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Selective breeding could improve the juvenile quality of Chinese mitten crab *Eriocheir sinensis*

Xiaodong Jiang^{1,2,3}, Haining Wang^{1,2,3}, Ying Cui⁴, Yongxu Cheng^{1,2,3}
Xugan Wu^{1,2,3*}

¹Key Laboratory of Freshwater Aquatic Genetic Resources of the Ministry of Agriculture and Rural Affairs, Shanghai Ocean University, Shanghai, 201306, China;

²National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, 201306, China;

³Shanghai Collaborative Innovation for Aquatic Animal Genetics and breeding, Shanghai Ocean University, Shanghai, 201306, China

⁴State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai, 200062, China)

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Abstract

Chinese mitten crab *Eriocheir sinensis* is an important aquaculture species in China, and a selective breeding program has been operated for the economic traits of growth and different maturity time of this species since 2011. This study was aimed to evaluate the juvenile quality of two selected *E. sinensis* lines (i.e. early-maturing strain: EM, late-maturing strain: LM), pond-reared juveniles without selection (PR) and wild-caught juveniles (WC). The results showed that: (1) Significant growth difference were only observed in females that the SGR of EM juveniles and the survivor of WC juveniles were both significantly higher than the PR, and the intermolt period of WC was significantly lower than the PR. Although EM juveniles had relatively shorter intermolt period and higher WGR than the LM, they both exhibited better growth performance than the PR, in terms of higher WGR and SGR. (2) Hepatopancreatic AKP activities of PR juveniles were significantly lower than other three populations for both genders, and hepatopancreatic MDA content was significantly higher in the WC juveniles. Moreover, hemolymphatic levels of ACP and T-AOC of both EM and LM juveniles were significantly higher than other two populations. (3) The injection of *Aeromonas hydrophilia* resulted in the highest and lowest cumulative mortalities to the WC and PR juveniles respectively, mortalities of EM and LM juveniles were similar and located between WC and PR juveniles. In conclusion, juvenile quality of EM and LM strains obtained certain promotion after three generations of selection for growth and different maturity time.

Introduction

The Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853, is an indigenous and economically important freshwater crab widely distributed throughout the eastern region of China (Wu et al., 2018a). The annual aquaculture production of *E. sinensis* has increased from about 17, 500 t in 1993 to 756, 877 t in 2018 in China (Wu et al., 2018a). Based on the life cycle of *E. sinensis*, the culture of this crab is divided into three discreet stages during two years: larval or hatchery stage (2–4 months, to produce megalopae) in brackish water, coin-sized juvenile culture stage (8–10 months, to produce coin-sized crabseed for the following growout) and adult growout culture (7–10 months, to produce market-sized adult crabs) in freshwater (Cheng et al., 2008). Juvenile quality is widely linked to the physiological condition, culture performance, immunity and disease resistance of juvenile crustacean (Racotta et al., 2003). Juvenile quality of *E. sinensis* reared under artificial conditions is always variable, the high-quality juvenile is characterized by faster growth, high survival and good ability to adapt environmental variations, while the poor-quality juvenile generally has high mortality, poor growth and pathogen carriers (Zhang et al., 2013). Therefore, it is important to improve juvenile quality and to obtain maximum yield and profit for the following culture of adult *E. sinensis*.

Traditionally, there was no commercial-scale hatchery production of *E. sinensis*, all forms of crab culture depended on the limited natural juvenile supply and subject to heavy fishing pressure in China (Sui et al., 2009). Declining in natural *E. sinensis* resources had been attributed to over-exploitation and environmental degradation, including siltation and pollution. To satisfy the growing market demands of *E. sinensis*, researchers and farmers had been making progress towards effective development of broodstock management and hatchery techniques, and the aquaculture industry of *E. sinensis* experienced good expansion after wild broodstock was successfully applied for juvenile production in commercial hatcheries since the 1990's (Cheng et al., 2008). However, many commercial hatcheries are used to select small adult *E. sinensis* as broodstock for the consideration of saving broodstock cost, which may have negative impacts on culture performance of pond-reared *E. sinensis* populations, i.e. slow growth rate and high percentage of precocious juveniles (He et al., 2014). Moreover, because of lacking *E. sinensis* juveniles in the basin of Yangtze river, the juveniles from the basins of Liaohe and Oujiang were introduced to the Yangtze basin for aquaculture purpose between 1990 and 2005, and such introductions and culture activities undoubtedly had led to germplasm hybrids among different geographical populations in Yangtze river basin, and negatively affected their culture performance (Liu et al., 2015; Wu et al., 2018b).

