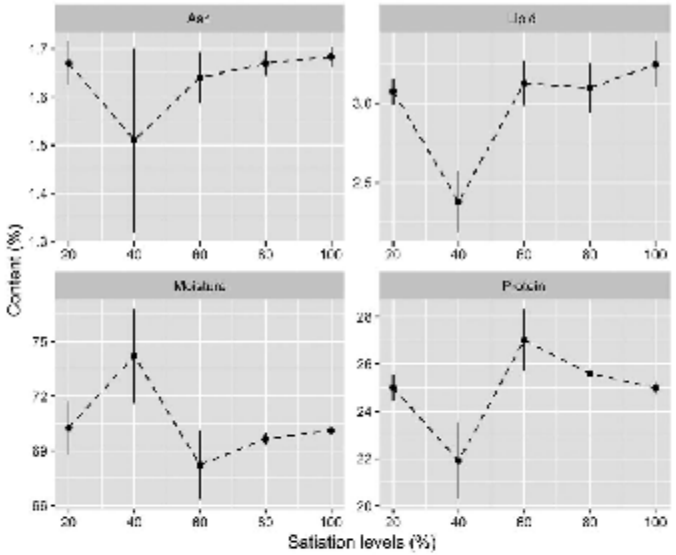

FIGURE 3 Line plots showing the contents of the ash, lipid, moisture and protein in the muscle of *Procambarus clarkii* among five different feeding levels. Data are shown as mean ± *SE*

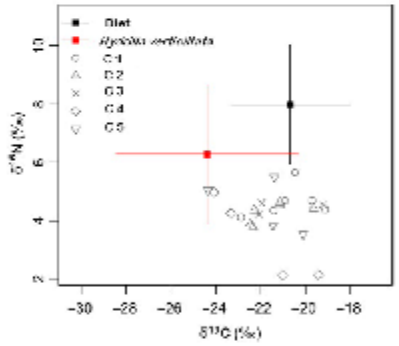

FIGURE 4 Stable isotope plots of nitrogen-carbon showing isotopic signatures of artificial diet and *Hydrilla verticillata* (mean ± *SD*) and *Procambarus clarkii* fed at different feeding levels. Abbreviations are as follows: C1, crayfish fed at 100% satiation; C2, crayfish fed at 80% satiation; C3, crayfish fed at 60% satiation; C4, crayfish fed at 40% satiation; and C5, crayfish fed at 20% satiation

TABLE 3 Mean percentage contributions (95% confidence interval) of the artificial diet and *Hydrilla verticillata* to the diets of *Procambarus clarkii* analysed using Bayesian stable isotope mixing model

Crayfish from different treatments	Food sources (%)	
	Artificial diet	*Hydrilla verticillata*
100% satiation	72.16 (33.09–100)	27.84 (0–66.91)
80% satiation	60.34 (26.14–95.82)	39.66 (4.18–73.86)
60% satiation	62.57 (21.71–100)	37.43 (0–78.29)
40% satiation	49.74 (7.02–90.04)	50.26 (9.96–92.98)
20% satiation	50.69 (6.30–89.33)	49.31 (10.67–93.70)

at a lower cost. Muscle composition could also reach a good level of quality at a lower production cost by reducing the amounts of artificial diet. This scenario might be particularly true for juvenile crayfish, which are very susceptible to under- and overfeeding. Generally, insufficient feeding leads to stunted growth and failure to attain desirable market sizes (Abrunhosa & Kittaka, 1997; Meng, Zhang, & Zhang, 2006; Pina, Nieves, Ramos-Brito, Chavira-Ortega, & Voltolina, 2005), while overfeeding increases input costs and reduces crayfish growth due to feed waste and pollution (Abidi & Khan, 2014). Data from our experiment indicated that a similar crayfish production was achieved at 60%, 80% and 100% satiation levels. This result suggested that feeding levels could be reduced to a proper degree without affecting the production. Similar results have been previously reported in *M. amblycephala* (Xu et al., 2016), *Acipenser transmontanus* (Deng et al., 2003; De Riu et al., 2012), *Salmo salar* (Sun et al., 2016), *Sebastes schlegeli* (Md Mizanur et al., 2014) and *Acipenser medirostris* (Zheng, Deng, De Riu, Moniello, & Hung, 2015). These studies indicated that the greatest weight gain was obtained when the feeding levels were below 100% satiation and our study was in accordance with these results.

4.2 | Effects of feeding levels on muscle composition of *P. clarkii*

Muscle composition results showed that *P. clarkii* fed to 60% satiation had a similar muscle composition as *P. clarkii* fed to 80% and 100% satiation. However, when crayfish were fed to 40% satiation, the lipid and protein contents in the muscle decreased significantly. This result suggested that although *P. clarkii* consumed more *H. verticillata*, it could not be a complete substitute for the artificial diet, likely due to its low protein and lipid contents (2.57% and 0.44% respectively) (Zhang, Zhang, & Ren-Fu, 2016). This result could also explain why *P. clarkii* showed growth retardation when feeding levels were reduced to 20% satiation. Many authors have reported similar effects of reduced feeding levels on body protein (El-Saidy & Gaber, 2005; Mihelakakis, Tsolkas, & Yoshimatsu, 2002; Van Ham et al., 2003) and lipid contents (Abdelghany & Ahmad, 2002; Hung, Conte, & Hallen, 1993; Wang, Kong, Li, & Bureau, 2007).

Furthermore, in this experiment, muscle moisture and ash contents were not significantly affected by feeding levels. Some of these results were consistent with those of previous studies showing that moisture content was not significantly affected by feeding levels, for instance, in *P. olivaceus* (Kim, Kang, Kim, & Kim, 2007). However, several studies on fish have reported that reducing feeding levels significantly increased ash content (Khan, Ahmed, & Abidi, 2004; Van Ham et al., 2003). In our study, we observed no significant differences in ash content among all treatments. One of the possible reasons is that fish in previous studies are fed only artificial diets, while *P. clarkii* in our study could also compensate with *H. verticillata* macrophytes when feeding levels are reduced. Another possible explanation is that in some studies, authors reduce feeding levels to lower rates than ours, which may more or less relate to the differences in ash content. Furthermore, the whole-body chemical composition was previously analysed, while in our study, only tail muscle composition was considered, which might also be the reason why the ash content was not significantly affected by feeding levels.

4.3 | Food contributions estimation

In this study, stable isotope analysis suggested that *H. verticillata* was an important component of crayfish diets, especially when crayfish were fed to 40% and 20% satiation. However, we found considerable interindividual isotopic variability within treatments in this study. One possible explanation for this variability is that when feeding levels are reduced, some individuals could have better access to the artificial diet than others due to the dominant hierarchy, whereby the subdominants compensated their lower rank in hierarchy by consuming less efficient alternative resources, such as *H. vertivillata* (Ahvenharju & Ruohonen, 2006). Such food resource differentiation could potentially explain the considerable uncertainty in crayfish isotopic values and the large 95% confidence intervals in food contributions found in this study. However, without studies to identify how the variability affects the estimation of food contributions, we cannot completely conclude that the food contributions in this study are estimated with a high precision. Although the food contribution results remain to be assessed precisely, they suggest a shift from the artificial diet to *H. verticillata* when feeding levels decrease. The results also suggest that using stable isotope data along with the mixing model to quantify the contributions of natural food items to the overall nutrition budget of crayfish may be a step forward in supporting cost-effective aquaculture.

4.4 | Application of reduced feeding levels in aquaculture

Feeding levels generally play an important role in aquaculture productivity and costs. Overfeeding generally results in higher production costs and has negative effects on fish or crayfish growth. Currently, lower feeding levels are recommended by many authors, and some studies suggest that natural food items in ponds can save up to 24.79%–50% of the artificial feed, for instance, in *L. vannamei* culture (Gamboa-Delgado, Pena-Rodriguez, Ricque-Marie, & Cruz-Suarez, 2011; Lara, Hostins, Bezerra, Poersch, & Wasielesky, 2017; Roy, Davis, & Whitis, 2012). Results from numerous experiments on fish have also demonstrated that reducing feeding levels to 65% satiation for *Scophthalmus maximus* (Van Ham et al., 2003), and to 90% satiation for *P. olivaceus* (Cho et al., 2007) does not reduce their production. This approach offers an incentive for farmers to reduce the inputs of artificial diets, and save a high percentage of production costs (Khan & Abidi, 2010; Nunes & Parsons, 1999).

Our results showed that the feeding level could be reduced to 60% satiation without significantly affecting the growth performance and muscle composition of *P. clarkii*. Moreover, the natural food items *H. verticillata* in ponds could potentially provide supplementary nutrition benefits to crayfish. Previous studies have also highlighted the crucial roles of natural food items in promoting shrimp growth, for instance in *Macrobrachium rosenbergii* (Correia, Pereira, Apolinario, Horowitz, & Horowitz, 2002; Correia, Pereira, Silva, Horowitz, & Horowitz, 2003), *Litopenaeus stylirostris* (Cardona et al., 2015) and *Marsupenaeus japonicus* (Arapi, Sadikaj, Malollari, Papa, & Kolaneci, 2012).

