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中国农业出版社
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ISBN 978-981-19-1711-0 ISBN 978-981-19-1712-7 (eBook)
https://doi.org/10.1007/978-981-19-1712-7

Jointly published with China Agriculture Press
The print edition is not for sale in China (Mainland). Customers from China (Mainland) please order the print book from: China Agriculture Press.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Golden pompano *Trachinotus ovatus* (Linnaeus, 1758) belongs to the family of Carangidae and is widely cultured in the Asian-Pacific region. Due to fast growth, high flesh quality, and suitability for cage culture, golden pompano has been considered a good candidate for aquaculture. As a result of increasing domestic and international markets, the total production of golden pompano reached 140,000 MT in 2020 in China. Although artificial breeding of this species has been successfully achieved and hatchery rearing practices have been established, there is a lack of research to address hatchery managers' issues during the ontogenetic development in the early life of golden pompano.

In the past decade, marine fish larviculture techniques have quickly developed. Feeding regimes and culture systems have been modified to improve rearing performance and efficiency in finfish hatcheries. While the use of live food organisms, especially at the initial feeding stage, is still obligatory in the culture of most marine fish larvae. The use of super-intensive rotifer culture using algal paste has improved cost-effective operation in hatcheries. Weaning of fish larvae to inert diets at a later development stage is easily achieved by co-feeding of inert diets and live food. Although larval deformity and cannibalism are still the major issues, system innovation and management strategies have dramatically reduced their adverse impact on larval fish rearing.

This book focuses on the physiological and molecular changes during the early ontogeny of *T. ovatus* and hatchery and nursery practices. We aim to provide comprehensive references for the larval rearing of this species. In this book, the first seven chapters update the recent development in the hatchery technology in *T. ovatus*, including feed and feeding, environmental manipulation, hatchery management, and fingerlings in transportation. Starting from the eighth chapter, we attempt to decode the possible molecular mechanisms underlying fish development and response to environmental changes and discuss the transcription and expressions of growth and development-related genes in *T. ovatus*. At the end of this book, functional feed additives to the diet and antibiotic usage of *T. ovatus* juveniles are discussed.

In the preparation of this book, all the contributors responded positively when they were invited to contribute chapters. We express our sincere gratitude to all contributors for their enthusiasm and commitment to contributing chapters and valuable information.

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Contents

Chapter 1
Ontogenetic Development of the Digestive System in Golden Pompano *Trachinotus ovatus*

Zhenhua Ma, Gang Yu, and Jian Guang Qin

Abstract The digestive ontogeny of *Trachinotus ovatus* from hatch to 32 days post-hatch was reviewed in this chapter. The development of digestive system in *T. ovatus* can be divided into three stages: stage I starting from hatching and ending at the onset exogenous feeding (3 DPH), stage II starting from first feeding and ending at the formation of gastric glands in fish stomach (15 DPH), and stage III starting from the appearance of gastric glands and continuing onward. The specific activity of lipase, trypsin, and amylase in fish increased rapidly from the exogenous feeding to 5–7 DPH and followed by random fluctuations. Pepsin activity was firstly detected on 15 DPH, and the specific activities increased with fish age. The dynamics of enzyme activity reflected the structural development in fish digestive system. After the formation of gastric glands in the fish stomach, the enzyme activities incline to be stable. According to the development of the digestive system, weaning of *T. ovatus* larvae can be initiated at 15 DPH. This chapter will improve our understanding of *T. ovatus* ontogeny during the larval phase and supply the feeding and weaning protocol for this important economic fish in hatcheries.

Keywords Ontogenetic development · Digestive system · Enzyme activity · *Trachinotus ovatus*

1.1 Introduction

The larval phase of marine fish has been deemed the main "bottleneck" limiting fingerling production to the grow-out facilities (Tucker 1998; Joan Holt et al. 2007). Inappropriate feed uptake can lead to massive mortality in this stage (Kohno et al.

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_1

1997; Hunt von Herbing and Gallager 2000; Yufera and Darias 2007; Ma et al. 2012b). During the initial feeding stage, larval fish experience a period of mixed nutrition and progressively transfer from endogenous nutrition to exogenous nutrition. During this transaction period, fish tissues, organs, and systems undergo structural or functional transformation or development. Therefore, knowledge on fish nutritional requirement, digestive performance, and live food supply will improve survival, growth, and nutrition supplement to fish larvae in hatchery rearing environment (Ma et al. 2012b; Hu et al. 2018).

The digestive system in most of the marine fish larvae is immature at hatching and will undergo a series of developments to have a fully and complete functional digestive system (Canino and Bailey 1995; Chen et al. 2006a). Determine the stage of developmental process when a mature digestive system can be formed is practically important in hatchery practices (Ma et al. 2012a). An accurate understanding of the main developmental events timing in fish larvae could guide us to conclude the proper time to wean fish larvae to formulated diets (Cahu and Zambonino-Infante 2001; Baglole et al. 1997). Furthermore, knowledge on the morphological and developmental differences of the digestive system in the larval stage will promote a further understanding on the species-specific digestive abilities and nutritional physiology of larval fish and can be applied to correspond the physiological developments with feeding practice and rearing protocol (Mai et al. 2005; Chen et al. 2006a, b; Lazo et al. 2011).

Previous studies have revealed that fish larvae generally have plenty of levels of digestive enzymes to digest live feeds at first feeding but probably difficult to digest formulated diet (Cahu and Zambonino-Infante 2001; Kolkovski 2001). Evidence indicates that marine larval fish can swallow formulated diet in their early life, but cannot digest it and mostly die without complete digestion of the diet (Beccaria et al. 1991; People Le Ruyet et al. 1993; Koven et al. 2001). The defeat of adaption of formulated diet in larval fish may be caused by insufficient dietary composition and low digestibility, especially when the digestive system of fish larvae is immature (Govoni et al. 1986; Cahu and Zambonino-Infante 2001; Chen et al. 2006b; Rønnestad et al. 2013). Evidence has proposed that the availability of digestive enzymes is an essential criterion for larval fish to survive on a formulated feed (Yufera et al. 2000; Kolkovski 2001; Yufera and Darias 2007). Without enow number of enzymes in the digestive system, food particles cannot be fully digested. Accordingly, understanding of the ontogenetic development of digestive enzymes in larval fish is critical to choose a proper weaning regime to a formulated diet in larva cultivation (Chen et al. 2006b; Lamarre et al. 2007; Ma et al. 2012a).

The aim of this chapter was to review the structural changes and development of digestive enzymes during early development of indoor cultured *T. ovatus* from hatch until metamorphosis, focusing on the digestive physiology of fish larvae, and pave the way for the improvement of golden pompano hatchery management level.

1.2 Growth and Development Pattern of *T. ovatus*

Data described in this chapter derived from our previous study on the digestive functionality in *T. ovatus* (Ma et al. 2014). On 1 DPH, the standard length of larval *T. ovatus* averaged at 3.06 mm (Ma et al. 2014). The average growth rate (AGR) and specific growth rate (SGR) were 0.49 mm/day and 5.88%/day, respectively. At hatching, the digestive tract of *T. ovatus* was a straight tube lying dorsally to the yolk sac, and incipient liver and pancreas were not presented at this stage (Ma et al. 2014). Incipient liver and pancreas come into view and become distinguished on 3 DPH. Mouth opening of fish was observed at the 2 DPH night. First feeding activity was detected on 3 DPH, and rotifers can be easily identified in the midgut of larval fish (Ma et al. 2014). On 4 DPH, yolk sac was completely absorbed, and supranuclear vacuoles (SNV) was formed. Oil globules were completely absorbed on 7 DPH, and the mixed feeding was carried out between 3 and 7 DPH. The standard length between 3 and 7 DPH had no significant difference ($P > 0.05$).

Gastric glands were firstly found on 15 DPH. The gastric glands consist of single-type secretory cells beneath the epithelium between the cardiac and pyloric regions (Ma et al. 2014). The number of gastric glands increased with the increasing of fish age, and gastric glands were found clustered in the cardiac and fundic regions (Ma et al. 2014). In the meantime, rudimentary pyloric caeca were found on the anterior midgut. Between 17 and 18 DPH, the stomach could be divided into cardiac, fundic, and pyloric regions. With the increasing of fish age, the fundic region was elongated and formed the largest section of the stomach. The number of SNVs in the hindgut decreased and cannot be observed after 19 DPH (Ma et al. 2014). The number of lipid vacuoles increased in posterior midgut on 18 DPH.

Similar to most marine fishes, the digestive system development in *T. ovatus* can be divided into three stages in accordance with the morphological and histological characters. Stage I began with hatching and finished before commencing exogenous feeding on 2 DPH. At the end of stage I, the yolk sac was partially depleted, the mouth opened, and the intestine was able to adapt to the exogenous feeds. The duration of stage I in *T. ovatus* is similar to bluefin leatherjacket *Thamnaconus modestus*, yellowtail kingfish *Seriola lalandi*, and coral trout *Plectropomus leopardus*, where nutrition is singly endogenous in the first 2 days after hatching (Chen et al. 2006a; Guan et al. 2013), but is shorter than common dentex *Dentex dentex* L., where nutrition is singly endogenous in the first 4 days after hatching or even longer (Santamaria et al. 2004; Liu et al. 2013).

Stage II started from onset first feeding (3 DPH) and ended at the formation of gastric glands. The stage II is the momentous stage in fish life as larva needs to quickly develop their feeding ability to accept the exogenous feeding, and failures will affect other physiological functions, leading to malnutrition and even death (Segner et al. 1993; Chen et al. 2006a; Ma et al. 2012b; Xu et al. 2021). In stage II, *T. ovatus* experienced a mixed nutrition period which fish gradually transferred from endogenous feeding to exogenous feeding. Within this transaction, the digestive system of *T. ovatus* was developed in structure and function to accommodate to

exogenous feeding before yolk-sac reserves were completely absorbed. Thus, the digestive tract of larval fish was further differentiated, and the supranuclear vacuoles and lipid vacuoles appeared in the hindgut and midgut on 4–5 DPH after depletion of yolk residuals. In stage II, *T. ovatus* fed on *Artemia* nauplii and rotifers for nutritional resource. Similar to most marine larval fish, significant transformation in the digestive system and speedy depletion of yolk-sac and oil globules may cause starvation or malnutrition if suitable feed is unavailable (Chen et al. 2006a).

Stage III started from the appearance of gastric glands and the pyloric caeca in the stomach of fish. This characteristic can be used as the standard to separate larvae from juveniles in marine fish (Baglole et al. 1997; Rønnestad et al. 2013) and also can be used as the point in time to start weaning fish to formulated diet (Ma et al. 2012b). The formation of this critical organs is species-specific and may also be accomplished with unsynchronized pepsinogen-secretion (reviewed by Rønnestad et al. 2013). For example, it takes 33 days in haddock *Melanogrammus aeglefinus* L. from hatch to stage III (Hamlin et al. 2000), while it takes 22 days in dover sole *Solea solea* (Boulhic and Gabaudan 1992), and 15 days in yellowtail kingfish *Seriola lalandi* (Chen et al. 2006a). Similar to yellowtail kingfish, it took 15 days in *T. ovatus* to enter stage III, indicating that the *T. ovatus* larvae belong to fast development species.

1.3 Changes of Digestive Enzyme Activity in Larva *T. ovatus*

The total activity of trypsin was not observed before 1 DPH and gradually increased with the increasing of fish age, after initial feeding (Ma et al. 2014). The first rapid increase was found on 15 DPH which coincided with the feeding of *Artemia* nauplii ($P < 0.05$). The total activity of trypsin reached the maximum at 19 DPH and was not significantly different afterward ($P > 0.05$). The specific activity of trypsin increased significantly on 7 DPH compared before ($P < 0.05$). Then, the specific activity of trypsin reduced on 13 DPH and increased significantly on 15 DPH ($P < 0.05$). Afterward, the specific activity gradually reduced to a low level on 32 DPH, which was similar to the level in newly hatched larvae. The total activity of amylase was not observed before first feeding and remained at low until 13 DPH (Ma et al. 2014). The activity progressively increased from 3 DPH to 17 DPH. The total activity peaked at 26 DPH ($P < 0.05$) and then kept at this level until the end of the experiment ($P > 0.05$). The specific activity of amylase increased rapidly on 3 DPH at the initial feeding ($P < 0.05$). After that, the activity reduced gradually from 5 DPH to 32 DPH (Ma et al. 2014).

The total activity of pepsin was first spotted on 15 DPH (Ma et al. 2014). Afterward, an upward trend was seen in the total activity of pepsin with fish age before 17 DPH. The total pepsin activity increased significantly from 19 DPH to 32 DPH ($P < 0.05$). The specific activity of pepsin was firstly detected on 15 DPH

(Ma et al. 2014). Then, the activity increased with the increasing of fish age and reached to the maximum level on 32 DPH. The total activity of lipase was not significantly different from 1 DPH to 19 DPH. The total activity of lipase gradually increased from 22 DPH to 36 DPH. The specific activity of lipase was increased rapidly from newly hatched larva to 5 DPH ($P < 0.05$) which was cohering with fish feeding on live feeds. After the first maximum, the specific activity gradually increased until the end of this experiment. On 32 DPH, the specific activity of lipase was 24.7 mU/mg protein, which was 120 times higher than that on 1 DPH (Ma et al. 2014).

Previous studies have demonstrated that the activity of some digestive enzymes in marine fish larvae can be detected before exogenous feeding (Zambonino Infante et al. 2008; Zambonino Infante and Cahu 1994b; Cara et al. 2003; Kolkovski 2001). The development of digestive enzymes in fish larvae can determine the timing of offering exogenous feeds to fish larvae (Gawlicka et al. 2000). In *T. ovatus*, the specific activity of trypsin, amylase, and lipase was spotted before the onset exogenous feeding, and a rapidly increasing trend was found between 3 and 7 DPH. In Pacific threadfin *Polydactylus sexfilis*, the amylase activity can't be quantified at hatch, but can be detected before the first feeding (Kim et al. 2001). The activities of trypsin, amylase, and lipase were significantly stronger than those in the newly hatched larvae in some species such as white sea bream *Diplodus sargus* and yellowtail kingfish (Cara et al. 2003; Chen et al. 2006b). In larval Eurasian perch *Perca fluviatilis*, the specific activities of trypsin and amylase can be found after hatch and increased greatly in the first few days after exogenous feeding (Cuvier-Peres and Kestemont 2002). These results suggest that digestive enzymes of fish larvae are at a low level before the start of exogenous feeding, while their activities rise to a high level during exogenous feeding. Similarly, the start points of trypsin, amylase, and lipase in *T. ovatus* are triggered by internal mechanisms, rather than by dietary stimulation, but reaching a relatively high level may be associated with food stimulation.

The developing patterns of digestive enzymes of *T. ovatus* like lipase and amylase were strongly associated with the structural changes of the digestive system identified in the histological studies. The significant increase of specific activities of lipase and amylase before exogenous feeding is similar to species like crimson snapper *Lutjanus erythropterus* and yellowtail kingfish (Chen et al. 2006b; Cui et al. 2018). The fluctuations in specific enzyme activities reflected functional development in the digestive tract and associated glands of fish larvae. After the appearance of gastric glands, the digestive system became functional in acidic digestion, and the specific activities of these digestive enzymes remained constant, while the total enzyme activities increased gradually with age. In crimson snapper, yellowtail kingfish *Seriola lalandi*, and walleye pollock *Gadus chalcogrammus* larvae, high specific activities of the trypsin and amylase were corresponded to the transitional period from endogenous to exogenous feeding (Cui et al. 2018; Chen et al. 2006b; Oozeki and Bailey 1995). Evidences have indicated that the increase of the digestive enzyme activities in the early larval phase could be genetically programmed and is not led by external food (Zambonino Infante and Cahu 1994a; Ma et al. 2014). The

developmental pattern of the enzyme activity from fluctuation to relatively stable in a late stage may be related to structural and physiological alterations in the larvae during metamorphosis (Chen et al. 2006b). Similar to the findings reported by Chen et al. (2006b), the development of larval *T. ovatus* shows the concurrence between the digestive enzyme activities and the morphological development of the digestive system.

In *T. ovatus*, the developmental pattern of trypsin activities varied from other digestive enzymes. Two maximums were recorded on 7 and 15 DPH, and then the specific activity reduced progressively. During the ontogenetic development of *T. ovatus*, the total enzyme activity showed a progressive increasing trend and followed by a decreasing trend afterward. The pattern of trypsin activities observed in *T. ovatus* larvae is similar to European sea bass larvae, in which the trypsin-specific activity progressive increased to 23 DPH followed by a rapid decrease but increased again on 40 DPH to the level when the exogenous feeding started (Zambonino Infante and Cahu 1994a). Meanwhile, the development patterns are varied in many other species. In Eurasian perch larvae, both specific and total activity of trypsin sustained to increase as fish larvae grew for 30 days (Cuvier-Peres and Kestemont 2002). In *T. ovatus*, the maximum specific activity of trypsin occurred on 15 DPH when the gastric glands formed and pepsin activity was detected. Afterward, the specific activity of trypsin is reduced with the increase of the pepsin activity. The sustaining reduction of trypsin activity after metamorphosis and the increase of pepsin activity in *T. ovatus* indicate that protein digestive enzyme has been transformed from trypsin to pepsin, and the stomach becomes functional (Chen et al. 2006a, b; Segner et al. 1994; Guerreiro et al. 2010). The digestive system of the *T. ovatus* larvae has the physiological conditions to receive the compound inert diet on 15 DPH, and weaning should begin at this point and afterward.

1.4 Conclusion

In conclusion, *T. ovatus* belongs to the fast-growing species as the standing of digestive ontogeny of fish larvae phase is relatively short. The specific activities of amylase and lipase were stable, and the total activity of these two enzymes increased gradually from 18 DPH. The relatively low specific of amylase reflects the carnivorous nature of *T. ovatus* juveniles and the limited capacity to digest carbohydrates.

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Chapter 2
Food Consumption, Ingestion, and Selectivity of Golden Pompano *Trachinotus ovatus* Larvae Under Various Rotifer Densities

Zhilu Fu and Zhenhua Ma

Abstract In this chapter, rotifer densities, food selectivity, and color preference during the rotifer feeding stage of *Trachinotus ovatus* were reviewed. *T. ovatus* larvae were reared under four rotifer densities (1, 10, 20, and 40 rotifers mL^{-1}), two types of live preys (rotifer and copepod nauplii), four colors of rotifer (blue, red, green, and purple), and five tank colors (black, green, white, pink, and dark red). The study was conducted between 1 day post hatching (DPH) and 5 DPH. In the feeding density of 20 rotifers mL^{-1}, the specific growth rate and survival rate were maximum. In terms of food ingestion, both 10 rotifers mL^{-1} and 20 rotifers mL^{-1} at 5 DPH and 9 DPH are significantly higher than other rotifer densities ($P < 0.05$), but there was no significant difference in each density at 7 DPH ($P > 0.05$). The rotifer consumption was no significant difference in each density at 7 DPH ($P > 0.05$), but the rotifer utilization efficiency had a significant difference and decreased with the increase of rotifer density ($P < 0.05$). The rotifer density of 1 rotifer mL^{-1} not only reduced food ingestion during the early stage but also delayed diet switch from rotifer to copepod nauplii. Dark tank background colors such as black and dark red could increase the food ingestion of larvae. Meanwhile, when fed with green rotifers, the food ingestion of larvae is significantly higher than that of other colors ($P < 0.05$). In the present study, the rearing of larval *T. ovatus* is suggested using dark wall color tanks with a feeding density of 10–20 rotifers mL^{-1} during the initial feeding stage. This chapter proposes a management protocol to use suitable type and

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_2

quantity of live food to feed *T. ovatus* larvae in a hatchery rearing condition, which could be applicable to the rearing of larval fish in other marine fish species.

Keywords Food consumption · Food ingestion · Food selectivity · Growth · Survival rate · *Trachinotus ovatus*

2.1 Introduction

In most species of marine fish, the digestive system of larvae is immature at hatching or immediately afterward (Falk-Petersen 2005). The success of ontogenic development is affected by different factors, including larval feeding capacity, water characteristics, and environmental temperature. The planktivorous nature of larval fish (Cox and Pankhurst 2000) at early life causes them to choose suitable species as living prey alongside fish ontogeny to attain the needed nutrition after the depletion of reserve yolk (Ma et al. 2012; Yúfera and Darias 2007). After the completion of yolk absorption, the survival, as well as the growth of fish larvae, is determined by the quality, quantity, and timing of external supply of food (Gisbert and Williot 1997; Ma et al. 2012; Yúfera et al. 2000). A key standard in fisheries recruitment and aquaculture production hence is fish larvae being successful in capturing live prey in the first few days post-hatching (Chesney 2005; Cushing and Horwood 1994). With respect to larval fish hatching, light can interact with the color of tank walls to interfere with prey capture. Light has a key influence in mediating the feeding behavior of larval fish since most of them are visual feeders (Monk et al. 2008). Some studies have investigated how the food consumption efficiency of fish larvae is influenced by the color of the tank they are bred in (reviewed by Ma et al. 2013a). There has been enough evidence of the fact that the choice of the color of the tank wall is rather species-specific. For instance, black tank walls are preferred by turbot, herring, and milkfish (Howell 1979; Duray 1995; Blaxter 1968), while the same does not support the survival and growth of haddock larvae (Downing and Litvak 2000). Besides the color of the tank walls, the intake of food in yellowtail kingfish larvae can also be affected by prey color (Ma and Qin 2012). Various rearing systems have been designed for the culture of *Trachinotus ovatus* larvae.

In fish rearing, feeding strategies and the holding system can impact fish survival and can led to body abnormalities. Hence once a species of live prey is determined to be suitable for the feeding of marine larvae, the feed is to be appropriately delivered to fulfil the nutritional requirement of fish larvae (Slembrouck et al. 2009). During the first feeding period, the capability of larvae to locate, select, hunt, handle, and ingest a food item is relatively constrained. A common strategy to increase food encounters and intake in larval fish is to increase the live food density in the environment (Slembrouck et al. 2009). But much research has pointed out that the marine fish are fed at live prey densities that far exceed the ability of the larvae to consume (Rabe and Brown 2000), leading to low food utilization efficiency and a waste of live food (Ma et al. 2013b). Additionally, overfeeding does not lead to improved survival and growth, and this claim has been proven to be true in other

studies (Temple et al. 2004; Ma et al. 2013b; Duffy et al. 1996). Long-term exposure to a high prey density can be harmful to fish since an extended period of feeding at a prey density that is higher than their need may prompt fish larvae to overfeed, prolong the time of gut evacuation, and reduce digestion efficiency (Temple et al. 2004; Johnston and Mathias 1994; Boehlert and Yoklavich 1984).

A well-known member of the family Carangidae, *T. ovatus*, is extensively cultured worldwide. Owing to its fast growth, appropriateness for cage culture, and superior flesh quality, *T. ovatus* is recognized as an excellent aquaculture species. Recently, there has been successful artificial breeding of this species, but several issues associated with managing *T. ovatus* larvae during larval rearing are still not clear. In China, copepods and rotifers are the major live feed for larval rearing of *T. ovatus*, but the optimal prey density is not known. This chapter covers the investigation of four major issues on whether (1) live prey density determines fish survival and growth rates, (2) food selectivity is dependent upon prey density, (3) prey density regulates the consumption of fish larvae, and (4) food ingestion by fish larvae depends on the color of the tank walls as well as the prey type. The answers to these fundamental questions will improve the efficiency and overall success of larval fish rearing hatcheries and the overall efficiency of fish larvae in utilizing live prey during the initial feeding stage.

2.2 Growth, Gut Content, Consumption, and Utilization of Food in *T. ovatus* Larvae Under Varying Rotifer Densities

Twelve 500-L tanks were used to stock 3-day-old fish larvae such that each tank has 60 fish per liter. The rotifer densities in the larval rearing tanks were set at 1, 10, 20, and 40 rotifers mL^{-1} with three replicates each. Three feeding times were scheduled during a day at 08:00, 12:00, and 17:30 h. Larval fish consumed rotifers from 3 to 9 DPH. For measuring growth, every 2 days, ten larvae were randomly collected from each tank. On every sampling day, larval fish were collected using a siphon tube vertically in triplicate from the uppermost, central, and lowermost locations of each tank. The collected samples from one tank were placed together in a 10-L bucket, followed by a random sample of fish larvae from the bucket. Length measurements were taken to the closest 0.05 mm, measurement from the upper jaw to the end of the notochord on a dissecting microscope using an ocular micrometer.

Assessment of the food ingestion was carried out by sampling fish larvae on the third, fifth, seventh, and ninth days post-hatch. On the day of sampling, larval fish were fed in the morning for 2 h. Afterward, ten larval fish were collected randomly from each tank. Live food was also collected from the tank to determine food density in the environment. Larval fish were subjected to anesthesia using 0.5% AQUI-S (AQUI-S New Zealand Ltd.) and preserved with buffered neutral formalin at a

concentration of 10% in sampling containers. Quantification of food ingestion was done using these preserved fish larvae. After the experiment, a count of all surviving fish gave an estimate of the survival rate. Growth was estimated in percent per day by specific growth rate (SGR) making use of the expression: SGR = 100 ($\ln SL_f - \ln SL_i$)/Δt (Hopkins 1992), where the initial and final larval fish standard length (mm) is represented respectively by SL_i *and* SL_f and Δt stands for a time duration between sampling days. The entire digestive tube was extracted from each fish larvae and then dissected at 100× magnification on a dissecting microscope. A count of all the live food items and their taxonomy groups were recorded. The number of trophy and mastax was taken to estimate the number of rotifers consumed by larval fish, whereas the entire body structure of copepods and eye pigments were considered proof of food ingestion.

The gut evacuation time was investigated using the following method on the seventh day post-hatching. Ten hours before this trial, 300 fish were kept in 500-L tanks in triplicate, and these fish were completely deprived of food. Prior to feeding, the empty digestive tract was checked by sampling ten fish for the analysis of gut content. Rotifers were fed to fish larvae for 2 h at a density of 20 rotifers mL^{-1}. Post feeding, gut content analysis of ten larvae sampled from every tank was carried out, and 100 larvae in triplicate were translocated to separate tanks with a capacity of 500 L to measure gut evacuation time. The tanks for the gut evacuation experiment had an identical environment to the growth trial except without food supply. From each gut evacuation tank, ten fish larvae were sampled every hour for analyzing gut contents, and the trial lasted 8 h. The 0.5% AQUI-S was used to anesthetize fish larvae, and the number of rotifers in the gut of individual fish was used as the number of food intake.

Elliott and Persson (1978) described the following equations: $F = (S_t - S_0 e^{-Rt})R/(1 - e^{-Rt})$ to determine the consumption rate, where S_t is the food quantity in the gastrointestinal tract after a time t, F is the rate of consumption of food per hour, S_0 is the amount of food initially contained in the stomach, and R is the rate of gut evacuation. The R is determined by using another equation (Elliott 1972), $R = (\ln A - \ln Y_x)/X$, or $Y_x = Ae^{-RX}$, where X represents time interval, Y_x is the number of food items in the digestive tract, and A is the value of Y_x at the intercept of the regression line and the ordinate. The magnitude of Y_x at the y-axis and the intercept of the regression line are represented by A.

The percentage of food eaten in comparison to the total count of food items is referred to as food utilization efficiency (FUE, %) (Ma et al. 2013b), FUE = 100 ($F \times t \times N_f$)/N_{total}, where N_{total} represents the total number of food, the number of food hourly eaten by larval fish is given by F, t stands for the feeding time of the larvae (4 h for this investigation), and N_f represents the total number of fish larvae in the tank.

A notable influence on the growth of pompano larvae was made by prey density ($P < 0.05$, Fig. 2.1). The *T. ovatus* larvae fed 1 rotifer mL^{-1} and 40 rotifers mL^{-1} exhibited low SGR compared to those fed in 10 rotifers mL^{-1} and 20 rotifers mL^{-1} ($P < 0.05$). The treatment with 40 rotifers mL^{-1} and 1 rotifer mL^{-1} or 20 rotifers mL^{-1} and 10 rotifers mL^{-1} did not reveal any significant difference ($P > 0.05$). The

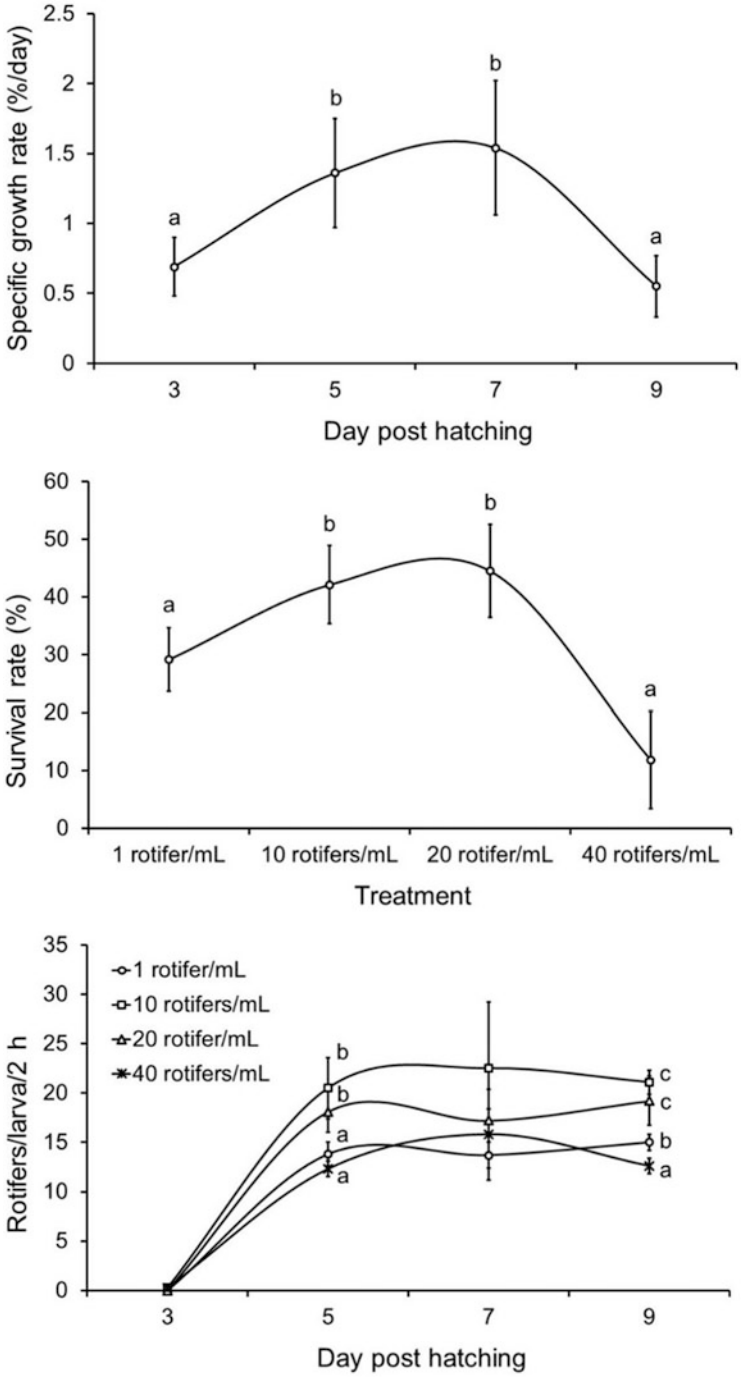

Fig. 2.1 Survival rate, growth rate, and food ingestion for larval *Trachinotus ovatus* fed 1, 10, 20, and 40 rotifers mL^{-1} from 1 to 9 DPH

value of SGR in the fish fed in 20 and 10 rotifers mL^{-1} was nearly twice higher ($1.56 \pm 0.44\%$ per day, and $1.32 \pm 0.33\%$ per day) than those fed in 40 and 1 rotifers mL^{-1} ($0.55 \pm 0.18\%$ per day, and $0.63 \pm 0.16\%$ per day). A notable influence of the prey density was also observed on the *T. ovatus* larvae survival rate ($P < 0.05$, Fig. 2.1). In comparison to the larvae in 10 rotifers mL^{-1} and 1 rotifer mL^{-1} ($P < 0.05$), the *T. ovatus* larvae in the 20 rotifers mL^{-1} and 10 rotifers mL^{-1} treatments had a notably high survival rate.

Figure 2.1 shows the food consumption potential of *T. ovatus* larvae at first feeding. The initial feeding occurred in the morning of 3 DPH, but each fish is ingested in 0.025 rotifers on average. Feeding treatments ($P > 0.05$) were not found to be significantly different. With an increase in the age of the fish, the consumption of food by the larvae continuously increased. On the fifth day following hatching, the total count of rotifers consumed by larvae reared in 10 rotifers mL^{-1} and 20 rotifers mL^{-1} was considerably high compared to those cultured in 1 rotifer mL^{-1} and 10 rotifers mL^{-1} ($P < 0.05$, Fig. 2.1). On 7 DPH, there was no significant difference in food consumption of larval fish between feeding treatments ($P > 0.05$). The pattern of consumption of food by fish larvae in different feeding treatments on 9 DPH was similar to that on 5 DPH. Meanwhile, larvae in the 10 rotifers mL^{-1} and 20 rotifers mL^{-1} treatments were notably increased compared to those cultured in 1 rotifer mL^{-1} and 10 rotifers mL^{-1} ($P < 0.05$). Least value for food consumption is presented in the 40 rotifers mL^{-1} treatment group as (12.77 ± 0.68) rotifers per larva in 2 h.

The estimate for the food intake was made individually for each larva. On the seventh day post-hatching, the food consumption of larvae peaked to 44–60 rotifers per day. However, it did not appear to be influenced by the rotifer densities ($P > 0.05$, Fig. 2.2). The rotifer density impacted food utilization efficiency in tanks ($P < 0.05$, Fig. 2.2). With the rise in rotifer densities, the efficiency of food utilization showed a significant decline from 65.4% to 3.2% ($P < 0.05$, Fig. 2.2).

2.3 Food Selectivity of *T. ovatus* Larvae Under Different Feeding Densities

Measurement of fish larvae inclination toward rotifers and copepod nauplii in terms of selection of food in the first feeding period was made on 5, 7, and 9 DPH. The siblings of the same cohort larvae were cultured in 12,500-L tanks. The density of rotifers in the tanks containing fish larvae was set at four different levels including 1, 10, 20, and 40 rotifers mL^{-1} with three replicates. At the beginning on the fifth day post-hatching, copepod nauplii were put into three enclosures in situ and were fed to fish three times a day at a density of 1 nauplii mL^{-1}. Fish were fed on each sampling day for 2 h before sampling. From each rearing tank, ten fish were collected for determining feed selectivity. Quantification of the prey items in the fish gut and the tank was done independently. By using the method elaborated by

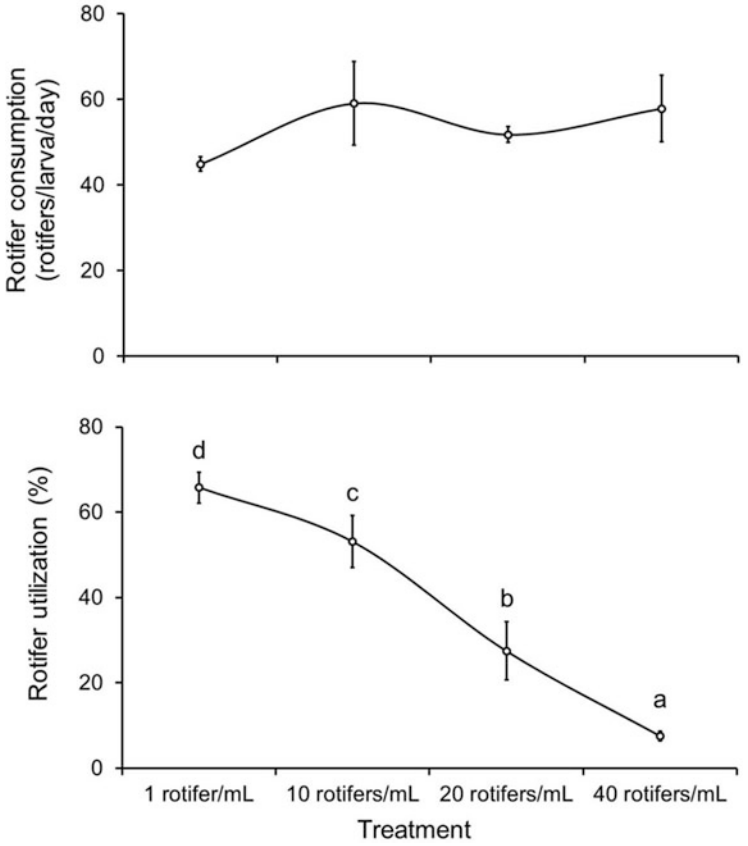

Fig. 2.2 Rotifer consumption and utilization efficiency on 7 DPH

Ivlev (1961), prey selectivity was estimated: Selectivity $= (r - p)/(r + p)$. The prey type in each fish (%) is given by r, whereas the prey types in the rearing medium (%) is given by p. The index ranges from −1.00 representing total abstinence to +1.00, indicating exclusive consumption.

Measurement of the lengths of the lower and upper jaw was made to ascertain the jaw gape dimension with an eyepiece micrometer and a dissecting microscope. Calculations were made using the following equations. Gape opening of $45^\circ = \sqrt{U^2 + L^2 - 2UL\cos 45}$ (1); gape opening of $90^\circ = \sqrt{U^2 + L^2}$ (2), where L is the lower jaw length and U is the upper jaw length (Qin and Fast 1997).

The mouth opening of *T. ovatus* larvae was measured individually, and the standard length range was found to be between 3.00 and 5.70 mm. An increase in mouth gape opening 45° and gape opening 90° of *T. ovatus* larvae was observed when the standard length increases (Fig. 2.3). The minimal and maximal mouth gape opening 45° were 205 μm and 302 μm, respectively, and minimal and maximal gape opening 90° were 377 μm and 557 μm, respectively. The corresponding standard

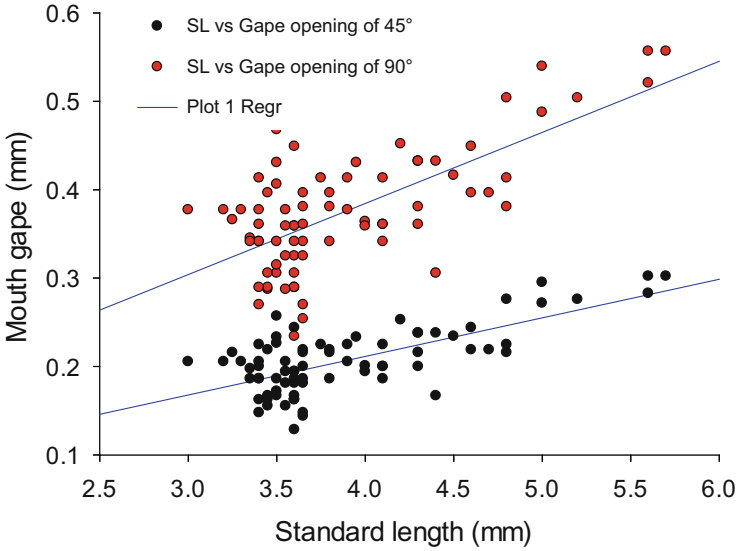

Fig. 2.3 Association between the standard length of fish larvae and the mouth gape. SL represents the standard length

length for the minimal mouth gape opening was 3.00 mm, and the corresponding standard length was 5.70 mm for maximal mouth gape opening.