Genetics and selective breeding are considered as essential tools to improve the economic traits of survival, growth rate and disease resistance for shrimps (Preston et al., 2004; Sae-Lim et al., 2013). During the last two decades, several breeding programs have been conducted on *E. sinensis* through mass selective breeding for growth performance (Wang et al., 2018). Our research team has initiated a selective breeding program for the traits of different mature time (i.e. Early maturity and lately maturity) and growth performance since 2011 (Cheng et al., 2016), which has reached the 3rd generation of juvenile crabs by the end of 2018. The previous studies have reported the effects of selective breeding on morphological characters (Jiang et al., 2018) and juvenile culture performance (Wang et al., 2016b) for the previous generations of our selective populations, this study was conducted to evaluate the culture performance during the beginning of the adult culture stage, immune parameters and disease resistance of two genetically-selected strains of juvenile *E. sinensis* when comparing to normal pond-reared population without genetic selection. Wild-caught *E. sinensis* juveniles from the Yangtze river were reported to have better culture traits, stronger immunity and higher disease resistance compared to pond-reared juveniles, thus wild-caught juveniles are taken into consideration and served as a control population in this study either (Jiang et al., 2017).

Materials and Methods

Experimental juveniles

The selected *juveniles* used in this experiment came from the long-term selection program for different maturity time of *E. sinensis*, i.e. early-maturing strain (EM) and late-maturing strain (LM). EM and LM strains were maintained by Shanghai Ocean University and were respectively selected based on pond-reared and wild-caught *E. sinensis* in 2011. To prepare broodstock for the EM strain, 1000 males and 1800 females of pond-reared *E. sinensis* above 185g and 135g respectively were collected from Shanghai Yuchi aquaculture farm at Chongming Island. Wild *E. sinensis* containing 900 males and 1800 females above 195g and 145g respectively were collected from the Yangtze River and served as broodstock for the LM strain. The larval hatching and rearing of this breeding program were operated in the breeding center in the Rudong county of Jiangsu Province, and megalopae after desalination were transported to the Chongming research station of Shanghai Ocean University for further cultivation of juvenile and adult *E. sinensis* in freshwater ponds. EM and LM populations were managed in a 2-year reproductive cycle, all steps were repeated until the 3rd generation of selected populations are produced by 2016. Detailed information on production and maintenance of two selected strains used in this study is described in Cheng et al. (2016), and the detailed operation of the hatchery, juvenile and adult culture for this breeding program were followed to He et al. (2015).

Commercially used broodstock were purchased from Jiangsu province to produce pond-cultured juveniles without selection (PR). Hatchery conditions and culture management of PR juveniles were maintained similar to the selected populations mentioned above. In late January of 2017, randomly selected juveniles from each population were transported to indoor circular tanks and would be used in subsequent experiments after acclimatizing to the experimental conditions and facilities.

Wild-caught (WC) juveniles were collected from the estuary of the Yangtze River (32°11'N, 119°27'E) in late January of 2017 with commercial trawlers. Collected WC juveniles were maintained in two indoor circular tanks (1 m in diameter, 0.5 m³ in water volume) equipped with circulating freshwater. To maintain their wild-origin nutritional status, WC juveniles were not fed until experiment.