In addition, reducing the feeding levels of the artificial diet might help save production costs. For instance, according to our survey in the farmland of Qianjiang (31,349 m^2), the annual cost of artificial diet is $10,796, and it accounts for 50.5% of total production costs (unpublished data, see Supplementary S2). If *P. clarkii* is fed to 60% satiation, then 40% of the cost (about $4,318 per year) of the artificial diet will be saved, with additional benefits for water quality. With this study, we hope to encourage crayfish farmers to reduce feeding levels and increase natural food items such as macrophytes in culture ponds to maximize crayfish production while reducing production and environmental costs. With this study, we also hope to encourage further scientific works aiming at refining feeding strategies of aquatic species and limiting feeding amounts, while considering the contributions of natural food items in aquaculture.

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CONFLICT OF INTEREST

None. The funding sponsors had no roles in the design of the study; in the collection, analysis or interpretation of data; in the writing of the manuscript; nor in the decision to publish the results.

AUTHORSHIP

Shiyu Jin and Tanglin Zhang conceived and designed the study; Shiyu Jin, Yan Ren and Jixin Yu conducted the experiment; Shiyu Jin and Lisa Jacquin drafted the initial manuscript and subsequent corrections; Sovan Lek contributed to the data analysis; Wei Li, Sovan Lek, Jiashou Liu, Zhongjie Li and Tanglin Zhang provided guidance for data analysis, provided critical feedback on the manuscript and approved the final manuscript; Tanglin Zhang agreed to be accountable for all aspects of the work and to solve any problems involved in the accuracy and integrity of any part of the work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Chapter 6

Effects of protein levels on crayfish growth and muscle composition

Growth performance and muscle composition response to reduced dietary protein levels of juvenile red swamp crayfish *Procambarus clarkii* (Girard, 1852)

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Abstract

High dietary protein inputs in aquaculture can lead to suboptimal growth and increased production costs. Red swamp crayfish, *Procambarus clarkii*, is one of the most noteworthy species in China, but little information is available concerning their dietary protein requirements under practical pond farming conditions where crayfish also derive a substantial part of their dietary needs from natural foods. In this study, we tested the effects of two dietary protein levels (26% and 30%) of two artificial diets on growth performance and muscle composition of juveniles *P. clarkii* in eight concrete ponds cultured with the macrophyte *Hydrilla verticillata* (four replicates for each treatment). The results found that no significant differences were observed in growth performance of *P. clarkii* when they fed with different dietary protein levels diets. Muscle composition analysis revealed that *P. clarkii* fed to 26% protein level diet had significantly higher crude protein and ash contents than that fed to 30% protein level while dietary protein levels had no significant influences on the lipid content in crayfish muscles. Stable isotope analysis suggested a shift in crayfish diets to *H. verticillata* when dietary protein levels decreased. With this study, we hope to encourage crayfish farmers to reduce dietary protein inputs and maximizing the use of natural foods uch as macrophytes in cultured ponds to maximize crayfish yields and reduce production costs.

Keywords: *Procambarus clarkii*; Growth performance; Muscle composition analysis; Principal component analysis; Stable isotope analysis

1. Introduction

Aquaculture has been one of the fastest growing animal-food sectors, and global aquaculture production was 110.2 million tones, with the sale values estimated at USD 243.5 billion (FAO, 2018). China, the world's largest producer, produces more than one-third of global fish production, largely depending on its fast developmental aquaculture industry (Ottinger et al., 2016). However, the rapid growth of aquaculture has already raised many issues related to environmental impacts, among which high organic and nutrient loadings in fish or crayfish aquaculture water bodies is perhaps the most serious (Talbot and Hole, 1994). As reported, most aquaculture wastes were ultimately from dietary inputs, especially from high protein levels diets, containing nutrients and numerous organic compounds (e.g. ammonium, phosphorus, dissolved organic carbon and organic matter) (Cho and Bureau, 2001; Crab et al., 2007). This high organic and nutrient loadings resulted in water deterioration, pathogenic microorganisms occurrence, and fish or crayfish hypoxia or even death (Chávez-Crooker and Obreque-Contreras, 2010). Therefore, management of aquaculture wastes must be approached by improving feed utilization and feeding strategies to develop sustainable aquaculture which addresses allocation of dietary inputs to maintain sustainability and productivity of aquaculture systems under environmental capacity (Hasan, 2000).

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), originating from northeastern Mexico and south-central United States, was introduced to China in 1929 (Li et al., 2012). Due to its high adaptability, rapid growth, short life cycles, and highly commercial values, *P. clarkii* aquaculture has developed rapidly in recent years and it has become the most noteworthy freshwater species both commercially and academically. The annual production was up to more than 0.85 million tons in 2016, constituting 41.94% of China's freshwater shrimp aquaculture production (Fisheries Department of Ministry of Aquaculture, 2017). It has become one of the most significant freshwater fishery products in China, and without doubt, the aquaculture of *P. clarkii* will continue to play an important role in the national supply of crayfish in the future. However, the intensive crayfish aquaculture suffered a lot from water

quality deterioration and disease outbreaks. For instance, more than 80% of farmed shrimp or crayfish production loss was due to white spot syndrome virus (WSSV) infection (Chen et al., 1997; Du et al., 2007; Zhan et al., 1998). It is thus highly demanding to reduce dietary inputs and improve aquaculture management to avoid aquaculture wastes, potential eutrophication, as well as a mass of economic loss (Person, 1991). However, any measures towards sustainable development must consider the feeding biology, the nutritional requirements of the cultured species, and the economics of both feed and fish production.

P. clarkii is omnivorous, and it has a diverse diet in the wild such as macrophytes, detritus, periphyton, benthos, plankton, agriculture by-products and microbially enriched detritus (Alcorlo et al., 2004; Correia, 2003; Gutierrez-Yurrita et al., 1998). Among these foods, it prefers fresh macrophytes, which have been proved to be an important part of its diet (Cronin et al., 2002; Smart et al., 2002). Among factors influencing its feeding preferences, amounts of phenolic compounds in macrophytes was the foremost instead of protein and phosphorus amounts (Cirujano et al., 2004). In contrast, it did not show preferences for any animal preys (Gherardi and Barbaresi, 2007).

However, the feeding preferences varied with life stages, with a preference from carnivorous (juvenile crayfish) over herbivorous (pre-adults and adults) (Correia, 2003). However, in intensive aquaculture systems, artificial diets are the main foods resources for crayfish and it accounts for more than 50% of the total aquaculture costs (Keckeis and Schiemer, 1992; Wong et al., 2016). As a primary component in diets formulation, protein is, of course, one of the most expensive components and highly determines diets prices (Huner and Meyers, 1979). High protein inputs in culture systems led to water pollution, low dissolved oxygen levels and decreasing feed efficiency and immune systems, which thus resulted in huge economic loss and waste outputs (Craig et al., 2017; Henry and Fountoulaki, 2014; Martinez-Cordova et al., 2003; Velazco-Vargas et al., 2014). Conversely, low dietary protein inputs resulted in low fish or crayfish growth rates and failure to reach commercial sizes (Craig et al., 2017). Thus, scientific feeding strategies minimizing feeds and production costs while

maintaining aquaculture production and environmental capacity to a sustainable level are crucial to the economic success and sustainability of *P. clarkii* aquaculture (Cho and Bureau, 2001). Optimal dietary protein requirements were relatively well investigated for juvenile *P. clarkii*, which confirmed that optimal dietary protein levels were 24%-30% (Hai and Jie, 2012; Jover et al., 1999; Ling et al., 2012; Wu et al., 2007; Xu et al., 2013a; Zhang et al., 2012). However, these studies of dietary nutrient requirement were under laboratory-controlled conditions, and the results from these studies could not be fully applied to pond culture conditions since many cultured organisms also derived a substantial part of nutrition from natural foods. This is particularly true for *P. clarkii*, which is capable of feeding various natural foods (e.g. macrophytes, detritus, periphyton, benthos, plankton, and microbially enriched detritus) (Alcorlo et al., 2004; Correia, 2003; Gutierrez-Yurrita et al., 1998) while little information exists concerning their dietary protein requirements under practical pond farmimg conditions where natural foods also contribute to crayfish growth. In this respect, identifying and quantifying the contribution of natural foods to the diets of *P. clarkii* in ponds will be helpful to advance scientific feeding management strategies, which will improve production efficiencies in *P. clarkii* aquaculture.