When the larval *T. ovatus* were co-feeding copepod nauplii and rotifers, the rotifer density had a significant effect on prey selectivity ($P < 0.05$, in Fig. 2.4). When copepod nauplii were initially included in the tanks containing *T. ovatus* larvae on 5 DPH, the larvae selected rotifers instead of copepods nauplii in the 1 rotifer mL^{-1} treatment. However, copepod nauplii were selected in the 10, 20, and 40 rotifers mL^{-1} treatments. The trend was similar on the seventh day post-hatching. On the ninth day post-hatching, copepod nauplii turned out to be the prey of choice in the 10, 20, and 40 rotifers mL^{-1}, and *T. ovatus* selected copepods against rotifers in these treatments. However, larvae continued to select rotifers against copepods nauplii even on the ninth day post-hatching in 1 rotifer mL^{-1} treatment.

2.4 Effect of Tank Color, Rotifer Color on the Food Consumption of Larval *T. ovatus*

Five hundred fish were kept in 500-L tanks in triplicates and deprived of food for 10 h prior to the experiment. On 5 DPH, 20-L containers with white, black, red, pink, and green colored walls were used to stock 40 fish each. The dissolved oxygen was maintained near saturation in each bucket using an air-stone. The temperature of the water was 28.5 °C. Light intensity was kept at 2400 lux. The experimental buckets

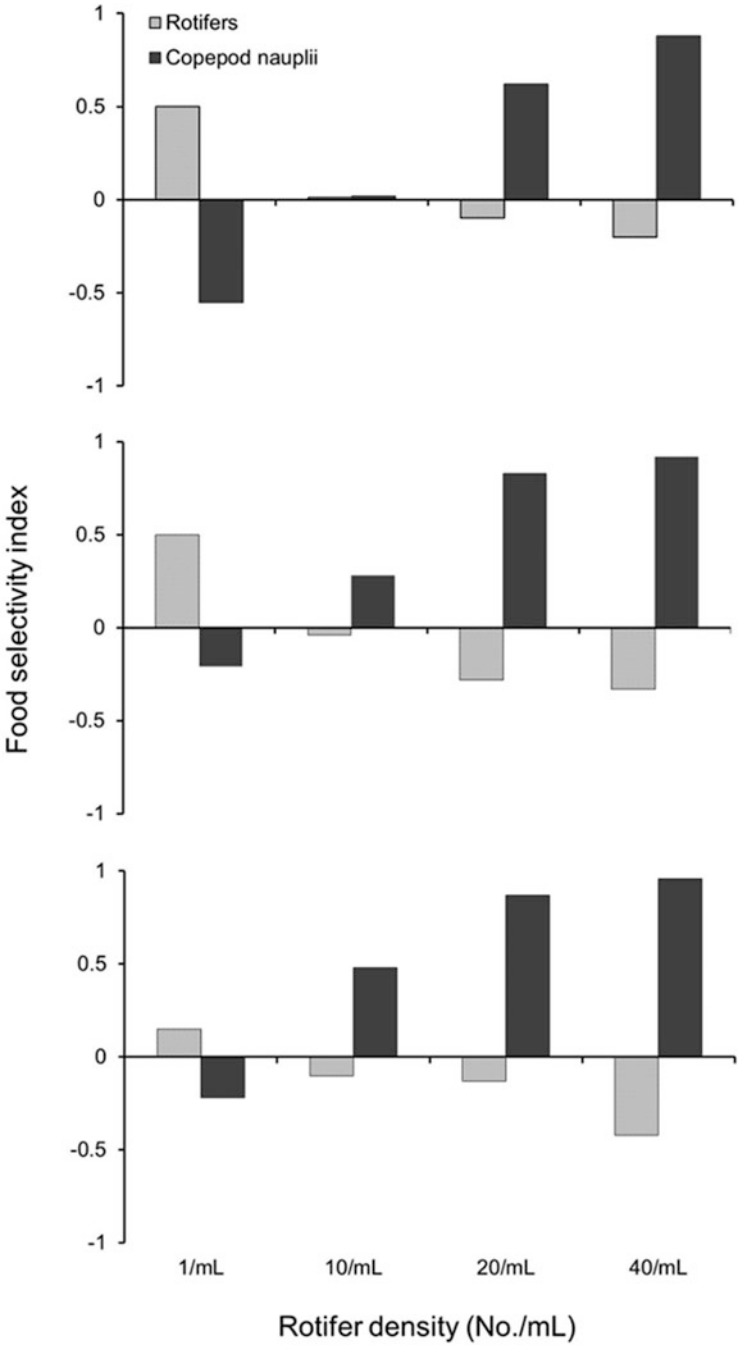

Fig. 2.4 *Trachinotus ovatus* larvae food selectivity indices

were filled with DHA Protein Selco-enriched rotifers (INVE Aquaculture) at a density of 10 rotifers mL^{-1}. Feeding time of 1 h was given to fish larvae. Afterward, ten larval fish were collected at random from every container. Fish larvae were subjected to anesthesia using 0.5% AQUI-S (AQUI-S New Zealand Ltd.). Larval fish were collected and stored into buffered neutral formalin (10%) once they were settled at the bottom of the sampling containers. The amount of ingested food was quantified using these fish larvae.

A total of 240 larval fish were stocked into 12 20-L containers with black walls on the fifth day post-hatching for the feeding trial of prey color. Prey colors were in green, blue, purple, and red in triplicate. Dissolved oxygen was maintained using an air-stone in every container. The water temperature was maintained at 28.5 °C, and 2400 lux was set as the value of light density. Soft Gel Paste™ Food Color (green, blue, purple, and red AmeriColor, Placentia, CA, USA) was fed to rotifers for 12 h before being introduced into the fish container. The trial period lasted 1 h to allow an adequate time of feeding. Afterward, ten *T. ovatus* larvae were randomly collected from each tank. Fish larvae were subjected to anesthesia using 0.5% AQUI-S (AQUI-S New Zealand Ltd), and then fish samples were stored in 10% neutral buffered formalin after allowing them to settle at the bottom of sampling vials. The quantity of food ingested was then estimated using these fish larvae.

The tank colors significantly influenced the food ingestion of larval *T. ovatus* ($P < 0.05$, Fig. 2.5). The highest value for food consumption occurred in the black tanks and dark red tanks, while the white tanks gave the lowest food ingestion. The difference in food consumption by larval *T. ovatus* between black, green, and dark red tanks or between white and pink tanks was not significant ($P > 0.05$, Fig. 2.5).

Rotifer colors also impacted food consumption of fish larvae. The green rotifers consumed by *T. ovatus* larvae were significantly higher than those of the purple, red, and blue rotifers ($P > 0.05$, Fig. 2.5). No notable differences were observed among purple, red, and blue rotifers ($P < 0.05$).

Prey density is another critical factor making the first feeding successful in marine larval fish (Ma et al. 2013b). There is a rise in foraging success with the rise in prey density (Houde and Schekter 1980; Laurence 1974; Munk and Kiorboe 1985) until the density reaches an asymptote level (Klumpp and Von Westernhagen 1986; Houde and Schekter 1980). A number of aspects that relate closely to the first feeding success in *T. ovatus* larvae have been investigated in the current study. A comprehensive understanding of the relationship between feeding performance and the growth of fish can essentially identify the factors governing larval fish survival.

With respect to the relationship between food density and food intake (Shaw et al. 2006; Gotceitas et al. 1996; Ma et al. 2013b) as well as fish ontogeny, different species vary to a great extent (Dou et al. 2000; Ma et al. 2013b; Parra and Yufera 2000). In the current work, food consumption of larval fish was notably influenced by the density of rotifers in the initial feeding duration. More rotifers were consumed by larval fish under 10 and 20 rotifers mL^{-1} compared to those in 1 and 40 rotifers mL^{-1} on the fifth and ninth days post-hatching. Earlier investigations have suggested that prey density has the potential to limit feeding rates of fish as a consequence of an increase in searching time (Houde and Schekter 1980). Thus

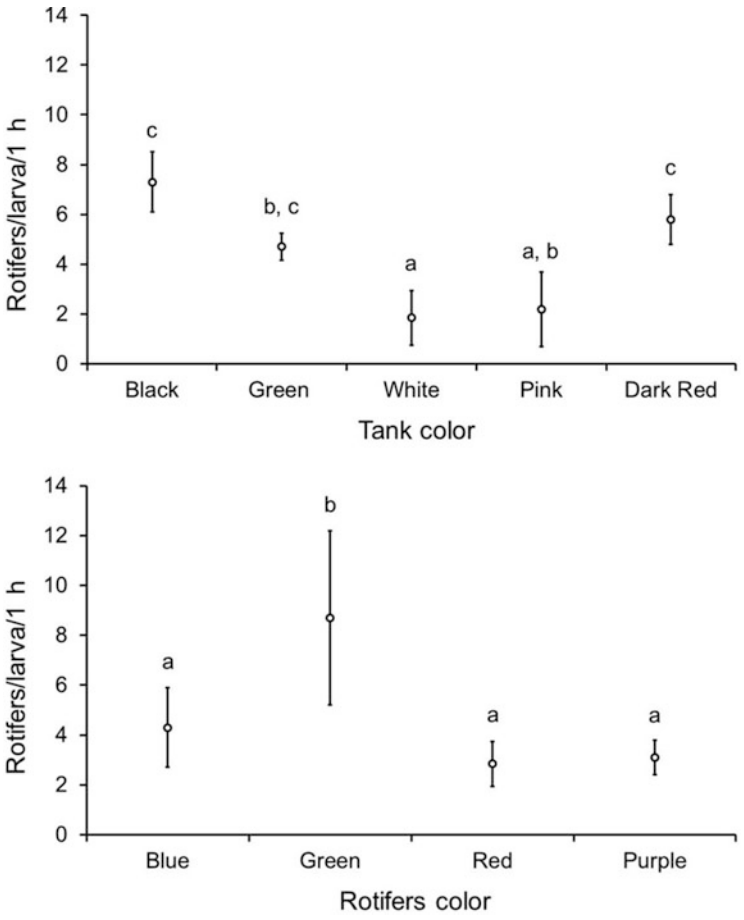

Fig. 2.5 Effects of rotifer color and tank colors on the food ingestion of fish larvae on 5 DPH

one can easily explain why fish larvae consumed fewer rotifers in the 1 rotifer mL^{-1} treatment than in the 10 and 20 rotifers mL^{-1}. The growth and survival of fish fed 1 rotifer mL^{-1} in the present study were lower than those in 10 and 20 rotifers mL^{-1}. Such results indicate that the density of 1 rotifer mL^{-1} has a negative impact on larval *T. ovatus* during ontogenetic development. This outcomeis supported by other studies that suggest that fish at a low prey density will spend more energy in swimming and searching for prey (Ruzicka and Gallager 2006), subsequently resulting in low survival and slow growth (Slembrouck et al. 2009).

A good volume of research suggests that the survival and growth of larval fish increase with the rise in prey density (Chigbu et al. 2002; Ma et al. 2013b; Wellington et al. 2010) until the prey density becomes exceedingly high and deteriorate water quality (Puvanendran and Brown 1999). In the current study, the performance of larval fish in 40 rotifers mL^{-1} exhibited a considerable impact on

prey density. Food ingestion, growth, and survival were inhibited in the 40 rotifers mL^{-1} treatment, suggesting that the feeding density has exceeded the limit of maximal prey feeding density. The results are similar to *Gadus morhua* (cod) larvae when the prey density was increased (Puvanendran and Brown 1999). The release of metabolites from the prey alters water quality and causes a reduction in larval survival and growth (Houde 1975). Therefore, an excessively high prey density could lead to a decline in the larval ability to capture prey (Laurel et al. 2001).

Prey selectivity is a vital factor for the optimal supply of live feed to fish larvae since fish change food preference as they grow (Ma et al. 2013b; Shaw et al. 2006). Previous research has shown that fish tend to adjust feeding patterns to fulfil their change of energy requirements (Bromley and Adkins 1984; Boujard and Medale 1994; Grove et al. 1978). In the current investigation, when fish reached 3.5 mm length, the mouth opening of fish larvae (gape opening 45° and 90°) became ready to capture prey of a larger size. The outcomes of the food selectivity test in this investigation suggest that on 5 DPH the fish in the 10, 20, and 40 rotifers mL^{-1} treatments began to adjust their feeding on larger copepod nauplii whereas fish fed 1 rotifer mL^{-1} kept selecting against copepods. In the light of optimal foraging theory, fish are faced with the choice of selecting prey only when excess food is available in the environment (O'Brien et al. 1976; Werner and Hall 1974; Krebs 1978). If fish are exposed to a low prey density environment, food choice of larval fish could be altered in during the period of co-feeding (Ma et al. 2013b). In this investigation, the increase in prey density from 10 to 40 rotifers mL^{-1} influenced food selection. The larval fish in the treatments with 10, 20, and 40 rotifers mL^{-1} could eat copepods after a 5-day co-feeding period. Even then, the batch of larval fish fed one rotifer mL^{-1} did not select copepod nauplii. Hence, it suggests that with the increase in fish growth, the larval fish began to select larger prey. However, the time of switching to larger prey may be affected by prey density.

Estimation of food supply and consumption is highly necessary for developing an optimal feeding scheme in fish larvae rearing (Ma et al. 2013b). Until now, the processes of food intake of larval fish including *Solea senegalensis* (sole) (Pedro Cañavate et al. 2006), flounders (*Pleuronectes ferruginea*, *Paralichthys dentatus*, *Rhombosolea tapirina*) (Shaw et al. 2006; Getchis and Bengtson 2006; Rabe and Brown 2000), *Seriola lalandi* (yellowtail kingfish) (Ma et al. 2013b), and *Cynoscion nebulosus* (spotted seatrout) (Wuenschel and Werner 2004) have been rigorously estimated. However, relatively little information is available regarding food intake of *T. ovatus*. This work focused on quantifying the food consumption of *T. ovatus* using different feeding schemes. Within the density range employed in the current work, the food consumption of *T. ovatus* larvae was independent of the density of rotifers. These findings corroborate with the results of early reports by Letcher and Bengtson (1993) and Ma et al. (2013b) that food consumption of silverside fish *Menidia beryllina* and larval yellowtail kingfish is not significantly affected by prey density.

Although many techniques have been employed to reduce the usage of live food by switching to micro-particulate diets, fish in marine hatcheries are still hindered by the high production cost of live feed (Holt et al. 2011; Callan et al. 2003). Hence an

understanding of the food utilization efficiency (FUE) is of utmost value when attempting to optimize production efficiency. Larval fish food intake requires quantification in order to increase FUE (Shaw et al. 2006; Dou et al. 2000). But information on the ratio of consumed food relative to the total supply in the tank is rather insufficient. The FUE of larval yellowtail kingfish decreases with the increase in live feed density according to a recent study by Ma et al. 2013b. In the current work, the FUE of rotifer reduced from 65% in the 40 rotifers mL^{-1} to 10% in the 1 rotifer mL^{-1} treatment. Base on the survival, growth, and FUE of larval *T. ovatus*, we suggest that the rotifer density should be kept at 10–20 rotifers mL^{-1} in the tank between 2 and 9 DPH.

In larval fish rearing, there is an interaction of ambient light with the wall color of the tank, thus affecting the efficiency of prey capture along with fish ontogeny. Generally, fish growth and survival are high in containers having dark walls (Chesney 2005) in comparison to those having light-colored walls. The exact preference for color is species-specific. Species like *Chanos chanos, Morone saxatilis*, and *Morone chrysops* prefer black walls (Martin-Tichaud and Peterson 1997; Denson and Smith 1996; Duray 1995), and tanned color is preferred by *Epinephelus suillus* (Duray et al. 1996), whereas light green color is preferred by *Colossoma macropomum* (Pedreira and Sipauba-Taveres 2001). The current study shows that more rotifers were consumed by fish in the dark red and black tanks than those in light-colored tanks, suggesting that *T. ovatus* larvae prefer dark walls during first feeding. In addition, food ingestion of larval fish is also influenced by prey color. Ma and Qin (2012) found that brown rotifers increased the rate of feeding of yellowtail kingfish. This work also shows that compared to the blue, purple, and red treatments, the number of rotifers consumed by larval fish fed green rotifers was considerably high, indicating that the first feeding *T. ovatus* larvae find green food particles more attractive than food particles in other colors.

2.5 Conclusion

The regulation of density of rotifers during the initial feeding period of larval fish can result in improved survival and growth. When the density of rotifers is 10–20 mL^{-1}, the growth and survival of larval fish have no significantly affection. Although fish grew slightly slower at 1 and 40 rotifers mL^{-1} than at 10 and 20 rotifers mL^{-1}, a relatively high rate for the survival of fish was obtained after feeding fish larvae with 10 or 20 rotifers mL^{-1}. The density of 1 rotifer mL^{-1} did not only reduce food intake initially, but it delayed the timing of diet switch from rotifers to copepod nauplii. In the early feeding stage, *T. ovatus* larvae consumed a large number of rotifers in dark tanks especially when the prey color was green. Considering the influence of live food ingestion, food utilization efficiency, food selection, prey color, fish growth, and survival during the rotifer feeding stage, we recommend that larval *T. ovatus* be reared at a feeding density of 10–20 rotifers mL^{-1} in dark tanks.

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Chapter 3
Weaning Regimes for Golden Pompano *Trachinotus ovatus* Larvae

Wei Fang, Zhenhua Ma, and Jian Guang Qin

Abstract This chapter covers the digestive ontogeny of *T. ovatus* from hatch to 32-day post-hatch (DPH). The development of the digestive system in *T. ovatus* can be divided into three stages: stage I starting from hatching and ending at the onset of exogenous feeding (3 DPH), stage II starting from first feeding and ending at the formation of gastric glands in the fish stomach (15 DPH), and stage III starting from the appearance of gastric glands and continuing onward. The specific activities of lipase, trypsin, and amylase in fish increased sharply from the exogenous feeding to 5–7 DPH. The pepsin activity was detected on 15 DPH, and the specific activities increased with fish age. The dynamics of enzyme activity reflected the structural development in the fish digestive system. After the formation of gastric glands in the stomach, the enzyme activities became stable. Depending on the development of the digestive system, the larvae of *T. ovatus* can begin weaning at 15 DPH. This chapter updates the improved understanding of the ontogeny of *T. ovatus* during the larval phase and provides the protocol of feeding and weaning for this economically important fish in aquaculture.

Keywords Ontogenetic development · Digestive system · Enzyme activity · *Trachinotus ovatus*

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_3

3.1 Introduction

Upon hatching, the digestive system of marine fish larvae is immature and undergoes major changes before fully having the function of food digestion (Cahu and Zambonino-Infante 2001; Hu et al. 2018; Ma et al. 2012). In spite of advances in larval fish culturing technology, most of marine fish hatcheries still depend on live feed such as rotifers *Brachionus* spp. and *Artemia* sp. as feed for early larvae of fish (Hamlin and Kling 2001; Sorgeloos et al. 2001; Hu et al. 2018). In practice, live feed supply is usually required beyond metamorphosis until larvae are weaned onto formulated diet (Alves et al. 2006; Ma et al. 2014; Cui et al. 2017). Nevertheless, the long-term use of live feed is costly and may lead to malnutrition due to unbalanced nutrition, and live feed does not meet the nutritional requirement for larval fish (Le Ruyet et al. 1993; Baskerville-Bridges and Kling 2000; Callan et al. 2003). Thus, the weaning of fish larvae at their early stage is essential.

Weaning with artificial feed instead of live feed is a gradual process during the larval stage. In aquaculture practice, most temperate marine fish, such as *Lutjanus erythopterus* and *Seriola lalandi*, usually begin weaning after metamorphosis (Cui et al. 2017; Ma et al. 2014). Marine fish can make weaning easier to manage and more effective if compound diet is introduced early (Baskerville-Bridges and Kling 2000; Hart and Purser 1996). It is likely to have a negative impact on the growth and survival of fish if artificial feed is introduced too early because larvae of fish have no ability to digest artificial feed (Andrade et al. 2012; Cahu and Zambonino-Infante 2001; Ma et al. 2014). The co-feeding protocol for fish larvae with both live and artificial feed has been developed in finfish hatchery to improve poor digestion of artificial feed at the early stage of development. The proportion of live feed is gradually reduced during the weaning stage, and the co-feeding strategy allows the fish larvae to receive artificial feed earlier in terms of nutrition (Rosenlund et al. 1997; Engrola et al. 2009b). The growth, survival, and the quality of marine fish species can be significantly improved by co-feeding such as *Sciaenops ocellatus* (Lazo et al. 2000), *Rhombosolea tapirina* (Hart and Purser 1996), and *Solea senegalensis* (Engrola et al. 2009a).

The response of fish to nutrient supply can be assessed by RNA/DNA ratio that has been used as an indicator for somatic tissue growth (Bailey et al. 1995; Buckley et al. 1999; Gwak et al. 2003). Since the amount of DNA per cell is almost constant, cross-species fish growth can be measured by the RNA/DNA ratio as a consistent measure (Pilar Olivar et al. 2009; Gwak and Tanaka 2001), whereas the quantity of RNA reflects the amount of protein synthesis in cells (Höök et al. 2008; Tanaka et al. 2008). Because the nutritional condition is related to feeding success and food supply (Tanaka et al. 2008), fish with an adequate nutrition supply may have a higher RNA/DNA ratio than malnourished fish (Boyd and Tucker 1992; Gronkjer et al. 1997).

Although fish can consume artificial diets in their early period of development, this does not guarantee the success of the artificial diet digestion and absorption, because the gut of fish may be filled with artificial diet and death (Cahu and

Zambonino-Infante 2001). Therefore, introduction time of artificial diet of fish larvae should be determined according to the development of digestive system (Cahu and Zambonino-Infante 2001). In some studies, the histological structure degrades when fish larvae are malnourished (Yufera et al. 1993; Chen et al. 2007). Midgut cells' height is a histological indicator of fish nutrient supply as evidenced by the early development of *Seriola lalandi* (Chen et al. 2007) and *Theragra chalcogramma* (Theilacker and Watanabe 1989).

The *Trachinotus ovatus* belongs to the Carangidae family and is extensive cultured in the Asia-Pacific region. We examined the development of the digestive system in previous studies; in particular, attention is paid to pepsin secretion and the appearance of gastric glands in the stomach (Ma et al. 2013). In this chapter, we discuss the suitable time of weaning for *T. ovatus*, aiming to improve fish survival and establish a cost-effective feed regime for the fish that are commercially important.

3.2 Weaning Scheme Design

On 12 DPH, the fish larvae were randomly divided into 12, 300-L experimental tanks (20 fish L^{-1}) for early weaning trial, which began on 13 DPH. The daily water exchange rate was 300% volume per day. Put an air stone in each tank to keep adequate dissolved oxygen and uniform the distribution of microalgae, rotifers, and *Artemia* nauplii. The weaning stage lasted for 10 days, including 5 days of co-feeding and 5 days of live feed combined with the introduction of artificial feed. Only feed the fish larvae with artificial diet after completion of weaning (Fig. 3.1).

Weaning treatments were composed of the same feeding protocol but began at four start time schemes after hatch: (1) start at 13 DPH and end at 22 DPH (W13), (2) start at 16 DPH and end at 25 DPH (W16), (3) start at 19 DPH and end at 28 DPH (W19), and (4) start at 22 DPH and end at 31 DPH (W22 as control); each group had

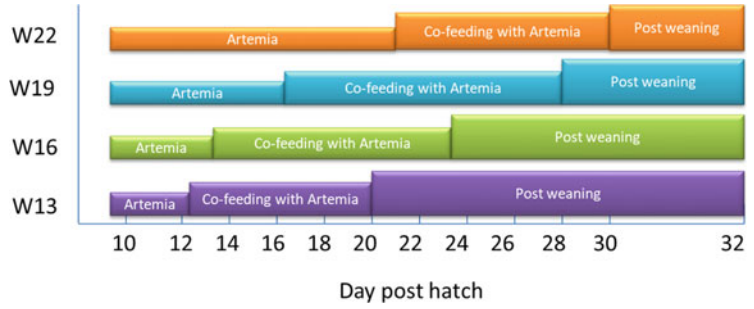

Fig. 3.1 Four co-feeding and weaning treatments to wean pompano *T. ovatus* larvae from 13 DPH to 32 DPH with separate commencing time by 3 days. W13: 13–22 DPH, W16: 16–25 DPH, W19: 19–26 DPH, and W22: 22–33 DPH (Ma et al. 2015)

three replicates. Each group has weaning interval for 3 days (i.e., 13, 16, 19, and 22 DPH, Fig. 3.1).

3.3 Growth, Survival, and Variation of RNA/DNA Ratios

The standard length (SL) of *T. ovatus* larvae on 1 DPH was 3.38 ± 0.17 mm (mean ± SD). When the weaning experiment was 13 DPH, the average SL of fish was 5.14 ± 0.44 mm. In the end, fish of W22 and W19 treatments increased significantly compared to fish in the W13 and W16 treatments ($P < 0.05$).

The specific growth rates of fish larvae of W13 and W16 treatments decreased significantly compared to fish in the W19 and W22 treatments, which were 7.32% day^{-1} and 7.82% day^{-1}, respectively ($P < 0.05$, Fig. 3.2). No significant difference of final survival rate of fish was found in W22 and W19 treatments, which were 85.17% and 86.5%, respectively ($P > 0.05$). No significant differences of final rates of fish were found in W13 and W16 treatments ($P > 0.05$), but it significantly decreased compared to fish in the W19 and W22 treatments ($P < 0.05$, Fig. 3.2).

Weaning time can significantly affect the growth of marine fish larvae (Curnow et al. 2006; Engrola et al. 2007). Inappropriate weaning time may lead to starvation of fish because of poor food digestion and absorption (Hu et al. 2018; Ma et al. 2012, 2014). There is no adequate nutrient supply during weaning period, and fish will use the energy stored in the body to maintain basic metabolism and to distribute less energy to growth, causing slow growth during the weaning period as reported in sand bass *Paralabrax maculatofasciatus* (Civera-Cerecedo et al. 2008) and *Senegalese sole* (Engrola et al. 2007). In *T. ovatus*, weaning time significantly affects the growth of fish larvae. The specific growth rates of fish of W19 and W22 groups were significantly higher than those of W13 and W16 groups. As we suggested in a previous study, *T. ovatus* is a fish with rapid development (Ma et al. 2013), and a functional digestive system appears at about 15 DPH. Higher fish growth rates were

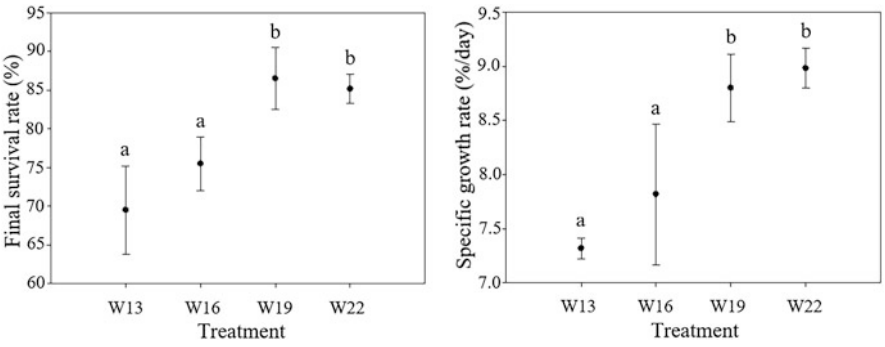

Fig. 3.2 Different weaning times, specific growth rate, and survival rate of *T. ovatus*. Different letters represent significant difference ($P < 0.05$). Abbreviations refer to Fig. 3.1 (Ma et al. 2015)

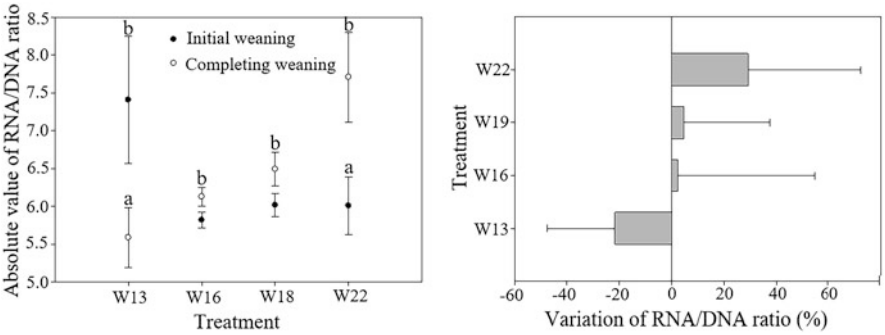

Fig. 3.3 The initial and final RNA/DNA ratios and variation of RNA/DNA ratios (VRD) in *T. ovatus* larvae during the weaning period. Different letters represent significant difference ($P < 0.05$). Abbreviations refer to Fig. 3.1 (Ma et al. 2015)

found in the W19 and W22 groups and may be associated with the function of the digestive system development.

During the ontogenetic development, the time of weaning regulated the RNA/DNA ratio ($P < 0.05$, Fig. 3.3). RNA/DNA ratio of fish of the W13 group at the end of weaning revealed a downregulating trend, and the ratio decreased from 7.4 to 5.6. A significant increase of the RNA/DNA ratios of fish after weaning was found in W16, W19, and W22 groups ($P < 0.05$, Fig. 3.3), and there was no significant difference in RNA/DNA ratios among these groups ($P > 0.05$, Fig. 3.3) though the mean variation of RNA/DNA ratios increased with the starting time of co-feeding and weaning (Ma et al. 2015).

The RNA/DNA ratios are used to understand growth pattern of many fish species during ontogenetic development (Pepin et al. 1999; Gwak et al. 2003; Höök et al. 2008). The RNA/DNA ratio of larval fish can be used to evaluate the fish nutritional condition because it is associated with food availability (Esteves et al. 2000; Diaz et al. 2011). The RNA content of larval fish such as turbot *Scophthalmus maximus* and herring *Clupea harengus* decreased when first feeding was not properly conducted (Clemmesen 1987). Previous studies have confirmed that the RNA/DNA ratio can be used to assess diet adequacy (Ben Khemis et al. 2000; Mendoza et al. 2008). In *T. ovatus*, weaning time can influence the change of the RNA/DNA ratio. When weaning was completed, the RNA/DNA ratio of fish of the W13 group indicated a reducing trend, which may suggest that weaning time has a negative impact on nutritional condition and growth of fish.

However, the positive variation of RNA/DNA ratios of fish was found in W16, W19, and W22 groups, which may indicate that fish adapt for artificial diet during weaning.

3.4 Height of Midgut Epithelial Cells

The epithelial cell height in the midgut of fish larvae is an excellent histological indicator to assess their nutritional condition (Gwak et al. 1999) because the form of enterocyte cells in the fish intestine can be changed by starvation (Domeneghini et al. 2002). The histological changes of fish larvae starvation vary with fish species and duration (Theilacker and Porter 1995; Gisbert et al. 2004; Ma et al. 2012). In larval yellowtail kingfish *Seriola lalandi*, the response of the midgut epithelium height to starvation occurred only before 33 DPH, and subsequently, there was no significant difference between normal fed and starved fish (Chen et al. 2007). In *T. ovatus*, the starting time of weaning significantly affected the intestinal epithelium, especially in the W13 treatment (Fig. 3.4). The height of epithelial cells of midgut of *T. ovatus* was decreased compared to the fish in the control group, from 15 DPH (W22), which is similar to the reports of Hamza et al. (2007) and Ostaszewska et al. (2005) where artificial diet is used to feed fish larvae. Starvation may cause a decrease of epithelial cell heights owing to incapability to digest artificial feed during the period of early weaning. However, the use of artificial diet results in low cell height because of intestinal epithelium damage (Hamza et al. 2007).

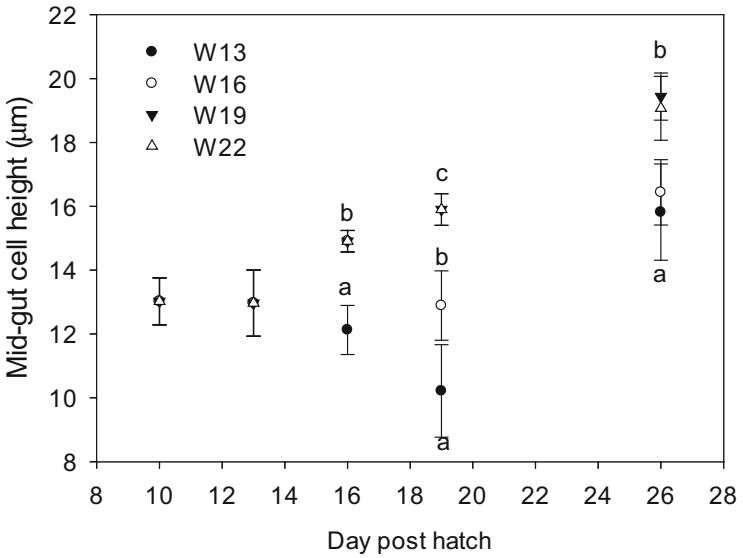

Fig. 3.4 The change of midgut cell heights of *T. ovatus* larvae in different weaning groups. Different letters represent significant difference ($P < 0.05$). Abbreviations refer to Fig. 3.1 (Ma et al. 2015)

3.5 Survival and Jaw Malformation

The co-feeding of live feed and artificial diet has been suggested as an effective program to improve the survival of larvae fish during weaning (Engrola et al. 2009a; Nhu et al. 2010; Clay et al. 2011). For example, whether artificial diet or *Artemia*, the alligator *Atractosteus spatula*'s survival rate is about 60% (Mendoza et al. 2008). However, the alligator's survival reached 95% when the alligator larvae were co-fed with 20% *Artemia* and 80% of artificial diet (Mendoza et al. 2002). In *T. ovatus*, co-feeding and weaning of fish larvae were successfully conducted. The survival rate of fish of W19 and W22 groups was higher (>85%) at the end of the experiment.

Nutrition is a significant factor affecting skeleton deformity (Cahu et al. 2003; Cobcroft et al. 2004; Sandel et al. 2010). During the period of co-feeding and early weaning, any inappropriate feeding protocol may lead to malnutrition of fish larvae.

The co-feeding time and early weaning time of marine fish larvae can affect the quality of fish such as skeletal malformation (Baskerville-Bridges and Kling 2000; Hamlin and Kling 2001). For example, compared with longer co-feeding and weaning time, shorter co-feeding and the time of weaning can improve spinal malformation incidence in southern flounder *Paralichthys lethostigma* (Faulk and Holt 2009). Similarly, during the weaning of pikeperch *Sander lucioperca*, a high malformation rate was found in the earlier weaning treatment (Kestemont et al. 2007). In this study, weaning time had no significant effects on jaw malformation ($P > 0.05$, Fig. 3.5). The jaw malformation rates were 15.54%, 11.78%, 14.00%, and 11.54% in the W13, W16, W19, and W22 treatments, respectively. The time of

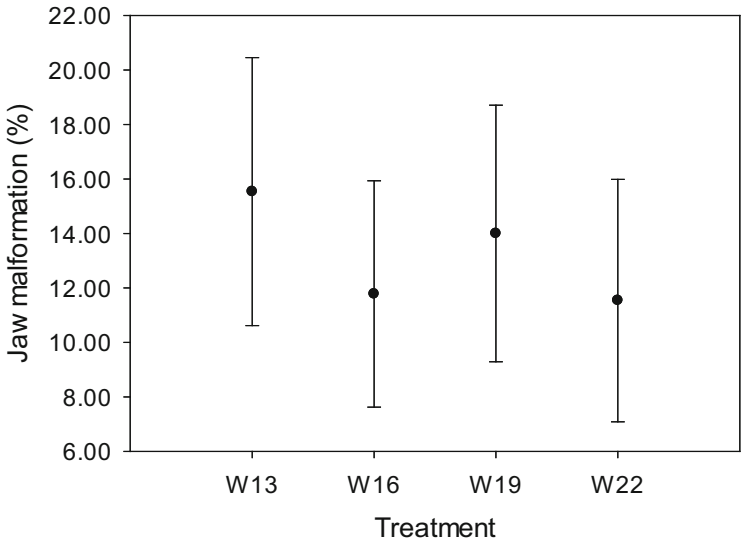

Fig. 3.5 The jaw malformation of *T. ovatus* larvae of different weaning groups. Different letters represent significant difference ($P < 0.05$). Abbreviations refer to Fig. 3.1

co-feeding and weaning cannot significantly affect the jaw malformation of larval *T. ovatus*. The existing evidence indicates that any of the current co-feeding and weaning regimes in this study can supply adequate nutrient to *T. ovatus* larvae during their development.

3.6 Conclusion

The *T. ovatus* larvae can be weaned from live feed to artificial diet after 16 DPH without affecting the fish growth and survival or increasing jaw deformity. However, the introduction of artificial diet before 16 DPH may affect the fish growth, survival, and nutritional condition. Although some *T. ovatus* larvae could be weaned on 13 DPH, we suggested the optimal time of weaning for *T. ovatus* larvae should be from 16 to 22 DPH.

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Chapter 4
Skeleton Development and Malformation of Hatchery-Reared Golden Pompano *Trachinotus ovatus*

Tao Zhang, Zhenhua Ma, and Ming Luo

Abstract Skeletal anomalies in farmed fish affect animal welfare and economic values in aquaculture, but little is known on skeletal ontogeny and anomalies in species of the Carangidae family. In this chapter, the skeleton development and malformation of golden pompano *Trachinotus ovatus* are discussed. Both caudal complex and vertebral column development in golden pompano started around 7–9 DPH, accompanied by the calcification of hemal arches, neural arches, and the occurrence of caudal elements. Uroneural is the last element of caudal complex which formed on 18 DPH. The fish showed at least one type of malformation over 33% under the artificial rearing condition.

The severe jaw deformities in fish were clearly observed on 3 DPH. Subsequently, the total incidence of jaw deformity rate was 9.6–46.6%, including intermediate and severe jaw deformity. The rate of jaw deformity of fish was the highest on 11 DPH (46.6%). The intermediate jaw deformity rate reached the peak value (36.4%) on 26 DPH. This chapter provides basal information as a reference of the malformation and skeletal ontogeny of golden pompano. The results of this study can be used to control the production quality of golden pompano.