Growth performance

The culture experiment was conducted at the Chongming research station of Shanghai Ocean University. In early February of 2017, 60 juveniles (half male and half female, mean weight 5.70±0.26g) selected from each population were stocked individually in plastic cages (24 × 17 × 17 cm³) and then were distributed into 6 rectangle tanks (depth = 12cm, volume = 18.4L). Juveniles in each tank were distributed to follow the randomized block design that containing 10 juveniles from each of the four populations. The tanks were supplied with filtered freshwater at a flow rate of 3 L/min and aeration was equipped around the tank to maintain sufficient dissolved oxygen. Cultural illumination was provided by fluorescent lamps under the photoperiod of 12L/12D. In addition, all tanks were supplied by a heat-exchange system that allowed maintaining the water temperature within 18-20 °C throughout the culture experiment. Juveniles were fed once a day at 16:00, daily feeding rate (Xinxin #1 diet for adult crabs with 40% protein and 10% lipid) was initially set at 3% of body weight and residual diets were siphoned out every 3 days. During the experiments, water quality parameters were monitored daily and maintained within optimal ranges (pH: 7-8.5, ammonia-nitrogen < 0.4mg/L, nitrite < 0.05mg/L). Juveniles were weighed (precision=0.01g) by an electronic balance (JY202, Shanghai Puchun Measure Instrument Co., Ltd., Shanghai, China) at the 4th day after their respective molt. Parameters for evaluating growth performance were calculated with following formulae after all survived juveniles finished their first molt:

$$\text{Weight gain rate (WGR, \%)} = 100 \times (W_f - W_i) / W_i;$$

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = 100 \times (\ln W_f - \ln W_i) / D.$$

$$\text{Survival} = 100 \times (\text{Initial no. of crabs} - \text{No. of dead crabs}) / \text{Initial no. of crabs}.$$

Where W_f and W_i mean final and initial individual weight (g), D is the intermolt period from the initiate of experiment to their first molt.

Antioxidant capacity and immunity

20 individuals were sampled from each population to analyze antioxidant capacity and immunity. The hemolymph was drawn from the base of the third pereopod with a 1-mL sterile syringe and was centrifuged at 10,000 rpm for 20 min at 4°C, the supernatant was stored at -80°C for subsequent analysis; Collected hepatopancreas were weighted and added to physiological saline solution in a proportion of 1:5 (w/v), and then were centrifuged at 10,000 rpm for 10 min at 4°C, the supernatant was taken and stored at -80°C until analysis as well.

The level of alkaline phosphatase (AKP), acid phosphatase (ACP), superoxyde dismutase (SOD), peroxidase (POD), total antioxidant capacity (T-AOC) and malondialdehyde (MDA) in hemolymph and hepatopancreas were determined with a spectrophotometer (T6 New Century, Beijing Purkinje General Instrument Co., Ltd, Beijing, China) and commercial detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The detailed measurement procedure was conducted according to the manufacturer's guidelines.

Challenge test and histological analysis

To evaluate the disease resistance of juveniles from different dietary treatments, *Aeromonas hydrophilia* Y-2-L-1 was used as pathogenic bacteria. After the acclimation period, crabs were injected intraperitoneally by 1.04×10^8 cfu/ml of bacterial suspension at the doses of 40.0 μ l/g according to the preliminary experiments. 120 injected individuals of each population were transferred into triplicate tanks with filtered water in a quarantine room. Cumulative mortalities were monitored and recorded daily for 7 days following the infection. To identify whether the mortality was confirmed to be due to the *A. hydrophilia* bacteria, hepatopancreas of the dead juveniles were sampled for routine bacteriological examination.

Two days after injection, hepatopancreas of two survived juveniles from each tank were sampled for histological analysis. After fixing in Bouin's solution for 12h, the tissues were dehydrated in ethanol, cleared in xylene and embedded in paraffin according to standard histological techniques. Serial sections of 5-7 μ m were cut using a rotary microtome (Leica RM2125RTS, Leica Microsystems, Bannockburn, IL, U.S.A.) and then were stained with hematoxylin-eosin. The histological characters were observed under a light microscope (Leica DM2500, Leica Microsystems) equipped with an automated Leica digital camera system (Leica DFC295, Leica Microsystems) and image manager software (Leica Application Suite version 3.8.0, Leica Microsystems).