In this study, we hypothesized that reduction in dietary protein levels to a proper level would not negatively influence the growth and muscle composition of *P. clarkii*, and that crayfish would compensate with more natural foods to maintain their growth when dietary protein levels reduced. To test this hypothesis, we placed 720 juvenile *P. clarkii* in eight concrete ponds cultured with widely-planted macrophyte *Hydrilla verticillata* in many crayfish cultured ponds in China. Then they were fed with two different protein levels artificial diets, which were chosen based on the dietary protein levels from previous studies and what have been commonly-used by farmers in crayfish aquaculture in China. Thus, the current study was conducted to: (1) investigate the effects of reduced dietary protein levels on the growth performance and muscle composition of juvenile *P. clarkii*; (2) quantify the contributions of artificial diets and *H. verticillata* to crayfish growth when dietary protein levels reduced. The study will hopefully provide scientific knowledge for farmers to refine

feeding strategies and improve the sustainability and productivity of *P. clarkii* aquaculture.

2. Materials and Methods

2.1. Experimental design

Juvenile *P. clarkii* (4.82 ± 0.15 g, 60.03 ± 0.52 mm, mean ± SE, no significant differences among treatments for crayfish sizes at the beginning of the experiment) were obtained from ponds at the Selection and Reproduction Center of Crayfish, Qianjiang, Hubei Province, China. A 50-day feeding experiment was conducted in eight experimental concrete ponds (90 juveniles per pond of 9 m^2), following the European Directive 2010/63/EU for animal experiments. There were four replicate ponds for the two treatments (26% and 30% protein levels). Before the experiment, crayfish were acclimated to the culture conditions for one week. At the beginning of the experiment, healthy juveniles were collected and randomly allocated to 8 concrete ponds. The running water flow rates in ponds were approximately 7 L/min, and constant aeration was supplied to each pond. Water depth was maintained at approximately 27 cm and *H. verticillata* was planted in 35 polyethylene flowerpots (0.44 m diameter) in each pond and used as both shelters and foods for *P. clarkii* with the coverage of 60% in each pond. The water temperature, pH, and dissolved oxygen (DO) were measured by a YSI probe (Yellow Springs Instruments, Yellow Springs, OH, USA). The concentrations of ammonia nitrogen, nitrite, chemical oxygen demand, total nitrogen, total phosphorus, and chlorophyll-a were determined using standard methods (APHA, 1992). Water quality parameters for all ponds (mean ± SE) were within the ranges of crayfish growth throughout the study: temperature 27.27 ± 1.06 ℃; DO 4.33 ± 0.70 mg/L; pH 9.3 ± 0.05; ammonia nitrogen 0.1400 ± 0.005 mg/L; nitrite 0.0472 ± 0.006 mg/L; total nitrogen 1.0609 ± 0.020 mg/L; total phosphorus 0.0445 ± 0.003 mg/L; chemical oxygen demand 8.8048 ± 0.100 mg/L; and chlorophyll-a 14.5477 ± 0.340 μg/L.

2.2. Feeding management

Throughout the experiment, crayfish were fed twice daily (8:00 and 18:00) in the two experiments.

A plastic pallet (30 × 15 cm) was placed at the bottom of each pond, and the artificial diet was placed on it (Fig. 1). For each feeding practice, crayfish were fed with excess weighted artificial diet. After their feeding activities stopped within one hour, the remaining artificial diet was removed, dried and reweighted (Van Ham et al., 2003). We then calculated the amount of artificial diet that was consumed by crayfish. The experiment ended after 50 days, when the majority of the males achieved a non-growing, and sexually active form.

2.3. Sample collection

At the end of the experiment, all crayfish were starved for 24 h and then collected for growth performance parameters measurement. Ten males and ten females from each pond (80 crayfish for each treatment) were randomly sampled for muscle composition analysis and chill-killed using an ice-water bath. The tail muscles were removed from the shells and stored at -20 ℃ for muscle composition analysis. Samples of two individuals from each pond were also chill-killed and maintained for stable isotope analysis.

2.4. Growth performance

Parameters for growth performance such as survival, final length (*L*), final weight (*W*), gonad weight, liver weight, and muscle weight were recorded and calculated as follows:

Survival (%) = $100 \times (N_t / N_0)$

Specific growth for weight (SGR_W, %, per day) = $100 \times [\ln(W_t) - \ln(W_0)] / T$

Specific growth for length (SGR_L, %, per day) = $100 \times [\ln(L_f) - \ln(L_0)] / T$

Gonadosomatic index (*GSI*, %) = $100 \times W_g / W_t$

Hepatosomatic index (*HSI*, %) = $100 \times W_l / W_t$

where N_t is the final number of *P. clarkii* per treatment, and N_0 is the initial number of *P. clarkii* per treatment; W_t is the final weight of *P. clarkii*, and W_0 is the initial weight of *P. clarkii*; L_f is the final length of *P. clarkii*, and L_0 is the initial length of *P. clarkii*; W_g is the gonad weight of *P. clarkii*, and W_l is the liver weight of *P. clarkii*; and *T* is the number of experimental days.

2.5. Muscle composition analysis

Crayfish muscle and diets were analysed for protein, lipid, moisture, and ash contents. Protein content was determined using the Kjeldahl method (N × 6.25) (William, 1980) with a 4800 Kjeltec Auto Analyzer (FOSS Tecator, Haganas, Sweden). Lipid content was determined by chloroform-methanol extraction (Folch, Lees & Sloane Stanley, 1957). Moisture content was determined by placing a 1-g sample into a convection oven (105 °C) for 2 h and drying it to constant weight (William, 1980). Ash content was determined by placing a 1-g sample combusting at 550 °C in a muffle furnace for approximately 10 h (William, 1980).

2.6. Stable isotope analysis

In total, 16 crayfish (four males and four females from each treatment), three artificial diet samples and four *H. verticillata* samples were collected at the end of the experiment and were analysed for their carbon and nitrogen isotope ratios, respectively. Muscle samples of crayfish (one male and one female randomly chosen from each pond, eight individuals for each treatment) were oven dried at 60 °C for at least 48 h to constant weight and were very finely ground (< 200 μm). All samples were processed for $\delta^{15}N$ and $\delta^{13}C$ isotopes by the Department of Earth System Science, Tsinghua University, Beijing, China (Alfaro, Thomas, Sergent & Duxbury, 2006). Approximately 3-mg samples were combusted, gasses analysed by gas chromatography and continuous flow-mass spectrometry (MAT-253, Thermo Fisher Scientific, USA). Samples were referenced to pre-calibrated C_4 sucrose, which was cross-referenced to the Vienna PeeDee Belemnite standard. The reference standard of $\delta^{15}N$ was atmospheric N_2 and measured to a precision of ± 1%. The isotope values for $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) were according to the following equation:

$$\delta^{13}C\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 1000$$

$$\delta^{15}N\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 1000.$$

2.7. Statistical analyses

The pairwise permutation test was carried out to test differences of survival among treatments. Students' t-tests were used to analyze the differences in other growth parameters, muscle composition, and crayfish $\delta^{13}C$ and $\delta^{15}N$ values of the two

treatments. Kruskal-Wallis test was used to analyze differences in $\delta^{13}C$ and $\delta^{15}N$ values of two artificial diets and *H. verticillata*. Growth performance parameters were also analyzed by principal component analysis (PCA). For the stable isotope data, we calculated the contributions of diet and *H. verticillata* to the growth of *P. clarkii* using the "SIAR" package in R. All analyses were performed by *R* version 3.3.2, and the significance level was set to 0.05.

3 Results

3.1Growth performance

The growth parameters such as survival, *W*, *L*, *GSI*, *HSI*, SGR_W, SGR_L, and muscle weight of males and females are shown in Table 1. Crayfish survival were 84.45% and 70.56% for 26% and 30% treatments, and there were no significant differences among the treatments (Students' t-test, $t = 2.06$, $P = 0.09$).

Most specifically, for males, no significant differences in growth parameters except muscle weight were observed among the treatments (Students' t-test, *W*: $t = 1.25$, $P = 0.22$; *L*: $t = 1.72$, $P = 0.10$; SGR_W: $t = 1.18$, $P = 0.25$; SGR_L: $t = 1.70$, $P = 0.10$; *GSI*: $t = 1.27$, $P = 0.22$; *HSI*: $t = 0.29$, $P = 0.77$; muscle weight: $t = 2.30$, $P = 0.03$). The muscle weight in the 26% treatment was significantly higher than the 30% treatments (Students' t-test, muscle weight: $t = 2.30$, $P = 0.03$).

Table 1 Growth performance parameters for female and male *Procambarus clarkii* fed at different protein levels diet (mean ± SE).

	Treatment	
	26% Protein level	30% Protein level
Survival (%)	84.45 ± 3.77	70.56 ± 5.60
Males		
W (g)[a]	25.29 ± 1.23	23.34 ± 0.94
L (mm)[b]	87.52 ± 1.49	84.37 ± 1.07
GSI (%)[c]	0.071 ± 0.013	0.051 ± 0.007
HSI (%)[d]	7.79 ± 0.35	7.91 ± 0.23
SGR_W (%, day^{-1}) [e]	3.28 ± 0.10	3.12 ± 0.08
SGR_L (%,day^{-1}) [f]	0.75± 0.0.03	0.68 ± 0.03
Muscle weight (g)	2.09 ± 0.10[a]	1.80 ± 0.07[b]
Females		
W (g)	22.79 ± 1.37	21.66 ± 0.69
L (mm)	90.46±2.03	90.00 ± 0.89

GSI (%)	0.38± 0.035	0.34 ± 0.039
HSI (%)	9.89 ± 0.15	10.05± 0.29
SGR_W (%,day^{-1})	3.06 ± 0.12	2.98 ± 0.06
SGR_L (%,day^{-1})	0.81 ± 0.04	0. 81 ± 0.02
Muscle weight (g)	2.38 ± 0.11	2.37 ± 0.06

Values in the same row sharing the same superscript are not significantly different (P > 0.05).