Keywords Skeleton development · Malformation · *Trachinotus ovatus*

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_4

4.1 Introduction

In marine finfish aquaculture industry, skeleton deformities associated with low survival and slow growth are the major bottleneck in the production of fingerlings (Sfakianakis et al. 2004; Ma and Qin 2014; Cheng et al. 2018). In practice, rearing of deformed fish is meaningless as the market value of these fish is low (Ma et al. 2016). To meet the demand of the fish industry, skeleton malformations have been investigated on several commercial species in the past decades. Up to present, commercially cultured species have been reported with the occurrence of skeletal malformation including Senegal sole (Gavaia et al. 2002), gilthead seabream (Prestinicola et al. 2013), red seabream (Miioru et al. 2002), European sea bass (Boglione et al. 1993), yellowtail kingfish (Cobcroft et al. 2004), and crimson snapper (Cheng et al. 2018). In order to understand the causes of skeletal malformation in marine finfish, research on the general development of skeletal structure is required to reduce the appearance of malformation in fish during ontogenetic development. Furthermore, comprehensive knowledge on fish osteology will provide a basic understanding of functional development in fish larvae, improve fish rearing protocols, detect skeletal deformities, and identify the potential factors regulating skeletal malformations (Koumoundouros and Sfakianakis 2001; Blaxter 1988; Fukuhara 1988; Haga et al. 2011; Ma et al. 2014a).

The golden pompano *Trachinotus ovatus* belongs to the family of Carangidae and is widely cultured in Asia-Pacific regions. In 2020, China has an estimated 100,000 metric tons of commercial production of golden pompano. Up to present, the life cycle of golden pompano has been closed and successfully explored several key aspects about larval rearing (Ma et al. 2014b, 2015a, b). However, high deformities (>30%) and low survival rate (<13%) have seriously hindered the golden pompano's profits in the early stage (Ma et al. 2016). In this chapter, the skeletal ontogeny of golden pompano is reviewed, and then jaw malformation and osteological malformation are discussed. This chapter provides references on the golden pompano larvae's malformation of jaw and osteological, and the results can be available for quality control of fingerling production in this species.

4.2 Skeleton Development

During the yolk sac stage, golden pompano larvae have a straight spinal notochord running the entire length of the body. In this period, the notochord (Nc) was the only visible axial supporting structure, and no vertebral elements were found. The neural arch was the first visible elements of the vertebral, firstly appeared on 7 days post hatch (DPH, Fig. 4.1a). The development of the arch begins with the intramembranous ossification of two buds on either side of the notochord. When the two buds joined together, an arch was formed. Mineralization extended from the base of arches, resulting in the formation of the center surrounded by the notochord.

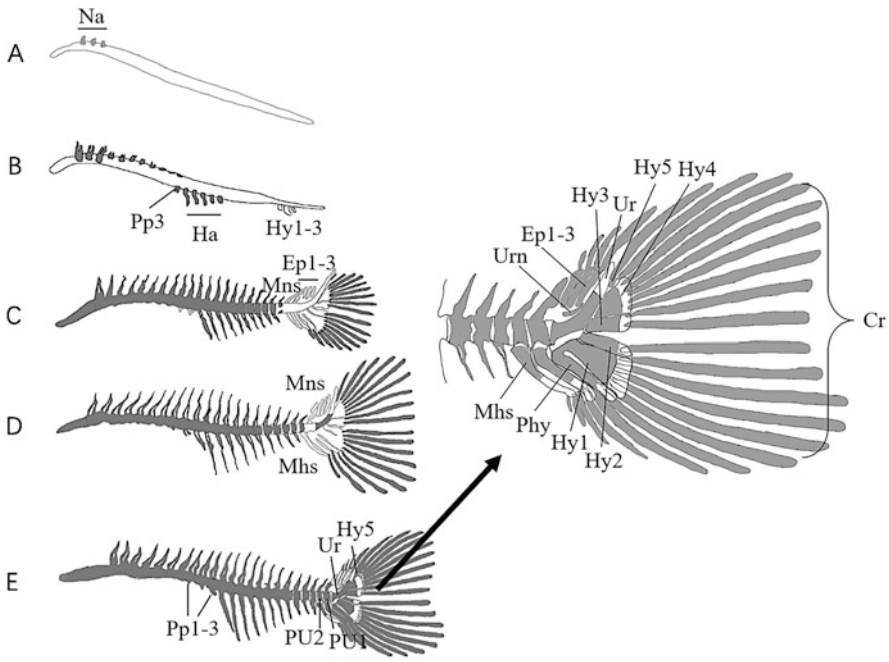

Fig. 4.1 The golden pompano's development of the caudal complex, caudal fin rays, and vertebral column. (**a**) 7 DPH, (**b**) 9 DPH, (**c**) 13 DPH, (**d**) 15 DPH, and (**e**) 18 DPH. Dark areas are the cartilage (except the notochord), and light areas are ossified skeletons. Abbreviations: *DPA* days post hatching, *Hy* hypural, *Mns* modified neural spine, *Phy* parhypural, *Ur* urostile, *Cr* caudal fin rays, *Ep* epural, *Mhs* modified hemal spine, *Nc* notochord, *PU* preural vertebrae (Zheng et al. 2016)

After 7 DPH, the vertebral ossification extended to the caudal fin. In this period where the first parapophysis (Pp3) is observed, at this stage, it is consistent with the spines of hemal in the abdomen of the vertebral column. More ossified hemal spines and neural spines were formed on 11 DPH. In addition, ossification occurred when the vertebral column extend from the anterior area to the posterior area (Fig. 4.1b).

Since 13 DPH, mineralization gradually approached to the posterior part of the vertebral column. At this stage, the ossified parapophyses 2–3 were seen on the abdominal side of the vertebral column (Fig. 4.1c). Urostyle started its bending upward. The vertebral trunk column happened for further mineralization. Most of the vertebral column was mineralized, but no affiliated elements associated with the unique plate were found at the urostyle end (Fig. 4.1d). The angle between the urostyle and the vertebral trunk was close to the last stage on 19 DPH (Fig. 4.1e).

In 18 DPH, the urostyle's metamorphosis and flexion were completed. All vertebral elements were formed and mineralized approximately here except for the tip of the vertebral trunk.

The *Trachinotus ovatus* caudal complex consists of a parhypural (Phy), five hypurals, two haemal spines (Hs), three epurals, and a neural spine (Ns).

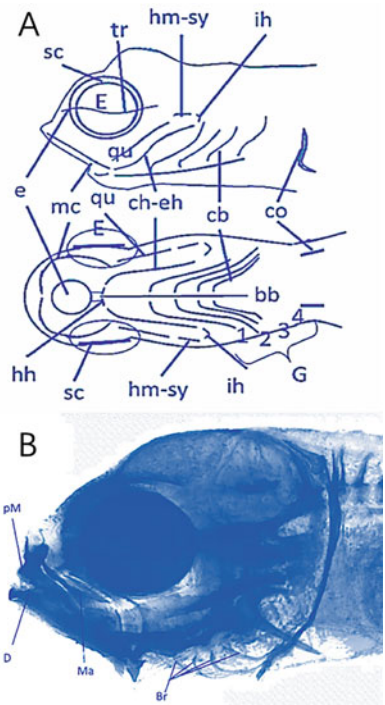

Fig. 4.2 (**a**) Normal larvae's skeletal structure at 5 DPH, diagrammatic of the cephalic cartilages. Abbreviations: *cb* ceratobranchial cartilage, *co* coracoid-scapula cartilage, *E* eye, *hh* hypohyal cartilage, *ih* interhyal cartilage, *nt* notochord, *sc* sclerotic cartilage, *bb* basibranchial cartilage, *ch-eh* ceratohyal-epihyal cartilage, *e* ethmoid plate, *G* gill arch, *hm-sy* hyomandibular–sympletic cartilage, *mc* Meckel's cartilage, *qu* quadrate cartilage, *tr* trabecula cartilage. (**b**) Golden pompano jaw's skeletal status at 11 DPH. Abbreviations: *Ma* maxilla, *Br* branchiostegal rays, *pM* premaxilla, *D* dentary (Ma et al. 2016)

Mineralization was not observed at the margin of the hypurals (Hy) and epurals, but was developed and mineralized at the caudal skeletons on 18 DPH (Ep, Fig. 4.1). An uroneural (Urn) was observed around the corner of upward urostile. The upward bent urostyle (Ur) and the first four (1–4) hypurals form a unique plate. Epurals 1–3 and hypural 5 remain separated from the urostyle. Instead, the modified neural spine (Mhs) and modified haemal spines (Mhs) significantly extend from the distal distinct cartilaginous ridges appear branch. There are a total of 25 caudal fin rays formed in the caudal area, and all of these rays are almost symmetrically attached to the unique plate. The neural spines and the epurals are articulated with four rays, hypurals 1–4 with 13 rays, the hypural 5 with two rays, the parhypural, and the haemal spines with 6 rays, presenting 13 rays on the upper side and 12 rays on the ventral side.

The golden pompano's first four gill arches developed on 5 DPH (Fig. 4.2a). The gill arch contained the basibranchial, ceratobranchial, and hypobranchial cartilages. At the anterior end of the hypobranchial cartilage, the hypohyal cartilage developed and was joined to a pair of ceratohyal-epihyal cartilage. The quadrate cartilage head was connected to the Meckel's cartilage and the hyomandibular-sympletic cartilage, which is connected with the interhyal cartilage (Fig. 4.2a). The ethmoid cartilage and trabeculae cartilage formed as neurocranium's structures during this period. It's easy to spot from the side of the fish. The trabecular cartilages, which connect to the posterior end of the ethmoid cartilages, extended to the midcourt line of the two eyes.

In this stage the coracoid-scapula cartilage appeared. The ossification process of jaw elements of fish was recorded around 7 DPH. At the 11 DPH, most of jaw element ossification such as premaxilla (pM) and maxilla (Ma) and dentary (D) was finalized on 11 DPH (Fig. 4.2b).

4.3 Jaw Malformation

The golden pompano's normal and malformed jaws are presented in Fig. 4.3. All larvae fish's jaws were normal on 1 DPH, but jaw deformities were observed on 3 DPH. Both intermediate and/or severe malformation can be observed since 3 DPH, with an overall incidence of jaw malformations ranging from 9.6% to 46.6%. Severe malformation of the peak appeared on 5 DPH at 3.26 mm standard length (SL). The

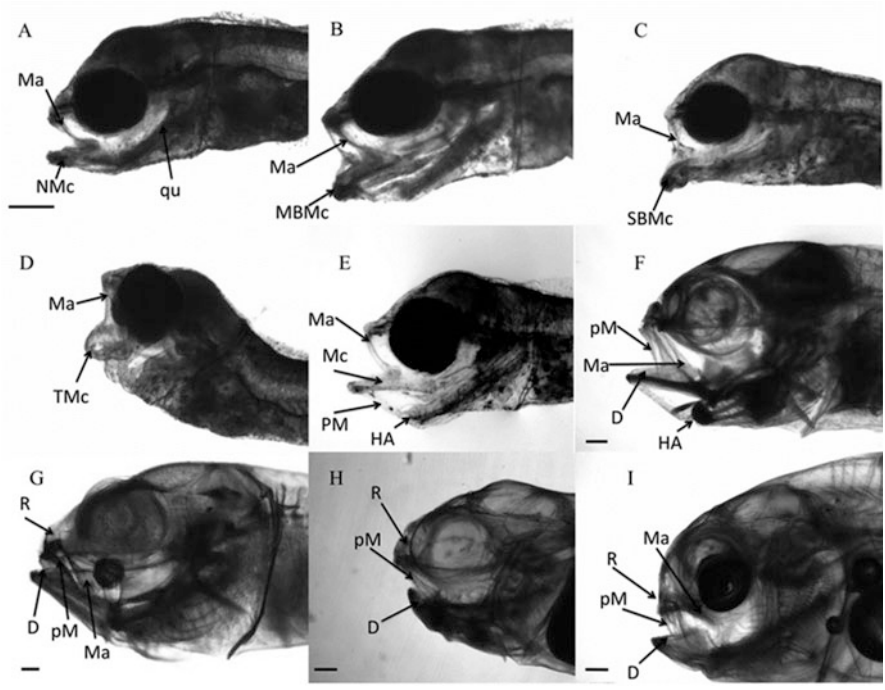

Fig. 4.3 Head view of different developmental stages of *Trachinotus ovatus* larvae (Ma et al. 2016). (**a**) Normal jaw structure (5 DPH). (**b**) Mediate bending on Meckel's cartilage (5 DPH). (**c**) Severe bending on Meckel's cartilage (5 DPH). (**d**) Twisted lower jaw (5 DPH). (**e**) Lowered hyoid arch (5 DPH). (**f**) Lowered hyoid arch (16 DPH). (**g**) Normal jaw structure (17 DPH). (**h**) Shorten lower jaw (22 DPH). (**i**) Pugheadness (29 DPH). Abbreviations: *NMc* normal Meckel's cartilage, *Nr* nasal region, *MBMc* mediate bending Meckel's cartilage, *SBMc* severe bending Meckel's cartilage, *TMc* twisted Meckel's cartilage, *HA* hypohyal arch, *pM* premaxilla, *PM* protractor muscle, *ma* maxilla, *R* rostral. Scale bars are 200 μm (**a**–**g**), 400 μm (**h**), and 625 μm (**i**)

peak value of severe malformation appeared on 5 DPH at 3.26 mm standard length (SL), and 25% larvae presented with serve jaw malformation. On 11 DPH (SL = 4.33 mm), the total proportion of jaw malformation peaked at 46.6%, while on 26 DPH (SL = 11.70 mm), the intermediate jaw malformation (where the main elements of the jaw were malformed, but the jaws could still perform basic functions) peaked at 36.4%.

One of the most common jaw malformations is curved Meckel's cartilage, which has been described in species such as Asian sea bass *Lates calcarifer* (Cobcroft and Battaglene 2013), crimson snapper *Lutjanus erythropterus* (Cheng et al. 2018), yellowtail kingfish *Seriola lalandi* larvae (Cobcroft et al. 2004), and Atlantic halibut *Hippoglossus hippoglossus* (Morrison and MacDonald 1995). In golden pompano, the curved Meckel's cartilage of jaw occurred on 3 DPH, which is similar to the timing of occurrence in yellowtail kingfish (Cobcroft et al. 2004) and crimson snapper (Cheng et al. 2018). There are two types of curved Meckel's cartilage malformation in golden pompano, moderate bent type and severe bent type. Both mediate bent and severe bent Meckel's cartilages can be found on 5 DPH. Fish with twisted Meckel's cartilage is normally accompanied with a serious delay of skeletal ontogeny. In Asian sea bass, on 18 DPH, the twisted Meckel's cartilage becomes obvious (Fraser and Nys 2005). The causes of this type malformation are due to the shortened twisted dentary. Meckel's cartilage, also is a unilateral jaw structure of a single side (Fraser and Nys 2005; Ma et al. 2016).

The timing of the onset of jaw malformation is species dependent. For example, the curved Meckel's cartilage can be detected when the first jaw malformation occurs in Meckel's cartilage of *Hippoglossus hippoglossus* around 19 DPH (Morrison and MacDonald 1995). In other species such as striped trumpeter *Latris lineata* larvae, on 44 DPH, jaw malformation is detected and characterized by a permanent obstacle of the closing mouth by an abnormal joint between the premaxilla and maxilla (Cobcroft et al. 2001). But in yellowtail kingfish *Seriola lalandi* (Cobcroft et al. 2004) and crimson snapper *Lutjanus erythropterus* (Cheng et al. 2018), the curved Meckel's cartilage is observed as early as 4 DPH.

In golden pompano, the jaw malformation occurred as early as 3 DPH. As fish larvae's jaw malformation is related to malnutrition in broodstocks (Cobcroft et al. 2004), larval golden pompano's early jaw malformation may be associated with brood fish nutrition; however, the relationship between jaw malformation and broodfish nutrition in fish warrants further investigation. Besides, tank wall color may also affect jaw malformation in striped trumpeter larvae (Cobcroft et al. 2012), but it is unclear whether the color of tank is associated with the jaw malformation of larval golden pompano. Fraser and Nys (2005) believe that the time variation of jaw deformity occurrence may be related to the differences of ontogenetic development. For instance, the deformity in the larval Japanese eel *Anguilla japonica* is detected upon the completion of the yolk sac (Kurokawa et al. 2008). Jaw malformation occurs after bone formation on 30 DPH (Cobcroft et al. 2001). In golden pompano, cartilage malformation was found on 3 DPH, and this suggests that the rapid development of golden pompano may lead to jaw malformation occurrence in their early life.

4.4 Osteological Malformation

The abnormal distribution of larval golden pompano is presented in Table 4.1 and Fig. 4.4. Percentage of golden pompano with normal development accounted for 21–62%, and the rest of fish showed various degrees of deformities. Around 22–44%

Table 4.1 Abnormalities were found in total deformed cases of larval golden pompano from 11 DPH to 31 DPH (% of total deformities, Zheng et al. 2016)

DPH	Atrophy	Malposition	Fusion	Scoliosis	Lordosis
11	50.00	0.00	0.00	0.00	50.00
14	0.00	50.00	0.00	0.00	50.00
16	6.25	31.25	6.25	25.00	31.25
19	20.00	0.00	40.00	0.00	40.00
22	18.75	0.00	62.50	0.00	18.75
26	22.22	0.00	77.78	0.00	0.00
29	21.05	0.00	52.63	0.00	26.32
31	21.62	10.81	48.65	0.00	18.92

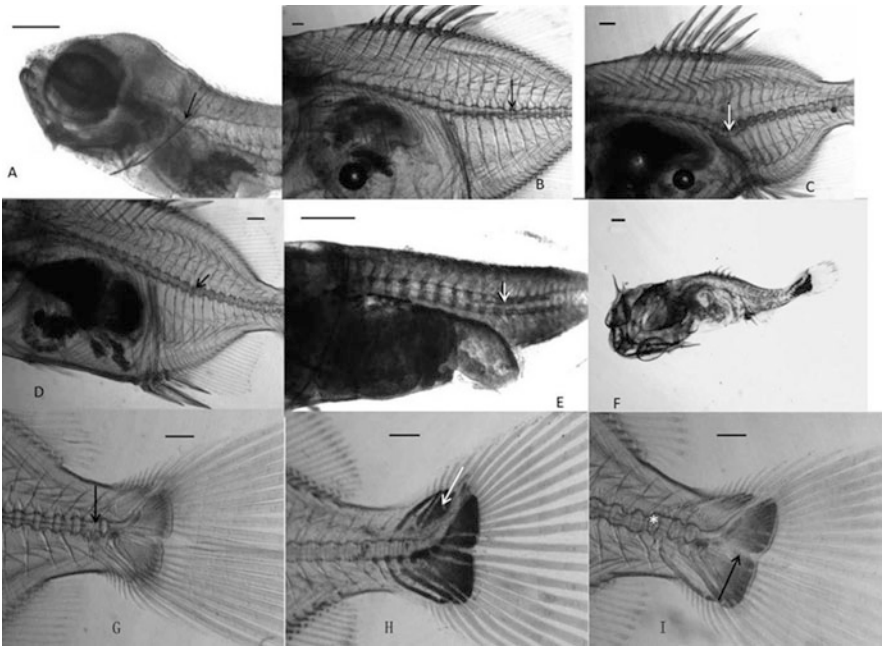

Fig. 4.4 Typical malformation found in different body parts of golden pompano (Zheng et al. 2016). (**a**) Notochord deformity on 3 DPH. (**b**) Malposition of vertebrae on 31 DPH. (**c**) Severe deformed vertebral column individual on 31 DPH (V-sharp). (**d**) Atrophy of vertebrae on 31 DPH. (**e**) Fusion between vertebrae on 11 DPH. (**f**) Scoliosis (16 DPH). (**g**) Preural vertebrae deformities. (**h**) Epural deformities. (**i**) Hypural deformities. Scales: 200 μm

of the deformities is characterized by only one type of deformity, and more than one type of deformity occurred in 10–40% of them. Scoliosis was only found on 16 DPH (25%).

Lordosis ranges from 18.75% to 50% and is characterized by a V-shaped backbone behind the swim bladder (Table 4.1). There were fusions between 14 DPH, 16 DPH, and 31 DPH of vertebrae. Atrophy peaked at 22.22% on 26 DPH, and the minimum value of 6.25% was found on 16 DPH. The incidence of malformations ranged from fish, with only one type of anomaly to multiple deformities from 16 DPH to 31 DPH. The number of normal development species accounted for 21–62%, and the rest of fish showed different degrees of deformities. Therefore, 22–44% of the malformation presented only one type of deformity, and 10–40% of them showed multiple malformations.

The malformations in the vertebral column and caudal complex are the most common observed areas in commercial cultured finfish (Negm et al. 2013; Cheng et al. 2018). In golden pompano larvae, the average vertebral malformation rate was around 18.72%, and this value is consistent with other species such as Pandora *Pagellus erythrinus* (Boglione et al. 2003), gilthead sea bream *Sparus aurata* (Boglione et al. 2001; Prestinicola et al. 2013), and zebra fish *Danio rerio* (Ferreri et al. 2000). In golden pompano, malformations of the vertebral column were the most frequently observed deformity under artificial rearing condition, in which the results are similar to the results reported in striped trumpeter *Latris lineata* (Fernández et al. 2009; Haga et al. 2011).

Although the causes of skeleton deformities have been gradually recognized, skeletal malformations are still one of the major issues reducing the development of marine finfish aquaculture (Cobcroft and Battaglen 2013). Various factors can cause skeleton malformations (Kessabi et al. 2013). The malformation has been observed as early as 4 DPH in yellowtail kingfish *Seriola lalandi*, and this may suggest that the nutrition reservation of broodstock is a key factor regulating the skeletal development during the early stage of fish (Cobcroft et al. 2004; Ma et al. 2014a). The triggering factors of the deformity in the caudal complex are still unknown (Haga et al. 2011). Moreover, vertebral deformities are often associated with swim bladder deficiencies (Daoulas et al. 1991). Vitamin A deficiency could induce vertebral column deformities, including lordosis, scoliosis, and kyphosis (Negm et al. 2013).

4.5 Conclusion

This chapter discusses the skeletal development and malformation of golden pompano during their early life. Severe jaw deformities were observed in larval golden pompano on 3 DPH. From 3 DPH onward, there were both intermediate and severe jaw malformations, and the total incidences of larval fish jaw malformations were 9.6–46.6%. Severe jaw malformation peaked on 5 DPH, and severe jaw malformation occurred in 25% of the larvae. The total percentage of jaw malformation peaked at 46.6% on 11 DPH, while the maximum of intermediate malformation was 36.4%

on 26 DPH. Five hypurals, three epurals, two haemal spines (Hs), one parhypural (Phy), and one neural spine (Ns) form the caudal complex of golden pompano. More than 33% of the experimental fish showed at least one type of malformation. Before the feeding for the first time, the deformities are discovered, suggesting that larval fish nutrition deficiency is likely to be the cause of these malformations. The results of this chapter can provide a reference for the study of skeletal malformation in intensive aquaculture of golden pompano in the future, and it can also be used to study the production quality control of golden pompano.

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Chapter 5
Nutrition and Temperature Regulate Rearing Performance of Golden Pompano *Trachinotus ovatus* Larvae

Qibin Yang, Gang Yu, Jian Guang Qin, and Zhenhua Ma

Abstract Nutrition and temperature are the primary factors affecting development of fish larvae during the early feeding stage. This chapter discusses how nutrient enhancement and ambient temperature affect the rearing performance of golden pompano *Trachinotus ovatus*. *Artemia* nauplii enriched with Algamac 3080 enhanced fish growth and reduced malformation. Fish fed *Artemia* nauplii enriched by *Nanochloropsis* achieved high survival but high jaw malformation. The water temperature of 26–29 °C enhanced growth and survival, while 23 °C was too low for both parameters. Jaw, vertebral column, and caudal vertebra deformity significantly increased at 33 °C. Therefore, the temperature range of 26–29 °C is optimal, and temperature >33 °C and <23 °C may have adverse impacts on fish performance. Information presented in this chapter will improve hatchery management on the production efficiency of golden pompano fingerlings. These findings may also apply to other similar species.

Keywords Nutrition · Temperature · Rearing performance · *Trachinotus ovatus*

5.1 Introduction

Malformation adversely impacts the market value of marine fish (Cobcroft and Battaglene 2009, 2013; Sandel et al. 2010; Ma et al. 2014c). Malformed fish are usually sold at a low price or manually removed during the production phase, thus significantly increasing the production cost (Koumoundourous et al. 1997). In addition, the deformation has adversely affected fish's growth and survival, food conversation, swimming ability, and susceptibility to stress and pathogens

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_5

(Koumoundourous et al. 1997; Andrades et al. 1996; Boglione et al. 2001). Such impact can greatly add production cost on marine fish aquaculture. Existing evidence indicates that malformation during larval development occurs on over 27% of fish, leading to severe mortality at the grow-out phase (Andrades et al. 1996). Although the rearing environment (such as temperature, water current, tank color, salinity, dissolved oxygen) (Koumoundouros et al. 1999, 2001; Sfakianakis et al. 2004; Hattori et al. 2004; Okamoto et al. 2009; Georgakopoulou et al. 2010; Owen et al. 2012), genetic factors (Ferguson and Danzmann 1998; Castro et al. 2007), pesticides, and parasites (Liang et al. 2012; Kusuda and Sugiyama 1981; Liu et al. 2012) have been identified to be associated with boney malformation, and more and more evidence has indicated the nutritional factors in the process of larval fish rearing as a direct result of the deformation of the fish (Afonso et al. 2000; Andrades et al. 1996; Sandel et al. 2010; Cahu et al. 2003a).

In the past decades, the requirements of essential fatty acids in fish larvae have been widely studied (Izquierdo et al. 1992; Morais et al. 2007; Kjørsvik et al. 2009). The long-chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid 20:5n-3 (EPA), docosahexaenoic acid 22:6n-3 (DHA), and arachidonic acid 20:4n-6 (ARA) play critical roles in growth, survival, and stress resistance in most marine fish larvae (Watanabe 1993; Bell and Sargent 2003; Faulk and Holt 2003). As the essential fatty acids in marine fish larvae, the dietary requirements of EPA, DHA, and ARA have been well quantified in fish nutrition (Hamre et al. 2002; Bell and Sargent 2003; Faulk et al. 2005). DHA plays a vital role in neutral membrane structure and functions (Sargent et al. 1999a; Copeman et al. 2002), and its requirement differs among fish species (McEvoy et al. 1998; Planas and Cunha 1999; Copeman et al. 2002). The abundances of DHA and EPA in cell membranes serve as a major source of energy to absorb fat-soluble vitamins (A, D, E, and K) and as precursors for prostaglandin hormones (Sargent et al. 1999a, b; Rezek et al. 2010). The DHA/EPA ratio has been considered an index to determine the optimal levels for these fatty acids in the growth and development of fish larvae (Koven et al. 1993; Tocher et al. 1997; Rodriguez et al. 1998). Since a deficiency or excess of DHA and EPA in the diet can affect survival and malformation of fish larvae (Sargent et al. 1999b), it is necessary to provide an adequate amount of these essential fatty acids through dietary manipulation (Palmtag et al. 2006).

Temperature is vital for the larval fish early development, because it can adjust the fish metabolism and feeding behavior (Ma 2014; Kestemont and Baras 2001). In aquaculture, temperature directly influences the size of fish larvae at hatching, the age and size at yolk absorption, growth, survival, feeding, and digestion in fish larvae (Martinez-Palacios et al. 1996; Rombough 1996; Jobling 1997; Fielder et al. 2005; Bustos et al. 2007). Besides, several studies have demonstrated that high mortality and abnormality of fish larvae are attributed to inappropriate temperature (Ørnsrud et al. 2004; Lein et al. 1997; Ludwig and Lochmann 2009). Furthermore, the way yolk energy is utilized varies between incubation temperatures. In many species, if the temperature is higher than the optimal level, the size at the onset of exogenous feeding can be smaller than in other temperatures, thereby contributing to complication in larval fish rearing, especially in species with small larvae. Within the

temperature range of fish tolerance, the increase of temperature accelerates ontogenetic development, but a high temperature may reduce fish survival. At high temperature, yolk-sac absorption of fish larvae is fast, but the period of endogenous feeding is short (Dou et al. 2005; Bustos et al. 2007; Ma 2014). Therefore, choosing the appropriate temperature is essential to improve the growth and survival of fish larvae in hatcheries.

Golden pompano *Trachinotus ovatus* has been used as a new candidate species for aquaculture. Although the life cycle of golden pompano has been closed and several key aspects such as food and feeding, development of the larval digestive system, and the weaning protocols have been successfully explored (Ma et al. 2014a, b, d), high malformation of golden pompano during the early development stage has severely reduced the production efficiency (Ma et al. 2014c). Chapter 4 has identified the position, type, and frequency of skeletal and jaw malformations in hatchery rearing of larval golden pompano. Nonetheless, factors causing malformations are still unclear in this fish. This chapter discusses the nutritional enhancement and ambient temperature on the rearing performance of golden pompano under a hatchery culturing condition. These chapter results provide better understanding on how nutritional enhancement and temperature regulate larval fish development. This chapter contributes to developing management strategies to improve this fish and other similar species in the hatchery production efficiency.

5.2 Nutritional Enhancement Regulates Fish Growth and Survival

Feed of DHA and EPA in larval fish is vital for the growth of fish (Rezek et al. 2010). As dietary DHA levels increased, improved fish growth has been observed in striped jack *Caranx vinctus* (Takeuchi et al. 1996), yellowtail *Seriola quinqueradiata* (Furuita et al. 1996), and Japanese flounder *Paralichthys olivaceus* (Izquierdo et al. 1992). The growth response of fish larvae to different enrichment products varies among species. For instance, the larval growth of striped bass *Morone saxatilis* and gilthead seabream *Sparus aurata* is not affected by feeding *Artemia* nauplii enriched with Algamac 2000 or PL-Cr (DHA-rich phospholipid extract of *Crypthecodinium* sp.), but the growth of halibut *Hippoglossus hippoglossus* larvae fed *Artemia* nauplii enriched with DHA Seleco is slower than those fed with PL-Cr (Harel et al. 2002). In golden pompano, fish growth was enhanced when fish larvae were fed with *Artemia* nauplii enriched with Algamac 3080 or *Nannochloropsis* (Fig. 5.1). Fatty acid composition of enriched and unenriched *Artemia* nauplii is shown in Table 5.1.

Fish treated with Algamac 3080 had the best SGR, which is consistent with the higher dietary DHA levels in the treated live feed. As a sensitive growth and nutritional condition indicator (Islam and Tanaka 2005; Zehra and Khan 2013), the RNA/DNA ratio indicates that better growth performance occurred in the

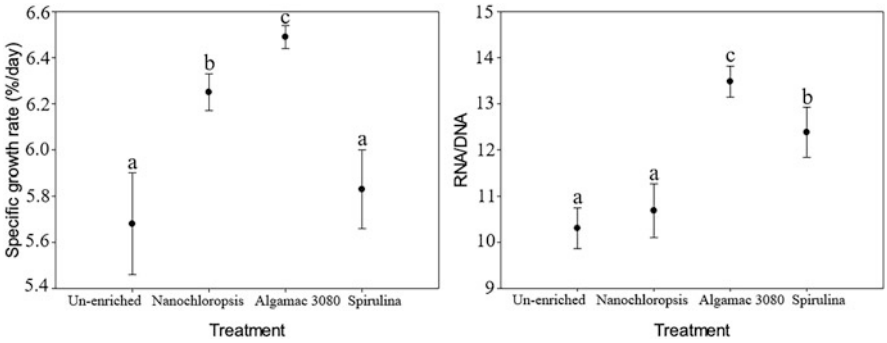

Fig. 5.1 Specific growth rate and RNA/DNA of golden pompano in different nutrient enhancements (Ma et al. 2016)

Table 5.1 Fatty acid composition (% of total fatty acids) of enriched and unenriched *Artemia* nauplii (Ma et al. 2016)

	Unenriched	Nanochloropsis	Algmac 3080	Spirulina
14:0	1.14 ± 0.21^{a}	0.80 ± 0.10^{a}	1.18 ± 0.32^{a}	0.94 ± 0.07^{a}
16:0	16.71 ± 2.4^{a}	12.13 ± 0.55^{a}	18.74 ± 4.68^{a}	13.46 ± 0.80^{a}
16:1n-7	0.44 ± 0.05^{b}	0.51 ± 0.09^{c}	0.25 ± 0.24^{a}	0.52 ± 0.01^{c}
18:0	5.88 ± 0.52^{ab}	6.12 ± 0.24^{b}	6.42 ± 0.44^{b}	5.1 ± 0.14^{a}
18:1n-9	9.27 ± 0.74^{a}	11.71 ± 0.72^{b}	9.74 ± 1.48^{b}	9.59 ± 0.32^{ab}
18:1n-7	19.44 ± 2.1^{a}	21.83 ± 1.75^{a}	19.87 ± 1.55^{a}	20.45 ± 0.19^{a}
18:2n-6	2.62 ± 0.41^{ab}	2.82 ± 0.30^{ab}	2.01 ± 0.86^{a}	3.74 ± 0.22^{b}
18:3n-3	0.32 ± 0.11^{a}	0.80 ± 0.45^{ab}	0.32 ± 0.10^{a}	0.91 ± 0.04^{b}
20:1n-9	0.61 ± 0.04^{b}	0.43 ± 0.03^{a}	0.35 ± 0.20^{a}	0.62 ± 0.09^{b}
20:4n-6 (ARA)	1.22 ± 0.10^{ab}	1.16 ± 0.03^{a}	1.30 ± 0.07^{b}	1.10 ± 0.10^{a}
20:5n-3 (EPA)	6.28 ± 0.87^{a}	8.49 ± 0.49^{b}	7.23 ± 1.00^{ab}	6.31 ± 0.46^{a}
22:6n-3 (DHA)	0.45 ± 0.06^{a}	1.87 ± 0.08^{b}	2.56 ± 0.31^{c}	1.74 ± 0.24^{b}
DHA/EPA	0.07 ± 0.00^{a}	0.22 ± 0.02^{b}	0.36 ± 0.09^{c}	0.28 ± 0.06^{b}
EPA/ARA	5.10 ± 0.31^{a}	7.31 ± 0.23^{b}	5.62 ± 1.07^{a}	5.75 ± 0.10^{a}
DHA/ARA	0.37 ± 0.02^{a}	1.61 ± 0.11^{b}	1.96 ± 0.13^{b}	1.62 ± 0.37^{b}
Total n-3	7.05 ± 1.04^{a}	11.16 ± 0.86^{c}	10.11 ± 0.79^{b}	8.96 ± 0.26^{b}
Total n-6	3.84 ± 0.05^{ab}	3.98 ± 0.33^{ab}	3.31 ± 0.79^{a}	4.84 ± 0.32^{b}
Total n-7	19.88 ± 2.15^{a}	22.35 ± 1.84^{a}	20.11 ± 1.78^{a}	20.97 ± 0.20^{a}
Total n-9	9.87 ± 0.70^{a}	12.14 ± 0.75^{b}	10.09 ± 1.28^{a}	10.21 ± 0.23^{a}
Total saturated	23.73 ± 3.13^{a}	19.05 ± 0.22^{a}	26.34 ± 5.44^{a}	19.50 ± 0.73^{a}
Total poly unsaturated	40.64 ± 4.39^{a}	49.63 ± 3.78^{a}	43.61 ± 4.63^{a}	44.97 ± 0.60^{a}

Different letters represent significant differences ($P < 0.05$)

treatment of Algamac 3080. However, the fish growth in the treatments of *Nannochloropsis* and *Spirulina* is not consistent with their RNA/DNA ratios and dietary DHA levels (Fig. 5.1). As Faulk et al. (2005) suggested, such inconsistency

may be possibly due to the difference in the protein content or amino acid profiles of live prey fed with different enrichment formulas.

Highly unsaturated fatty acids, especially DHA, EPA, and ARA, are necessary to marine fish growth, development, and survival (Cahu et al. 2003a; Sargent et al. 1999b; Rezek et al. 2010). In order to develop lipid-enriched food of fish larvae, the requirements of essential fatty acids of fish larvae have been extensively researched by using live bait enriched with different oils and micronutrients, and the aim is to enhance the content of essential fatty acids of live bait (Takeuchi 1997; Sargent et al. 1997; Izquierdo et al. 2000). However, excessive content of lipid or unbalanced composition of lipid classes leads to low growth and skeletal malformation in species such as gilthead seabream *Sparus aurata* (Salhi et al. 1999), Atlantic cod *Gadus morhua* (Kjørsvik et al. 2009), yellowtail kingfish *Seriola lalandi* (Ma and Qin 2014), and Atlantic halibut *Hippoglossus hippoglossus* (Olsen et al. 2000). Enrichment did not change the DHA/ARA ratio, but *Artemia* nauplii enriched with Algamac 3080 resulted in a higher DHA/EPA ratio (0.36:1, Table 5.1). The high DHA/EPA ratio treated with Algamac 3080 resulted in rapid growth but low survival. In contrast, the unenriched and *Nannochloropsis* treatments showed better survival where the DHA/EPA ratio was 0.07:1–0.22:1. Low fish survival rate of Algamac 3080 treatment supports the claim of a previous study that a high DHA/EPA ratio and a high DHA content may reduce the survival of larval fish (Planas and Cunha 1999) as the composition of unbalanced lipid classes in the diet affects the digestion and absorption of fatty acids in fish larvae (Salhi et al. 1997, 1999).

5.3 Malformation

Skeletal malformation on fish in marine aquaculture is a recurrent issue (Ma and Qin 2014; Ma et al. 2014c), and skeletal malformation negatively affects fish quality in commercial production via suppressing fish growth and survival (Andrades et al. 1996; Boglione et al. 2001). The abnormalities in fish larvae can have sublethal (Barahona-Fernandes 1982; Cobcroft et al. 2001) or lethal effects on fish larvae (Boglione et al. 2013) as the distorted mouth shape would affect the efficiency of food ingestion (Pittman et al. 1989), while notochord anomalies in newly hatched larvae can severely affect fish swimming (Boglione et al. 2013). Jaw malformations are a common type of skeletal malformation, and there are many different forms (Cobcroft et al. 2001) that have been frequently found in both wild-caught and artificially reared marine fish (Boglione et al. 2001). Izquierdo et al. (2010) believed that PUFA play a key role in bone formation, and the composition of fatty acid composition in bone and cartilage can also be affected by dietary lipids (Watkins et al. 1997; Kokkinos et al. 1993; Liu et al. 2004). As the dietary lipids are primarily from live feeds, enrichment on live feeds may affect jaw malformation. In golden pompano, enrichment significantly affected jaw malformation. Fish fed *Artemia* nauplii enriched with Algamac 3080 or *Spirulina* showed two times lower in jaw

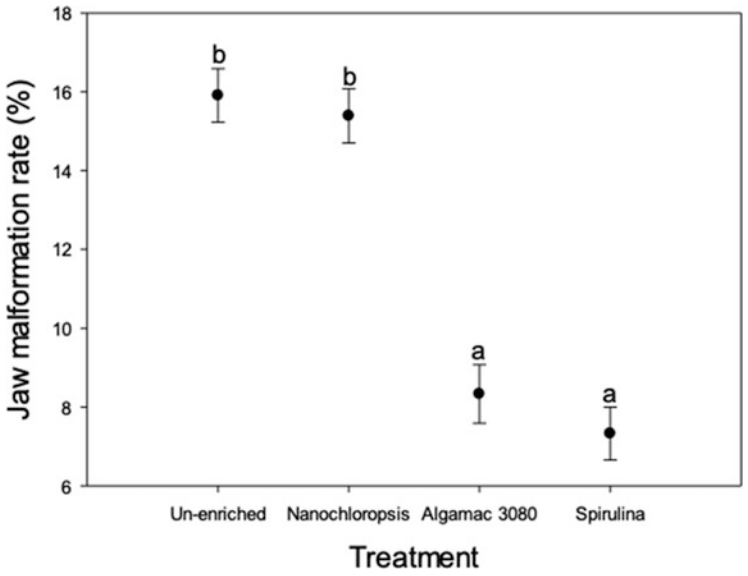

Fig. 5.2 Jaw malformation of golden pompano fed with *Artemia* nauplii enriched with different nutrient enhancement (Ma et al. 2016)

malformation than those fed unenriched *Artemia* nauplii or *Artemia* nauplii enriched with *Nannochloropsis* (Fig. 5.2). The results of low jaw malformation treated by Algamac 3080 and *Spirulina* may indicate that such nutrient enrichment is sufficient to meet the needs of jaw development during this stage.