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software package. The data are presented as mean \pm standard error (SE), and homogeneity of data variance was analyzed using Levene's test. Growth date Statistical analysis was conducted using one-way ANOVA and differences determined using Tukey's multiple range test: a *P* value of < 0.05 was considered statistically significant. When homogeneity of variances was not achieved, data were subjected to the Kruskal-Wallis H nonparametric test, followed by the Games-Howell nonparametric multiple comparison test. Two-way ANOVA was also used to identify and separate the effects of gender, selection and their interaction.

Results

Growth performance

Table 1 showed the culture performance of juvenile *E. sinensis* during the beginning of the adult culture stage. The significant difference was only observed in female juveniles that the SGR of EM and the survivor of WC were significantly higher than the PR, and the intermolt period of WC was significantly lower than the PR. As for difference between selected and non-selected populations, EM and LM juveniles both exhibited better growth performance than the PR, in terms of higher WGR (6.33-13.66%) and SGR (16.30-47.82%). As for difference between two selected populations, EM juveniles had a relatively shorter intermolt period and higher WGR than the LM. According to the two-way ANOVA, selective

breeding had significant effects on the intermolt period and SGR of juveniles, but no significant interaction between gender and selection was observed.

Table 1 Effect of selective breeding on growth performance of juvenile *E. sinensis*.

Items	Intermolt period	WGR	SGR	Survivor
Male				
EM	37.67±7.27	53.20±16.50	1.15±0.33	65.00±14.43
LM	41.13±5.71	55.54±12.66	1.17±0.32	62.50±21.65
WC	36.68±8.94	46.20±16.19	1.09±0.43	66.67±15.02
PR	40.33±7.14	46.94±17.59	0.95±0.27	41.67±8.33
Female				
EM	34.53±6.17 ^{ab}	56.30±12.30	1.36±0.41 ^b	55.83±4.17 ^{ab}
LM	40.70±6.63 ^{bc}	52.75±11.18	1.07±0.31 ^{ab}	54.17±4.17 ^{ab}
WC	32.21±8.11 ^a	50.89±9.03	1.26±0.45 ^{ab}	78.33±11.02 ^b
PR	45.21±5.17 ^c	50.55±12.42	0.92±0.24 ^a	43.33±8.33 ^a
P-value				
Gender	0.629	0.540	0.185	0.412
Population	0.000	0.185	0.009	0.052
Interaction	0.138	0.485	0.369	0.351

Note: Data were presented as mean ± SD (n=6); Values in the same column without a common letter are significantly different ($P < 0.05$); EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection.

Antioxidant capacity and non-specific immune indices

Figure 1 presented the antioxidant and immune indices in the hepatopancreas of juvenile *E. sinensis*. The AKP activities of PR juveniles were significantly lower than other three populations for both genders ($P < 0.05$). Moreover, SOD activity of EM juveniles is significantly higher in the males, but a significantly higher level of this index was observed in the WC individuals in the female ($P < 0.05$). Despite for males or females, no statistical differences were observed in ACP and T-AOC levels among different populations.