[a]W: final weight (g).

[b]L: final length (mm).

[c]GSI: gonadosomatic index (%) = 100×(gonad weight, g)/(final weight, g).

[d]HSI: hepatosomatic index (%) = 100×(liver weight, g)/(final weight, g).

[e]SGR_W: specific growth for weight (%/day) = 100×[ln(final weight)-ln(initial weight)] / experimental days.

[f]SGR_L: specific growth for length (%/day) = 100×[ln(final length)-ln(initial length)] / experimental days.

Female *P. clarkii* fed to diet with 30% protein level showed no significant differences in all parameters (Students' t-test, *W*: $t = 0.74$, $P = 0.47$; *L*: $t = 0.21$, $P = 0.84$; SGR_W: $t = 0.60$, $P = 0.55$; SGR_L: $t = 0.14$, $P = 0.89$; *GSI*: $t = 1.06$, $P = 0.30$; *HSI*: $t = 0.50$, $P = 0.62$; muscle weight: $t = 0.10$, $P = 0.92$).

PCA was performed to summarize the main trends in the growth performance of both males and females in the two treatments (Fig. 1). PC1 included *W*, *L*, SGR_W, SGR_L, and muscle weight, explaining 56.19% of the variance among samples. PC2 mainly separated females and males into two groups by *GSI* and *HSI*, explaining 30.26% of the variance. The two components explained 86.44% of the total variance. Considering both males and females, Fig. 1 illustrates that crayfish fed to 26% treatment had on significant differences in all parameters when compared with those of 30% treatment.

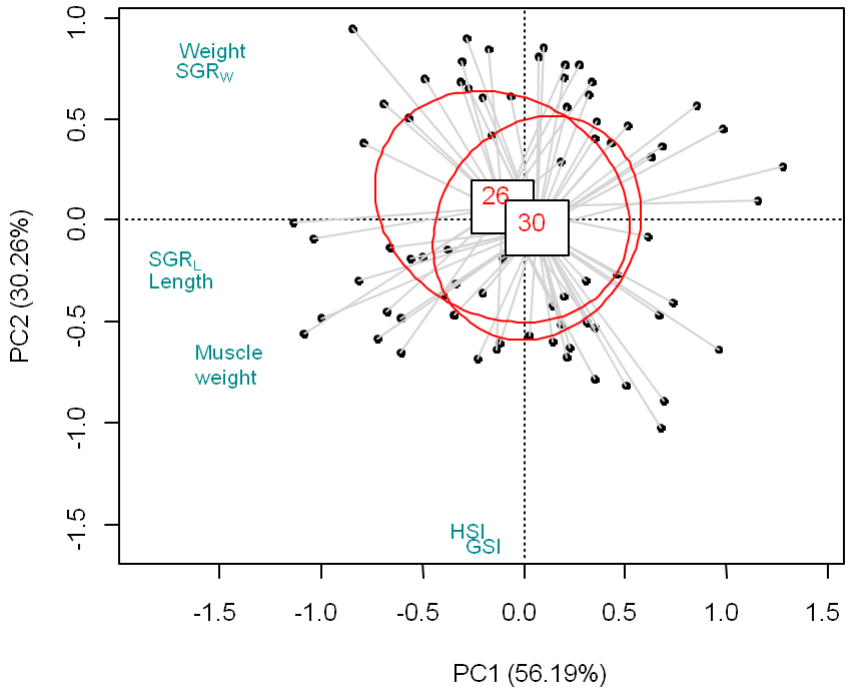

Figure 1. Principal component analysis (PCA) of growth parameters for both male and female *Procambarus clarkii* fed with different protein level diets. Each point represents a specific crayfish. Abbreviations are as follows: 26: 26% protein level, and 30: 30% protein level. GSI: gonadosomatic index, HSI: hepatosomatic index, SGR_W: specific growth for weight, SGR_L: specific growth for length.

3.2 Muscle composition

The ash, lipid, moisture, and protein contents of *P. clarkii* in the two different treatments are shown in Fig. 2. *P. clarkii* fed to 26% protein level diet had significantly higher crude protein and ash contents than that fed to 30% protein level (Students' t-test, crude protein: $t = 4.47$, $P < 0.001$; ash: $t = 5.67$, $P < 0.001$). While the moisture content of *P. clarkii* in the 26% treatment was significantly lower than that of in the 30% treatments (Students' t-test, moisture: $t = -4.37$, $P < 0.001$). Dietary protein levels had no significant influences on the lipid content in crayfish muscles (Students' t-test, crude lipid: $t = -1.47$, $P = 0.17$).

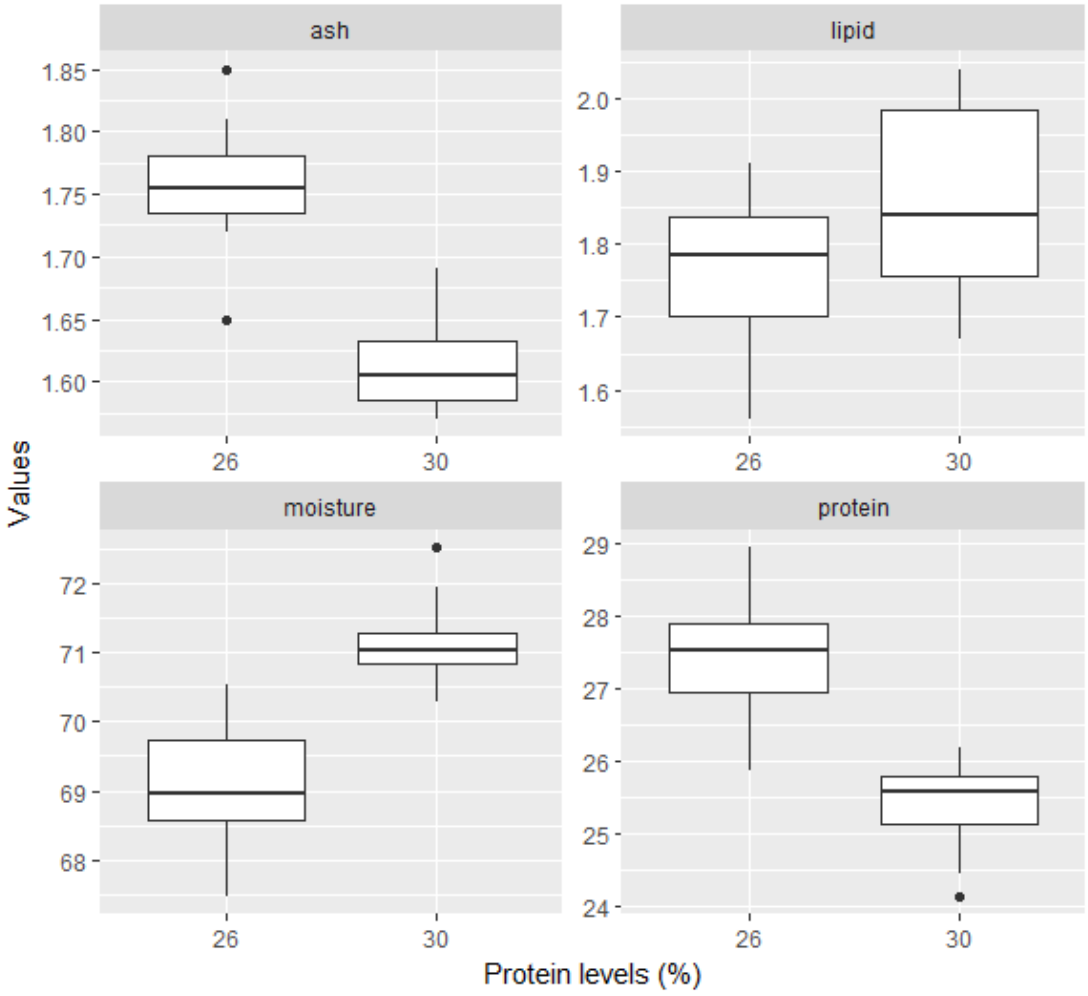

Figure 2. Box-plot showing the variations in percentages of the ash, lipid, moisture, and protein of *Procambarus clarkii* among two different protein levels. Box-plot representation: the horizontal line inside the box represents the median, and the lower and upper borders of the box represent the 25th and 75th percentiles. The upper and lower whiskers indicate the maximum and minimum range of the data excluding outliers.