Up to present, the relationship between the deficiency of essential fatty acids and the development of skeletal anomalies is poorly understood (Boglione et al. 2013).

Hamre et al. (2002) suggest that insufficient intake of n-3 HUFA in *Artemia* nauplii may cause abnormal development of fish larvae. Recent evidence has demonstrated that fatty acids, such as EPA, DHA, and ARA, play a key role in the development of bone.

For example, a 50% reduction in deformed fish was found in diet supplement with higher levels of DHA (Izquierdo et al. 2010), and changes in dietary ARA/EPA may indirectly affect osteoblast development and bone metabolism (Berge et al. 2009). In golden pompano, skeletal malformation was improved in the treatment of Algamac 3080, and this result is in line with high levels of DHA in feed. The results of this study indicate that 2.56% DHA level may be suitable for the larval golden pompano's skeletal development. Vertebral column malformation and caudal complex malformation are the most frequently reported body deformity in commercially cultured species (Negm et al. 2013). Up to present, little is known on the causes triggering the deformity in the caudal complex (Haga et al. 2011). In the literature, vertebral deformities are often associated with swim bladder abnormality (Daoulas et al. 1991; Chatain 1994), but vitamin A deficiency can also induce vertebral column deformities (Negm et al. 2013). In golden pompano, the highest vertebral

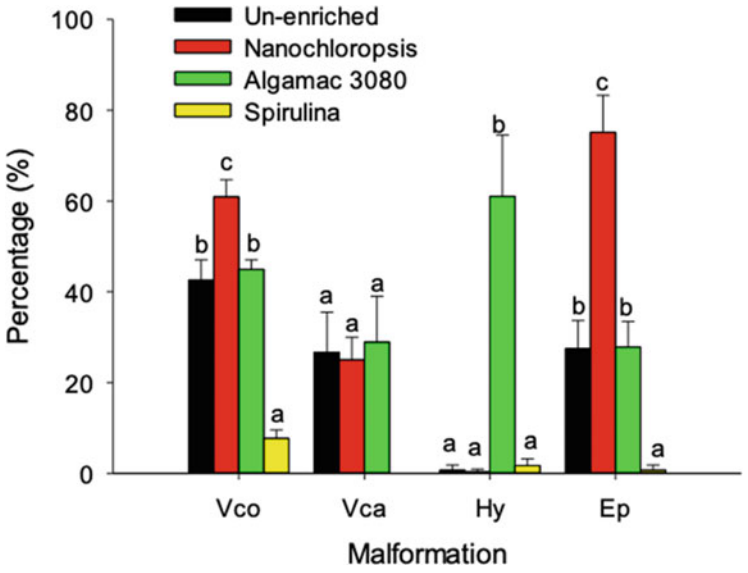

Fig. 5.3 The vertebral column (Vco), caudal vertebra (Vca), hypural (Hy), and epural (Ep) malformations and vertebral malformation of larval golden pompano of four enrichment treatments on 27 DPH (Ma et al. 2016)

column (Vco) malformation (60.9%) and epural (Ep) malformation (75.1%) were observed in the fish fed *Artemia* enriched with *Nannochloropsis*, and the lowest Vco malformation (7.7%) and Ep malformation (0.7%) were found in the treatment of *Spirulina* enrichment. Significantly higher hypural malformation (61.0%) was observed in the treatment of Algamac 3080 enrichment than in other treatments (Fig. 5.3). This result may suggest that nutrient enhancement in *Artemia* nauplii affects vertebral deformities, and the impact of nutritional components in *Artemia* nauplii on larval fish development warrants further study in future.

5.4 Temperature Affects Fish Performance

Temperature affects fish growth, metabolism, and food intake, and the effects of temperature on body growth have been recorded in many larval fish species including nase *Chondrostoma nasus* (Keckeis et al. 2001), striped trumpeter *Latris lineata* (Choa et al. 2010), haddock *Melanogrammus aeglefinus* (Martell et al. 2005), Australian snapper *Pagrus auratus* (Fielder et al. 2005), yellowtail kingfish *Seriola lalandi* (Ma 2014), and Atlantic halibut *Hippoglossus hippoglossus* (Lein et al. 1997). In golden pompano, the newly hatched golden pompano grow slowly in the first 9 days. After 12 DPH, the size of the different temperature treatment of fish larvae of differences began to emerge. Temperature has an effect on fish growth on

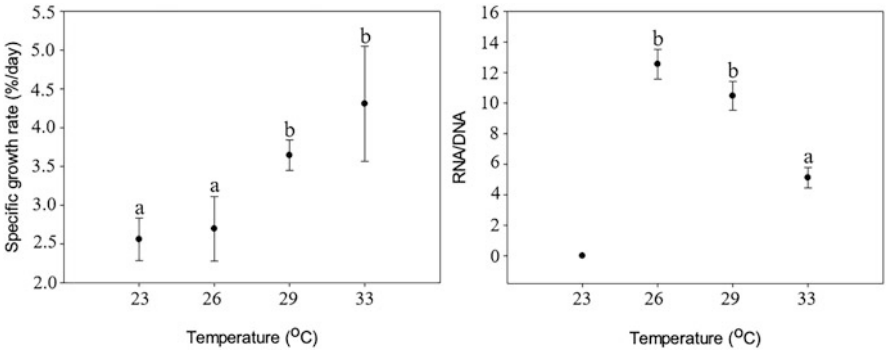

Fig. 5.4 Specific growth rate of standard length and RNA/DNA ratio of golden pompano larvae reared at 23, 26, 29, and 33 °C (Yang et al. 2016)

18 DPH, which accelerates when the temperature increases from 29 to 33 °C (Fig. 5.4).

The rapid growth of golden pompano at high temperature may be related to the improvement of larval feeding and digestive function after 15 DPH, as Ma et al. (2014b) found that the gastric glands and goblet cells appeared in the intestines of larval golden pompano after 15 DPH at the temperature of 27–29 °C. As the Florida pompano *Trachinotus carolinus* (Riley et al. 2009), the mouth gape height of 1.05 mm should allow larval golden pompano to ingest *Artemia* nauplii and other similar particle sizes by 12 DPH.

Therefore, the significant differences in fish size between thermal treatments after 18 DPH may be also related to the use of more energetic food from 9 DPH onward. Riley et al. (2009) found that the growth trajectories of *T. carolinus* could vary substantially between progenies, with some progenies exhibiting slower growth and others much faster growth.

The RNA/DNA ratio has been proven as a sensitive indicator of growth and nutritional condition in fish larvae and juveniles (Buckley et al. 1999; Islam and Tanaka 2005; Zehra and Khan 2013). Previous studies have demonstrated that water temperature and food availability can affect the RNA/DNA ratio of fish larvae (Goolish et al. 1984; Mathers et al. 1992). In golden pompano, the RNA/DNA ratio of fish significantly dropped when temperature increased from 29 to 33 °C at 18 DPH (Fig. 5.4). Reduction in RNA/DNA ratio can be regarded as a result of nutrient deficiency in fish larvae (Tanaka et al. 2008). The reduction of RNA to DNA ratio of fish at 33 °C may indicate a slow growth status of fish larvae at 18 DPH. At the end of this study, the high SGR of a few larger fish may be due to cannibalism. However, the reason for the lower RNA/DNA ratio at 33 °C is not clear.

Fish mortality frequently occurs at the key period of nutritional transition from endogenous to exogenous nutrition (Ma et al. 2012; Otterlei et al. 1999). During the period of transitional feed, when food supply and photoperiod are within the range of fish requirements, the temperature can strongly impact fish survival (Gardeur et al. 2007; McGurk 1984; Kamler 1992; Ma 2014). For instance, mortality is closely

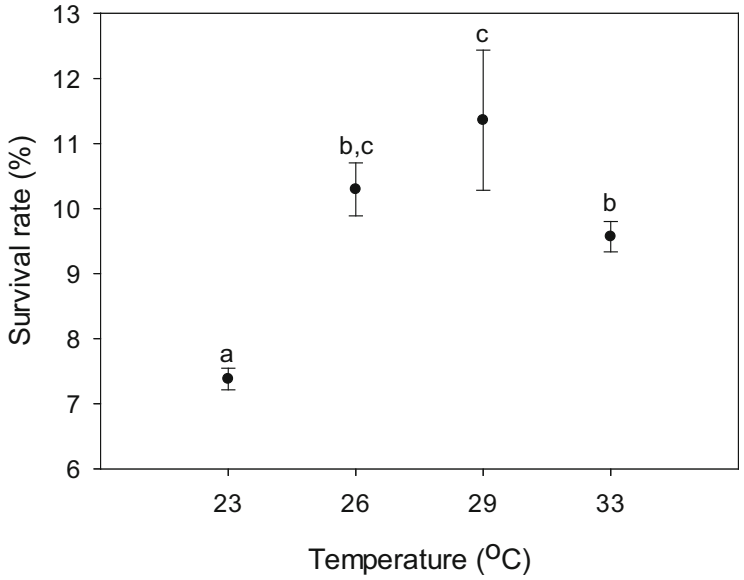

Fig. 5.5 Survival rate of golden pompano larvae at 23, 26, 29, and 33 °C (Yang et al. 2016). (The letters of "temperature" in the *x*-axis is crowding)

related to temperature in the larvae and juveniles of *Pangasianodon hypophthalmus* (Baras et al. 2011), *Seriola lalandi* (Ma 2014), *Glyptocephalus cynoglossus* (Bidwell and Howell 2001), and *Inimicus japonicus* (Wen et al. 2013). Ma (2014) suggests a temperature-sensitive period where mortality is likely to occur during the early ontogeny of golden pompano. In golden pompano, survivals of fish larvae were low in all the temperature treatments (Fig. 5.5). There could be many reasons why survival rates were so low, including egg quality, the inadequacy of feeding schedules, or cannibalism (May 1974; Baras and Jobling 2002; Baras et al. 2011; Ma et al. 2012). Nevertheless, there is some clear-cut tendency here that the survival of golden was highest at 29 °C and declined at lower or warmer temperatures, with 23 °C being unsuitable for their rearing.

As one of the primary physical factors, the water temperature can affect early morphological abnormalities during the ontogenetic development of fish larvae (Seikai et al. 1980, 1986; Klimogianni et al. 2004). Evidence indicates that high water temperature can increase the percentage of malformation during the ontogenetic development in fish species such as tilapia *Oreochromis mossambicus* (Wang and Tsai 2000), Senegalese sole *Solea senegalensis* (Dionisio et al. 2012), and gilthead seabream *Sparus aurata* (Georgakopoulou et al. 2010). The present study demonstrates a significant temperature effect on the vertebral column and caudal vertebra malformation in golden pompano. This result is consistent with similar findings in *Dicentrarchus labrax* (Sfakianakis et al. 2006). In golden pompano, temperature associated abnormity in the vertebral column of the larval golden pompano was the most prevalent malformation (Fig. 5.6). Additionally, the

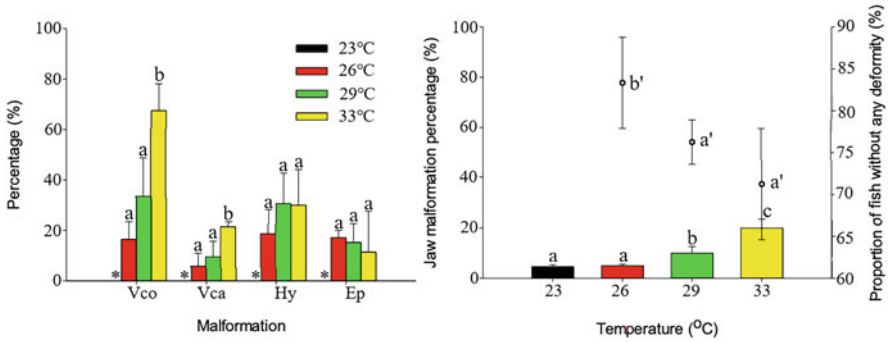

Fig. 5.6 Malformation of golden pompano larvae reared at 23, 26, 29, and 33 °C. *Vco* the vertebral column malformation, *Vca* caudal vertebra malformation, *Hy* hypural malformation, *Ep* epural malformation (Yang et al. 2016). You should indicate the meaning of bar diagram and scattered diagram (i.e., jaw malformation vs. without deformity) in the right diagram

V-shaped malformation accounted for >50% of the whole deformations. A previous study indicates that the absence of swim bladder or its malfunction could account for over 50% of V-shaped deformations in fish (Daoulas et al. 1991). Hemal lordosis can also result from the exposure to high flow velocities (Divanach et al. 1997) or turbulent flows (Kihara et al. 2002; Kranenbarg et al. 2005). In the present study, deformities frequently occurred at all temperatures, suggesting that other factors may also prevail in the present study causing deformity. Future research is required to further identify these factors.

The caudal fin complex is one of the most sensitive parts of the skeletal system in fish, and deformities can occur even in the range of normal rearing temperature (Takeuchi et al. 1998; Haga et al. 2002, 2011). Rates of deformities can increase with increasing temperature, but this is not consistent in gilthead seabream *Sparus aurata* (Fernández et al. 2008; Georgakopoulou et al. 2010). In golden pompano, however, the proportion of fish with deformed caudal fin complex did not vary significantly with water temperature (Fig. 5.6). Jaw malformation is a significant concern in fish culture because it affects fingerling quality (Von Westernhagen 1988). Frequently, as the temperature of water rises, the proportion of larval fish exhibiting jaw deformities increases (Alderdice and Velsen 1971; von Westernhagen 1974; Bolla and Holmefjord 1988; Lein et al. 1997), and we observed the same pattern in golden pompano. This temperature-dependent pattern is often attributed to higher oxygen (Rombough 1997) and nutritional requirements at high temperatures, which may not be met unless feed with higher energy or protein contents is provided (Cahu et al. 2003a, b; Ma 2014).

5.5 Conclusion

In summary, *Artemia* nauplii enriched with Algamac 3080 can enhance fish growth performance and reduce malformation. Fish fed *Artemia* nauplii enriched with *Nanochloropsis* showed high survival but with high jaw malformation. Nutritional enhancement in *Artemia* nauplii can significantly affect the performance of golden pompano larvae. The water temperature of 26–29 °C enhanced growth and survival, while 23 °C was too low for both. The deformity of the jaw, vertebral column, and caudal vertebra significantly increased at 33 °C. Therefore, the temperature range of 26–29 °C is optimal, and temperature >33 °C and <23 °C may have adverse impacts on fish performance. Future study should focus on refining the optimum dietary enrichment and rearing temperature in golden pompano larvae to improve growth and survival and decrease boney malformation of fish larvae.

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Chapter 6
Physical Responses of Golden Pompano *Trachinotus ovatus* to Rearing Salinity

Shengjie Zhou, Mingyang Han, Rui Yang, and Jing Hu

Abstract The physiological status of euryhaline teleost is regulated by environmental salinity through different mechanisms. This chapter discusses the salinity to the juvenile golden pompano *Trachinotus ovatus* (Linnaeus 1758) rearing performance impact.

Rearing salinity significantly affected fish growth and the RNA/DNA ratio. When the salinity was 34‰, the fish growth rate and RNA/DNA ratio were higher. The effect of salinity on pepsin activity was not significant. However, rearing salinity had a significant effect on α-amylase activity. The α-amylase activity of fish reared at the salinity of 10‰ was significantly lower than fish at the salinity of 34‰. Raising salinity has significant effects on FCR of juvenile golden pompano. The FCR of fish cultured at the salinity of 10‰ was five times higher than the FCR of fish reared at 34‰. The GPX activity was highest when the salinity was 26‰ and lowest when the salinity was 34‰. The activities of SOD of fish reared at 18‰ and 34‰ were significantly higher than those reared at 10‰ and 26‰. The lowest activity of Na^+K^+-ATPase was obtained in fish at 34‰, while the highest activity of Na^+K^+-ATPase was obtained when fish at 18‰. Juvenile golden pompano can be reared above 26‰ without affecting fish performance, and the salinity $<$18‰ is not suitable for the growth of juvenile golden pompano.

Keywords Salinity · Rearing performance · Digestive enzyme activity · Antioxidant enzyme · Na^+K^+-ATPase · *Trachinotus ovatus*

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_6

6.1 Introduction

Salinity is the most important environmental factor affecting aquatic habitats, and it has been involved in many studies regarding on its impact on fish growth performance (Rubio et al. 2005). Previous studies have suggested that environmental salinity can change physiological activities such as feed intake (Rubio et al. 2005), metabolic rate (Dutil et al. 1997), activity of enzyme (Moutou et al. 2004), and feed conversion rate (Alava 1998), which are closely linked to the fish growth. In practice, the growth performance of fish is better under moderate salinity conditions, but the underlying mechanisms are still controversial (Moutou et al. 2004; Baeuf and Payan 2001).

The enzyme analysis of digestive has been considered a reliable method to understand the digestive process and nutrition condition of fish (Ueberschär 1988; Ma et al. 2014). Previous studies have demonstrated that changes of salinity can alter the enzyme activities of digestive in species such as *Salmo gairdnerii* (Colin et al. 1985), *Sparus sarba* (Kelly et al. 1999), *Centropomus parallelus* (Tsuzuki et al. 2007), and *Sparus aurata* (Moutou et al. 2004). Such variation of digestive enzyme activities can significantly affect the growth of fish (Tsuzuki et al. 2007). Since proteinases can catalyze the hydrolytic degradation of proteins, it plays a crucial role in living organism's growth and survival (Klomklao 2008). Alpha-amylase is an important enzyme for carbohydrate digestion and is involved in carbohydrate metabolism of energy supply (Papoutsoglou and Lyndon 2003). As fish require more metabolic energy for osmoregulation, a higher α-amylase activity may indicate energy spending in the process of osmoregulatory. The α-amylase and pepsin activities have been used to explore the influence of salinity digestibility to fish (Yan and Wu 2010).

Although ambient salinity can affect fish physiological condition via different mechanisms, these underlying mechanisms are not well understood (Arnason et al. 2013). When ambient salinity is approaching the physiological tolerance limit, fish may be stressed, and the system of immune defense may be compromised (Harris and Bird 2000). The relationship between salinity variation and fish immune defense has been paid much attention (Zhang et al. 2011; Choi et al. 2013; Arnason et al. 2013).

Scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are the main components of physiological antioxidant protection of marine fish and play an important role in the immune defense system of marine fish (Winston and Di Giulio 1991; Halliwell and Gutteridge 1996). Within the physical process, SOD promotes the dismutation of two O^{2-} molecules to H_2O_2 and O_2, and CAT and GPX convert H_2O_2 to H_2O. The inadequate antioxidant defenses to combat reactive oxygen species can lead to oxidative stress (Martinez-Alvarez et al. 2002). Nevertheless, knowledge about the response of antioxidant enzymes to salinity of marine fish is still limited.

Golden pompano *Trachinotus ovatus* has been identified as a good aquaculture candidate species due to its fast growth, high flesh quality, and suitability for cage

farming. In South China, most golden pompano farming is carried on small farms in marine and brackish environments using discontinuous and non-quantified methods. During the rearing period of golden pompano, salinity variations are often associated with low growth, disease outbreak, and massive mortality. In this chapter, the effects of environmental salinities (10‰, 18‰, 26‰, and 34‰) on juvenile golden pompano (wet weight 3.24 ± 0.14 g) during the grow-out phase are discussed, aiming to increase the production efficiency of commercial farming of golden pompano.

6.2 Growth and Survival of Golden Pompano Under Different Salinity

Fish adaptations to salinity vary among pompano species. For instance, the recommended low salinity range is 15–25‰ for *T. blochii* (Kalidas et al. 2012), 12–19‰ for *T. carolinus* (Moe et al. 1968), and 10–20‰ for *T. marginatus* (Costa et al. 2008). In golden pompano, juveniles showed a reasonable survival rate at 18‰, 26‰, and 34‰, suggesting a good adaption of this species within this salinity range. A previous study suggests that fish adaption to ambient salinity changes is life stage-dependent (Aliume et al. 1997) with some metabolic restraints (Peters et al. 1998; Rocha et al. 2007). Although some marine fish species can tolerate a wide range of salinity gradient changes, the consumption of metabolic energy during osmotic regulation is unavoidable (Woo and Kelly 1995; Moser and Miller 1994; Tseng and Hwang 2008). Even in species with lower metabolic rates, osmoregulation seems to consume a high proportion of the available energy, ranging from 20% to 50% of the total energetic expenditure (Baeuf and Payan 2001).

Maximum growth would occur in an isosmotic environment (10 ± 2‰) because of low osmoregulatory energy demand (Brett 1979), but optimal salinity for fish growth is species-specific. For example, the optimal growth salinity is 55‰ for *Chanos chanos* (Swanson 1998) and 14‰ for *Gadus morhua* (Lambert et al. 1994). In contrast, the growth of *Acanthopagrus butcheri* is not significantly affected by the rearing salinity from 0‰ to 12‰ (Partridge and Jenkins 2002), and salinity in the range of 5–35‰ has no effect on the growth of *Centropomus parallelus* (Tsuzuki et al. 2007). In golden pompano, the growth of juvenile fish was sensitive to the rearing salinity, and the highest growth rate was recorded in fish cultured at 34‰ (Table 6.1). The lowest growth rate was observed in fish cultured at the salinity of 10‰. These results indicate that the growth of juvenile golden pompano is reduced at lower salinity. The RNA/DNA ratio is used as an indicator of the fish's growth potential when sufficient food is provided to young fish under laboratory conditions (Tanaka et al. 2007). In juvenile golden pompano, culture salinity had a significant effect on the RNA/DNA ratio (Table 6.1). Since the diet, food availability, feeding scheme, and environmental conditions were the same across treatments, the salinity should cause the RNA/DNA ratios change. Higher RNA/DNA ratio is under the

Table 6.1 Initial and final mean body weights, specific growth rate (SGR), survival, RNA/DNA, pepsin activity, amylase activity, and FCR of juvenile golden pompano at different salinities (Ma et al. 2016a)

	10‰	18‰	26‰	34‰
Phase 1 (24 days)				
Initial weight (g)	3.11 ± 0.35^{a}	3.21 ± 0.41^{a}	3.19 ± 0.52^{a}	3.43 ± 0.59^{a}
Final weight (g)	3.21 ± 0.52^{a}	3.45 ± 0.48^{a}	3.72 ± 0.39^{a}	4.19 ± 0.86^{a}
Phase 2 (30 days)				
Initial weight (g)	3.21 ± 0.52^{a}	3.45 ± 0.48^{a}	3.72 ± 0.39^{a}	4.19 ± 0.86^{a}
Final mean weight (g)	4.64 ± 0.18^{a}	6.34 ± 0.75^{b}	6.38 ± 0.43^{b}	12.22 ± 2.43^{c}
SGR (%/day)	1.23 ± 0.11^{a}	2.01 ± 0.27^{b}	1.79 ± 0.21^{b}	3.54 ± 0.21^{c}
Survival (%)	66.07 ± 9.74^{a}	$82.04 \pm 6.32^{a,b}$	94.28 ± 3.71^{c}	87.12 ± 0.64^{b}
RNA/DNA	7.69 ± 3.32^{a}	$11.85 \pm 1.32^{a,b}$	12.85 ± 0.83^{b}	$15.84 \pm 2.38^{b,c}$
Pepsin activity (mU/mg protein)	366.64 ± 72.42^{a}	349.52 ± 26.38^{a}	355.92 ± 76.17^{a}	362.72 ± 55.43^{a}
Amylase activity (mU/mg protein)	2.82 ± 0.53^{a}	7.97 ± 4.68^{a}	20.46 ± 4.49^{b}	20.16 ± 2.98^{b}
FCR	8.66 ± 0.44^{c}	6.58 ± 1.02^{b}	5.02 ± 0.74^{b}	2.50 ± 0.53^{a}

Different letters of the same row represent a significant difference ($P < 0.05$)

condition of high salinity farmed and higher RNA/DNA ratio, and high specific growth rate is the same.

6.3 Digestive Enzyme Activities of Golden Pompano Under Different Salinity

The alternation of ambient salinities can lead to the changes of digestive enzyme activities (Moutou et al. 2004; Woo and Kelly 1995). This effect may further affect the digestion and absorption of dietary protein (Tsuzuki et al. 2007). Previous studies have also evaluated the relationship between growth rate and digestive enzyme activities of fish at different salinity, and a correlation is shown between growth and target digestive enzymes. Previous studies have evaluated the fish growth rate under different salinity and the relationship between the activity of digestive enzymes and indicated the growth and the correlation between target enzymes (Moutou et al. 2004; Woo and Kelly 1995). In larval golden pompano, the activities of amylase in fish at 26‰ and 34‰ salinities were higher than those at 10‰ and 18‰ salinities, and also the growth rate of fish at 34‰ was higher than fish at 10‰. But the existing literature does not support that amylase activity corresponds to fish growth. Therefore, it may be worth further investigating the relationship between amylase activity and fish growth.

The FCR of cultured fish is different under different environmental salinity, and the response of feed conversion ratio to salinity is species-specific (Partridge and

Jenkins 2002). For example, when *Gadus morhua* are reared at salinities of 7‰, 14‰, and 28‰, the best FCR was obtained at 14‰ (Lambert et al. 1994), but better FCR can be achieved when fish were reared at 24‰ in *Acanthopagrus butcheri* (Partridge and Jenkins 2002). However, compared with the treatment groups with salinity of 8‰, 18‰, and 38‰, *Carassius auratus* reared at salinity of 28‰ could obtain the best FCR (Klaoudatos and Conides 1996). In juvenile golden pompano, the FCR of fish increase with the increase of ambient salinity, and the optimal FCR was observed when fish group is reared at 34‰ (Table 6.1). Coincidently, higher amylase activity was also found when fish were reared at 34‰. This could indicate that the digestibility is increased when fish are cultured at 34‰.

6.4 Antioxidant Enzyme and Na^+K^+-ATPase Activities of Golden Pompano Under Different Salinity

Ambient salinity can change fish metabolism and result in different survival rates. The alternation of antioxidant enzyme activities in fish may be caused by a hypo-osmotic shock (Roche and Boge 1996). In juvenile golden pompano, the GPX activity in fish liver gradually increased, when the ambient salinity was between 10‰ and 28‰, while the CAT activity of the liver presented a gradually declining trend. Similar results have also been reported by Wilhelm Filho et al. (1993) and Martinez-Alvarez et al. (2002). The activity of SOD of fish at 28‰ salinity was significantly lower compared to 34‰ salinity (Fig. 6.1). Furthermore, when fish were reared in the salinity of 28‰, the highest GPX activity and the lowest CAT activity were also observed, and the final survival rate of fish at 28‰ was significantly higher than in other treatments. This may indicate that the salinity of 28‰ is more suitable for the juvenile golden pompano's basal metabolism.

The Na^+-K^+-ATPase (NKA) actively transports Na^+ out and K^+ in animal cells among the transporters that modulate ion fluxes (Post and Jolly 1957), and NKA generally involved in the maintenance of an internal hypo-osmotic state during changes in environmental salinity. NKA activity in the osmoregulatory organ is accompanied by the change of ambient salinity (Hirose et al. 2003; Burg et al. 2007; Marshall 2002). In juvenile golden pompano, after 30 days of the study, the NKA activity of fish was corresponding to the rearing salinity. Compared to the control group, fish reared at the salinities of 18‰ and 10‰ showed higher activity of NKA (Fig. 6.1). This result is consistent with the previous research results (Madsen et al. 1996; McCormick 1995; Morgan et al. 1997). Under low salinity treatment, NKA activity was higher, and SGR was lower, indicating that low salinity of 10–18‰ was not suitable for the physiology of juvenile pompano.

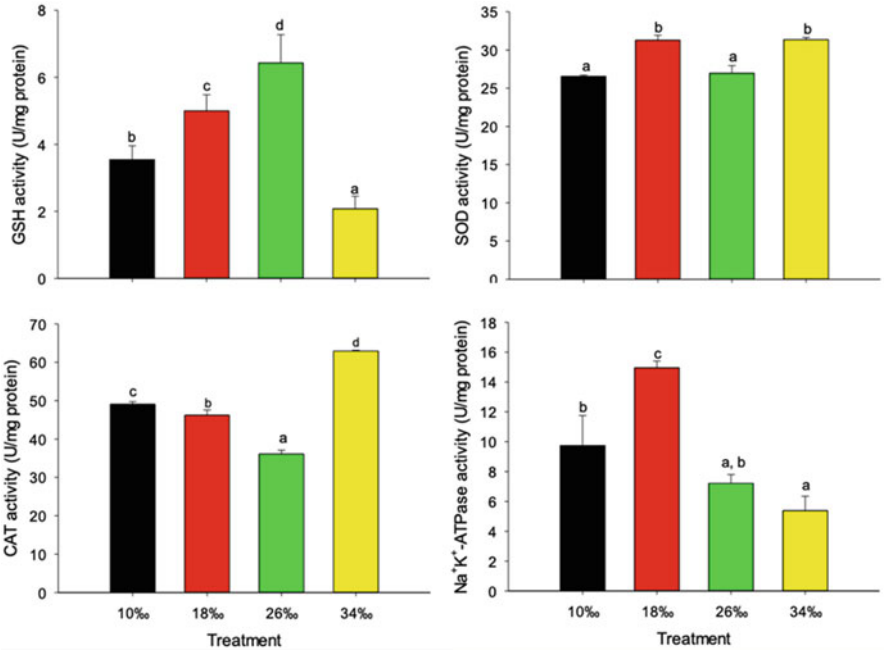

Fig. 6.1 The GSH, SOD, CAT, and Na^+K^+-ATPase activities of juvenile golden pompano cultured at 10‰, 18‰, 26‰, and 34‰ salinities. Different letters represent significant difference ($P < 0.05$) (Ma et al. 2016b)

6.5 Conclusion

Ambient salinity has significant effects on fish growth and RNA/DNA ratio. When the salinity was 34‰, the growth rate and RNA/DNA ratio of fish were higher. The FCR of fish cultured at the salinity of 10‰ was five times higher than the FCR of fish reared at 34‰. The activities of NKA and antioxidant enzymes corresponded with fish survival. Fish have a higher survival rate when salinity is 26‰. Juvenile golden pompano can be raised above 26‰ without affecting the performance of fish, while salinity <18‰ is not suitable for the juvenile golden pompano growth.

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Chapter 7
Physiological Responses of Golden Pompano *Trachinotus ovatus* Larvae Fingerlings in Transportation

Jiawei Hong, Zhenhua Ma, and Tao Zhang

Abstract The golden pompano *Trachinotus ovatus* has an outstanding farming performance and industrial advantages in aquaculture due to its great taste and short production cycles. At present, it has gradually become an important fish in aquaculture around the world. The transportation of golden pompano is a common but a vital component in its aquaculture production operation, especially for fingerlings. The success of fish transportation can affect the production cost to a large extent, which even directly determines the subsequent production profit. Generally, transportation in oxygenated polyethylene bags is the most common method. But in the transportation process from a hatchery to a fish grow-out farm, high mortality has been a serious problem. Up to present, there is no protocol for live transportation to follow based on scientific evidence on this species. Therefore, there is a strong need to know what happens to *Trachinotus ovatus* fingerlings in transportation, which means to understand the physiological responses of fish to water quality changes and stressors during transportation, so that we can determine optimal condition for the transport of live fish. In this chapter, we reviewed the results of previous studies on the physiological response of *Trachinotus ovatus* fingerlings to the transportation conditions and sought after the responding patterns of liver and serum biochemical parameters in fingerlings under transportation stress. Finally, we developed suitable protocols to optimize the transport success of *Trachinotus ovatus* fingerlings.

Keywords Transportation · Physiological responses · Water quality · Liver biochemical parameters · Serum biochemical parameters

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_7

7.1 Introduction

Generally, closed-container and open-container transporting systems are mostly adopted in live fish transportation (Berka 1986; Das et al. 2015; Zhang et al. 2019). The closed-container system is commonly used for fish transportation including golden pompano *Trachinotus ovatus*. The oxygen-filled polyethylene bags are the most common method for live fish transportation (Gomes et al. 2006; Wang et al. 2019). The polyethylene bag is sealed, and the initial environmental condition largely determines the living condition of fish at the end. Meanwhile, it is also more likely to lead to a cumulative effect of some metabolic products excreted by fish compared with the open system with controlled conditions. This cumulative effect can eventually lead to deterioration of water quality. Combining with other stress factors, such as noise, vibration, and damage by handling, the integrated stress could weaken the health status of fish (Gomes et al. 2003; Leggatt et al. 2006; Pan et al. 2010). During transportation, the common methods to reduce stress include using pure oxygen, low temperature, or anesthetic (King 2009; Islam and Hossain 2013), but the lack of a precise and scientifically proven transporting protocol can still cause high mortality. Consequently, it is essential to develop a transporting protocol that is suitable for transportation of golden pompano *Trachinotus ovatus* fingerlings.

When the internal environment inside a fish body is out of balance due to stress, the nervous system and endocrine system will play a vital role to restore harmony inside the fish body by increasing oxygen consumption and immune ability (Nomura et al. 2009). Despite the innate effort of regulation to resume the balance of the internal environment, a disorder of physiological and biochemical functions, impaired immune capacity, and even mortality may occur during transportation (Barton 2002; Tort 2011). Besides, serum biochemical indexes, oxidative stress indexes, and immune parameters are generally used as quantitative indexes of physiological responses to stress on fish. Therefore, in this chapter, we first report our recent research progress on the understanding of transportation duration and the side effects associated with time and then summarize the stress response measured by different physiological parameters. Finally, we address the establishment of transport protocols. This chapter reveals the pattern of liver and serum biochemical parameters of golden pompano fingerlings under transportation stress and provides the theoretical basis to optimize the protocols for fingerling transportation.

7.2 Transportation Time and Associated with Fish Stress

The stressors of transportation include low oxygen, noise, shaking, high density, and physical damage by handling. Every single stressor can induce the physiological response of fish suffering from corresponding one. For example, López-Patiño et al.

(2014) reported that when *Oncorhynchus mykiss* was under the stress of handling, the level of hormone rose in the body, which influences on the production of glycogen and lipid in the liver. Wysocki et al. (2006) placed *Lateolabrax japonicus* and *Cyprinus carpio* in a noise environment, and then the content of cortisol in serum has surged.

High fish density is often used in transportation, but it is the most common source of stress. The physical interaction between fish will increase the risk of body damage to scales and make fish more susceptible to pathogen infections (Braun and Nufier 2014; Ruane and Komen 2003). Simultaneously, high fish density can deteriorate water quality in a closed environment during transportation. Concerning the deterioration of water quality, fish may be infected by disease pathogens during transportation.

It is manifested that stress can be caused by the change of water temperature, low dissolved oxygen, pH fluctuation, and accumulation of carbon dioxide and ammonia (Singh et al. 2004; Urbinati et al. 2004; Gomes et al. 2006). Considering the duration required for fish transportation, we first determined the optimum transporting time of *Trachinotus ovatus* fingerlings by monitoring the variation of some water quality parameters in the process of transportation.

The simulated transportation experiment was carried out with the sealed and oxygenated method. Seven transportation times were set up, namely, 0, 4, 8, 12, 16, 20, and 24 h. Three replicates were set in each group, and the fish density was 9 kg/m^3. The 0 h group was treated for the control. At the end of the experiment, the HACH probe (HACH, HQ40d, USA) was immediately put into the bag to determine pH, DO (dissolved oxygen), and temperature. After that, a 500-mL water sample was immediately taken from each bag and stored in the water sampling bottle for further determination of ammonia nitrogen concentration (NH_3-N). Besides, the survival rate of fish was recorded.

The results showed that the transporting time was significantly correlated with the water quality and fish survival. Temperature showed a trend of decrease first and then increase (Fig. 7.1a), but it was at a low level in the treatment groups than in control. The temperature reached the lowest value of 17.1 °C at 16 h and reached the highest value of 26.1 °C at 24 h. There was no significant change in dissolved oxygen concentration in water over time ($P > 0.05$, Fig. 7.1b). And pH decreased gradually with the time of transportation and reached 6.81 at 24 h (Fig. 7.1c). With the extension of transporting time, the ammonia nitrogen content in the water increased gradually and reached the maximum value of 0.068 mg/L in 24 h (Fig. 7.1d). When the transporting time is less than 8 h, the survival rate of *Trachinotus ovatus* fingerlings was 100%. However, when the transportation time lasted more than 12 h, the survival rate gradually reduced to zero (Table 7.1).