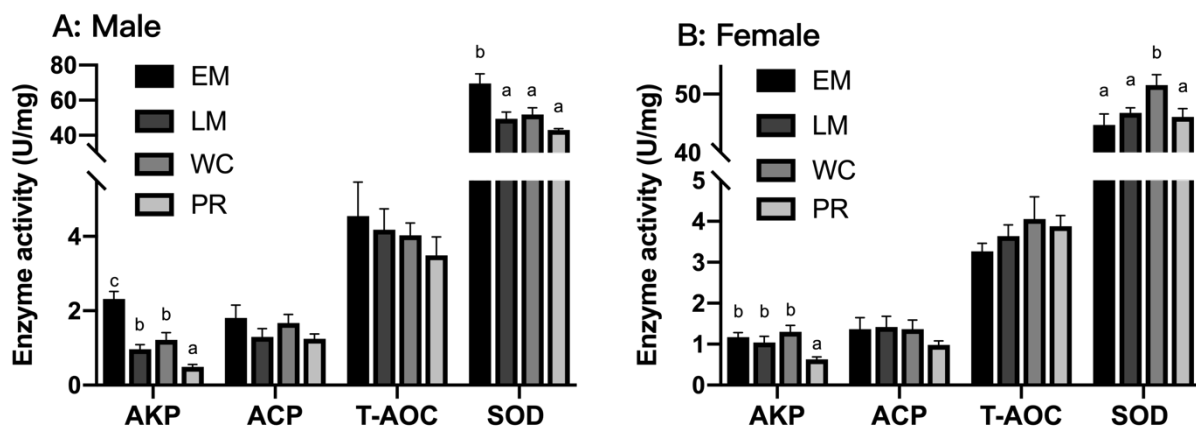


Figure 1 Effect of selective breeding on non-specific immune and antioxidant indices in hepatopancreas of male (A) and female (B) juvenile *E. sinensis*. EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection. The data are expressed as the means ± standard error (SE). Different letters above bars of the same series indicate significant differences among treatments ($P < 0.05$).

The antioxidant ability and immunity parameters in the hemolymph of juveniles were presented in **Figure 2**. Within males, levels of ACP and T-AOC of both EM and LM juveniles were significantly higher than other two populations, and LM showed significantly higher SOD activity than the PR ($P < 0.05$). Moreover, the significantly higher levels of POD were observed in WC juveniles. As for females, the ACP and POD activities of WC individuals were significantly higher than the PR, No significant difference was observed in the AKP, SOD, and T-AOC levels ($P > 0.05$).

Figure 3 showed the MDA contents in the hepatopancreas and hemolymph of juveniles. The significantly higher contents of MDA were observed in hepatopancreas of WC juveniles for both males and females ($P < 0.05$). Moreover, the MDA content in the hemolymph of EM and WC individuals were significantly higher than the LM ($P < 0.05$).

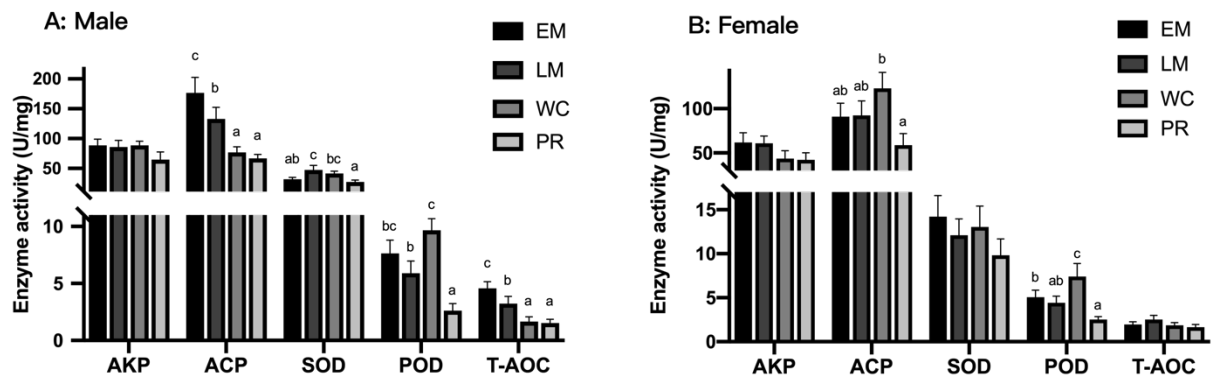


Figure 2 Effect of selective breeding on non-specific immune and antioxidant indices in haemolymph of male (A) and female (B) juvenile *E. sinensis*. EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection. The data are expressed as the means \pm standard error (SE). Different letters above bars of the same series indicate significant differences among treatments ($P < 0.05$).