3.3 Stable isotope analysis

The $\delta^{13}C$ and $\delta^{15}N$ values of *P. clarkii* from the different treatments are shown in Fig. 3 and Fig. 4. The $\delta^{13}C$ and $\delta^{15}N$ values were not significantly different in the two treatments (Students' t-test, $\delta^{13}C$: t = -2.39, P = 0.03; $\delta^{15}N$: t = 0.06, P = 0.95).

Individuals exhibited variability in their isotopic signatures in 26% and 30% treatments, which were -20.94‰ and -20.12‰ for $\delta^{13}C$, 4.77‰ and 4.76‰ for $\delta^{15}N$, respectively. The mean $\delta^{13}C$ and $\delta^{15}N$ values were -20.82‰ and 4.27‰ for 26% treatment, and -20.09‰ and 4.96‰ for 30% treatment, respectively. *H. verticillata* had lower $\delta^{13}C$ and $\delta^{15}N$ values than did the artificial diet, at -25.19‰ and 2.88‰, respectively. The $\delta^{13}C$ and $\delta^{15}N$ values of the artificial diet and *H. verticillata* were significantly different (Kruskal-Wallis test, $\delta^{13}C$: $\chi^2 = 6.71$, $P = 0.03$; $\delta^{15}N$: $\chi^2 = 8.02$, $P = 0.02$).

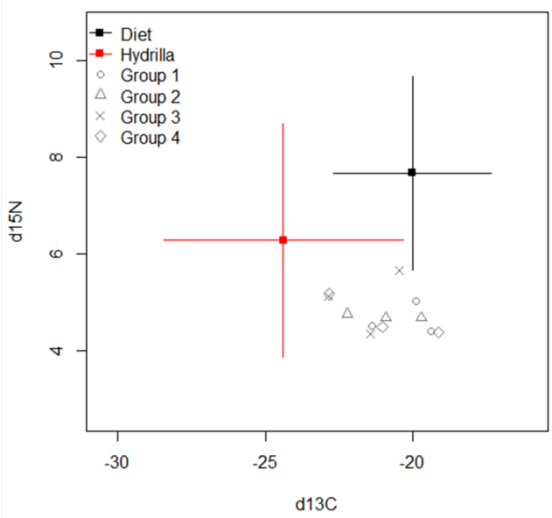

Figure 3. Stable isotope plots of nitrogen-carbon showing isotopic signatures of artificial diet with 26% protein level, *Hydrilla verticillata* and *Procambarus clarkii* (mean ± SD). Group 1, Group 2, Group 3, and Group 4 represent the four replicates in 26% treatment.

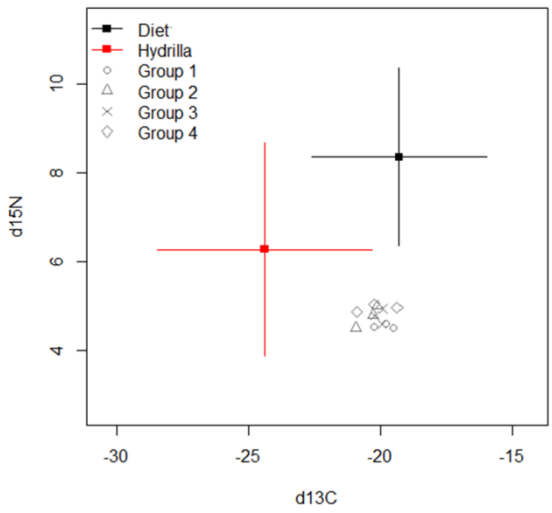

Figure 4. Stable isotope plots of nitrogen-carbon showing isotopic signatures of artificial diet with 30% protein level, *Hydrilla verticillata*, and *Procambarus clarkii* (mean ± SD). Group 1, Group 2, Group 3, and Group 4 represent the four replicates in 30% treatment.

Table 2 Mean percentage contributions (95% confidence interval) of artificial diets and *Hydrilla verticillata* to the diets of *Procambarus clarkii* in 26% and 30% treatments

Treatments	Replicates	Foods contributions (%)	
		Artificial diet	*Hydrilla verticillata*
26%	1	66.34 (22.60 – 100)	33.66 (0 – 77.34)
	2	59.17 (18.52– 98.92)	40.83 (1.08 – 81.49)
	3	52.10 (13.13 – 90.49)	47.90 (9.51 – 86.87)
	4	54.65 (10.85 – 96.99)	45.35 (3.01 – 89.15)
30 %	1	65.62 (25.37 – 100)	34.38 (0 – 74.63)
	2	60.25 (23.68 – 98.30)	39.75 (1.70 – 76.32)
	3	63.61 (24.87 – 100)	36.39 (0 – 75.13)
	4	61.15 (19.11 – 100)	38.85 (0.06 – 79.89)

The Bayesian mixing model results revealed that *H. verticillata* was an important component of crayfish diet. The mean contribution of *H. verticillata* increased from 37.34% to 41.93% when the dietary protein levels decreased from 30% to 26%, although 95% confidence intervals overlapped (Table 2).

4 Discussion

4.1Effects of dietary protein levels on growth performance of *P. clarkii*

The current study demonstrated that reducing the dietary protein level of the artificial diet to a level of 26% did not significantly affect the growth performance of crayfish. This suggested that feeding *P. clarkii* to a dietary protein level of 26% could ensure crayfish production at a good level with fewer costs. Although based on the laboratorial experiments, without considering the contribution of macrophytes, many previous studies confirmed that the growth of *P. clarkii* did not benefit from high dietary protein levels. For instance, juvenile *P. clarkii* obtained the best growth rate when they fed with a diet of 27% protein level (Wu et al., 2007). Others suggested that optiaml dietary levels for juvenile crafish were 24% - 30% (Hai and Jie, 2012; Ling et al., 2012; Xu et al., 2013a; Xu et al., 2011; Zhang et al., 2012). Similar results have also been found on other species such as *Macrobrachium americanum* (Méndez-Martínez et al., 2017), *Ctenopharyngodon idella* (Xu et al., 2016), *Cherax quadricarinatus* (Cortés-Jacinto et al., 2003), *Macrobrachium carcinus* (Benítez-Mandujano and Ponce-Palafox, 2014), and *Litopenaeus vannamei* (Shahkar et al., 2014). All of these studies showed that excess dietary protein levels have negative effects on the growth of cultured organisms.

For intensive aquaculture operation, artificial diets may make up more than 50% of thte production costs (Keckeis and Schiemer, 1992; Wong et al., 2016), and the diets prices highly depend on the proportion of protein. In order to harvested crayfish at desirable market sizes in the shortest time, farmers tended to use high protein levels artificial diets. However, high protein inputs in culture systems caused water pollution, low dissolved oxygen levels and decreasing efficiency of food absorption and immune systems, which thus resulted in huge economic loss (Craig et al., 2017; Henry and Fountoulaki, 2014; Martinez-Cordova et al., 2003; Velazco-Vargas et al., 2014). Considering the similar production of the two treatments in the current study, we suggest reducing the dietary protein levels to 26% to maintain aquaculture production at minimum economic losts which not only brings numerous benefits to farmers but

also is the key to improving the economical and environmental sustainability of crayfish culture.

4.2Effects of dietary protein levels on muscle composition of *P. clarkii*

Muscle composition results showed that *P. clarkii* fed to 26% protein levels had significantly higher crude protein and ash contents than that fed to 30% protein level while no significant influences on the lipid content. This suggested that reducing dietary protein level to 26% would not have negative effects on crayfish muscle composition. Crude protein contents in muscle tended to decrease with the increase of dietary protein levels. This result was consistent with what has been reported on crayfish *Astacus leptodactylus* (Ghiasvand et al., 2012) and crab *Portunus trituberculatus* (Jin et al., 2013), however, it was not accordance with several previous studies. Some found that muscle crude protein content tended to increase with increase in dietary protein levels significantly for *P. clarkii* (Li, 2012; Yu, 2011), *Cherax quadricarinatus* (Pavasovic et al., 2007), and *Macrobrachium americanum* (Méndez-Martínez et al., 2017) while others demonstrated that dietary protein levels had no significant differences on muscle composition for *P. clarkii* (Ling et al., 2012), *C. quadricarinatus* (Thompson et al., 2004), *Macrobrachium carcinus* (Benítez-Mandujano and Ponce-Palafox, 2014), *Litopenaeus vannamei* (Hu et al., 2008), and *Macrobrachium nipponense* (Zhang et al., 2017). Crude lipid content was not significantly affected by dietary protein levels in the present study, which was in agreement with those reported for *P. trituberculatus* (Huo et al., 2014), and *C. quadricarinatus* (Thompson et al., 2004) but disagreed with studies reported in *P. clarkii* (Li, 2012; Su et al., 2009; Xu et al., 2013a), *A. leptodactylus* (Ghiasvand et al., 2012), *P. trituberculatus* (Jin et al., 2013), *M. carcinus* (Benítez-Mandujano and Ponce-Palafox, 2014), *M. nipponense* (Zhang et al., 2017), and *M. americanum* (Méndez-Martínez et al., 2017). The ash and moisture contents showed opposite tendency with the increase of dietary protein levels in the current study. In contrast, most studies found no significant differences in ash and moisture contents with dietary protein levels increasing (Catacutan, 2002; Ghiasvand et al., 2012; Hu et al., 2008; Huo et al., 2014; Jin et al., 2013; Méndez-Martínez et al., 2017; Wu et al., 2007;

Zhang et al., 2017). Many factors could affect the muscle compostion content of cultured organisms. For instance, the protein and ash contents are size-dependent, and lipid content tends to increase with sizes and be affected by life stages and energy intake (Shearer, 1994). The different diet formulation also had significant effects on muscle composition contents. The study has proved that diets containing energy from carbohydrate produced higher body protein levels than diets containing the same amount of energy from lipid (Shearer, 1994). In addition, other factors such as feeding amounts, temperature, salinity, and protein digestibility of crayfish would result in the different amounts of protein, lipid, carbohydrate and energy crayfish obtain, and thus cause the differences in their muscle composition. Taken together, our results of muscle composition analysis indicates the use of high dietary protein levels would be unnecessary when a high abundance of natural foods are present in the culture systems. It is concluded that a high protein input through farming period is not the best feeding strategies for this crayfish.