During transportation, the main product from fish respiration is carbon dioxide, which can make the water from weak acid to a gradual decrease of the pH value (Parodi et al. 2004; Paterson et al. 2003). In this experiment, the pH value decreased with the increase of transporting time, which is consistent with the previous research results. In this study, the temperature and fish metabolism will be reduced by putting two 500-mL ice bags in each box (King 2009). The temperature is maintained at a

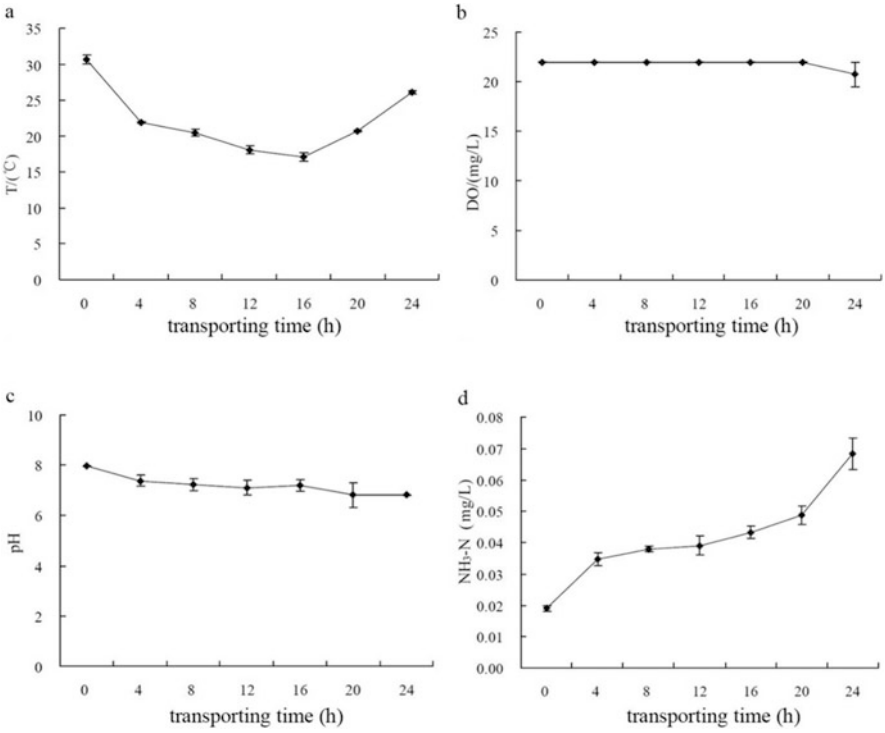

Fig. 7.1 Effects of transporting time on water quality: (**a**) temperature; (**b**) DO (dissolved oxygen); (**c**) pH; (**d**) NH_3-N

Table 7.1 Effects of transporting time on the survival rate of *Trachinotus ovatus* fingerlings

Transporting time	0 h	4 h	8 h	12 h	16 h	20 h	24 h
Survival rate (100%)	100	100	100	50	0	0	0

low level compared with the control even there was a slight rise at a later time. In addition, owing to the addition of pure oxygen to the plastic bags, dissolved oxygen was kept at saturation by high partial pressure in the bag. The DO level was much over the safe threshold for the fish at all time.

In the process of fish transportation, the NH_3-N content usually shows an increasing trend in the water with the extension of transporting time (Zeppenfeld et al. 2014; Bui et al. 2013; Salbego et al. 2015). In this experiment, the content of ammonia nitrogen in the transporting water increased with the increase of time and reached the maximum value after 24-h transportation, which is consistent with the previous results. The death of juvenile fish was related to increased ammonia nitrogen content. If the content of ammonia nitrogen is too high, the fish will be poisoned or even die. It is shown that a low pH value and a high NH_3-N content had a synergistic effect on the reduction of the binding capacity of hemoglobin to

oxygen. When the content of ammonia nitrogen was higher than 0.02 mg/L, it would produce strong toxicity and even endanger the life of juvenile fish. The content of ammonia nitrogen was higher than 0.04 mg/L from 12 h. It is possible that the transportation time is too long, and the excreta and mucus produced by fish metabolism are accumulated, which can be attached to the gills of fish to weaken the oxygen uptake capacity. Overall, according to the evidence gathered above, we recommend that the optimum transporting time of *Trachinotus ovatus* fingerlings be less than 8 h.

7.3 Physiological Responses During Transportation

7.3.1 Responses of Biochemical Parameters in the Liver

The excessive production of reactive oxygen species (ROS) is related to the damage caused by environmental stress. Excessive accumulation of ROS such as H_2O_2, -OH, and O_2^- is toxic to cells, but the system of antioxidant in animals can offset the negative effects of ROC (Elstner 1982). Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are protective enzymes in fish and play a key role in scavenging free radicals (Olsvik et al. 2005).

Lactate dehydrogenase (LDH) is one of the important enzymes involved in glycolysis and the citric acid cycle. High LDH values are generally related with liver disease, myocardial infarction, muscle injury, and blood disease. Generally, the lactic acid levels in muscle are low, but anaerobic metabolism and excessive exercise can cause it to increase.

7.3.2 Responses of Biochemical Parameters in the Serum

The stimulation of stressors can influence the composition of fish serum. The variation of the cortisol content of fish is one of the critical physiological indicators to evaluate stress responses (Fanouraki et al. 2011). When a fish suffers from stress, the hypothalamic–pituitary–adrenal (HTPA) axis rapidly responds and promotes the release of adrenocorticotropic hormone (ACTH), thus regulating the synthesis and release of cortisol hormone (Acerete et al. 2004; Kubokawa et al. 1999; Oyoo-Okoth et al. 2011). Meanwhile, under stress, an increase of cortisol secretion may indirectly lead to the formation of glucose (Lays et al. 2009). The increase of serum cortisol and glucose of fish is an important manifestation of stress response during transportation (Benovit et al. 2012; Robertson et al. 1988). In addition, the changes in creatinine and some enzymes can also help evaluate the stress response of fish.

Creatinine (CRE) which is a metabolite of muscle tissue can be excreted by the kidneys. It will accumulate in the body when kidney function is damage. Thus, in a

clinic, CRE detection is the standard method in diagnosing kidney disease (Valarmathi and Azariah 2003).

Glutamic pyruvic transaminase (GPT) is mainly synthesized in the liver and is one of the visible indicators of liver damage. When the liver function is impaired or pathological changes occur, GPT is continuously released into the blood, resulting in a continuous rise of GPT in serum. Glutamate oxaloacetate transaminase (GOT) is mainly found in myocardial cells and can be used to assess whether the myocardium is damaged. When cardiomyocytes are damaged, or their permeability increases, GOT is released into the blood (Costas et al. 2011).

7.4 Establishing Transportation Protocols Based on Physiological Responses

7.4.1 Fish Density

To assess the impact of fish density in the transportation of *Trachinotus ovatus* fingerlings, we used three densities (D1 = 9 kg/m^3, D2 = 12 kg/m^3, D3 = 15 kg/m^3) during the period of 8 h. The operating method of simulated transportation is the same as above. After the trail, T-SOD, LDH, CAT, and POD activities in the liver, CRE, GPT, and GOT activities in the serum were determined by commercial assay kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). Cortisol (COR) was determined by the cortisol assay kit (Jiangsu Kurt Biotechnology Co., Ltd., Yancheng, China) in accordance with the manufacturer's protocols. Glucose (GLU) was determined by a glucose assay kit (Shanghai Shengrong Biology Pharmacy Co., Ltd., Shanghai, China).

In liver tissues, there was no significant difference in the T-SOD activity of D1, D2, and D3 ($P > 0.05$, Fig. 7.2), while there was a significant difference in the T-SOD activity of D3 compared with the control ($P < 0.05$). The LDH activity of experimental groups was significantly higher than that of the control group (fish in the recirculating system, 12 kg/m^3), and there was no significant difference between D2 and D3. No significant difference was seen in the POD activity among D1, D2, and D3 groups, but all of them were significantly lower than in control. The CAT activity of the three groups was significantly lower than that of the control, and it did not show any significant difference between these three densities. As we can see, the density stress has led to the activation of the antioxidant system and an increase of CAT activity, but the stress was not high enough to cause any obvious response. In addition, LDH activity was proved to have an upward trend since the density of 9 kg/m^3 has imposed stress on fish. It seems that the stress effect has been reflected via the change of liver functions.

In the serum, the content of CRE in groups D1 and D2 was significantly lower than that in the control group, but the content of CRE in group D3 was significantly higher than that in the control group (Fig. 7.3). The serum COR levels in D1, D2, and

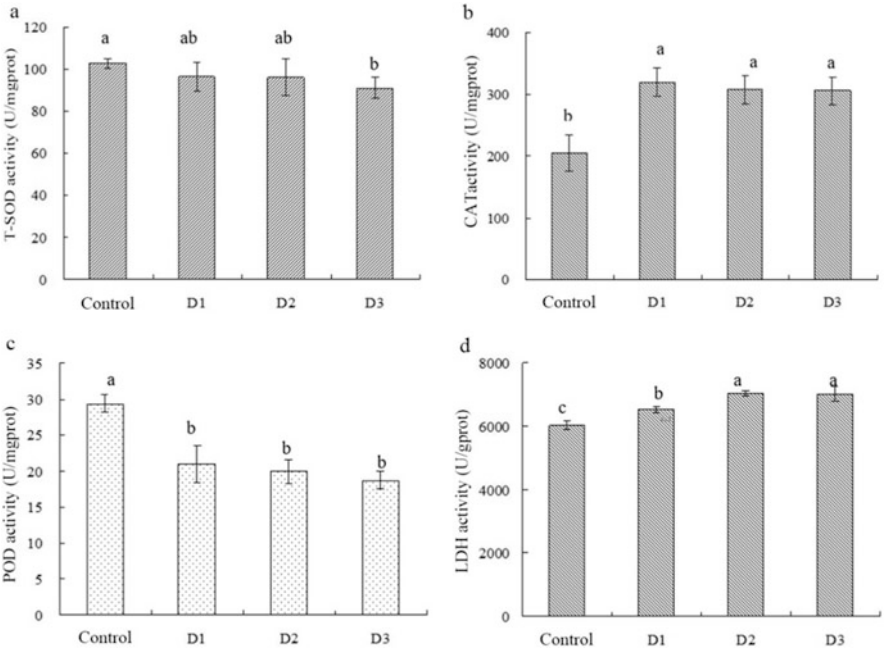

Fig. 7.2 The activities of T-SOD (**a**), CAT (**b**), POD (**c**), and LDH (**d**) of liver after transported at three fish densities: 9 kg/m^3 (D1), 12 kg/m^3 (D2), and 15 kg/m^3 (D3). Take fish in the recirculating system as control (control) (Liu 2019)

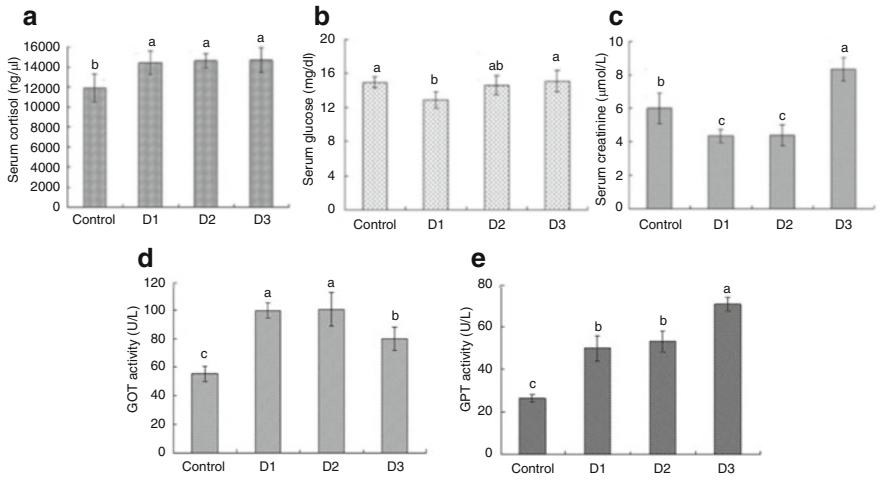

Fig. 7.3 Content of serum cortisol (COR) (**a**), content of glucose (GLU) (**b**), content of creatinine (CRE) (**c**), GOT (**d**), and GPT (**e**) activity under three transportation densities (Liu 2019)

D3 groups are not significantly different, but significantly higher than those of the control group. The content of GLU in D2 and D3 was not significantly different from that in control, while D1 was significantly lower than that in control. The activities of GPT and GOT in D1, D2, and D3 groups were significantly higher than those in control. Meanwhile, the GPT activity in D1 and D2 groups was significantly lower than that in group D3, but GOT activity was significantly higher than that in group D3. In brief, except for GLU, other indexes measured in the serum are all stimulated to rise. Considering the 100% survival rate at the end, the suitable transport density of *Trachinotus ovatus* fingerlings is set to 15 kg/m^3 within 8 h of using cryogenic, airtight, and oxygenated transportation.

7.4.2 Eugenol Anesthetic Concentration

Following up the density treatment, we tested the effect of different eugenol concentration (C1 = 5 mg/L, C2 = 7 mg/L, C3 = 9 mg/L) on physiological responses of *Trachinotus ovatus* fingerlings after transportation for 8 h at the density of 15 kg/m^3. The operating method of simulated transportation and the indexes measured were the same as above.

The serum LDH and T-SOD activities in control were significantly higher than those in other treatment groups ($P < 0.05$, Fig. 7.4). The CAT activity increased first and then decreased until that in the C3 group was significantly lower than the control ($P < 0.05$). The POD activity increased with the increase of eugenol concentrations. In the experiment, the liver T-SOD activity of the control was significantly higher than that of the treatment groups. Meanwhile, the CAT activity of treatment groups showed a downward trend, indicating that eugenol can alleviate the stress response of *Trachinotus ovatus* during transportation. Besides, when eugenol was applied to fish, the POD activity increased to eliminate excessive free radicals and reduce the oxidative stress damage to the body.

In the liver tissue, there was no significant difference in the CRE content between the control and C1 groups ($P > 0.05$, Fig. 7.5), and the counterpart in other groups increased significantly with the increase of eugenol concentration ($P < 0.05$). The COR content increased first and then decreased and was higher than the control. In contrast with COR, GLU decreased first and then increased, and both were lower than the control. The activities of GPT and GOT decreased first and then increased. After 8 h of transportation, the deterioration of water quality and the stimulation of eugenol may cause stress response to *Trachinotus ovatus*, resulting in a significant increase of serum CRE and COR. When the concentration was at a low level, the sedative effect of eugenol and the decrease of water temperature calmed down the fish, and the energy consumption of the body was decreased. Meanwhile, the blood circulation and fish metabolism also slowed down, resulting in a decrease of GLU content in the serum and a decrease of GPT and GOT activities.

Based on the experimental evidence, the concentration of eugenol should not exceed 7 mg/L within 8 h when *Trachinotus ovatus* fingerlings are transported under

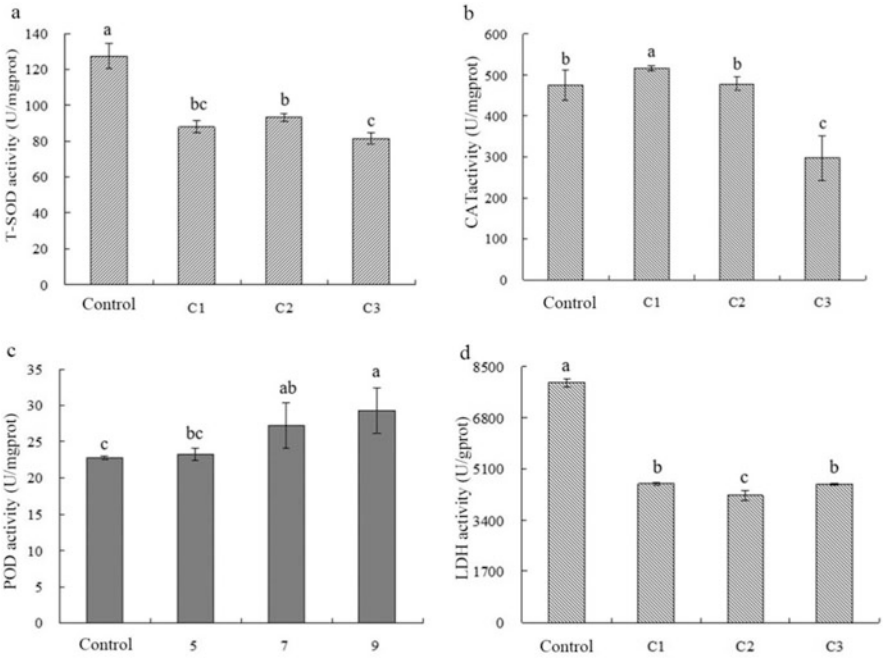

Fig. 7.4 T-SOD (**a**), CAT (**b**), POD (**c**), and LDH (**d**) activity of liver after transported at three eugenol concentrations: 5 mg/L (C1), 7 mg/L (C2), and 9 mg/L (C3). Take fish in the recirculating system as control (control) (Liu 2019)

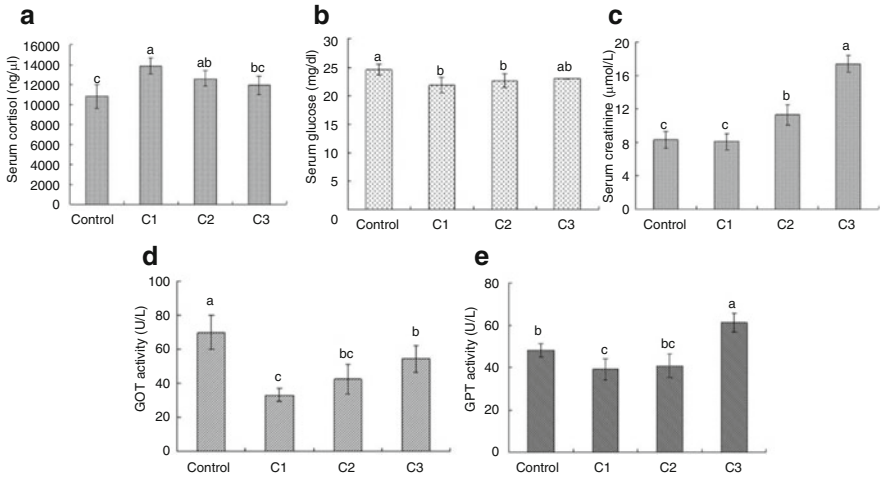

Fig. 7.5 Content of serum cortisol (COR) (**a**), content of glucose (GLU) (**b**), content of creatinine (CRE) (**c**), GOT (**d**), and GPT (**e**) activity under three eugenol concentrations (Liu 2019)

the conditions of anesthesia, low temperature, and airtight oxygenation at the density of 15 kg/m^3.

7.4.3 *Fish Density Under Eugenol Anesthetic in Transportation*

After identifying the optimal concentration of eugenol at 7 mg/L, we further tested the fish response using three densities (TD27 = 27 kg/m^3, TD54 = 54 kg/m^3, TD81 = 81 kg/m^3) under eugenol anesthetic for a transportation period of 8 h.

The liver T-SOD activity of TD54 and TD81 groups had no significant change, while the liver T-SOD activity of TD27 group was significantly decreased compared with the control group (RL, fish in the recirculating system, 54 kg/m^3) (Fig. 7.6). The activity of CAT was significantly different among the three density groups. With the increase of fish density, the CAT activity gradually increased but still significantly lower than the control. In contrast, the activity of POD showed an opposite trend with CAT. The activity of POD decreased gradually with the increase of fish density. Meanwhile, the activity of POD was higher than that of RL group. But the activity of LDH in the process of transportation is not affected by fish density significantly. In our study, antioxidant enzyme activity was not affected by transportation

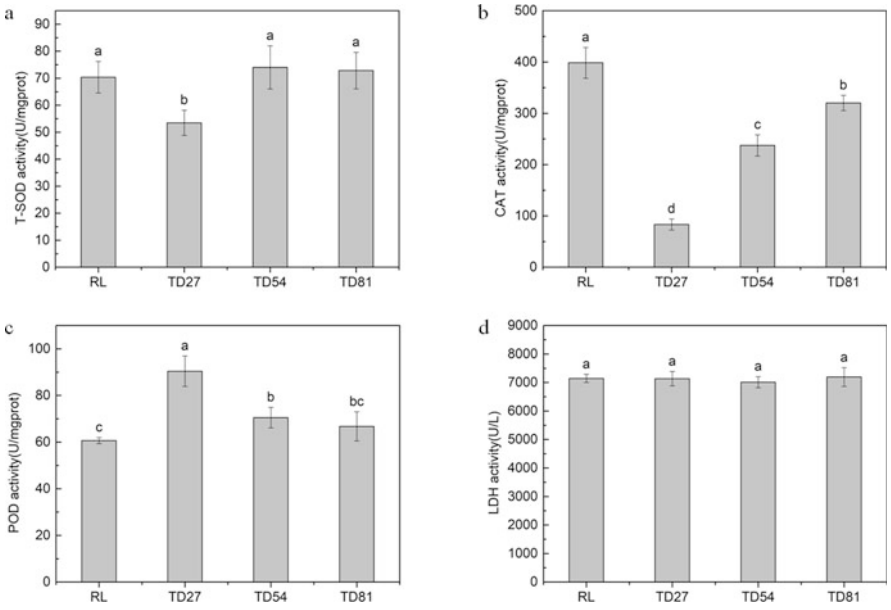

Fig. 7.6 The activity of T-SOD (**a**), CAT (**b**), POD (**c**), and LDH (**d**) in liver after transported at three fish densities: 27 kg/m^3 (TD27), 54 kg/m^3 (TD54), and 81 kg/m^3 (TD81). Take fish in the recirculating system as control (RL) (Hong et al. 2019)

significantly. The production of superoxide dismutase (SOD) is considered to be the first step to deal with oxyradicals. CAT and POD follow, converting the resulting free radicals (H_2O_2) into water and oxygen. The T-SOD showed that none of the all treatment had a significant response to the first defense of oxidative toxicity. Conversion of H_2O_2 was enough to stimulate POD and CAT to produce a certain degree of response. Contrary to the trend, CAT and POD are responsible for converting H_2O_2 into water and oxygen under a low level of stress, while CAT increases with the increase of stress to compensate for part of the POD function.

The content of creatinine in the serum was influenced significantly after 8-h transportation ($P < 0.05$, Fig. 7.7). Compared with the control group, the creatinine of serum of fish in three density groups was significantly decreased and gradually increased with the increase of density. The content of serum cortisol increased after transportation, but there was no significant difference among the three densities ($P > 0.05$). Serum glucose of fish in all three density groups was lower than that in RL group. The glucose content of these three treatments was slightly increased, but the glucose content of fish in TD81 group was significantly higher than that in TD27 group. The variation of serum GPT and GOT activities were similar, and the three transport density groups were higher than the control group. However, with the increase of fish density, GPT and GOP values gradually decreased, and the GPT and GOP values of TD81 treatment were the lowest. During transportation, the content of glycogen decreased, while the content of lactic acid increased. However, our results are inconsistent with previous studies. This may be due in part to the mitigating effects of eugenol addition and ambient low temperatures. In addition, transport stress led to a significant increase of serum cortisol, which is consistent with the previous study results on other teleost fishes. Manuel et al. (2014) found that the African sharp tooth catfish *Clarias gariepinus* increased serum cortisol four to five times after transportation stress. Gomes et al. (2006) found that a higher transport density led to higher mortality and serum cortisol in juvenile tambaqui *Colossoma macropomum*.

Congleton et al. (2000) also found that when the transport density reached its seasonal maximum (55–65 g/L), the cortisol of juvenile chinook salmon (*Oncorhynchus tshawytscha*) increased significantly. The physiological responses of different fish species under transport stress depend on species (Barton et al. 2003), which may explain the discrepancy between our results and other studies.

Increased fish serum cortisol and glucose stress reaction in the process of transportation are common (Robertson et al. 1988; Benovit et al. 2012). Although in our study, the blood glucose level of fish with three densities increased during transportation, it was significantly lower than that of the control group, indicating that low temperature and anesthesia treatment can reduce basal metabolism and improve the survival rate of fish during transportation. As for creatinine, with the increase of fish density led to a gradual accumulation of creatinine of fish, but the content of serum creatinine in three density groups was still lower than in the control group. This indicates that transportation stress has no effect on the kidney function of fish species at the design density, but the density of fish can affect the serum creatinine level. In addition, the GOT and GPT of the three transportation densities were higher than

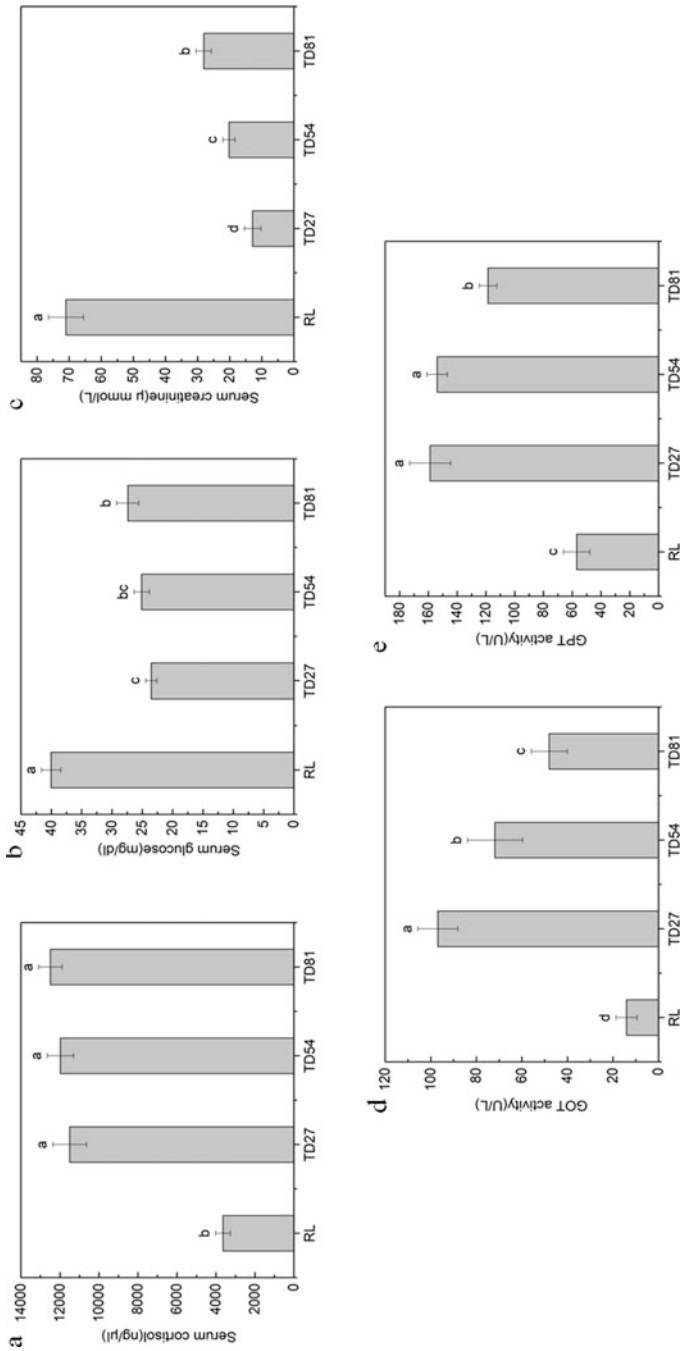

Fig. 7.7 The content of serum cortisol (COR) (**a**), the content of glucose (GLU) (**b**), the content of creatinine (CRE) (**c**), GOT (**d**), and GPT (**e**) activity under three transportation densities (Hong et al. 2019)

those of the control fish, indicating that the liver function of the three transportation densities was affected to some extent.

Therefore, based on the results of the present study, the transport density of *Trachinotus ovatus* larvae can be increased to 81 kg/m^3 within a transportation period of 8 h under anesthesia, cryogenic, airtight, and oxygenation.

7.5 Conclusion

Based on the evidence of physiological responses of *Trachinotus ovatus* fingerlings to transportation conditions, the following conclusions can be drawn: (1) Optimal transporting time of *Trachinotus ovatus* fingerlings is less than 8 h, (2) optimal transporting density of *Trachinotus ovatus* fingerlings is less than 15 kg/m^3, (3) optimal concentration of eugenol is 7 mg/L when eugenol is added as an anesthetic, and (4) transported for 8 h, with eugenol addition as an anesthetic at 7 mg/L. The optimal transporting density of *Trachinotus ovatus* fingerlings can reach 81 kg/m^3.

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Chapter 8
Transcriptional Response of Golden Pompano *Trachinotus ovatus* Larvae to Temperature

Mingyang Han, Zhengyi Fu, Zhenhua Ma, and Gang Yu

Abstract The molecular response pattern of golden pompano *Trachinotus ovatus* larvae under temperature stress is reviewed in this chapter. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that a large number of differentially expressed genes (DEGs) were related to metabolic processes, protein synthesis, and nutrient digestion and absorption. The differentially expressed genes were most highly enriched into the pathways, including protein digestion and absorption, fat digestion and absorption, starch and sucrose metabolism, vitamin digestion and absorption, and glutathione metabolism. High temperature may inhibit the secretion of type II collagen by downregulating the expression of Shh and Sox9a genes. This chapter advances our understanding of the response mechanism to high temperature in *Trachinotus ovatus* and guides relevant production practices in hatcheries.

Keywords Transcriptional response · Temperature stress · Skeletal development · *Trachinotus ovatus*

8.1 Introduction

Environmental factors are essential to the success of aquatic animal breeding. Temperature is one of the most vital environmental factors in fish since it plays a profound and controlling role in the growth, reproduction, metabolism, feeding behavior, and all metabolic processes of fish (Katersky and Carter 2005; Ma 2014; Somero 2010; Yang 2016). Although fish can deal with temperature changes by extensive biochemical, metabolic, and physiological acclimations within a suitable range (Xu et al. 2018), the inappropriate temperature can still have an adverse

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_8

impact. Low temperature may significantly reduce the growth rate and the activity of digestive enzymes in fish (Tang et al. 2018). A high or low temperature may lead to a high mortality rate for fish, whose physiological process is severely disrupted (Griffiths and Harrod 2007; Ibarz et al. 2010). Besides, the inappropriate temperature may also cause disease outbreaks because the immune functions of fish are affected by temperature (Chen et al. 2002; Ndong et al. 2007). Previous studies have revealed that the outbreaks of disease in aquaculture caused by bacteria are linked to rising ambient temperatures (Matanza and Osorio 2018). During the early development of fish larvae, temperature plays a vital role because fish larvae are more sensitive to the environment. The change of water temperature may lead to abnormal growth rate, low survival rate, and high deformity rate of fish larvae (Ornsrud et al. 2004a, b; Yang et al. 2016). With the vigorous development of industries and human activities, the frequency of abnormal changes in the environment caused by human intervention increases. The intensification of global warming makes the aquaculture industry face significant challenges. Some reports have shown that commercial farming of fish such as tilapia (*Oreochromis niloticus*) (Zerai et al. 2010), common carp (*Cyprinus carpio* L.) (Sun et al. 2019), and red seabream (*Pagrus major*) (Hwang et al. 2012) is adversely affected by temperature stress. The temperature-orientated stress has brought substantial economic losses to the aquaculture industry.

The golden pompano (*Trachinotus ovatus*) belongs to the family of Carangidae and is widely distributed in Asia-Pacific regions. The golden pompano is a popular and commercially valuable fish, which is broadly cultured in the coastal area of South China in recent years owing to fast growth and suitability for cage culture (Lin et al. 2012; Ma et al. 2014; Tan et al. 2016). The fry cultivation of golden pompano is usually carried out in spring and summer. There is a peak period of death between 3 and 7 days post-hatch (DPH) of golden pompano, especially during the first few days after initial feeding, but the mortality rate is generally less than 20% (Ou and Li 2017). It has been reported that the increase in water temperature results in an increase in mortality of the larvae at this stage (Wang et al. 2011). Furthermore, the experience of environmental adaptation in the early life of fish can subsequently alter their phenotypes and physiological responses to future environmental change (Scott and Johnston 2012). Due to climate change caused by global warming, water temperature and salinity in the shallow coastal water and intertidal zones are liable to fluctuate. In Hainan Province, the fry cultivation is mostly carried out in outdoor ponds, which was more susceptible to climate change, especially after several days of continuous high temperature. However, little is known about the responding mechanism of golden pompano larvae to the temperature change at this stage. As a species of warm-water fish, the growth and survival of golden pompano larvae are strongly dependent on water temperature, but the biochemical and molecular studies on the response to temperature stress are limited to a few proteins or genes (Allais et al. 2019; Ma et al. 2016a, b, c, 2017a, b).

Transcriptomics refers to the study of all transcripts of a particular cell, tissue, or organism at a particular stage of growth or development, or under a certain physiological condition (Wang et al. 2009). RNA-Seq is a method of transcriptome analysis using deep sequencing technology, allowing for a more efficient and clear

understanding of the entire transcriptome, including non-model organisms that lack complete genome sequencing (Ekblom and Galindo 2011; Qian et al. 2014). With the rapid development of the transcriptome technology, the emergence of RNA-Seq technology based on high-throughput sequencing provides a new and convenient method with a lower cost to understand various biological processes in fish and is now increasingly used in different areas of aquatic biology (Eissa and Wang 2016; Mu et al. 2014; Qian et al. 2014). A growing number of studies have used this technique to analyze the temperature stress response of fish, such as *Cyprinus carpio* (Sun et al. 2019), *Oreochromis niloticus* (Zhou et al. 2019), *Pagothenia borchgrevinki* (Bilyk and Cheng 2014), *Lates calcarifer* (Newton et al. 2013), *Acanthochromis polyacanthus* (Veilleux et al. 2015), and *Melanotaenia duboulayi* (Smith et al. 2013). The responses of extreme eurythermal and stenothermal fish species to acute and long-term exposure to temperature are gradually revealed (Logan and Buckley 2015). A large number of differentially expressed genes related to metabolism, development, and immunity under temperature stress are obtained. However, at the same time, the results also show that the mechanism by which fish respond to temperature stress is complex. Therefore, it is imperative to explore the response mechanism of different fish to temperature stress.

At present, the transcriptome responses of *T. ovatus* juveniles to different nutritional conditions have been studied (Lei et al. 2020; Liu et al. 2019), but the transcriptome responses of *T. ovatus* larvae to temperature have not been reported. This chapter addresses temperature stress on transcriptome responses in *T. ovatus* larvae by using RNA sequencing technology. The results will help improve the understanding of the molecular mechanism and biological basis of fish adaptation to temperature variation.

8.2 The GO and KEGG Analysis of Differentially Expressed Genes (DEGs) at Different Temperatures

This chapter discusses the transcriptome responses of *T. ovatus* to temperature based on our previous studies. The GO and KEGG analyses were conducted to determine the biological functions and pathways that are significantly correlated with DEGs at different water temperatures. DEGs were significantly enriched with 147 GO terms between the low temperature (LT) group and the medium temperature (MT) group, including 88 terms for biological processes, 30 terms for molecular functions, and 29 terms for cellular components. “Cellular component biogenesis” was the most significantly enriched terms in the biological process. “Organelle,” “intracellular organelle,” and “cytoplasm” were the predominant GO terms in the cellular component. The top three significantly enriched GO terms in the molecular function were “hydrolase activity,” “cation binding,” and “metal ion binding” (Fig. 8.1a).

Between the HT group and MT group, there were 152 significantly enriched GO terms, including 105 terms in biological process, 43 terms of molecular functions, and 4 terms in the cell components. In the biological process, “metabolic process,”

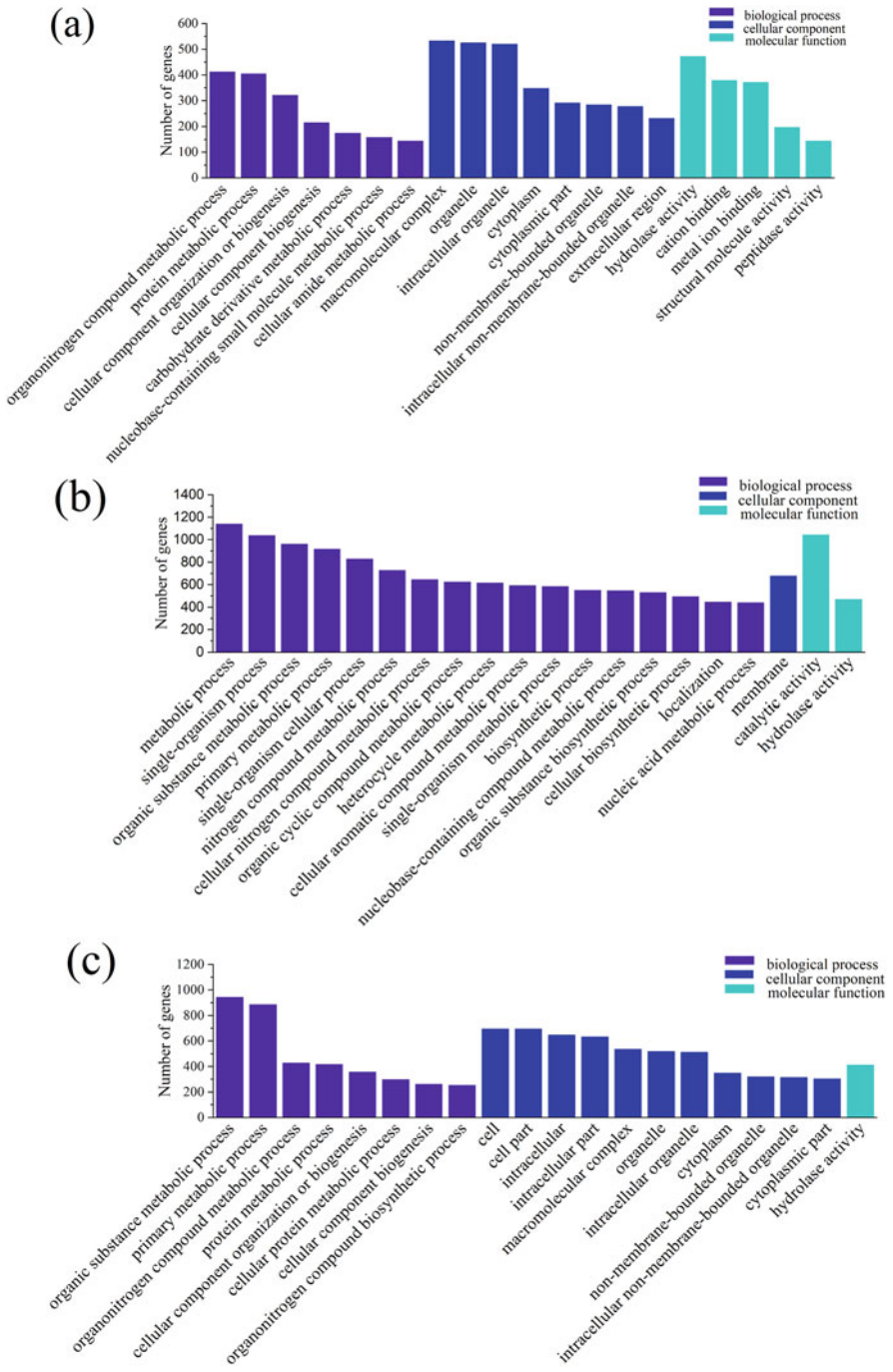

Fig. 8.1 GO classification of the DEGs. *BP* biological process, *CC* cellular component, *MF* molecular function. (**a**) LT group versus MT group; (**b**) HT group versus MT group; (**c**) LT group versus HT group

"single-organism process," "organic substance metabolic process," and "primary metabolic process" were the predominant terms. In the cellular component, "membrane" was the most significant terms. In the molecular function, "catalytic activity," "hydrolase activity," and "oxidoreductase activity" were dominant (Fig. 8.1b).

In comparing the LT group and the high temperature (HT) group, there were 164 significantly enriched GO terms, while 103 terms were enriched significantly in the biological process, 33 terms in the cell components, and 28 terms in the molecular functions. The GO terms of the "organonitrogen compound biosynthetic process," "protein metabolic process," and "cellular component organization or biogenesis" were the predominant terms in the biological process. "Macromolecular complex," "organelle," and "intracellular organelle" were the predominant GO terms in the cellular component. "Hydrolase activity" was the most significantly enriched terms in molecular function (Fig. 8.1c).