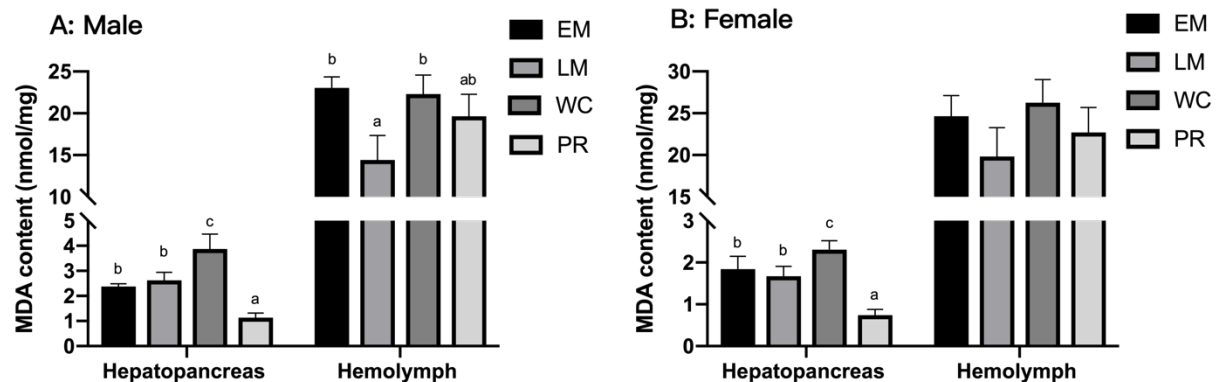


Figure 3 Effect of selective breeding on MDA contents in hepatopancreas and hemolymph of male (A) and female (B) juvenile *E. sinensis*. EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection. The data are expressed as the means \pm standard error (SE). Different letters above bars of the same series indicate significant differences among treatments ($P < 0.05$).

Challenge test and histological analysis

The mortalities of juveniles after injection of *A. hydrophila* were presented in **Figure 4**. The challenge test showed that cumulative mortalities kept increasing during the first 4 days and remained unchanged thereafter. Cumulative mortalities of the PR juveniles had been higher than other three populations, and the lowest mortality was observed in WC juveniles with the mean value of 40% by the end of the challenge test. Cumulative mortalities of EM and LM individuals were similar and were located between WC and PR individuals. However, no obvious differences were observed in histological structure of

juvenile hepatopancreas, besides relatively more blister-like cells in PR individuals (**Figure 5**).

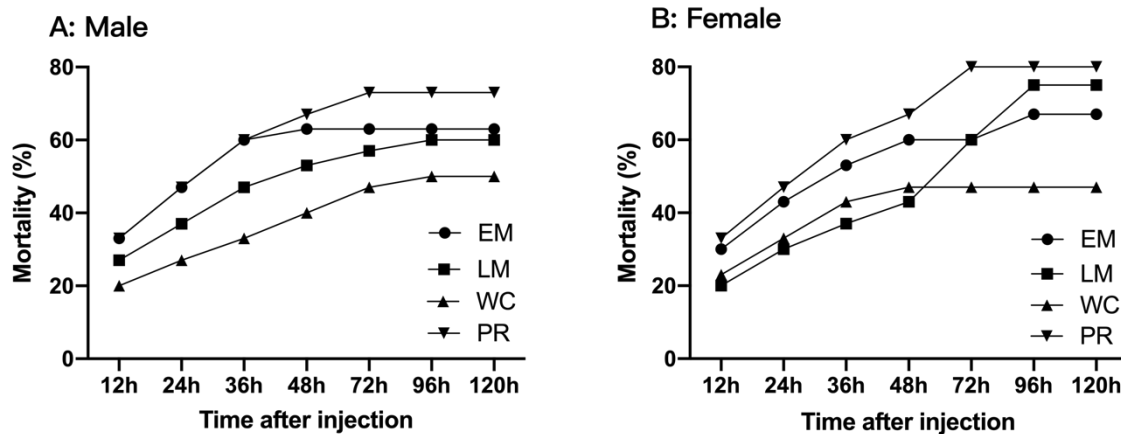


Figure 4 Accumulative mortality of male (A) and female (B) juvenile *E. sinensis* after injection with *Aeromonas hydrophila*. EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection.