4.3 Natural foods contributions and implications for sustainable aquaculture

In this study, stable isotope analysis showed that *H. verticillata* was an important component of *P. clarkii* diets, and its contributed to crayfish at a level of 37.34% and 41.93% when the dietary protein levels decreased from 30% to 26%. This confirmed our hypothesis that a reduction in dietary protein levels to a proper level would not negatively influence the growth and muscle composition of *P. clarkii* because of the supplementary nutrition from *H. verticillata*. Actually, besides *H. verticillata*, other natural foods have also been proved to contribute a greater proportion to the growth of *P. clarkii* such as benthic detritus, sediment, planktonic, zooplankton, and invertebrates (Alcorlo et al., 2004; Grey and Jackson, 2012; Gutierrez-Yurrita et al., 1998; Huner, 1981; Kreider and Watts, 1998). These studies confirmed that significant nutritional roles of natural foods played in intensive or semi-intensive crayfish culture can not be ignored. Such cases could also be found in other crayfish. For instance, in semi-intensive pond culture of *Cherax quadricarinatus*, the contribution of natual plants to crayfish growth could be up to approximately 44% (Joyce and Pirozzi, 2016). For *Paranephrops zealandicus*, terrestrial detritus constitited up to 58.3% of stomach

contents (Hollows et al., 2002). These results had important implications on the effective utilization of natural foods in crayfish culture, which also highlighted the benefits of natutal foods contributing to crayfish growth and reduced production costs.

However, when using the Bayesian mixing model calculating food resources contributions, considerable interindividual isotopic variability in each treatment was observed. This reflected that there existed food resources differentiation among crayfish in the current study (Grey et al., 2004). This could be attributed to the feeding preferences of *P. clarkii*, which have been proved to unrelated to nutritional value and foods availability (Gherardi and Barbaresi, 2007). Thus, although *P. clarkii* was provided with abundant and nutrient-enriched artificial diets, it still showed differences in foods utilization. However, without exact studies analyzing the possible effects of how interindividual isotopic variability influences the contributions of different foods sources, we are not sure that the contributions estimated in this study were highly precise. Anyway, they did suggest the important roles of natural foods provided in crayfish growth. Futhermore, the stable istope analyses are convenient means to quantify the contributions of natural foods in aquaculture, we suggest future studies on interindividual isotopic variability to provide valuable insights into feeding behaviour and niche breadth of crayfish, and probably of other organisms.

4.4 Implications of reduced dietary protein levels for sustainable aquaculture

At a management level, reducing current dependence of farming systems on high dietary protein inputs and maximizing utilization of natural foods as alternative and more sustainable sources of nutrition is of high significance to further reduce production costs (feed costs), and thus maintain profitability (Tacon, 1997). The nutritional and economic importances of natural foods have been well recognized in many crustacean species cultures with a consequent increase in ponds productivity and yield. For example, previous studies on *Cherax destructor* and *Litopenaeus stylirostris* have demonstrated the growth-enhancing effects of natural foods in ponds, which contributed to 28%—79% and 37—40% of their growth and thus helps save artificial diet costs (Cardona et al., 2015; Duffy et al., 2011). Similar cases could also be found in *Litopenaeus vannamei* (Gamboa-Delgado et al., 2011; Porchas-Cornejo et

al., 2012; Roy et al., 2012; Xu et al., 2013b), *Farfantepenaeus brasiliensis* (Emerenciano et al., 2012), *Cherax quadricarinatus* (Viau et al., 2012), Macrobrachium rosenbergii (Correia et al., 2002; Correia et al., 2003), *Litopenaeus stylirostris* (Cardona et al., 2015), and *Marsupenaeus japonicas* (Arapi et al., 2012). All these studies indicate that maximizing the use of natural foods in the overall nutritional budget of pond-cultured crayfish will not only improve crustacean species growth but also reduces production costs to a large degree. In this respect, it is important that farmers learned to be more efficient in their use of their available natural foods in ponds to maximum production profit. Since food and feeding involve large production cost, therefore further studies should be encouraged to reduce production costs through the utilization of natural foods and exploration sustainable feeding strategies.

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Please see general bibliography

Chapter 7

General discussion

The main aim of this thesis was to investigate the reproduction, population dynamics of commercial populations in China and explore the optimal artificial reproduction techniques and juveniles feeding strategies. I interpreted the reproductive pattern and population dynamics of *P. clarkii* in a commercial pond, and this part played a very important role in sustainable fishery management. I also tested the hypotheses that water manipulating could be an alternative to traditional artificial reproduction of *P. clarkii* and reducing inputs of artificial diets to a proper level would not affect crayfish growth and muscle composition due to the supplementary nutrition provided by natural food items. These studies will hopefully provide guidance for scientists, government, and farmers to make scientific aquaculture management and reduce production costs.

In this framework, I put forward that avoid sex selection during reproductive seasons and reducing fishing pressure on immature crayfish. High fishing pressure during reproductive season could influence long-term stock productivity. It may also cause death of offspring. Furthermore, reducing fishing on younger crayfish and selectively catching old crayfish will help to promote large-sized individuals and render crayfish culture more profitable. This would also offer more access to the environmental resources (e.g., food availability) for juveniles and then may result in faster growth. I also tried to manipulate water temperature to improve reproductive performance and embryonic development. The results showed similar effectiveness in inducing spawning when compared with traditional artificial reproduction techniques (eyestalk ablation and hormones injection), which confirmed our hypothesis. For the embryonic development, we found abnormalities when temperatures were above 29 ℃, which indicated that higher water temperature should not be applied to embryos management. Further, I built a developmental model to predict the embryonic development under various water temperatures. I finally showed possibility of reducing feeding levels and dietary protein levels in aquaculture management. For this study, reducing feeding level to 60% satiation and dietary protein level to 26% did not affect crayfish growth performance and muscle composition significantly. This was mainly because natural food items *H. verticillata*

in the ponds provided additional nutrition for crayfish and stable isotope analysis also confirmed this idea. However, the natural food items could not totally replace the artificial diets because of the low protein and lipid contents. This was the reason why crayfish fed to 20% satiation showed growth retard. Based on these results, I suggest reducing high inputs of artificial diets and maximizing use of natural food items to reduce production costs in aquaculture.

7.1 Reproductive time of *P. clarkii* in Qianjiang, China

In this thesis, we evaluated crayfish reproductive pattern by calculating gonadosomatic index (GSI), hepatosomatic index (HSI), and determining their ovarian development and fecundity. Finally, we found female *P. clarkii* spawned once yearly, and mostly spawning activities started from September to November. However, due to low water temperature, eggs in late autumn were probably prevented from hatching. Those eggs, having survived the harsh winter conditions, would be more likely to hatch in the next spring when the environment is favorable. Thus, in our studied area, we found two recruitment phases yearly, which were from October to November, and March to May. The delaying hatching could be an adaptive strategy of *P. clarkii* for unfavorable environmental conditions such as low water temperature in winter (Lass et al., 2005). Previous studies showed that it would take up to 130 days for *P. clarkii* eggs to be successfully hatched when water temperature was below 10℃ (Suko, 1954; Suko, 1956). In the present study, the mean water temperature was 13.58℃ and 10.03℃ in November and December, which confirmed that eggs released in late autumn were probably prevented from hatching by low water temperature. Accordingly, we found another crayfish recruitment in spring. Actually, in different places, different recruitment events were observed. For instance, there were two-yearly distinct recruitments in Italy (Scalici & Gherardi, 2007; Maccarrone et al., 2016), southern Portugal (Adao & Marques, 1993), Spain (Cano & Ocete, 1997; Alcorlo, Geiger & Otero, 2008), America (Sommer, 1984) and Japan (Suko, 1958), while only one recruitment occurred in central Portugal (Anastácio & Marques, 1995) and Germany (Chucholl, 2011). The crayfish ovarian development and embryonic development were related to various factors such as water temperature, habitat uses,

and food resources (Sastry, 1983; Harhoğlu and Farhadi, 2017), thus, the differences in plastic recruitment patterns were difficult to explain. In this thesis, we infer that the single spawning peak with two recruitment patterns is most likely driven by the low water temperature, but further studies are still needed to test it.