As for the KEGG pathway analysis, the top 20 pathways with the most significant enrichment were selected and displayed in Fig. 8.2. In the comparison between HT group and MT group, DEGs were enriched into 245 metabolic pathways. The significantly enriched pathways included protein digestion and absorption, chemical carcinogenesis, *Staphylococcus aureus* infection, metabolism of xenobiotics by cytochrome P450, drug metabolism–cytochrome P450, fat digestion and absorption, drug metabolism and other enzymes, starch and sucrose metabolism, retinol metabolism, vitamin digestion and absorption, and glutathione metabolism. Between the LT group and HT group, DEGs were enriched into 256 metabolic pathways, and 4 pathways were enriched significantly in the ribosome, phototransduction, cardiac muscle contraction, and protein digestion and absorption. Between the LT and MT groups, DEGs were enriched into 261 metabolic pathways, including significantly enriched pathways of the ribosome, phototransduction, cardiac muscle contraction, and protein digestion and absorption.

8.3 Effects of Water Temperature on Transcription, Translation, Protein Folding, and Degradation-Related Genes

Understanding the response of organisms to environmental changes in different ontogenetic periods is of great significance for ecological protection and sustainable development of related industries. Existing research points out that the potential threat of global warming to tropical species or warm-adapted species may be more prominent because their living water is close to the upper limit of the thermal tolerance (Pérez-Portela et al. 2020; Tomanek 2010). The aggravation of global warming and high temperature in summer will pose a potential threat to the farming regions of *T. ovatus*.

All organisms must respond to environmental stress (Buckley and Huey 2016; Logan and Buckley 2015). Under a stress condition, heat shock proteins (HSPs) play a vital role in maintaining cellular homeostasis (Deane and Woo 2011; Kayhan and

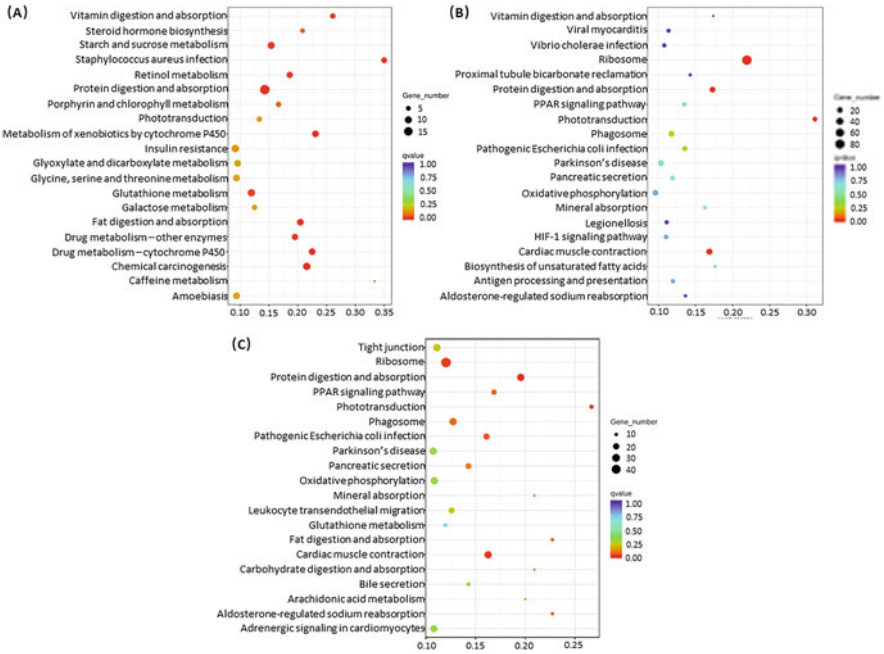

Fig. 8.2 KEGG pathways of DEGs. (**a**) HT group versus MT group; (**b**) LT group versus HT group; (**c**) LT group versus MT group

Duman 2010). For instance, HSP70 proteins are one of the most ubiquitous classes of chaperones, which have crucial functions in protein folding, maintenance of protein homeostasis, and enhancement of cell survival following a multitude of stresses (Murphy 2013). HSP90α is involved in the activation and maintenance of a wide range of regulatory and signaling proteins of cell proliferation, which is vitally important for maturation and activation of proteins (Neckers 2007). Many studies have indicated that thermal stress can increase in the expression level of HSPs in fish (Deane and Woo 2011; Ojima et al. 2005). The transcription level of *HSP70* was the highest in the MT group, and the lowest expression was observed in the HT group. Meanwhile, the transcription levels of *HSP90α* were downregulated with the increase of temperature (Fig. 8.3). This may indicate that when water temperature exceeds a certain range, the refolding efficiency of HSPs may not be high enough, or the ability of a larva to prevent protein damage and misfold through molecular chaperones such as HSPs may be inhibited (Parsell and Lindquist 1993). Another explanation may be that the increase in the synthesis of HSPs is completed, as the turnover of mRNA for HSPs is relatively rapid compared to the protein itself (Buckley et al. 2006; Logan and Buckley 2015).

In thermal stress, activation of other mechanisms, such as ubiquitin-mediated proteolysis, is often required to promote the degradation of damaged proteins and thus maintain cell homeostasis (Hatakeyama and Nakayama 2003; Kultz 2005;

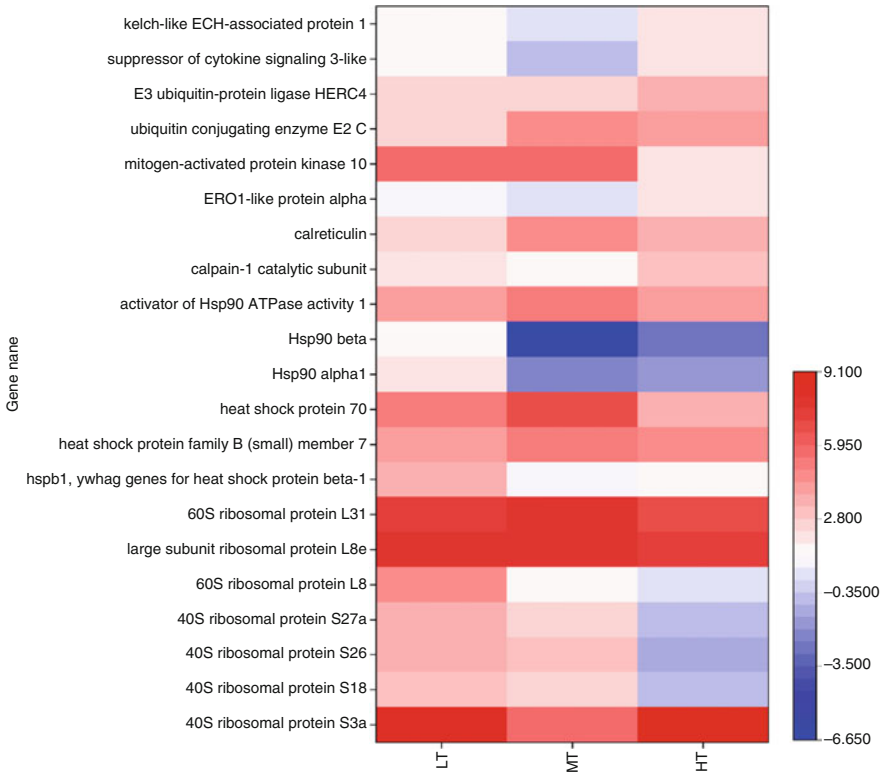

Fig. 8.3 Heat map of the DEGs related to transcription, translation, protein folding, and degradation of *T. ovatus* larvae at different temperatures. Log_2(RPKM) value for each gene has been taken the average of three values and is shown using a color scale

Pickart and Eddins 2004). Some genes belonging to the ubiquitin-mediated proteolysis pathway are upregulated in the MT group and the HT group, including ubiquitin-conjugating enzyme E2C, ubiquitin-protein ligase E3, suppressor of cytokine signaling 3-like, and kelch-like ECH-associated protein 1 (Fig. 8.3). At the same time, with the increase of temperature, many DEGs were enriched in the GO terms related to peptidase activity and hydrolase activity. This also indicates that the water temperature of 32 °C may harm *T. ovatus* larvae.

Compared with the LT group, DEDs enriched into the ribosome pathway were downregulated in the MT and HT groups (Fig. 8.3). The ribosome is the site of protein synthesis, and its primary function is to promote the mutual recognition of mRNA and rRNA and translate the nucleotide sequence on mRNA into the amino acid sequence on the polypeptide chain (Derenzini et al. 2017). Ribosomal protein is an essential component of ribosomes and plays a crucial role in the synthesis of proteins in cells. The results of this study are similar to those of Liu et al. (2013) who show that the ribosomal protein gene in gills of catfish treated with high temperature

was significantly inhibited. These results suggest that the general protein synthesis of the larvae may be inhibited under a too high temperature (Buckley et al. 2006). The study of Cai et al. for *Lateolabrax maculatus* similarly suggests that high temperature may decrease anabolism of protein (Cai et al. 2020).

8.4 Effect of Water Temperature on Metabolism-Related Genes

The metabolic response of *T. ovatus* larvae was significantly affected by high temperature. As a fundamental property of all organisms, metabolism is considered to be highly dependent on temperature and determine the rates of resource uptake or allocation to growth and reproduction (Ohlberger et al. 2012), and the effect of temperature on organism metabolism is species-specific. According to the research of Zheng et al. (2019), heat stress could lead to downregulation of transcription levels of some key enzymes in the glucose metabolism pathway and upregulation of enzymes related to fat metabolism in *Marsupenaeus japonicus*. In the heat stress study of zebra fish, the metabolic rate also increases with temperature (Vergauwen et al. 2010). The GO enrichment analysis showed that a large number of DEGs were enriched into metabolism-related items, including "metabolic process," "organic substance metabolic process," "primary metabolic process," "heterocycle metabolic process," "organic cyclic compound metabolic process," "organonitrogen compound metabolic process," "ATP metabolic process," "protein metabolic process," and "small molecule metabolic process." KEGG analysis showed that the pathways of starch and sucrose metabolism were significantly influenced at high temperature. In the HT group, the transcriptional levels of alpha-glucosidase, maltase-glucoamylase, and UTP-glucose-1-phosphate uridylyltransferase were significantly upregulated (Fig. 8.4). These results indicate that the metabolic activity of the larva under a high-temperature environment would be significantly enhanced. Compared with MT group, the "metabolism of xenobiotics by cytochrome P450," "drug metabolism–cytochrome P450," "drug metabolism and other enzymes," and "glutathione metabolism" pathway were significantly affected. The DEGs enriched to the glutathione metabolic pathway were all formed by upregulated genes (Fig. 8.4). Activation of the glutathione metabolic pathway can protect cells from oxidative damage and provide reducing substances for maintaining the internal environment of cells to resist high-temperature stress (Tate and Meister 1981). These results indicate that the larvae in the 32 °C water are more susceptible to stress from external substances.

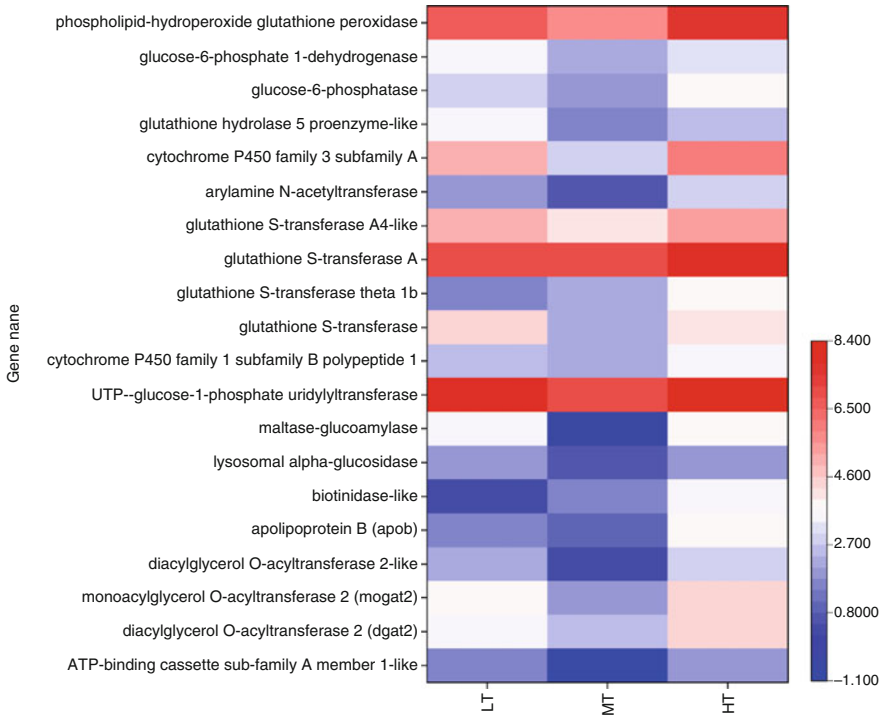

Fig. 8.4 Heat map of the DEGs related to the metabolism of *T. ovatus* larvae at different temperatures. Log_2(RPKM) value for each gene has been taken the average of three values and is shown using a color scale

8.5 Effects of Water Temperature on Genes Related to Digestion and Absorption

The first exogenous feeding *T. ovatus* usually starts at 3DPH, and the oil balls disappear completely at 7DPH (Ou and Li 2017). The larvae then transform from endogenous nutrition to exogenous nutrition. At this stage, adequate nutrition and energy are essential for the growth and survival of fish larvae. Temperature can affect the activity of digestive enzymes and the digestion efficiency of nutrient in fish (Yufera et al. 2019). With the increase of temperature, a large number of DEGs were enriched in pathways related to the digestion and absorption of nutrients, such as protein digestion and absorption pathway, fat digestion and absorption pathway, and vitamin digestion and absorption pathway. With the increase of temperature, the transcriptional levels of the trypsin (*PRSS*), chymotrypsin-like protease (*CTRL*), and carboxypeptidase (*CPA*, *CPB*) genes in this pathway were significantly downregulated, while the pepsin A (PGA) genes were significantly upregulated (Fig. 8.5). This may indicate that the increase in temperature enhanced the digestion of protein in the stomach of fish larvae. The DEGs enriched to the fat digestion and

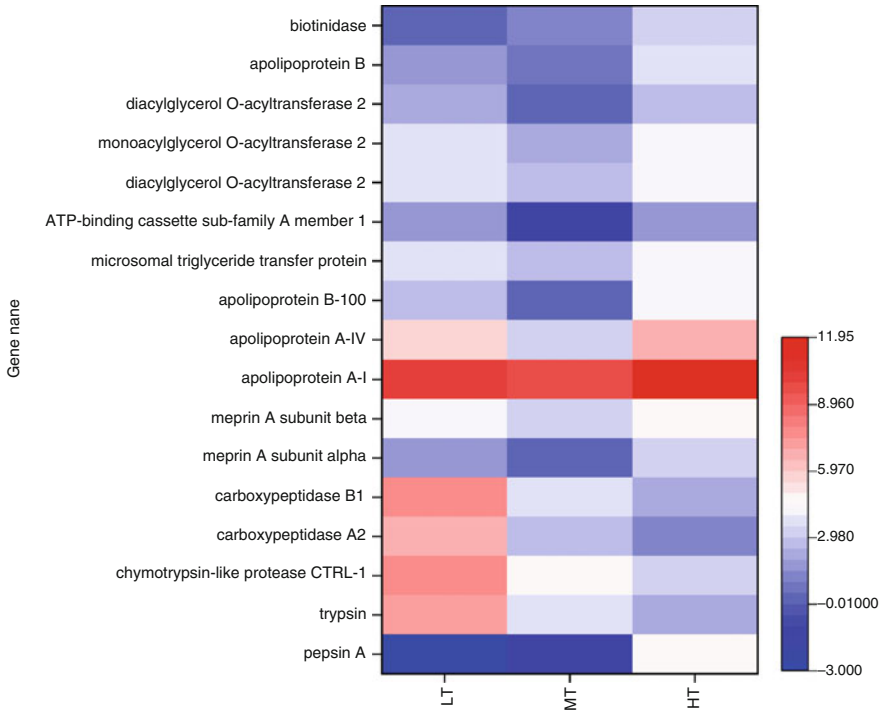

Fig. 8.5 Heat map of the DEGs related to digestion and absorption of *T. ovatus* larvae at different temperatures. Log_2(RPKM) value for each gene has been taken the average of three values and is shown using a color scale

absorption pathway were all formed by upregulated genes (Fig. 8.5). This may indicate that high temperature promotes the fat utilization of the larva to meet the higher energy requirement at high temperature. Notably, vitamin digestion and absorption pathway and retinol metabolism pathway were also significantly affected, indicating higher vitamin requirements at this high temperature. The results demonstrate at the molecular level that high temperatures may cause a significant increase in the nutritional need of *T. ovatus* larvae, which is consistent with the view of Cahu et al. (2003).

8.6 Effects of Water Temperature on Bone Development-Related Genes

The temperature can affect the bone development of the larva and even lead to bone malformation by promoting or inhibiting the expression of some genes related to bone development. Bone morphogenetic proteins (BMPs), the vital growth factors in transforming growth factor beta (TGF-β) superfamily, regulate the growth and

differentiation of osteoblasts and chondrocytes (Bragdon et al. 2011; Marques et al. 2016). For instance, BMP2 and BMP4 are involved in differentiation of chondrocytes to form cartilage (Mei and Xu 2005). Previous studies have shown that the expression of BMP2 and BMP4 in fish is upregulated due to the increase in temperature (Ma et al. 2016a, b, c). However, in the study of Han et al. (2020), although the expressions of BMP2 and BMP4 showed an upregulated trend, the differences in their expressions under different temperature treatments were not significant, which may be due to the single sampling event only at 8DPH (Fig. 8.6). The Sox gene family is a group of transcription factors that exhibit extensive spatiotemporal expression patterns during early development in vertebrates, involving many developmental processes and gender determination (Russell et al. 1996). Sox9 is the main gene that regulates chondrogenic differentiation and is found in all chondrogenic progenitor cells and chondrocytes except hypertrophic chondrocytes in mouse embryos (Akiyama 2008). Ytteborg et al. (2010) reported that the increase of temperature would result in the downregulation of sox9 gene in Atlantic salmon, thus inducing vertebral deformity. The study of Ma et al. (2017a, b) on *T. ovatus* also found that sox9 expression was downregulated at high temperature. In the study of Han et al. (2020) at 32 °C, the expression of sox9a was significantly lower than at 24 °C and 28 °C, which is in line with previous studies (Fig. 8.6).

Proteins in the Hedgehog (Hh) gene family of vertebrates act in a signaling pathway to transmit information for cell differentiation in embryogenesis and development (Bijlsma et al. 2004). Sonic Hedgehog (Shh) is a subgroup of Hh genes, the most widely expressed Hh protein in mammals and plays a key role in the induction of early chondrocyte differentiation (Bijlsma et al. 2004; Pusapati et al. 2018). The results of Han et al. (2020) showed that Shh expression in *T. ovatus* larvae was significantly downregulated at 32 °C (Fig. 8.6). This indicates that temperature teratogenicity of fish may be related to high temperature inhibiting the expression of Shh gene. However, some studies have shown that the increased temperature does not affect Shh expression in Atlantic salmon (Ornsrud et al. 2004a, b). Therefore, the

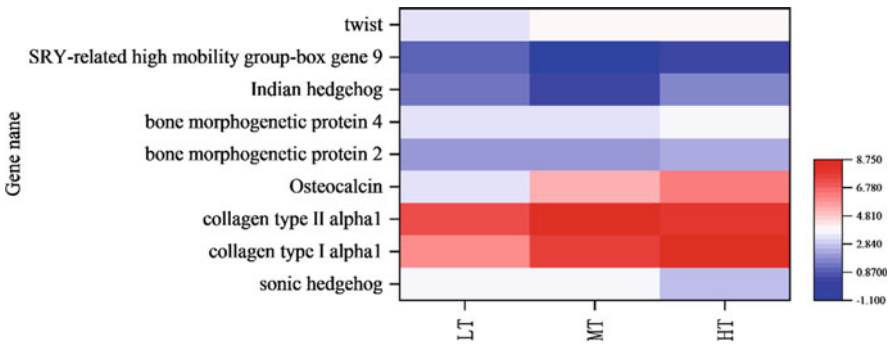

Fig. 8.6 Heat map of the DEGs related to bone development of *T. ovatus* larvae at different temperatures. Log_2(RPKM) value for each gene has been taken the average of three values and is shown using a color scale

regulation mechanism of temperature on Shh needs to be further studied. Shh can regulate the expression of col2a1 and runx genes, and Sox9 is the direct upstream gene of col2a1 (Lefebvre 2019; Lefebvre et al. 1997). In zebra fish, Sox9a can upregulate the expression of chondrogenic genes such as Col2a1, Runx3, and Runx2b (Dalcq et al. 2012). The study in *T. ovatus* indicates that the expression of col2a1 gene is significantly downregulated at high temperatures, while runx2 is not significantly different and has low expression levels under different temperature treatments (Fig. 8.6).

Therefore, we consider that high temperature can affect the nutrient metabolism and hedgehog signaling pathways of *T. ovatus* larvae and inhibit the secretion of type II collagen by downregulating the expression of Shh and Sox9a genes, which may lead to abnormal bone development. In addition, ocn is associated with bone mineralization (Riera-Heredia et al. 2018), and we found the expression of ocn gene is significantly upregulated when the temperature went up (Fig. 8.6). Some studies (Balbuena-Pecino et al. 2019) also show that increasing temperature could induce an upregulating response on ocn genes in the cultured bone-derived cells. However, more experiments are needed to verify the effect of temperature on ocn gene expression and explore the regulatory mechanism.

8.7 Conclusion

We believe that high temperature may lead to increased metabolism and nutritional requirements of golden pomfret larvae and affect the expression of genes related to bone development. RNA-seq based on high-throughput sequencing can reveal gene expression, pathway information, and regulatory mechanism involved in *T. ovatus* larvae under temperature stress. This study provides clues for understanding the response to high temperature in fish and guiding production practices.

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Chapter 9
IGF Genes in Golden Pompano *Trachinotus ovatus* Larvae

Xiaomei Wang, Donghao Li, and Xiangming Lin

Abstract Insulin-like growth factors (IGFs) include IGF I and IGF II. IGF I is an essential regulator of cell division, differentiation, embryonic development, and growth. IGF II, also known as somatomedin A, is mainly involved in the regulation of embryonic growth. Growth hormone may have different effects on IGF-II gene expression in different fish. This chapter reviews some studies on IGFs in fish larva and juvenile, analyzes the expressions of IGF I and IGF II at different time of ontogenesis and incubation temperature, and evaluates the effects of nutrition on IGF expression in *Trachinotus ovatus* larvae and juveniles. The expression of IGF I increased with the growth of larvae and juveniles, but the influence of water temperature was not significant. The water temperature influences the expression of IGF II. Its expression at 23 °C was significantly higher than that at 26 and 29 °C. The expression of IGFs in fish larvae on 28 DPH was not accompanied by nutrient manipulation. The time-dependent expression of IGF genes of larval fish is crucial to understand the ontogenetic development and larval fish growth in early life.

Keywords *Trachinotus ovatus* · Insulin-like growth factors · Ontogenesis · Temperature · Nutrition

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_9

9.1 Introduction

A group of polypeptide hormones and insulin-like growth factor (IGFs) have many functions such as cell growth, metabolic regulation, basic metabolism, and organ differentiation (Saltiel and Kahn 2001; Taniguchi et al. 2006). The function of IGFs is mediated through a complex intracellular signaling pathway, which depends on the binding of peptide to the corresponding receptor and the activation of the intrinsic receptor tyrosine kinase (Ozaki et al. 2013). The liver is the main organ to test IGF's expression in both mammals and fishes (Reinecke and Collet 1998; Schmid et al. 1999; Duan et al. 1993). IGFs are indicators of growth performance (Duan 1998; Reinecke et al. 2005; Moriyama et al. 2006). The relationship between the amount of IGFs and fish growth has been intensively investigated due to the fact that growth regulation is one of the essential concerns for fish in aquaculture (Reinecke and Collet 1998; de Jesus et al. 2002; Tanaka et al. 1998; Kaneko et al. 2011). However, it is unclear how IGF regulates fish growth at the molecular level, though the roles of IGFs have been studied from the perspectives of molecular biology, biochemistry, and physiology of various fish species (Reinecke et al. 2005; Duan 1998; Schmid et al. 1999).

IGFs have been cloned and analyzed in several fish species including Japanese eel *Anguilla japonica* (Moriyama et al. 2006), *Sarotherodon melanotheron* (Fan and Li 2011), Japanese flounder *Paralichthys olivaceus* (Tanaka et al. 1998), barramundi *Lates calcarifer* (Stahlbom et al. 1999), rainbow trout *Oncorhynchus mykiss* (Perrot and Funkenstein 1999), torafugu *Takifugu rubripes* (Kaneko et al. 2011), gilthead seabream *Sparus aurata* (Duguay et al. 1996), sea bass *Dicentrarchus labrax* (Terova et al. 2007), and milkfish *Chanos chanos* (de Jesus et al. 2002). Previous studies have indicated that nutrition supply, developmental stage, and seasonal changes could influence the level of IGFs in fish (Moriyama et al. 2000; Duan 1998; Perrot and Funkenstein 1999), and IGFs play a key role in regulating development, reproduction, somatic growth, and osmoregulation (Reinecke et al. 2005; Moriyama et al. 2006).

IGF I is a 70-amino-acid polypeptide, which is highly conservative, and the structural sequence is similar to both IGF II and insulin. The IGF I plays an important role in regulating cell division, differentiation, and embryonic development and growth (Patruno et al. 2008; Reinecke and Collet 1998). Besides, it can promote cell division and differentiation, promote protein synthesis, inhibit protein degradation, and regulate osmotic pressure of migratory fish (Zee et al. 1998; Ayaso et al. 2002). During the postnatal life of mammals, IGF I is an important mediator of growth hormone (GH) activity and in connection with fetal growth (Froesch et al. 1985; Daughaday and Rotwein 1989). IGF II is a pluripotent cell proliferation regulator, which plays a vital role in cell differentiation, proliferation, embryonic growth and development, and tumor cell proliferation (Rachmilewitz et al. 1992). In fish, both IGF I and IGF II act not only as a growth factor but also as a metabolic hormone, which can be detected in the brain, liver, gills, eye, gastrointestinal tract,

heart, kidney, pancreatic islets, spleen, skeletal muscle, and gonads (Vong et al. 2003; Ayson et al. 2002; Reinecke et al. 2005).

The golden pompano (*Trachinotus ovatus*) belongs to the Carangidae family, which grows fast and suits for cage culture, making it a good candidate for aquaculture. However, there is little information about IGFs in golden pompano. It is necessary to investigate the expression of IGF during fish ontogeny for its importance on growth. In this chapter, the expression of IGFs in golden pompano larvae was explored during fish ontogeny in the first 18 days post-hatch (DPH). Fish were exposed to different water temperature on 12 and 18 DPH and different nutrient manipulations on 28 DPH. The expression pattern of IGFs could provide necessary information to increase the understanding of the development and growth of golden pompano larvae and provide possible indicators for the measurement of growth potential in fish larvae.

9.2 Cloning and Sequencing of IGF I and IGF II cDNA in Golden Pompano

The primers designed for golden pompano cDNA cloning (Table 9.1) by Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) were based on unpublished transcriptome sequences (Illumina HiSeq2000, annotated by NR, KOG, Kegg, and Swissprot) and our laboratory's previous golden pompano sequence data.

IGF I cDNA identified from golden pompano was 742 bp long and contained an open reading frame of 558 bp encoding 185 amino acid (aa) residues (Fig. 9.1). The deduced IGF I protein consisted of the signal pro-IGF I (44–185aa) and peptide (1–43aa), which constituted domains B (29aa), C (10aa), A (21aa), D (8aa), and E (74aa). The six characteristic cysteine residues were conserved and involved in the disulfide bonds, including Cys50, Cys62, Cys88, Cys89, Cys93, and Cys102.

The IGF II cDNA identified from golden pompano was 768 bp long and contained an open reading frame of 648 bp encoding 215aa residues (Fig. 9.2). The deduced IGF II protein consisted of the signal peptide (1–47aa), and the mature protein including domains B (32aa), C (11aa), A (21aa), D (6aa), and E (98aa). The

Table 9.1 Primers for gene cloning and real-time PCR (Ma et al. 2016)

Primers	Sequence (5′–3′)	Amplicon sizes (bp)
IGF I-F	TCCTGTTCGCTAAATCTCACTT	
IGF I-R	TGTCCATTCGCTCCTTCC	742
IGF I-qF	CGCAATGGAACAAAGTCGG	
IGF I-qR	AGGAGATACAGCACATCGCACT	198
IGF II-F	TCCAACCAAATAACCCC	
IGF II-R	GACAAAGCTATAATCCCCTAG	768
IGF II-qF	GCAAAGACACGGACCCCACT	
IGF II-qR	CGAGGCCATTTCCACAACG	142

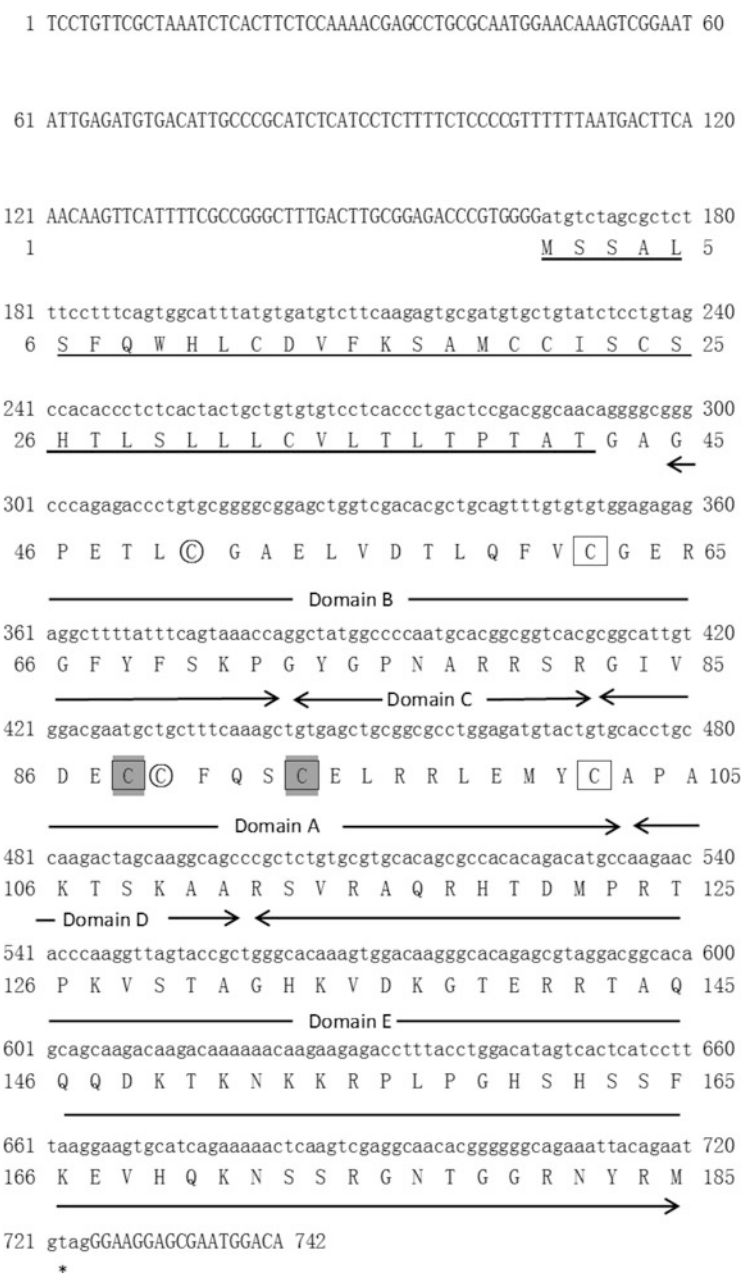

Fig. 9.1 Nucleotide and deduced amino acid sequence of golden pompano IGF I (Ma et al. 2016). The domains B, C, A, and D are marked. The three disulfide bonds formed by the six cysteine residues are shown as rectangles, circles, and shaded rectangles. The disulfide bonds are formed between symbols. The stop codon is denoted by an asterisk (Ma et al. 2016)

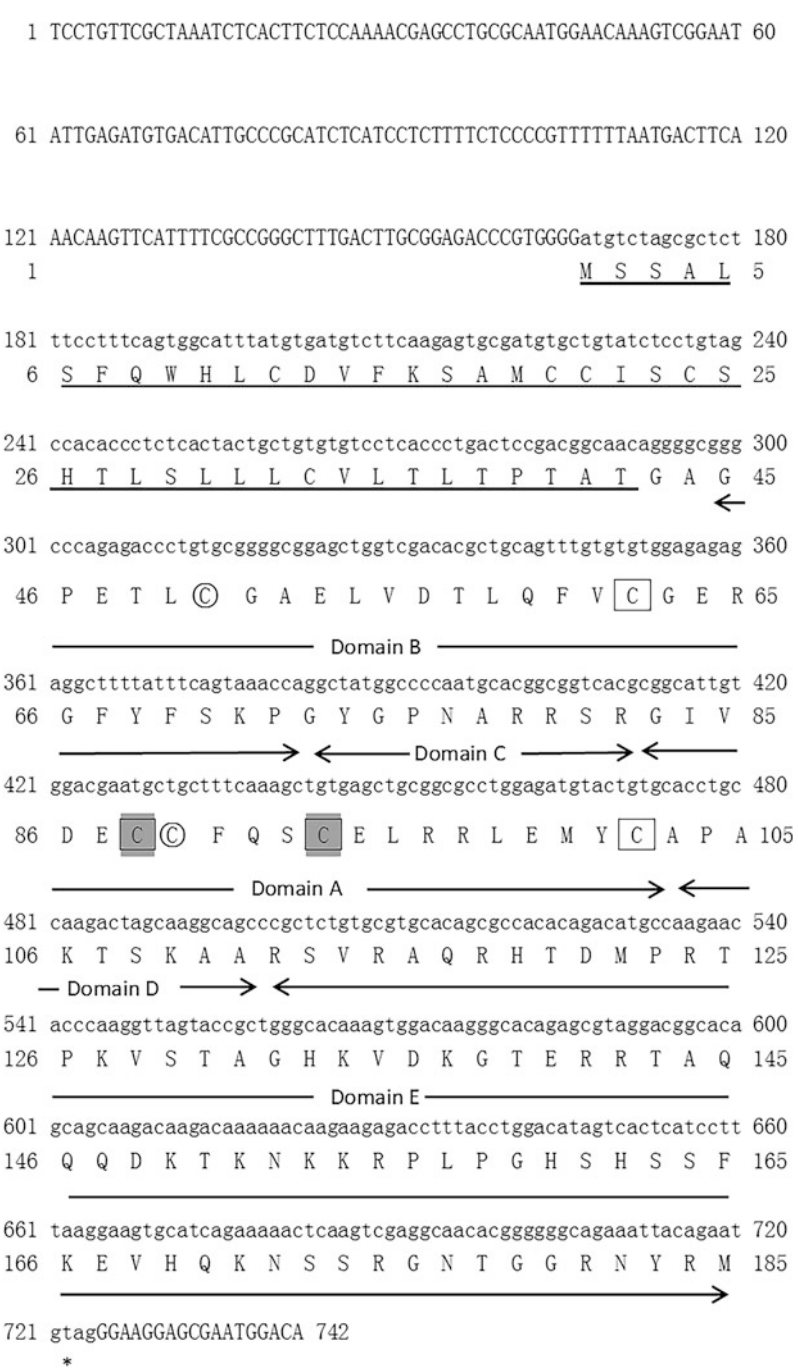

Fig. 9.2 Nucleotide and deduced amino acid sequence of golden pompano IGF II. The domains B, C, A, and D are marked. The three disulfide bonds formed by the six cysteine residues are shown as circles, rectangles, and shaded rectangles. The disulfide bonds are formed between symbols. The stop codon is denoted by an asterisk

six characteristic cysteine residues were conserved and involved in the disulfide bonds, including Cys56, Cys68, Cys96, Cys97, Cys101, and Cys110.

According to the result of sequence comparison, IGF I and IGF II deduced amino acid sequence of golden pompano were highly identified with those from other vertebrates and had a high degree of homology among the teleost (Fig. 9.3). The deduced amino acid sequences of golden pompano IGF I cDNA was 48.7–95.7% identified from other vertebrate species, with the highest percentage identified from *Sparus aurata* (95.7%) and *Lateolabrax japonicus* (95.7%), and the lowest percentage identified from *Mus musculus* (48.7%) and *Homo sapiens* (48.7%). Furthermore, compared to other vertebrate species, the amino acid sequences of IGF I from golden pompano, *Sparus aurata*, and *Lateolabrax japonicus* contained an extra amino acid sequence (KVSTAGHKVDKGTERRTAQQQDKTKNK) in the E domain.

The deduced amino acid sequence of golden pompano IGF II cDNA was 44.9–92.6% identified from other vertebrate species and the highest value identified from *Lateolabrax japonicus* and *Lates calcarifer* (92.6%) and the lowest value identified from *Bos taurus* (44.9%) (Fig. 9.3).

Use the neighbor joining method to construct a phylogenetic tree to evaluate the relationship between the golden pomfret and the IGF genes of other vertebrate species (Fig. 9.4). The results indicated that these species gathered into two groups, composed of IGF I and IGF II, respectively. The branches of the teleost species clustered together in each distinct group. The phylogenetic analysis of results was almost the same as the established phylogeny. The deduced amino acid sequences of golden pompano IGF cDNA were compared with those from other vertebrate species. The degree of homology was high among teleost by a comparative analysis.

9.3 Expression of IGFs During Early Development of Golden Pompano Larvae

On 1, 3, 5, 9, 12, and 18 DPH, ten fish per tank were sampled for size measurements, respectively. The expression levels of IGF genes in golden pompano larvae were analyzed by quantitative real-time PCR (qPCR). Gene-specific primer pairs of IGF genes (Table 9.1) were amplified in LightCycler480 II (Roche, Switzerland). EF-1α was used as an internal reference. The relative quantification (RQ) was calculated using the ΔΔCT (comparative threshold cycle) method (ΔCT = CT of target gene − CT of EF-1α, ΔΔCT = ΔCT of any sample − ΔCT of calibrator sample). The efficiencies of the primers (E) were EIGF I = 0.997, EIGF II = 1.004.