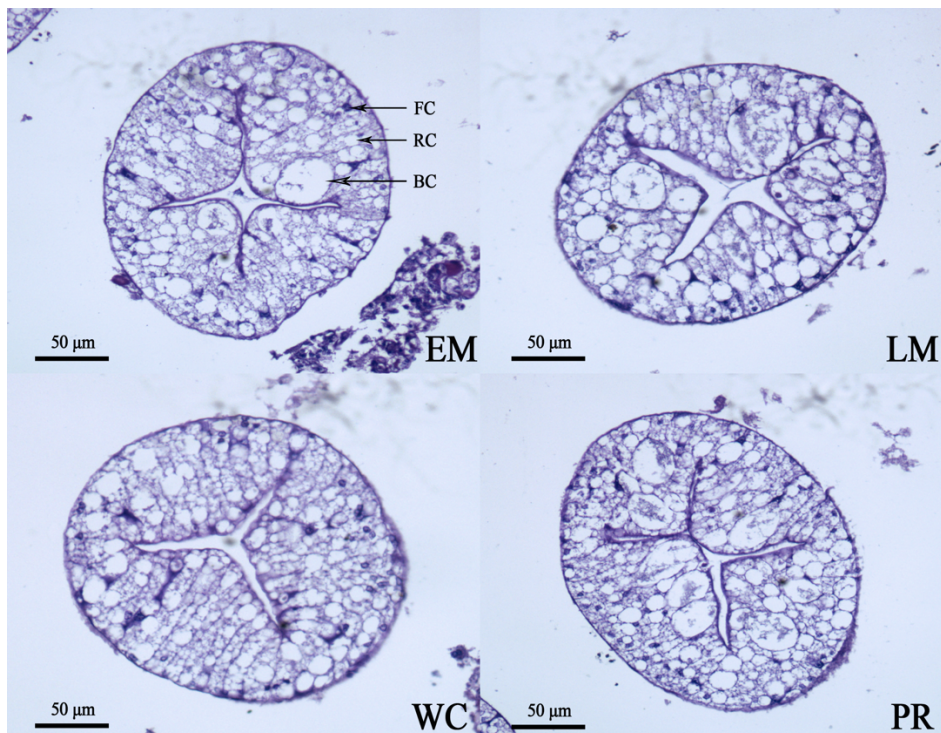


Figure 5 Histological structure of juvenile hepatopancreas after challenge with *A. hydrophila*. FC, fibrillar cell; RC, resorptive cell; BC, blister-like cell. EM, early-maturing strain; LM, late-maturing strain; WC, wild-caught juveniles; PR, pond-reared juveniles without selection.

Discussion

Growth performance

Crustaceans were reported to have a high heritability in body weight, genetically selective breeding has begun to play an increasingly important role in the improvement of their growth performance and perhaps to reduce environmental impact via increased feed efficiency (Rutten et al., 2005). Based on the two-stage culture of *E. sinensis* in artificial condition, body weights of each population were pre-selected at juveniles stage and then were selected at harvest, which would result in a higher selection response than the single-stage selection (Sae-Lim et al., 2013). After their first molt during the adult-culture stage,

EM and LM juveniles exhibited a 6.33-13.66% increase in WGR compared to the PR juveniles in the present study. The growth improvements in our study corroborate results from previous researchers: Ma et al. (2011) found that selected mud crab *Scylla paramamosain* exhibited an 18.00% increase in growth rate compared to unselected crab, and Li et al. (2013) reported a 20.12% selection response for growth of swimming crab *Portunus trituberculatus* after five generations' selection. In the case of growth performance between two selected strains, we found that the final mean weight of EM juveniles was significantly higher than that of LM individuals during the juvenile culture stage in a recent study (Wang et al., 2016b). In the present study, EM juveniles also exhibit better growth performance than LM individuals, in terms of shorter intermolt period and higher WGR, this will help them complete puberty molt and gonad development ahead of LM individuals.

The growth of crustaceans at any given stage is the consequence of molts, consisting of shedding the old exoskeleton and then taking up water or air to expand the new one. Variability in growth rate of crustaceans is manifested as long or short intermolt period depending on both intrinsic and extrinsic factors, such as temperature, photoperiod, diet, and salinity (Shen et al., 2012). In the present study, an obviously shorter intermolt period was detected within wild juveniles, this could result from the existence of different environmental factors existed among wild and pond-reared conditions, as have been described for other crustacean species (Kallio-nyberg et al., 2004). Accumulated temperature and nutrition are generally considered as the major parameters influencing the intermolt period (Chen et al., 2016; Daoud et al., 2010). Although wild and pond-reared juveniles were cultured under same temperature during the experiment, water temperature of the Yangtze River in the winter is generally 4-6 °C higher than artificial ponds, higher temperature accumulation could contribute to the shorter intermolt period of wild juveniles. Moreover, wild crabs in the Yangtze River always feed more during the wintering period due to higher water temperature, this could also be explained to the shorter intermolt period of wild juveniles.