7.2 Population dynamics of commercial *P. clarkii* in China

We estimated *P. clarkii* population dynamics including growth coefficient (*K*), growth parameter index (*Ø'*), total mortality rate (*Z*), natural mortality rate (*M*), fishing mortality rate (*F*), and exploitation rate (*E*) by using electronic length frequency analysis in R package "TropFishR" based on data of cephalothorax length (CTL). Finally, we found five growth cohorts for both females and males, and crayfish had faster growth rate but smaller sizes in the studies area. We then estimated total mortality rate (*Z*), natural mortality rate (*M*) and fishing mortality rate (*F*) of the commercial population, which were 1.93, 1.02, 0.91 $year^{-1}$ for females and 2.32, 0.93, 1.39 $year^{-1}$ for males, respectively. These results showed that the mortality of male crayfish was mainly caused by fishing. The estimates of exploitation rate (*E*) indicated that male crayfish were overexploited and under high fishing pressure, with the values of 0.47 and 0.60 $year^{-1}$ for females and males, respectively.

Length-frequency analysis showed the structure of commercial *P. clarkii* population is made up of five growth cohorts for both females and males. Among which, the first three growth cohorts were constituted of abundant younger crayfish, which were fast-growing individuals while the fourth and fifth growth cohorts were constituted of old individuals with extremely slow growth rates. When compared with other studies, we found that cohorts of *P. clarkii* varied considerably in numbers across populations. For instance, we found five cohorts in Portugal (Anastacio et al., 2009), six in Italy (Dörr & Scalici, 2013), seven in China (Huang et al., 2012), and eight and nine for males and females in Germany (Chucholl, 2011). The differences in growth cohorts estimation were probably caused by the differences in CTL sizes of *P. clarkii* among those studies. For instance, in our study, only crayfish with a CTL higher than 15.20 mm were captured, which were mainly attributed to trapping activities. Thus, the selectivity of sampling traps used in different studies might

partially affect the growth cohorts estimation to a certain degree. Therefore, it was possible that the CTL frequency analysis in our study only partially described the real population structure of the commercial *P. clarkii* population.

Furthermore, we also found that fishing mortality rate (*F*) and exploitation rate (*E*) of male *P. clarkii* were higher than females. Generally, natural mortality rate (*M*) has been widely used as the upper limit of *F* for sustainable fishing, which suggests that *E* should be less than 0.5 to prevent populations from overfishing (Gulland, 1971; Gulland, 1983; MacCall, 2009; Froese et al., 2016). However, in the present study, the estimated *E* of 0.60 for males was higher, indicating that the male *P. clarkii* was overexploited and under high fishing pressure. This might be related to the males-directed fishing selection during the reproductive period. During reproductive periods, female crayfish tended to stay in burrows for parental care to their offspring and it was hard to catch them (Gherardi & Barbaresi, 2000; Thiel, 2000; Dörr et al., 2006; Donato et al., 2018). And in order to maximize short-term catch rates and profitability, farmers intentionally targeted male crayfish during fishing activities and then more males were selectively harvested during the reproductive periods (Zhou et al., 2010). This males-directed selectivity may impose adverse effects on reproductive output since it causes difficulties in females finding mates. Similar cases were also found in crabs (Gray & Powell, 1966; Smith & Jamieson, 1991). Thus, in fishery management, the possible side effects of sex selection on reproductive success of the population should be considered (Zhou et al., 2010). Actually, overfishing causes damage and stress to crayfish, which negatively affected their growth and survival (Chopin & Arimoto, 1995). Even though some crayfish escape from fishing, they may be injured and die later due to physical damage. In such a situation, the fishing activities should be well monitored to protect the commercial *P. clarkii* population from further depletion. We thus suggest reducing fishing intensity on immature crayfish and avoid sex selection during the reproductive period to improve the overall sustainability of commercial *P. clarkii* populations.

7.3 Reasons why we did not determine age composition when analyzing population dynamics for crayfish?

Age and growth plays very important roles in fishery management, and knowledge of accurate age will help to know how fishing activities affect population growth dynamics and ecosystem services (Beamish & McFarlane, 1983). Normally, the age of aquatic animals can be determined through scales, bones, vertebrae and otoliths (Campana, 2001; Campana et al., 2006). However, for crustaceans, they underwent several times of molting during their life cycle and during intermolt, they grew rapidly. This provided limitations for studies their age composition because of the potential loss of calcified structures for each molting. In this situation, age studies of crustacean species are limited and the most common way is to use length-frequency analysis to estimate. Up to now, this methods have been used for lots of crustacean species such as crab *Trachypleus tridentatus* (Almendral & Schoppe, 2005), lobsters *Homarus americanus* (Gendron & Sainte-Marie, 2006) and *Panulirus ornatus* (Kienzle et al., 2012), crayfish *Pacifastacus leniusculus* (Fonseca & Sheehy, 2007) and *Cambarus hubbsi* (Larson & Magoulick, 2011), and shrimp *Pleoticus muelleri* (Castilho et al., 2012).

However, this method could estimate with high accuracy only when the studied species are satisfied with three assumptions: (1) they have restricted reproductive seasons; (2) they show significant annual growth; (3) population does not migrate. In this case, this method is suitable for short-lived species age determination (Hartnoll, 1982). Furthermore, the length-frequency analysis might estimate different results for the same population because this method depends highly on the cephalothorax length data which is strongly related to the selectivity of sampling traps. For instance, in the present study, only crayfish with a cephalothorax length higher than 15.20 mm were captured. Therefore, it was possible that this length-frequency analysis only partially described the real population age composition. This method could also be unreliable if the samples included high proportion of older individuals. When analyzing age composition, older crayfish with slower growth rates might group together with young individuals which show fast growth. This would finally affect the estimated results.

It’s why length-frequency analysis is applied to estimation of age composition of many shrimps with short lifespan. Although this method has drawbacks, the convenience of this method makes it still the main way to estimate age structures of crustacean species (Vogt, 2011).

7.4 Perspectives on temperature manipulation of crayfish reproduction

The ability to induce spawning spontaneously is a key step for large-scale production of juvenile crayfish in aquaculture. With the fast development of aquaculture, new technological advances in artificial reproduction are crucial to effective stock enhancement. As traditional artificial techniques, the eyestalk ablation and hormone injection have been extensively used for inducing spawning activities of crustacean species. According to several previous studies, the spawning rates for crayfish injected hormones ranged from 20% to 77.5% and when *P. clarkii* was treated with eyestalk ablation, spawning rates averaged 63.33% (Liu et al., 2014; Liu et al., 2013b; Zhang, 2011). However, these two methods often compromise with low survival (from 15.56% to 51.11%) for female crayfish in these studies. Such cases could also be found in other crustacean species, such as *Penaeus monodon*, *Penaeus vannamei*, and *Macrobrachium rosenbergii* (Vaca and Alfaro, 2000; Wei and Zhao, 1992; Wen et al., 2009). In the current study, results showed that water temperature manipulation could induce more than 50% crayfish spawning and the survival averaged 84% for all treatments. This indicated that temperature manipulation could be an efficient and more ethical alternative for crayfish reproduction compared to eyestalk ablation and hormones injection. Furthermore, the quality of eggs could be influenced by eyestalk ablation and hormones injection. The eggs’ quality is often reflected by egg sizes, egg shape and clarity, larval survival, percentages of fertilization, hatching, and abnormalities (Bourque & Phelps, 2007). However, this requires a series of long-term studies to evaluate the egg quality and this information is still limited now. Anyway, these results support that water manipulation is an efficient alternative technique of reproduction, and 21 ℃ – 25 ℃ is suggested for improving the the reproductive performance of female *P. clarkii*.

7.5 Higher water temperature should not be applied into *P. clarkii* embryos management

We have evaluated effects of five temperatures (17 ℃, 21 ℃, 25 ℃, 29 ℃ and 33 ℃) on *P. clarkii* embryonic development. Finally, we found that embryos showed abnormalities and subsequently died at 29 ℃ and 33 ℃. These abnormalities occurred during the early stages of embryo development (< 72h), which included abnormal cleavage, blastula lesions, punctured membranes, abnormal invagination of blastula, and gastrulation lesions. However, no abnormalities were observed in embryos at 17 ℃, 21 ℃, and 25 ℃.