IGFs are peptide hormones important to vertebrate's somatic growth (Kaneko et al. 2011). IGF I and IGF II are highly similar with a sequence and are the main isoforms of IGF. The structure and biological function of IGF I in somatic growth are partly conserved or conserved in teleosts (Wood et al. 2005; Moriyama et al. 2000). IGF II can promote postnatal growth in response to the nutritional state of teleosts

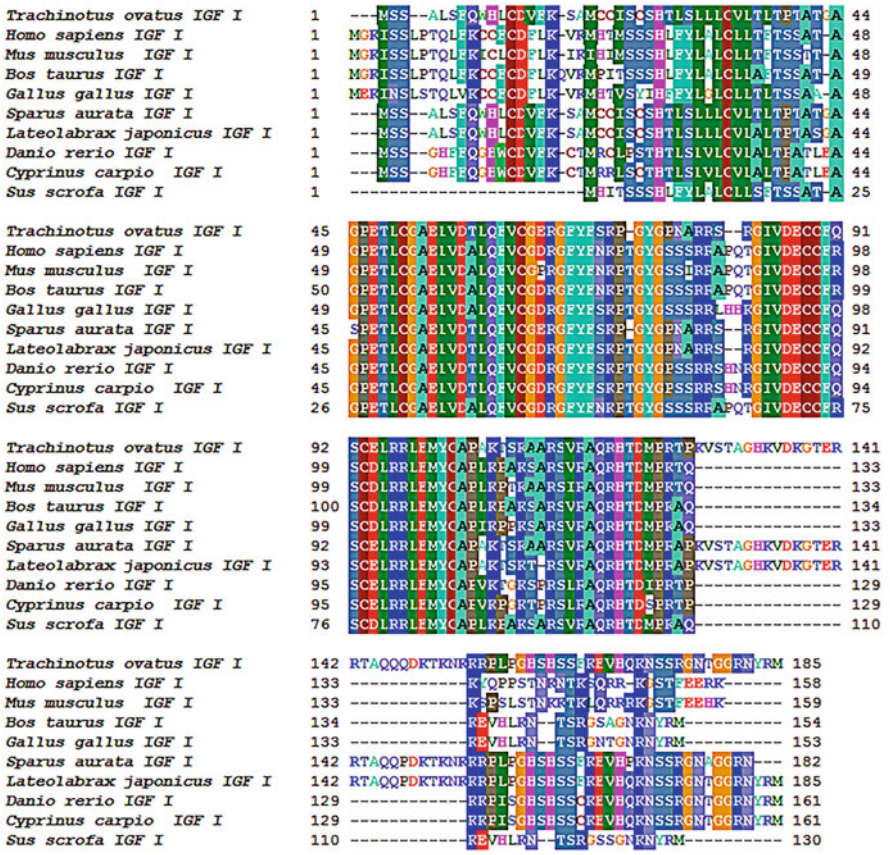

Fig. 9.3 Amino acid sequence comparisons of both golden pompano IGF I and IGF II with those from other vertebrate species revealed a high sequence identity. The IGF I and IGF II sequences from other species were downloaded from GenBank (http://www.ncbi.nih.gov). The references for IGF I and IGF II amino acid sequences are as follows: *Homo sapiens* (IGF I: NP_001104753.1, IGF II: NP_000603.1), *Bos taurus* (IGF I: NP_001071296.1, IGF II: NP_776512.2), *Mus musculus* (IGF I: NP_034642.2, IGF II: NP_034644.2), (IGF I: NP_999421.1, IGF II: NP_999048.1), (IGF I: NP_001004384.1, IGF II: NP_001025513.1), (IGF I: AEX60712.2, IGF II: AEX60713.2), *Cyprinus carpio* (IGF I: ABQ08938.1, IGF II: ADQ44896.1), *Sparus aurata* (IGF I: ABQ52656.1, IGF II: AAY46224.1), *Danio rerio* (IGF I: NP_571900.1, IGF IIa: NP_571508.1, IGF IIb: AAL06080.1), *Lates calcarifer* (IGF II: AAB64195.1), and *Salmo salar* (IGF II: NP_001117119.1)

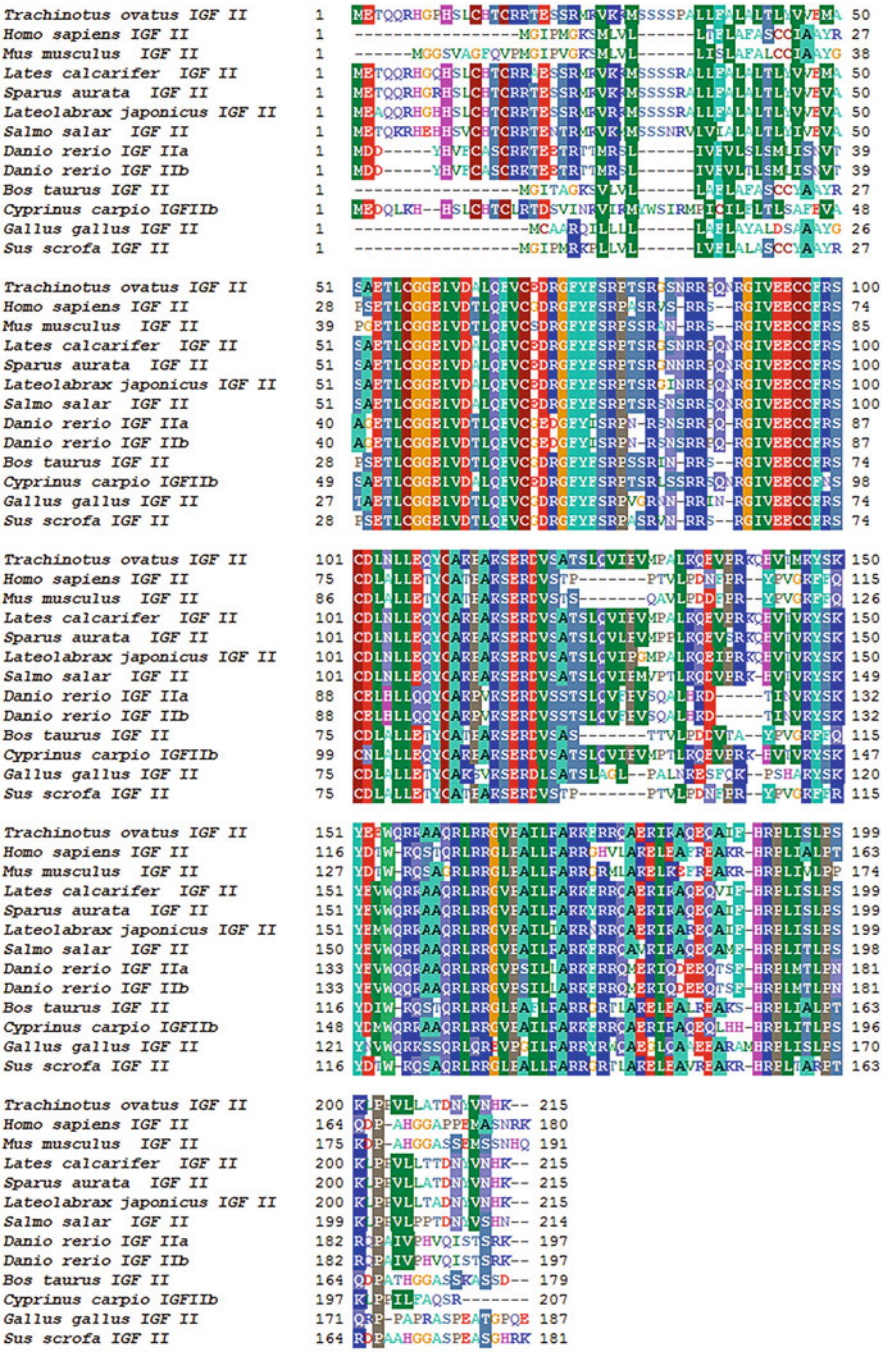

Fig. 9.3 (continued)

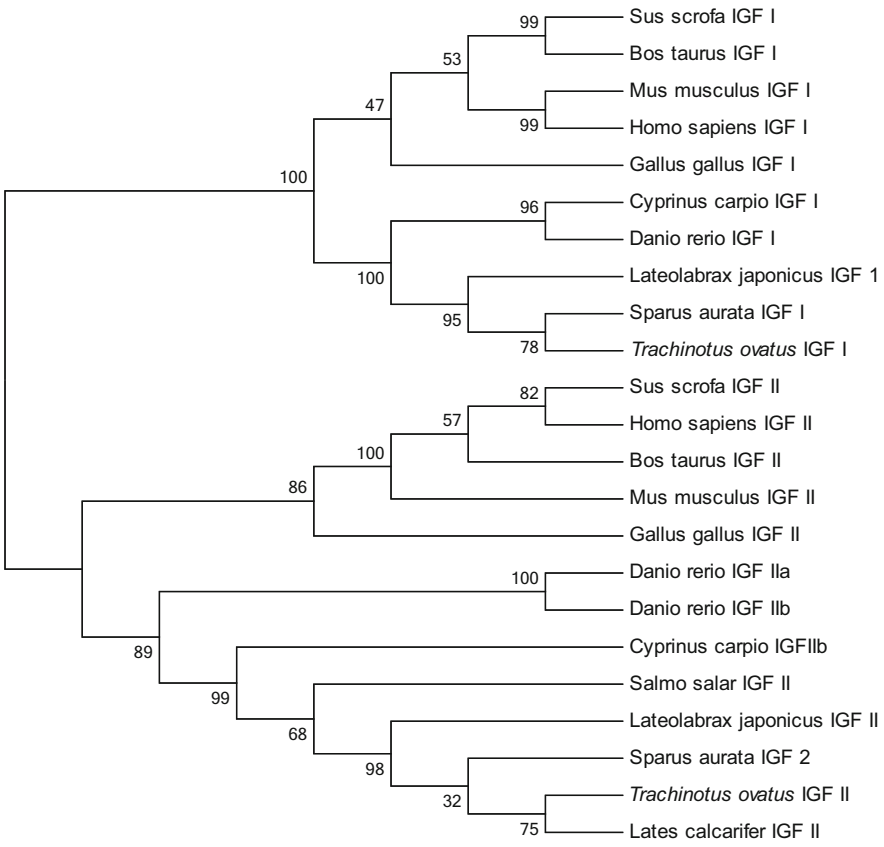

Fig. 9.4 The phylogenetic tree of IGF I and IGF II constructed by the neighbor-joining method

(Shamblott et al. 1995; Fox et al. 2010). In *Oncorhynchus kisutch*, IGF I and IGF II expression levels were elevated during rapid growth of the fish.

The IGF I relative expression at hatching did not show any significant difference with that on 1 DPH ($P > 0.05$, Fig. 9.5), but increased rapidly from 1 DPH to 3 DPH. From 3 to 5 DPH, the IGF I relative expression was similar and increased dramatically on 12 DPH. The IGF I expression of golden pompano larvae increased in the early development and maintained a relatively high level, which is the same as this species' rapid growth (Ma et al. 2014).

The relative expression of IGF II at hatching was significantly lower than that on 1 DPH ($P < 0.05$, Fig. 9.5). IGF II expression level increased and reached a peak on 3 DPH but significantly decreased while maintaining a relatively low levels until 18 DPH with the onset of first feeding. This change of IGF II expression may be due to different nutrient supplement, because most fish rely on endogenous nutrient supply before 3 DPH and on exogenous nutrient supply after 3 DPH (Shamblott et al. 1995; Fox et al. 2010).

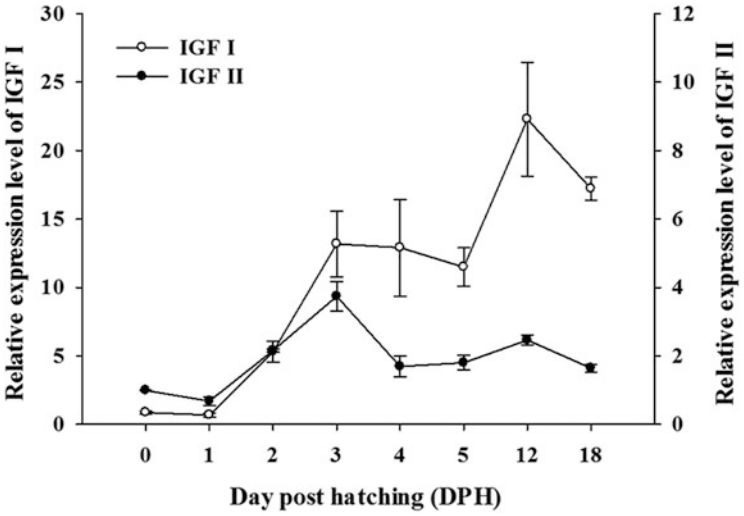

Fig. 9.5 Gold pompano relative expression of IGFs early larval development (Ma et al. 2016)

9.4 Effect of Water Temperature on IGF Expression

The growth and jaw deformity of golden pompano larvae were affected by water temperature on 18 DPH. The SGR of larvae reared at 29 °C was significantly higher than that at 23 and 26 °C ($P < 0.05$, Table 9.2). The jaw deformity of larvae reared at 26 and 29 °C was significantly higher than that at 23 °C ($P < 0.05$, Table 9.2). In all replicates, the survival rate of all treated fish was very low.

There was no significant difference in the expressions of IGF I among different temperatures ($P > 0.05$, Fig. 9.6) on 12 DPH and 18 DPH. However, in all temperature treatments, the expression of IGF I on 18 DPH was significantly higher than that on 12 DPH ($P < 0.05$). It seems that the water temperature does not affect the expression level of IGF I. In this study, fish were fed with both rotifers and *Artemia nauplii* before 12 DPH but solely with *Artemia nauplii* on 18 DPH. The IGF I expression increased as fish grew maybe due to the increase of feed intake as larvae develop (Duan et al. 2010; Martins et al. 2014). The results of IGF I expression to water temperature in this study were similar to Atlantic salmon (*Salmo salar*) (Hevrøy et al. 2013) and rainbow trout (*Oncorhynchus mykiss*) (Hevrøy et al. 2015).

Table 9.2 Specific growth rate and jaw deformity of larval golden pompano on 12 and 18 days post hatching (DPH) and 28 DPH

	23 °C	26 °C	29 °C
12 DPH specific growth rate (%/day)	2.06 ± 0.93^a	2.68 ± 0.85^a	3.47 ± 0.33^b
18 DPH specific growth rate (%/day)	2.56 ± 0.28^a	2.70 ± 0.42^a	3.64 ± 0.20^b
Jaw deformity (%)	4.55 ± 0.83^a	5.00 ± 0.64^a	10.00 ± 2.65^b

Different letters represent significant differences ($P < 0.05$)

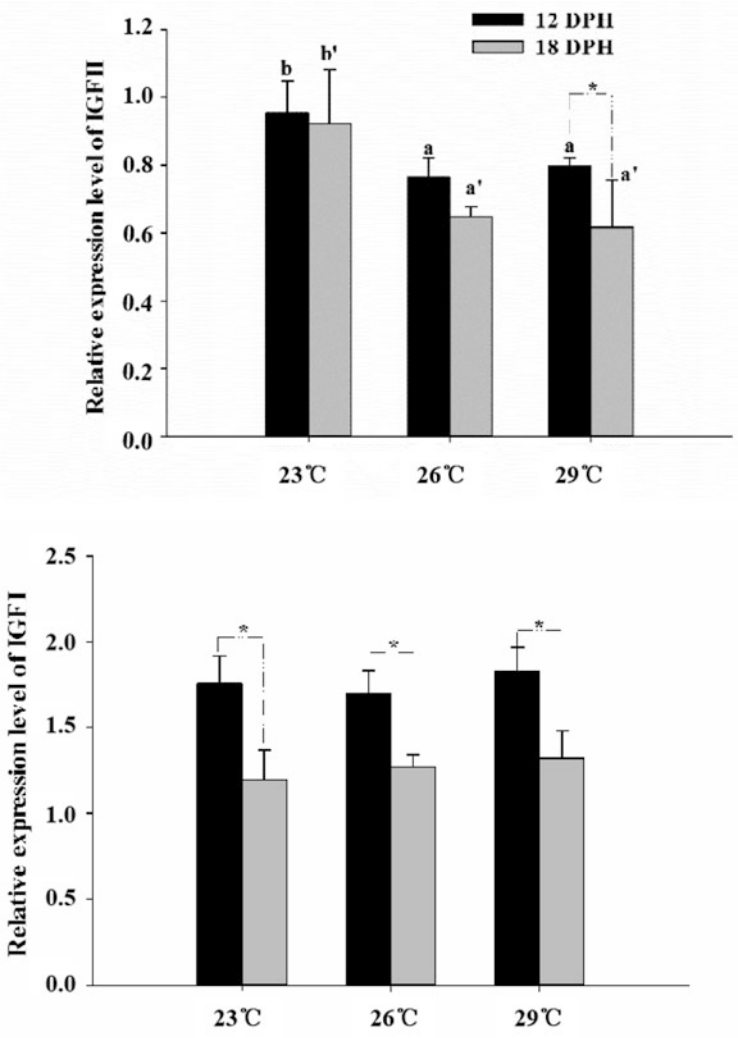

Fig. 9.6 Responses of IGF-1 and IGF-2 to the water temperature of 23, 26, and 29 °C on 12 DPH and 18 DPH in golden pompano larvae (Ma et al. 2016)

Compared with IGF I, the expression of IGF II in fish at 23 °C was significantly higher than that at 26 and 29 °C on 12 DPH ($P < 0.05$, Fig. 9.6), but there was no significant difference between that at 26 and 29 °C ($P > 0.05$). The expression of IGF II in fish at 23 °C on 18 DPH was also significantly higher than that of at 26 and 29 °C ($P < 0.05$). The IGF II expression on 18 DPH was only significantly higher than that on 12 DPH at 29 °C ($P < 0.05$). IGF II is involved in the growth process, and its expression is related to hyperplasia, hypertrophy, and cell differentiation (Duan et al. 2010). During fish growth, the expression of IGF II is more obvious than

IGF I (Peterson et al. 2004). Previous studies have shown that temperature and salinity affect IGF II expression of fish (Martins et al. 2014; Cnaani and Hulata 2013). The level of IGF II was significantly higher at 23 °C than that at 26 and 29 °C, indicating that temperature significantly affects the IGF II expression on 12 and 18 DPH. There is no positive correlation between the expression of IGF II and the growth rate of larval golden pompano, even though the growth rate was higher at 29 °C. However, the IGF II expression increases in higher water temperature in *Lophiosilurus alexandri* (Martins et al. 2014). The expression of IGF II tends to be higher in fast-growing catfish (Peterson et al. 2004). The IGF II expression level is associated with nutritional status rather than with the growth rate (Gabillard et al. 2003). The effect of temperature on IGF II mRNA expression varies with developmental stages (Gabillard et al. 2005).

9.5 Effect of Nutrient Manipulation on IGF Expression

There were three dietary treatments with three replicates for each treatment. *Artemia nauplii* were treated with three methods: (1) enriched with instant microalgal paste (*Nannochloropsis* sp., Qingdao Hong Bang Biological Technology Co., Ltd., Qingdao, China); (2) enriched with Algamac 3080® (Aquafauna, USA); and (3) the control, with no enrichment. Artemia cysts were produced from the Great Salt Lake, UT, USA (INVE Aquaculture). The fish in each tank were sampled for size measurement among nutritional manipulations at 0, 12 and 28 DPH, respectively. The results showed that the growth and survival of larval golden pompano was significantly affected by nutrient manipulations on 28 DPH ($P < 0.05$, Table 9.3).

Fish fed *Artemia nauplii* enriched with Algamac3080 showed the highest SGR, while fish fed non-enriched *Artemia nauplii* showed the lowest SGR. The highest survival was found in the treatment of *Artemia nauplii* unenriched ($P < 0.05$, Table 9.3), while the lowest survival was found in the fish fed Algamac 3080 enriched *Artemia* ($P < 0.05$).

The results showed that there was no significant difference in the expression of IGF I and IGF II in *Artemia* nauplii fed with non-enriched, enriched *Nannochloropsis*, and enriched Algamac 3080 on 28 DPH ($P > 0.05$, Fig. 9.7).

The IGFs play a key role in fish growth of somatic. The IGF I expression is related to nutritional conditions in hybrid striped bass *Morone chrysops* × *Morone saxatilis* (Picha et al. 2008), rainbow trout *Oncorhynchus mykiss* (Gabillard et al. 2006), channel catfish *Ictalurus punctatus* (Peterson and Waldbieser 2009), and yellowtail

Table 9.3 Specific growth rate and jaw deformity of golden pompano larvae on 28 days post-hatch (DPH) in nutrition treatments

28 DPH	Non-enriched	*Nannochloropsis*	Algamac3080
Specific growth rate (%/day)	5.68 ± 0.22^{a}	6.25 ± 0.08^{b}	6.49 ± 0.5^{c}
Jaw deformity (%)	15.91 ± 0.68^{b}	15.39 ± 0.69^{b}	8.33 ± 0.74^{a}

Different letters represent significant differences ($P < 0.05$)

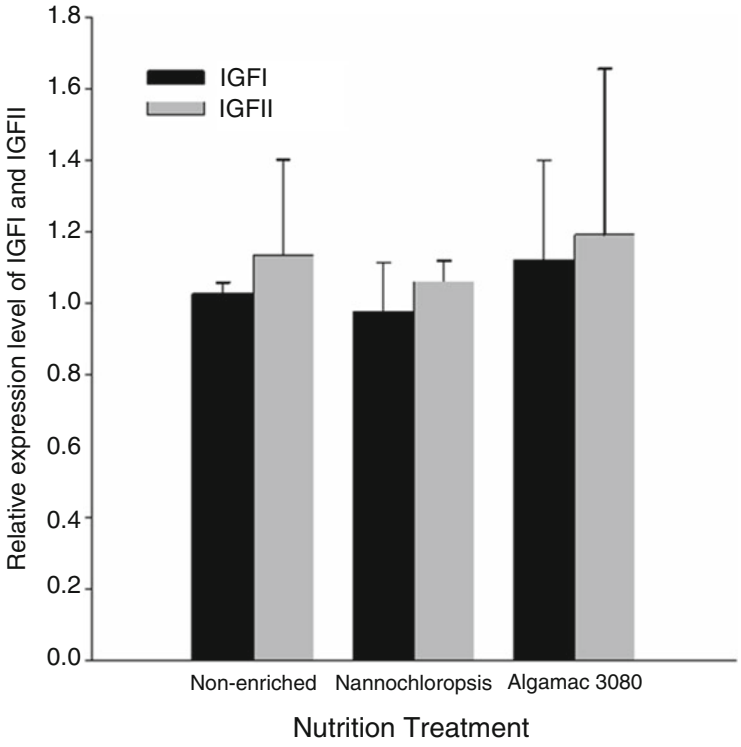

Fig. 9.7 Responses of IGF-1 and IGF-2 to nutrient manipulation of golden pompano *Trachinotus ovatus* larvae on 28 DPH

Seriola quinqueradiata (Fukada et al. 2012). Most of these studies have focused on the IGF I expression under different nutritional conditions (e.g., fasting and re-feeding). For instance, vitamins, lipids, and fatty acids can change the expression of IGFs (Villeneuve et al. 2006; Fernández et al. 2011).

In addition, the relationships between IGF expression and nutrients may be associated with the developmental stage of fish (Chen et al. 2000). The results of nutrient manipulations indicate that nutritional treatment affects the growth and survival of fish, but there were no significant differences in IGF I and IGF II expressions on 28 DPH in this study. The possible reason is that both IGF I and IGF II are not sensitive to nutrient treatment in the fish at this developmental stage. In all replicates, the survival rates were low, which needs further investigation to test if the number of fish in the tank can affect IGF expressions.

9.6 Conclusion

In this study, the golden pompano's IGF I and IGF II cDNA were cloned and analyzed during the early development. The IGF I and IGF II deduced amino acid sequences in golden pompano revealed a high degree of identification with other fish species. The deduced mature peptide regions of IGF I and IGF II cDNA were composed of five domains and included six cysteine residues. The cysteine residues were found in A and B domains of the IGF I of golden pompano, which are similar to other species. The A and B domains in IGF I play a key role in maintaining the tertiary structure (Reinecke et al. 1997; Fukada et al. 2012), which is crucial for binding receptor binding and proteins (Duguay et al. 1996; Fan and Li 2011). It is important to investigate the expression pattern of IGFs in fish larvae in early life, as it is helpful to understand the ontogenetic development and growth of larval fish. In this study, larval golden pompano's IGF I and IGF II cDNA were cloned and analyzed. The results indicate that the with the growth of the larval golden pompano, the fish expression increased. The IGF II expression of larval golden pompano was significantly affected by water temperature; however, the IGF I and IGF II did not respond to the nutrient manipulation.

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Chapter 10
High Water Temperature Induces Jaw Deformity and Bone Morphogenetic Protein (BMP) Gene Expression in Golden Pompano *Trachinotus ovatus* Larvae

Jing Sun, Zhengyi Fu, Zhenhua Ma, and Gang Yu

Abstract Deformity during the early development of golden pompano *Trachinotus ovatus* has significantly influenced the production capacity of a fish hatchery. However, factors leading to skeletal deformity in this species have never been assessed. In this chapter, the impact of rearing temperature on jaw deformity and BMP gene expression is discussed. Jaw deformity rate of fish larvae increased with the increase of ambient temperature, and the highest malformation rate was recorded at 33 °C. The expressions of the BMP4 and BMP5 genes were positively correlated to the occurrence of jaw malformation. The cultivating water temperature of *T. ovatus* larvae should be maintained at 26–29 °C. These findings will clarify the role of water temperature in influencing bone deformity in fish larvae and provide a reference point to optimize the environmental condition during the rearing process of golden pompano in hatcheries.

Keywords *Trachinotus ovatus* larvae · Water temperature · Jaw deformity · BMPs

10.1 Introduction

Temperature is one of the important factors affecting the early development of larval fish through regulating the feeding behavior and metabolism during larval development (Ma 2014; Kestemont and Baras 2001). Besides, studies have shown that unsuitable temperature can cause high mortality and deformity of larval fish (Lein et al. 1997; Ørnsrud et al. 2004; Ludwig and Lochmann 2009). The skeletal malformation is often related to slow growth and high mortality of fish larvae and has continually hindered the production of marine fish in the hatchery (Koumoundouros 2010; Boglione et al. 2013a, b). Jaw abnormality is not only a

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_10

factor leading to a high mortality rate of fish but also a factor reducing the market value of fish (Cobcroft et al. 2004; Barahona-Fernandes 1982; Ma et al. 2014c). Such malformations have been reported in commercial aquaculture of gilthead sea bream *Sparus aurata* (Prestinicola et al. 2013; Andrades et al. 1996), striped trumpeter *Latris lineata* (Cobcroft et al. 2012), and yellowtail kingfish *Seriola lalandi* (Cobcroft et al. 2004). Lein et al. (1997) suggested that unsuitable rearing temperature can cause jaw malformations in fish. Under inappropriate temperature, significant deformities of skeleton and gill cover have been reported in gilthead seabream *Sparus aurata* and cranial malformation in European sea bass *Dicentrarchus labrax* (Georgakopoulou et al. 2007). In pompano *Trachinotus ovatus*, more than 33% of fish in the same cohort display more than one type of deformities during the larval stage (Zheng et al. 2014; Ma et al. 2014c), but it is not clear whether the temperature can cause jaw malformations in this species. Consequently, in the process of pompano's larval ontogenesis, to study relationship between temperature and the jaw malformation is very necessary.

Skeletogenesis includes differentiation and proliferation of various cell types, such as osteoblasts, chondrocytes, osteoclasts, and osteocytes which determine the shape, size, and mineral composition of bone structure (Karsenty and Wagner 2002; Nijweide et al. 1986; Phan et al. 2004). The expression of genes mainly underlies the procedure of cell proliferation and differentiation but could also be changed by individual genetic characteristics and biological and nonbiological elements (Boglione et al. 2013a, b). Therefore, it is necessary to examine the structure of gene networks, which will provide an insight into the understanding of the underlying mechanisms of bone deformity. The biological and nonbiological factors could cause bone malformation, while the gene expression is the potential mechanism behind this factor. In some vertebrates, bone formation is controlled by bone morphogenetic proteins (BMPs) at different phases of cell development (e.g., maturing osteoblast, stem cells, hypertrophic chondrocytes, proliferative chondrocytes) (Hogan 1996a, b; Windhausen et al. 2015; Alaee et al. 2014). In the animal kingdom, the function and structure of BMPs are conservative. The function and structure of different BMPs in single species can be seen through their roles in various biological processes (Razdorov and Vukicevic 2012). For example, BMPs 1, 2, and 3 play an essential role in bone fracture repair because these proteins can stimulate the growth of osteoblasts (Grgurevic et al. 2011). BMPs 2, 4, and 6 can regulate skeletogenesis, in particular, chondrocyte differentiation into cartilage, and cell maturation in osteoblast lineages can lead to bone formation (Minina et al. 2001; Rickard et al. 1994; Wan and Cao 2005; Canalis et al. 2003). Although several studies have been conducted to test the expression of BMP genes in various fish species, most of these studies are focused on the changes during embryonic development (Myers et al. 2002; Palomino et al. 2014; Marques et al. 2014, 2015; Tiago et al. 2014). In marine fish, the studies on the expression of BMP genes after incubation and their biological function are limited to the test of nutrient effect such as lipids and vitamins (Villeneuve et al. 2005a, b, 2006). Recently, BMP genes have been used to evaluate the impact of high temperature on the bone abnormality of fish larvae (Ytteborg et al. 2010). The study on BMP expressions in the ontogeny of golden pompano can

contribute to the baseline data on the factors relevant to jaw deformity in fish larvae during osteogenesis.

The *T. ovatus* is an important economic species of the Carangidae family and is a potential species for aquaculture diversification (Guo et al. 2014). Although the digestive function develops early (Ma et al. 2014a, b) and the nutrient requirements of the first feeding *T. ovatus* larvae have been researched (Ma et al. 2014d), the information on the cause of deformity during the early developmental period is fragmental. Our previous studies have reported the type, position, and frequency of jaw and skeletal deformities in hatchery-reared *T. ovatus* larvae (Zheng et al. 2014; Ma et al. 2014c). However, factors leading to skeletal deformity in this fish have never been assessed. In this chapter, the impact of rearing temperature on jaw deformity and BMP gene expression is discussed. The results are derived from fish cultured at three constant temperatures of 26, 29, and 33 °C from hatching to 18 days post-hatch (DPH) in a hatchery.

10.2 Growth, Survival, and Jaw Deformity at Different Temperatures

The water temperature is a key to the success of fish hatchery production factors and can significantly impact the quality of fish larvae (Boglione et al. 2013b). The growth of *T. ovatus* larvae was significantly affected by temperature ($P < 0.05$, Fig. 10.1). The specific growth rates (SGRs) of fish increase with temperature elevation in the rearing facility. Temperature can affect metabolism, food intake, and growth of fish (Ma 2014; Jobling 1994), and the effect of temperature on larval growth of farmed fish species has been well documented including striped trumpeter *Latris lineata* (Choa et al. 2010), Australian snapper *Pagrus auratus* (Fielder et al. 2005), nase *Chondrostoma nasus* L. (Keckeis et al. 2001), yellowtail kingfish *Seriola lalandi* (Ma 2014), and haddock *Melanogrammus aeglefinus* L. (Martell et al. 2005). In *T. ovatus*, the rapid growth at high temperature probably is related to the high food intake and improved digestive mechanism as evidenced by the early appearance of gastric glands and goblet cells in the gut after 15 DPH at 27–29 °C (Ma et al. 2014b). The growth of fish larvae was expedited when fish were weaned from rotifers to *Artemia* nauplii. Like Florida pompano *Trachinotus carolinus* (Riley et al. 2009), the mouth gape of *T. ovatus* larvae reached 1.05 mm by 12 DPH, which enables the fish to ingest larger food particles such as *Artemia* nauplii. For this reason, the marked size difference in *T. ovatus* size between temperature treatments at 18 DPH may also be attributed to the use of enriched rotifers for high-calorie food from 9 DPH onward.

In both artificial and wild environments, fish will go through critical periods in ontogeny and shift from endogenous nutrition to exogenous nutrition (Otterlei et al. 1999; Ma et al. 2012). During the phase of feed transformation, when food provision and light condition are within the range of first feeding requirement for fish larvae,

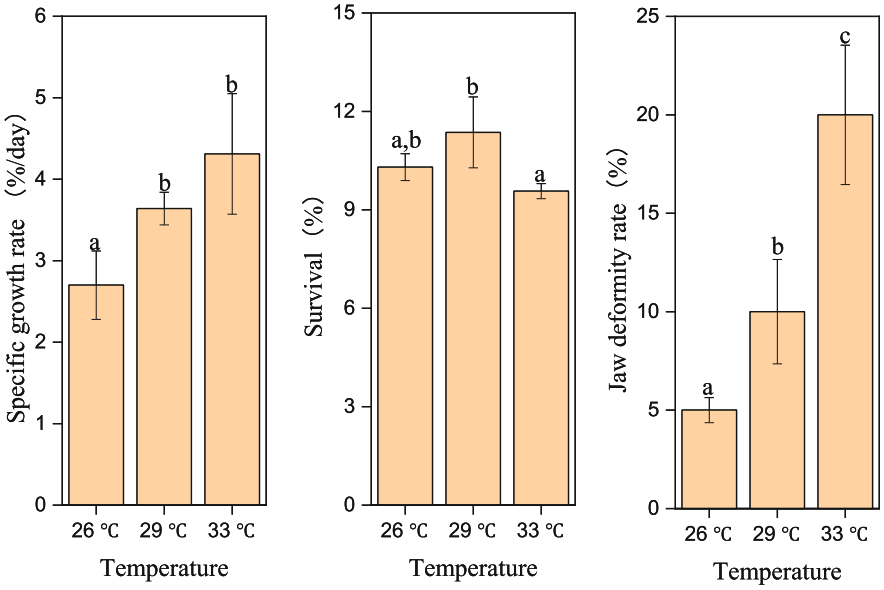

Fig. 10.1 Survival, specific growth rate, and jaw deformity rate of *T. ovatus* larvae cultured at 26, 29, and 33 °C. Means with the same letter are not significantly different ($P > 0.05$) (the symbol of "°C" is not shown in the *x*-axis of the figure) (Ma et al. 2016)

the temperature may be the most decisive factor for fish survival (Kamler 1992; McGurk 1984; Ma 2014; Gardeur et al. 2007). Previous research has indicated that mortality is temperature-dependent in the larvae and juveniles of *Pangasianodon hypophthalmus* (Baras et al. 2011), *Seriola lalandi* (Ma 2014), *Glyptocephalus cynoglossus* (Bidwell and Howell 2001), and *Inimicus japonicus* (Wen et al. 2013). Ma (2014) argues that there is a temperature-sensitive stage when mortality occurs in fish larvae during early development. In *T. ovatus*, the lowest survival rate was found when fish were reared at 33 °C (Fig. 10.1), suggesting that the highest level of temperature tolerance has reached for this species.

Jaw abnormality is a crucial point in fish culture because it impacts the quality of fingerlings for further grow-out (Von Westernhagen 1988). In *T. ovatus*, the rate of jaw deformities rose with the rise of temperature, and the maximum value occurred at 33 °C (Fig. 10.1). The temperature-dependent deformity has also been reported in other species such as Pacific herring *Clupea pallasi* (Alderdice and Velsen 1971) and Atlantic halibut *Hippoglossus hippoglossus* (Lein et al. 1997). The fast growth at temperature requires a high level of dissolved oxygen (Rombough 1997) and an adequate amount of nutritional supply. However, unless the feed contains high levels of energy, the fish may not grow very fast (Cahu et al. 2003a, b; Ma 2014). In addition, temperature could interfere with fish development by accelerating or postponing the development of the digestive system, which may be related to the increased rate of skeletal malformation at high temperature. In the present study, the fertilized eggs of *T. ovatus* hatched at 26 °C, and then yolk sac larvae were

acclimated to each of the experimental temperatures (26, 29, and 33 °C) for 5 h on 2 DPH. However, the rapid augment of ambient temperature from 26 to 29 °C or 33 °C may also induce jaw malformation.

10.3 Expression of BMP Genes at Different Temperatures

The growth of bone depends on the dynamic balance between the rate of cartilage generation and bone adherence (Breur et al. 1991). BMP2 and BMP4 genes are closely associated with protein synthesis for physiological activities in the crucial period of embryonic development, such as dorsal-ventral axis specification (Graff 1997), apoptosis (Glozak and Rogers 1996; Graham et al. 1994; Zou and Niswander 1996), and epithelio-mesenchymal interactions (Vainio et al. 1993). The BMP2 gene in zebra fish is correlated to the induction and maintenance of ventrolateral cells during the initial stage of development. However, a missense mutation of the BMP2b gene lead to the dorsalized phenotype of the zebra fish *swirl* mutant, which lacks the cardiogenic mesoderm (Kishimoto et al. 1997). Ytteborg et al. (2010) found that the expression of BMP2 increased when fish are under a high temperature condition. In *T. ovatus*, the expression of BMP2 was significantly affected by water temperature ($P < 0.05$, Fig. 10.2). Compared with the fish at 26 °C, the expression of BMP2 in fish showed a trend of increase at 29 °C (Fig. 10.2), which is consistent with the result reported by Ytteborg et al. (2010). However, the reason for low expression of BMP2 in fish at 33 °C remains unclear.

BMP4 plays a different role in the growth of some vertebrate species (Whitman 1998; Hogan 1996b; Dale and Johns 1999; Mehler et al. 1997; Shi and Massague 2003) and has been used to assess whether the BMP pathway is involved in nutrient deficiency of bone deformities (Villeneuve et al. 2005a, b, 2006) or environmental stress (Ytteborg et al. 2010). According to Villeneuve et al. (2006), the increase of BMP4 and RARγ expressions can diminish the number of osteoblasts for bone generation, and the damage of bone cells is counteracted by the interaction between retinoic acid and BMP4. In *T. ovatus*, the expression of BMP4 at 29 and 33 °C was significantly higher than those at 26 °C. Jaw deformities of fish at 29 and 33 °C were also significantly higher than those fish at 26 °C. This result is consistent with Ytteborg et al. (2010), such as the results of the study, namely, under the condition of high temperature raising, tend to increase the BMP4 gene expression. When the expression of BMP4 gene was upregulated, the incidence of jaw deformity increased (Villeneuve et al. 2006).