Previous studies suggested that mortality of crustacean mainly occurred during the molting period and may result from kinds of interaction between environments and their physiological states (Wang et al., 2016a). For example, abnormal photoperiod schedules could influence the regular synthesis or release of molt inhibiting hormone (MIH) and then result in heavy molt mortality to the crayfish *Orconectes virilis* (Aiken, 1969), and molting mortality of spider crab *Hyas araneus* was induced by long starvation (Anger and Dawirs, 1981). Wild juveniles exhibited obviously lower mortality than three pond-reared populations in this study, accumulated more energy reserves may contribute to their successful molting.

Non-specific immunity and disease resistance

Crustaceans are all subject to a wide range of pathogens including protozoans, bacteria, and viruses, both in the wild and in culture. Juvenile crustaceans are on their vulnerable life stage and especially appear to be the targets of some pervasive and consequential diseases. It is therefore the immunity and pathogen resistances are widely used to evaluate the juvenile quality.

AKP and ACP are two important phosphatases that regulate many essential functions in the immunity of all living organisms (Xue and Renault, 2000). SOD and POD are two key enzymes in the antioxidant defense system of living body, they can transform free radical to H₂O₂ and O₂ by a disproportionate reaction (Parihar et al., 1997). Obviously higher levels of AKP, ACP, SOD and POD in the hepatopancreas and hemolymph of EM and LM strains, associated with high survival after the challenge experiment, greatly exhibited the positive immunity and obtained with selective breeding for growth performance and different maturity time. The positive relationship between growth and disease resistance was similarly reported in other species, such as salmonids *Salmo salar* and halibut *Hippoglossus hippoglossus* (Beacham and Evelyn, 1992; Imsland et al., 2002). However, the 7.69-12.76% reduction in mortality relative to the non-selected juveniles were obviously lower than previous breeding response for disease resistance of shrimps (Argue et al., 2002; White et al., 2002), suggesting that selection for growth performance only promote horizontal

immunity and resistance, while selection for specific pathogen provides effective and vertical resistant to the corresponding pathogen.

Wild juveniles exhibited higher activities of SOD and POD than other three populations, this result is consistent with previous research on antioxidant capacity of wild Bluefin Tuna *Thunnus thynnus* and *P. trituberculatus* when comparing to those pond-cultured individuals (Ferrante et al., 2008; Jiang et al., 2012). In this study, wild juveniles also displayed obviously lower mortality rates in the pathogens challenge test than the pond-reared juveniles, as shown in other studies on disease resistance or predator avoidance of wild individuals (Congleton et al., 2000). The positive association between the immunity parameters and disease resistance of juveniles suggests that the nonspecific immune system is important for the resistance against pathogens. Other studies have also emphasized nonspecific immunity is particularly important in pathogen resistance (Cha et al., 2013). MDA is one of the most readily assayed end products of both enzymatic and non-enzymatic lipid peroxidation reactions, measurement of MDA provides a convenient index of lipid peroxidation (Devasena et al., 2001). There certainly exists a large number of pathogenic bacteria in the natural circumstances, the resulted imbalance between the production of reactive species and antioxidant defense might induce serious oxidative stress, as reflected by relatively higher MDA content in the hepatopancreas of wild juveniles.

In summary, this study confirmed that juvenile quality of EM and LM strains obtained promotion after three generations' selection for growth and different maturity time. Immunity and disease resistance of two selected populations are still inferior to the wild-caught individuals, indicating there is probably great space for juvenile quality of both selected populations to improve by adjusting selection intensity and breeding strategies. Further efforts in this breeding program will help to provide fundamental data for the production of superior juveniles with high quality.

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