In aquaculture, abnormality of embryos is one of the most serious problems, which is mainly due to suboptimal culture conditions (Cobcroft et al., 2001; Fraser and De Nys, 2005). For example, high temperature could induce abnormalities of embryos especially during cleavage, blastomere and gastrulation stages of many hatchery-reared species (Aritaki and Seikai, 2004; Huang et al., 2010; Sfakianakis et al., 2004; Wang and Tsai, 2000). In our study, *P. clarkii* exposed to the high temperatures (29 ℃ and 33 ℃) during embryonic development also showed abnormalities and ceased to develop while no abnormalities were detected at lower temperatures (17 ℃, 21 ℃, and 25 ℃). Similar phenomena have also been reported in many fish species such as *Solea senegalensis* (Dionísio et al., 2012), *Danio rerio* (Casper et al., 2015), *Vimba vimba* (Lugowska and Kondera, 2018), *Sparus aurata* (Georgakopoulou et al., 2010), and *Dicentrarchus labrax* (Georgakopoulou et al., 2007).

Furthermore, high water temperature caused mortalities of embryos (Lahnsteiner et al., 2012; Lugowska and Witeska, 2018). In our study, all the embryos failed to hatch above 29 ℃ while a previous study showed that 40% of the embryos of *P. clarkii* died at 30 ℃, and 100% died at 41 ℃ (Lv et al., 2004). We inferred that different maternal thermal history could be responsible for this discrepancy, which was considered as the most important factor influencing thermal tolerance, thus resulting in the different results of embryos thermal tolerance between the two studies (Lutterschmidt and Hutchison, 1997; Soundarapandian et al., 2014). There were also

studies showing that dynamic temperature changes helped to increase crayfish thermal tolerance (Beitinger et al., 2000; Heath, 1963; Hutchison and Ferrance, 1970; Mora and Maya, 2006). Thus, the reduced thermal tolerance of embryos might be also due to constant temperatures in the present study. Based on these results, we thus recommend performing embryos hatching at 25℃ and avoiding hatching temperatures higher than 29°C to perform balanced embryonic development.

7.6 Supplementary nutrition from natural foods cannot be ignored

Overfeeding and high dietary protein inputs in aquaculture can lead to suboptimal growth and increased production costs. For many crayfish, they are omnivorous and can feed on macrophytes, detritus, periphyton, benthos, and plankton in wild (Anderson et al, 1987; Nunes & Parsons, 1999; Soares et al., 2005; Gherardi & Barbaresi, 2008). In this thesis, we have confirmed that feeding levels could be reduced to 60% satiation, which would not impair crayfish growth performance and muscle composition. Reducing dietary protein levels to 26% could also have the similar effects. The stable isotope analysis also demonstrated that crayfish consumed more natural foods from the ponds when the feeding levels or protein levels decreased. This provides incentives for farmers to reduce the artificial diets input in aquaculture. Actually, lower feeding levels have been recommended by many authors, and some studies suggest that natural food items in ponds can save up to 24.79–50% of the artificial feed, for instance, in *Litopenaeus vannamei* culture (Roy, Davis & Whitis, 2012; Gamboa-Delgado, Pena-Rodriguez, Ricque-Marie & Cruz-Suarez, 2011; Lara, Hostins, Bezerra, Poersch & Wasielesky, 2017). Other studies on *Cherax destructor* and *Litopenaeus stylirostris* have also demonstrated the growth-enhancing effects of natural foods in ponds, which contributed to 28%—79% and 37—40% of their growth and thus helps save artificial diet costs (Cardona et al., 2015; Duffy et al., 2011). Studies on fish have also demonstrated that reducing feeding levels to 65% satiation for *Scophthalmus maximus* (Van Ham et al., 2003), and to 90% satiation for *Paralichthys olivaceus* (Cho et al., 2007) does not reduce their production.

Furthermore, natural foods in aquaculture systems help to save production costs. In intensive aquaculture systems, it is common that artificial diets account for more

than 50% of total aquaculture costs (Keckeis & Schiemer, 1992; Wong, Mo, Choi, Cheng & Man, 2016). If *P. clarkii* is fed to 60% satiation, then 40% of the cost (about $4318 per year) of the artificial diet will be saved. At a management level, reducing current dependence on high diets inputs and maximizing utilization of natural foods as alternative and more sustainable sources of nutrition is of high significance to further reduce production costs (feed costs), and thus maintain profitability. What's more, farmers will gain more profits from these feeding strategies. With this study, we also hope to encourage further scientific works aiming at refining feeding strategies of aquatic species and limiting feeding amounts, while considering the contributions of natural food items in aquaculture.

Chapter 8

Conclusions and perspective

Generally, the current study focused on the reproductive biology and ecological factors influencing reproductive performance, embryonic development, and juvenile growth of *P. clarkii*. Firstly, our results have highlighted that spawning activities of female *P. clarkii* took place from September to November with two recruitments yearly (a major one from October to November and the minor one from March to May) in Qianjiang, China. There were five growth cohorts for females and males in the commercial pond while male *P. clarkii* were overexploited and under high fishing pressure. Secondly, our study suggests that manipulating water temperature is an effective way to induce spawning in females and optimize embryonic development to improve larval production. We found that the optimal temperatures for improving *P. clarkii* reproductive performance were 21℃ and 25 ℃ and the optimal temperature for embryonic development was 25℃. We also built a temperature-dependent developmental model, which could help farmers to predict larval recruitment depending on their culture conditions. Thirdly, the study demonstrated that reducing the amounts of an artificial diet to a feeding level of 60% satiation did not significantly affect the growth performance and muscle composition of both male and female *P. clarkii*. Stable isotope analysis suggested a shift in crayfish diets to easily available *H. verticillata* when feeding levels decreased. Fourthly, reducing the dietary protein level of the artificial diet to a level of 26% would also not significantly affect the growth performance and muscle composition of crayfish.

Due to the big challenge in sustainable and continuous supply of juvenile crayfish to *P. clarkii* culture industry, further works on improving female reproductive output and embryos survival should be encouraged to promote aquaculture productivity and sustainable fisheries. For instance, embryos are generally sensitive to environmental conditions, and any huge changes in environment conditions will affect their developmental process and then have potential effects on juveniles growth and survival. Salinity, expected to influence crayfish embryos metabolic activities, developmental rates, yolk utilization efficiency, and other physiplogical processes, has significant implications on *P. clarkii* embryonic development and survival. Therefore, studies of potential salinity effects will be helpful to improve hatching

rates and embryonic development. These studies will be also applied in intensive embryos management for mass juveniles production.

Besides salinity, nutrition is playing vital role in improving juvenile crayfish growth and survival. The onset of exogenous feeding is crucial to juveniles survival. If failure in supplying sufficient food resources, high proportion of motality will occur during juvenile crayfish growth (Huner, 2002). There were numerous studies showing that cultured organisms survival could be up to 82% in Mugil cephalus, 91% in Sparus aurata, and 85% in Solea senegalensis if they were reared in an optimal conditions (e.g. abundant food resources) (Tamaru et al., 1994; Yúfera et al., 2005). Thus, understanding of nutritional roles and nutrient composition of feeds in the early ontogeny of *P. clarkii* is of primary importance in improving juvenile crayfish survival and designing inert artificial feeds for their first feeding in aquaculture. Furthermore, the first feeding is considered as the transition period from which the source of energy basic to support embryonic development changing from yolk reserves to exogenous feeding. In order to achive successful transition, understanding metabolic processes involved in food uptake, digestion and assimilation is also of high priority (Yufera and Darias, 2007). For example, to what level that lipid and protein can be absorpted after juvenile crayfish start to feeding exogenous feeds? How do pancreatic enzymes (trypsin, lipases, and amylase) activities response to different exogenous feeds? How do digestive regulatory peptides or hormones act together to influence crayfish digestive activities after their first feeding? Further studies on these parts are very necessary to build a bright scenario towards sustainable aquaculture.

From the perspective of sustainable aquaculture, increasing crayfish yield and reducing production costs with the tolerance of environment capacity has gained great concerns in recent years. Seperating crayfish eggs from female crayfish abdomen for artificial incubation provides new outlook to save adult crayfish management costs. Although this method excludes the social experience of maternal care, it is considered as an alternative to traditional crayfish reproduction. Stripping eggs from female crayfish for artificial reproduction reduces maternal egg brooding problems and also prevent transmission of pathogens from brood stock to offsprings (Pérez et al. 1999;

Seemann, 2014). There were previous findings showing that artificial incubation of freshwater crayfish eggs could have higher survival rates when compared with maternal incubation (Strempel 1974; Pérez et al. 1999). However, information on specific time of eggs stripping and eggs quality evaluation is still limited (Seemann, 2014). Further studies aiming at assessing the detailed differences between early stipped eggs and maternal hatching eggs in growth performance, survival, and ability to resist fungal infections are highly needed before this technique being applied in aquaculture on a large scale. In addition, during artificial incubation after stripping eggs from females, exploring antifungal drugs (e.g. formaldehyde, hydrogen peroxide, copper sulphate) to prevent eggs from fungal infection is also important in improving crayfish survival and hatching rate. The salt solution might also be used as an alternative way to control fungal infections. In this case, the general state of artificial eggs health can be greatly improved and mass of high quality juveniles can be supplied to the aquaculture industry by more further studies working on those aspects.

Chapter 9

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