Previous studies have demonstrated that the 60A subgroup (BMP5, 6, 7) is functionally supernumerary and that the collective expression of the 60A subgroup determines the functional change in the early fish development (Kim et al. 2001; Solloway and Robertson 1999). During endochondral ossification, BMP5 can stimulate the mesenchymal cells to coagulate into chondrocytes (Bailon-Plaza et al. 1999; King et al. 1994). Moreover, the mutated BMP5 gene can cause skeletal malformations, indicating the essentiality of BMP5 in skeletal development (Storm

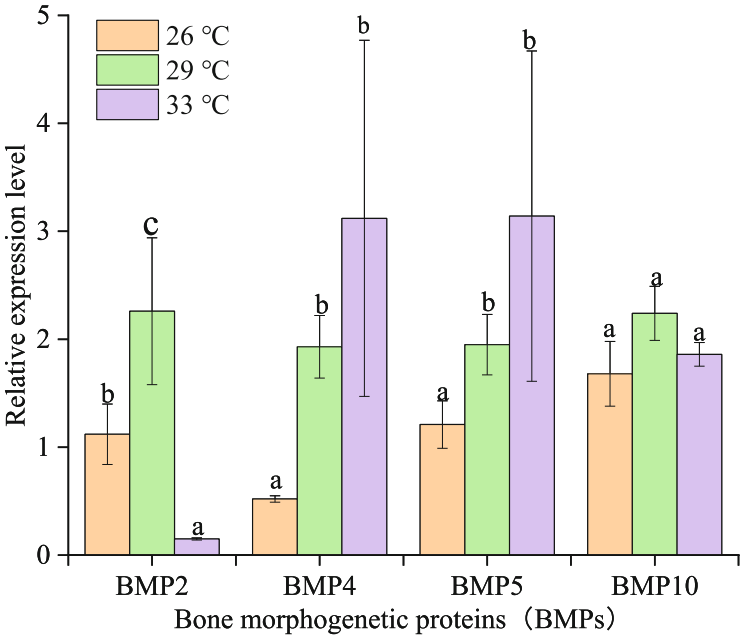

Fig. 10.2 Relative expression levels of bone morphogenetic proteins of *T. ovatus* larvae at different temperatures on 18 DPH. For BMP2, the reference was the 26 °C BMP2; for BMP4, the reference was the 26 °C BMP4; for BMP5, the reference was the 26 °C BMP5; for BMP10, the reference was the 26 °C BMP10. Means with the same letter are not significantly different ($P > 0.05$) (Ma et al. 2016)

et al. 1994; Kingsley et al. 1992; Wolfman et al. 2003). In *T. ovatus*, BMP5 expression patterns in fish is similar to the expression pattern of BMP4 (Fig. 10.2). Under 29 and 33 °C, the expression level of BMP5 in fish was significantly higher than that under 26 °C. Although the expression level of BMP5 and jaw abnormalities in *T. ovatus* increased with the increase of rearing temperature, there is no direct evidence to suggest that the expression of BMP5 can regulate the jaw abnormalities.

The BMP10 gene is mainly expressed in the heart of an adult but with a lower chance in the lung and liver (Neuhaus et al. 1999). During the period of heart development, BMP10 is expressed in the ventricular chamber, atrium, and trabeculae in *Bulbus cordis* (Neuhaus et al. 1999). In zebra fish, a comparatively high BMP10 expression occurs in the liver and heart, but low expression level can be observed in the kidney and brain (Bland 2001). In *T. ovatus*, feeding temperature had no significant effect on the expression of BMP10, indicating that 18 DPH was insensitive to the expression of BMP10 in ovate cells.

10.4 Conclusion

In summary, temperature significantly regulated the jaw development in larval *T. ovatus*. Jaw malformation rate in fish larvae increased with the increase of rearing temperature, and the highest malformation rate occurred in fish at 33 °C. To reduce massive malformation, we should control the rearing water temperature at 26–29 °C for *T. ovatus* larvae. Gene expression analysis indicates that the expression levels of BMP4 and BMP5 were positively correlated to the occurrence of jaw malformations, but the underlying mechanism needs further study.

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Chapter 11
Twist Gene in Golden Pompano *Trachinotus ovatus* Larvae

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Abstract In this chapter, the *twist* gene and its expression pattern in golden pompano are discussed. The golden pompano *twist* cDNA of 880 bp contains an open reading frame of 507 nucleotides encoding a protein of 168 amino acids. The protein has a molecular weight of 18.93 kDa. The expression of the twist gene increased with fish age after hatching and arrived at the maximum at 3 and 4 DPH. The highest expression level of the *twist* gene in fish tissues on 18 DPH occurred in the stomach and spleen, whereafter the kidney and brain. On 12 DPH, the expression of twist first increased and then decreased from 23 to 29 °C. On 18 DPH, the rearing temperature had no significant effect on the expression of the *twist* gene. The temporal variation of *twist* expression in fish larvae may enhance our understanding of bone ontogeny and formation in fish larvae, and the *twist* gene expression may serve as a useful indicator in larval rearing management of golden pompano to predict the occurrence of body malformation in the early stage of fish development.

Keywords *Twist* gene · Golden pompano *Trachinotus ovatus* · Gene expression · Ontogeny · Temperature

11.1 Introduction

Twist protein is a kind of nuclear transcription factor, containing a basic helix-loop-helix (bHLH) structure. Twist protein is a key to cell-type determination and differentiation. At the early developmental stage, twist protein can regulate osteogenesis (Lee et al. 1999; Hornik et al. 2004; Germanguz and Gitelman 2012). The *twist* gene is a small family and presents from the metazoan ranging to cnidarians to

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_11

human (Lee et al. 1999). In some species of vertebrates and invertebrates, the function of the *twist* gene is fundamental for development and survival, and *twist* mutants have embryonic lethality (Germanguz et al. 2007; Yeo et al. 2009). The *twist* gene is necessary for gastrulation and mesoderm formation and establishment of dorsoventral polarity and was initially recognized in common fruit fly *Drosophila melanogaster* (Simpson 1983; Thisse et al. 1987). The profile of twist gene expression has been reported in several species such as mice (Fuchtbauer 1995; Stoetzel et al. 1995), rats (Bloch-Zupan et al. 2001), frogs (Hopwood et al. 1989; Stoetzel et al. 1998), chicks (Tavares et al. 2001), Japanese rice fish (Yasutake et al. 2004), and zebra fish (Germanguz et al. 2007; Yeo et al. 2007). The *twist* gene plays a vital role in bone development. Several studies have demonstrated that the *twist* gene is expressed in primary osteoblastic cells and preosteoblasts (Murray et al. 1992; Rice et al. 2000). Torsion expression has been used as an indicator of axial mesodermal development, which is associated with spinal development The expression of *twist* has been used as an indicator of axial mesoderm development due to its relationship to vertebral column formation (Halpern et al. 1995; Yan et al. 1995).

Golden pompano *Trachinotus ovatus*, belonging to the phylum Chordata, class Actinopterygii, and family Carangidae, is an excellent candidate species in aquaculture with fast growth and is suitable for cage culture. However, body malformation is very common in *T. ovatus* larvae, and more than 33% larvae in a population have shown not less than one type of malformation (Ma et al. 2016; Zheng et al. 2016). In the research to explore the causes of *T. ovatus* malformation, a potential marker needs to be identified to predict the occurrence. To understand the expression pattern of early bone development, gene such as *twist* during fish ontogeny will shed a light to adjust fish malformation during early development. This chapter provides basic information about *twist* gene and twist protein of *T. ovatus* and prospects the expression pattern of the *twist* gene during the ontogeny of larval *T. ovatus* before the 18 days post-hatch (DPH) and the response of the *twist* gene expression level to water temperature on 12 DPH and 18 DPH. The results could provide a hint to understand the process of osteogenesis in golden pompano larvae.

11.2 Cloning and Sequencing of Twist Gene cDNA in Golden Pompano

The sequence of *T. ovatus* was based on unpublished transcriptome measured previously in our laboratory (Illumina HiSeq2000, annotated by NR, KOG, KEGG, and Swissprot). The genes cloning primers (forward 5-′-TCCCAGGAACCCATCTAT-3′; reverse 5′-CAACACGAAAGCACATCAG-3′; amplicon size: 852 bp) were designed with Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA).

The cDNA sequence length of *T. ovatus* twist gene (GenBank accession: KY204035) was 880 bp. The open reading frame (ORF) of the *twist* gene with

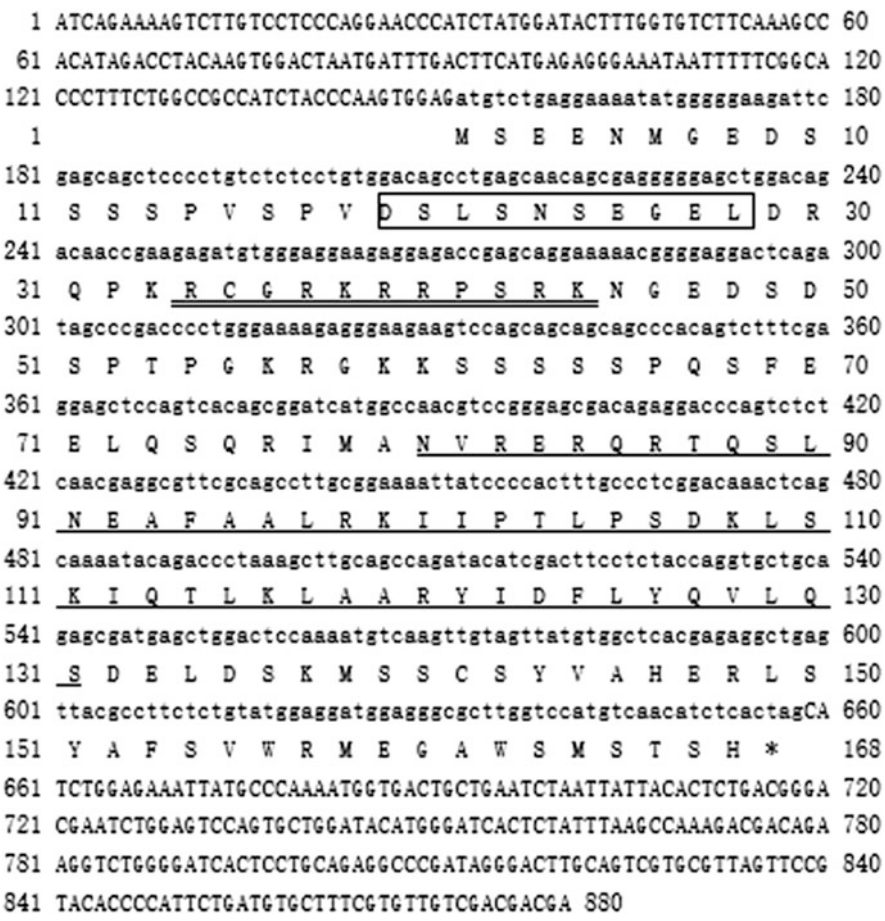

Fig. 11.1 The nucleotide sequence and deduced amino acids of the *T. ovatus* twist gene (underline: helix-loop-helix domains (HLH), double-underline: predictions of nuclear localization signals (NLSs), box: Granins signature) (Ma et al. 2018)

507 bp in length encoded a polypeptide of 168 amino acids (aa) (Fig. 11.1). The polypeptide has a molecular weight (Mw) of 18.93 kDa and theoretical isoelectric point (p*I*) of 9.14.

The bioinformatics analysis of the deduced amino acid sequence consisted of a Granins signature sequence (^{19}DSLSNSEgEL^{28}), nuclear localization signals (^{34}RCGRKRRPSRK^{44}), and a helix-loop-helix domain (^{80}N–S^{131}) (Fig. 11.2). The predicted secondary structure of the *T. ovatus* twist gene contained two helixes and one stand (Fig. 11.3). The molecular modeling of *T. ovatus* twist was shown in Fig. 11.3, containing two α-helixes. The deduced protein subcellular localization was predicted in the nucleus (0.960) by the PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

Fig. 11.2 The prediction of a conserved domain of the *T. ovatus* twist gene showing the Granins signature sequence (^{19}DSLSNSEgEL^{28}), nuclear localization signals (NLS: ^{34}RCGRKRRPSRK^{44}), and helix-loop-helix domain (^{80}N–S^{131}) (Ma et al. 2018)

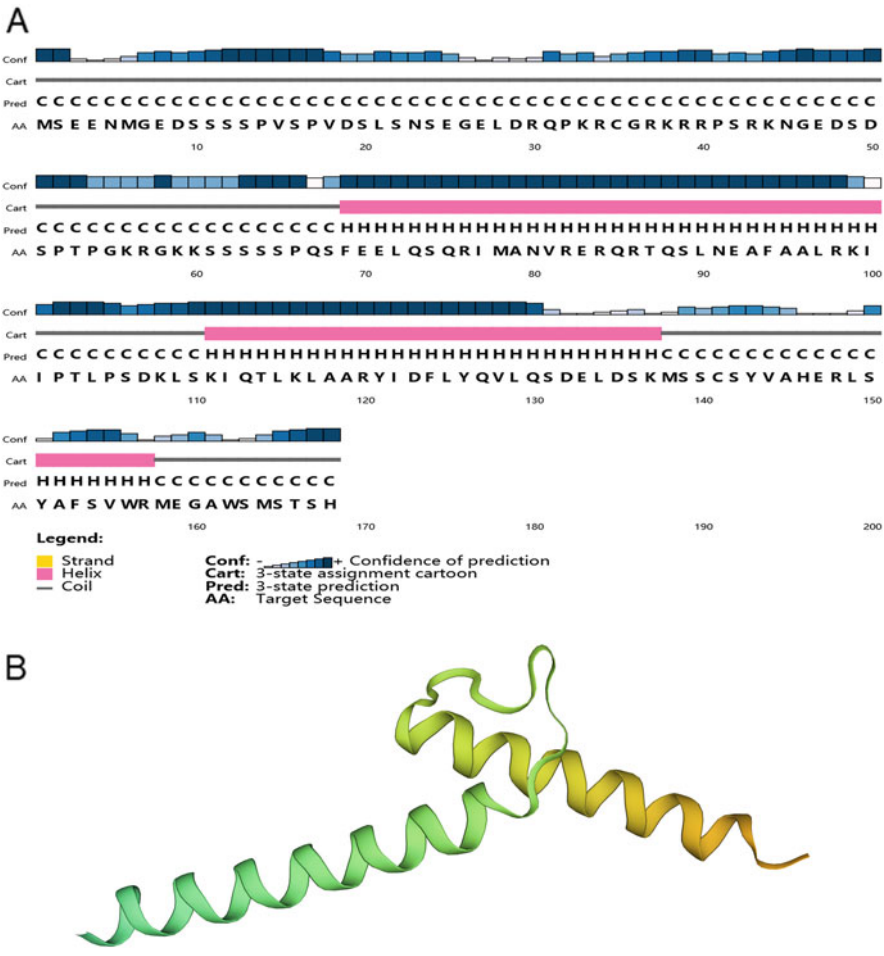

Fig. 11.3 The predicted secondary structure (**a**) and tertiary structure (**b**) of the *T. ovatus* twist gene (Ma et al. 2018)

11.3 Multiple Sequence Alignments and Phylogenetic Analysis

Table 11.1 shows the multiple sequence alignment results of the deduced amino acid sequences of the twist gene in *T. ovatus* and other species. The predicted amino acid sequence of the twist gene from golden pompano shared 98.8% identity with the twist 1 gene of *Oryzias latipes* (NP_001098177.1), 98.2% identity with the twist 1 gene of *Takifugu rubripes* (NP_001098069.1), and 77.1–84.5% identity with twist 2 gene of other species. A phylogenetic analysis of the twist gene comprised two main clusters, and the resulting neighbor-joining tree shows that the twist gene contains twist 1 and twist 2 clusters (Fig. 11.4). The deduced twist amino acid sequences of all species included the conserved helix loop and helix domain (Fig. 11.5).

All twist proteins belong to group "A" basic helix-loop-helix (bHLH) domain (Atchley and Fitch 1997). The function of twist protein is indicated by its expression pattern and is driven by the cell itself. The development expression histories of the conserved genes can provide evidence for the inference of gene function evolution (Germanguz et al. 2007). The *Twist* genes belong to the highly conserved family of crucial transcription factors, and their functions in directing the developmental program have diverged during evolution.

Table 11.1 Identity and similarity of twist gene homologue between the *T. ovatus* twist gene with other families (Ma et al. 2018)

Species	Homologues	Accession NO.	AA	Similarity (%)	Identity (%)
Trachinotus ovatus	Twist	Present study	168	–	–
Oryzias latipes	Twist 1	NP_001098177.1	168	98.8	95.8
Takifugu rubripes	Twist 1	NP_001098069.1	168	98.2	93.5
Danio rerio	Twist 1	NP_001017820.1	169	91.1	87.1
Gallus gallus	Twist 1	NP_990070.1	190	78.9	72.2
Rattus norvegicus	Twist 1	NP_445982.1	203	73.9	67.8
Mus musculus	Twist 1	NP_035788.1	206	72.8	66.8
Homo sapiens	Twist 1	NP_000465.1	202	74.8	68.6
Oryzias latipes	Twist 2	NP_001295933.1	164	84.5	77.6
Takifugu rubripes	Twist 2	NP_001098070.1	163	86.3	77.1
Danio rerio	Twist 2	NP_001005956.1	160	86.3	79.9
Gallus gallus	Twist 2	NP_990010.1	160	89.3	84.5
Mus musculus	Twist 2	NP_031881.1	160	88.7	83.3
Rattus norvegicus	Twist 2	NP_067723.1	160	88.7	83.3
Homo sapiens	Twist 2	NP_476527.1	160	88.7	83.3

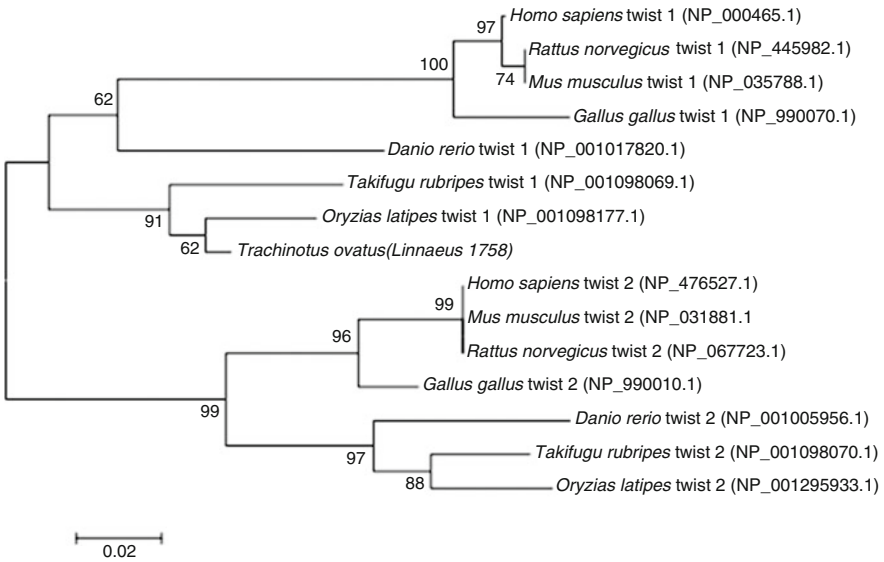

Fig. 11.4 Neighbor-joining tree based on phylogenetic analysis for amino acid sequences of *twist* gene family members (Ma et al. 2018)

11.4 Expression of Twist Gene

Approximately 300 mg (wet weight) larval fish were sampled from the rearing tanks in triplicate on 0, 1, 2, 3, 4, 5, 12, and 18 DPH. A sufficient number of individuals were collected. Total RNA was extracted using TRIzol (Invitrogen, USA). The Primer Premier 5 program (Premier Biosoft International, Palo Alto, CA, USA) was used for designing the primers of the *twist* gene (forward 5-′-GTGGGAGGAAGAGGAGACCG-3′; reverse 5-′-CGAGGGCAAAGTGGGGAT-3′; Amplicon size: 215 bp) and EF-1α (forward 5′-CCCCTTGGTCGTTTTGCC-3′; reverse 5′-GCCTTGGTTGTCTTTCCGCTA-3′; amplicon size: 101 bp). EF-1α was used as the internal reference and amplified. Quantitative real-time PCR (qPCR) was used to analyze the twist gene expression level in *T. ovatus* larvae. Gene-specific primer pairs for the twist gene (Table 11.1) were amplified in LightCycler480 II (Roche, Switzerland).

11.4.1 Expression of Twist During Fish Ontogeny

On 18 DPH, the expression level of the *twist* gene in *T. ovatus* larvae showed a trend of first increased and then decreased over time but was low at the time of hatch (Fig. 11.6). The expression level of *twist* increased with fish age and reached a higher level on 3 and 4 DPH (Fig. 11.6). Beginning from 5 DPH, the expression levels of

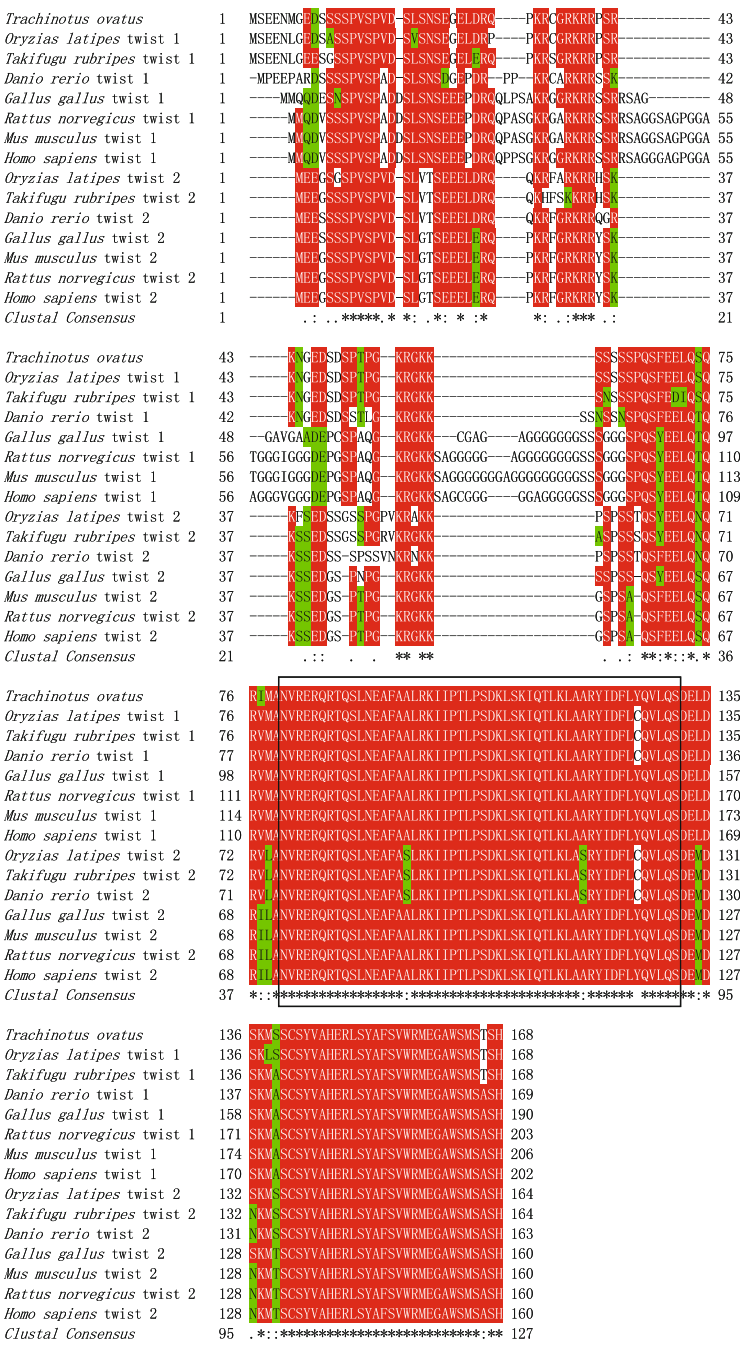

Fig. 11.5 Multiple sequence alignment of the deduced amino acid sequences of the twist gene. The conserved helix-loop-helix domain is boxed (Ma et al. 2018)

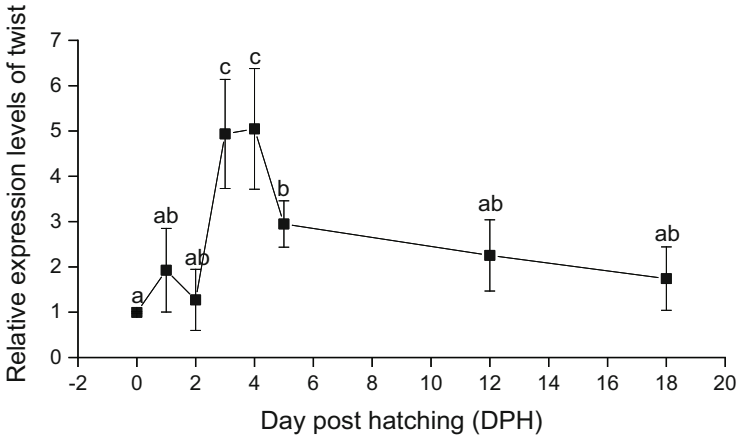

Fig. 11.6 Relative expression levels of the twist gene during the development of golden pompano larvae. Data with different letters indicate significant difference ($P < 0.05$) (Ma et al. 2018)

the *twist* gene in larval fish gradually reduced and became similar to the level of 1 DPH at 18 DPH ($P > 0.05$).

The *twist* gene can be involved in the development and differentiation regulation of many organs and tissues (Hebrok et al. 1997; Komori 2006; Singh et al. 2011; Soo et al. 2002). In the development of some vertebrates, such as chickens and mice, the expression of *twist* genes has shown a dual function: One is to prevent premature differentiation, and the other is for tissue specification (Fuchtbauer 2002; Scaal et al. 2002; Soo et al. 2002). Some studies have revealed that the *twist* genes can inhibit muscle and cartilage differentiation (Fuchtbauer 1995; Hebrok et al. 1994; Rohwedel et al. 1995). In murinae, the twist gene can inhibit myogenic differentiation, which is achieved by blocking the binding of MyoD to DNA, titrating E-proteins, and inhibiting transactivation by MEF-2 (Hamamori et al. 1997; Hebrok et al. 1997; Spicer et al. 1996). The expression pattern of the *twist* genes in embryonic development is important in function, and there have been a lot of related studies, but most of them are limited to the embryonic stage (Dill et al. 2007; Germanguz and Gitelman 2012; Germanguz et al. 2007; Yeo et al. 2009). In *Bombyx mori*, the expression of the *twist* gene can be detected from the egg to the embryo on the fifth day (Guo et al. 2011). In golden pompano, the expression level of *twist* genes increased significantly from hatch to 3 DPH and gradually reduced until 18 DPH. The high expression of the *twist* gene was consistent with the rapid growth period in the early development of larval golden pompano (Ma et al. 2014), indicating the quick formation of tissues and organs during this period.

11.4.2 Expression of Twist in Fish Tissues

In tissues, the level of *twist* gene expression in different organs of *T. ovatus* larvae on 18 DPH was in the descent order from spleen, stomach, brain, kidney, gill, head kidney, intestine, liver, muscle to heart (Fig. 11.7).

The *twist* genes have specifically expressed in some tissues and have the function of regulating muscle differentiation and mesoderm pattern (Baylies and Bate 1996; Castanon and Baylies 2002; Cripps et al. 1998). The expression of *twist* gene can be observed in hemolymph, epidermis, midgut, testis, ovary, and silk gland in *B. mori* (Guo et al. 2011). In some vertebrates, such as frog, chick, and mouse, pharyngeal arches and somites or sclerotome are the specific tissues for the expression of *twist* genes (Hopwood et al. 1989; Wolf et al. 1991; Li et al. 1995; Scaal et al. 2001; Tavares et al. 2001). In human, *twist* mutations are vital for development and pathophysiological change, because it can cause arrested osteoblastic differentiation and maintenance of an immature and neoplastic phenotype (Singh et al. 2011). In zebra fish, the expression of *twist* genes is identified in the head mesenchyme, intermediate mesoderm, body wall, somite, caudal gut, olfactory placode, branchial arch, caudal notochord, tail bud, hypochord, heart valve, and dorsal aorta (Germanguz and Gitelman 2012; Yeo et al. 2007, 2009). However, there is little research of the *twist* gene expression in different tissues of fish especially in early ontogeny. It is not yet known completely why spleen and stomach of larval *T. ovatus* were observed to have the higher expression of the *twist* gene. This kind of phenomenon may indicate that these organs develop fast as observed in other studies (Nieto et al. 1996; Shishido et al. 1993; Tavares et al. 2001), but the evidence remains to be investigated.

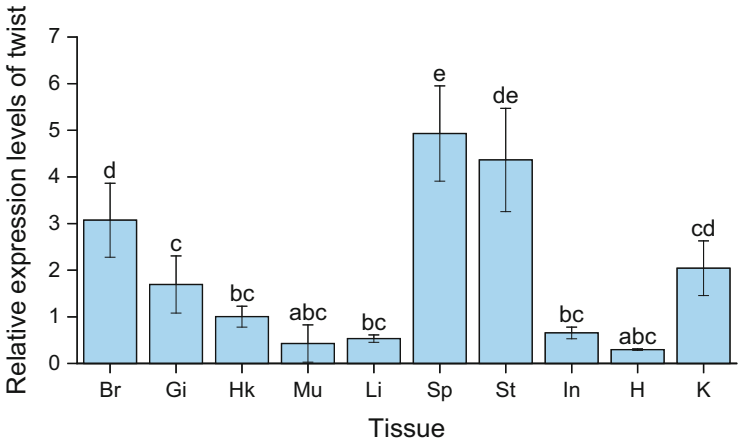

Fig. 11.7 Relative expression levels of the *twist* gene in different tissues of *Trachinotus ovatus*. Data with different letters indicate a significant difference ($P < 0.05$). Abbreviations: *Br* brain, *Gi* gill, *Hk* head kidney, *Mu* muscle, *Li* liver, *Sp* spleen, *St* stomach, *In* intestine, *H* heart, *K* kidney (Ma et al. 2018)

11.4.3 Response of Twist to Water Temperature

The *twist* gene expression in golden pompano of 12 and 18 DPH has a similar trend under the influence of water temperature, but the degree of change was different (Fig. 11.8). On 12 DPH, the maximum of *twist* gene expression level was observed in larvae at 26 °C ($P < 0.05$), and minimum of *twist* gene expression level was observed in larvae at 29 °C ($P < 0.05$). On 18 DPH, the expression level of *twist* in fish was not significantly affected by the water temperature ($P > 0.05$), but the highest and lowest expression levels occurred at the same temperature as 12 DPH. In each water temperature treatment, the expression level of *twist* has no significant difference between 12 and 18 DPH ($P > 0.05$).

The *twist* gene has been certified to control skeletogenic mesenchyme in zebra fish (Germanguz and Gitelman 2012), chick (Hornik et al. 2004), mice (Ishii et al. 2003), and human (Singh et al. 2011). The expression level of *twist* gene has an impact on osteogenic gene expression (Lee et al. 1999). Further evidence demonstrates that *twist* can interact with the DNA-binding domain of Runx2 to inhibit its function, without altering its expression (Bialek et al. 2004). In mice, the characters of *twist* genes in chondrogenesis and osteogenesis have been widely studied. Afanador et al. (2005) reported that the *twist* gene has a functional role in mice periodontal ligament, and its expression is transiently decreased by the occlusal hypofunction. In zebra fish, *twist* genes have been considered the major regulator of chondrogenesis (Hartmann 2009; Hinoi et al. 2006; Reinhold et al. 2006). This gene works as a negative regulator of osteoclastogenesis by inhibiting the expression of genes in the downstream of runx2 (Bialek et al. 2004). In Atlantic salmon, the *twist* gene expression level varies when skeleton malformation occurs in high temperature (Ytteborg et al. 2010). In golden pompano, the expression of *twist* in larvae reared at 26 °C which significantly increased as compared to those reared at 23 and 29 °C in

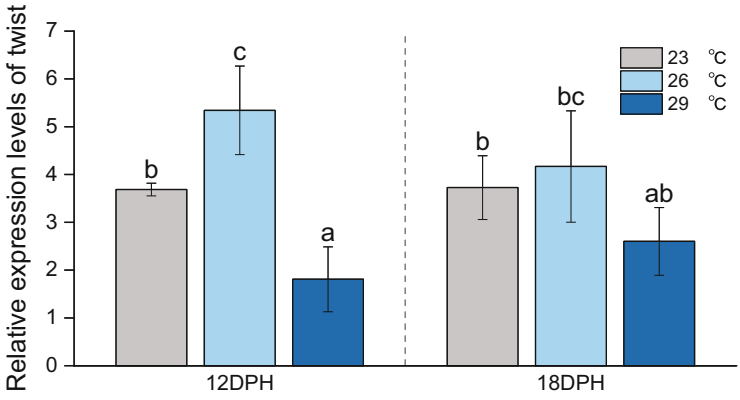

Fig. 11.8 Relative expression levels of the twist gene at different water temperatures on 12 DPH and 18 DPH in *Trachinotus ovatus* larvae. Data with different letters indicate significant difference ($P < 0.05$). "°C" is not shown in the legend (Ma et al. 2018)

some life stages. The expression level of the *twist* gene is consistent with increasing malformation of golden pompano larvae reared at 26 and 29 °C (Yang et al. 2016). These findings of *twist* may suggest that it may be a marker of skeleton malformation during fish ontogeny.

11.5 Conclusion

The length of cDNA sequence of *T. ovatus twist* gene (GenBank accession: KY204035) was 880 bp with an ORF of 507 bp, which encodes 168 amino acids. The twist gene expression showed high temporal and tissue preference at the stage of larval stage, with peak twist gene expression in 3–4 DPH and high expression in spleen and stomach. The expression of *twist* in golden pompano larvae showed high temporal and tissue preference, with peak expression in 3–4 DPH and high expression in spleen and stomach. Meanwhile, *twist* gene expression was significantly affected by water temperature on 12 DPH. These results would deepen our understanding of ontogenesis in the early stage of golden pompano larvae. In the field and a fish farming conditions, the *twist* gene expression of larval golden pompano can be used as a potential indicator for rapid assessment of environmental conditions affecting fish skeletal development.

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Chapter 12
Intestinal Fatty Acid-Binding Protein Gene (I-FABP) in Golden Pompano *Trachinotus ovatus* Larvae

Changlin Li, Maoshang Lin, Shengjie Zhou, and Rui Yang

Abstract Fatty acid-binding proteins (FABPs) belong to the multigene family with 14–16 kDa molecular mass and have the function of binding long-chain fatty acids in both vertebrates and invertebrates. The intestinal fatty acid-binding protein (I-FABP) is a small cytosolic protein and plays a critical character in intracellular fatty acid trafficking and metabolism in fish gut. In this chapter, the nucleotide sequence and expression level of I-FABP gene in *Trachinotus ovatus* larvae are discussed. The full-length cDNA of I-FABP spanned 815 bp and a 399 bp open reading frame encoding 132 amino acids. In the process of ontogenetic development, the expression of 18 DPH is the highest. The expression level of I-FABP gene is the highest in the intestine on 18 DPH, followed by eyes. On 18 DPH, the expression of I-FABP genes was significantly affected by environmental temperature and nutrition enhancement. Monitoring of I-FABP gene expression level could serve as a potential indicator, used in assessing nutrient supply and functional development of the digestive system in fish larvae.

Keywords Fatty acid-binding proteins · Golden pompano *Trachinotus ovatus* · Gene expression · Ontogeny · Nutrition enhancement

12.1 Introduction

The fatty acid-binding proteins (FABPs) are an important multigene family with 14–16 kDa molecular mass and involved in long-chain fatty acid uptake and deposition in animals (Alvite et al. 2008; Borchers et al. 1989; Kanda et al. 1989). The length of FABPs in different species varies from 126 to 137 amino acids (Chen and Shi 2009; Pelsers et al. 2005; Sharma et al. 2004). As members of intracellular

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_12

lipid binding protein superfamily, the primary function of FABPs is to defend cells from cytotoxic impacts of free fatty acids, engage in the transportation of free fatty acids for targeting specific metabolic pathways, engage in fatty acid signaling within the nucleus, and modify lipid metabolic enzymes (Besnard et al. 2002; Lowe et al. 1987; Storch and McDermott 2009). FABPs were named according to the mammalian tissue of their first isolation, such as the intestine, heart, liver, and myelin. According to previous studies, the existence of different FABP types with specific roles is related to the histological structure and physiological functions of different tissues (Banaszak et al. 1994; Veerkamp et al. 1991, 1993).

As a small cytosolic protein, the intestinal fatty acid-binding protein (I-FABP) plays a vital role in transportation and metabolism of intracellular fatty acids in fish gut (Her et al. 2004). It has been reported that the expression of I-FABP genes is essential evidence for intestinal differentiation in fish (Pierce et al. 2000), frogs (Chalmers et al. 2000), rats (Likic and Prendergast 1999), and humans (Sonnino et al. 2000). Previous studies have reported that the I-FABP gene expression level may be changed when the tissue is damaged and regeneration occurred (Schroyen et al. 2012; Simula et al. 2010). In finfish aquaculture, the I-FABP is broadly used as an indicator to study physiological function and nutrition response of fish (Overland et al. 2009; Venold et al. 2013; Yamamoto et al. 2007), but the specific function of I-FABP in fish larvae is not clear.

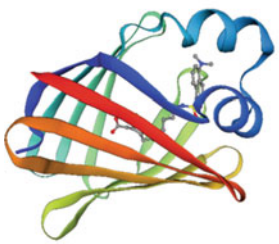
The golden pompano *Trachinotus ovatus*, a marine finfish in the Carangidae family, is an economic species and suitable for cage aquaculture (Ma et al. 2014). Substantial progress has been made in artificial breeding and culture technique of this species, but the low quality of juvenile fish is the biggest obstacle to expanding production of golden pompano in hatcheries (Ma et al. 2016; Zheng et al. 2016). The understanding of nutritional requirement and ontogeny of the digestive system of larval fish is helpful to improve the quality of fingerling and management of fish feeding (Ma et al. 2012). Since the I-FABP gene expression has been used as a useful indicator for feeding and intestinal functions in salmon and trout (Venold et al. 2012, 2013), this study was focused on the period of larval *T. ovatus* from hatching to taking shape of a functional stomach. The I-FABP gene expression pattern at different water temperature and nutritional conditions was investigated. The expression pattern of I-FABP genes could help us assess the metergasis of the digestive system in larval *T. ovatus* during early development stage and predict the occurrence of fish malformation in aquaculture using the expression of I-FABP genes as a potential indicator.

12.2 Cloning and Sequencing of I-FABP Gene cDNA

The Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used for designing the gene cloning primers (Table 12.1) based on the unpublished *T. ovatus* transcriptome sequences in our lab. Gene-specific primer pair sequences for the I-FABP gene were amplified in the LightCycler480 II system (Roche, Switzerland).

Table 12.1 Primer sequences of *Trachinotus ovatus* (Lin et al. 2017)

Primers	Sequence (5′–3′)	Amplicon sizes (bp)
I-FABP-F	GGCATGGCACAGTTCTT	689
I-FABP-R	CACTTTTCACAGGTTATTAGGT	
I-FABP-qF	CGGCTCCTGGAAAATTGATC	111
I-FABP-qR	ATGGTTATCTTGAGGTTGTCGTG	
EF-1α-qF	CCCCTTGGTCGTTTTGCC	101
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA	



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   1 TGCAAACAGTTCTGCCATTCAAAAAGAATACAGAGTCATTTGGCATGGATTATTGGGCAT  60
  61 GGCACAGTTCTTTCTATTCAAAACCACTGTGTTGCCCTATCAGCTGCAGTGCTAGATGTT 120
 121 GCCATGAGAAAATTTGAGCTTTAAACGGCCACATCAGCATTCAGATAGATGACGAGATAA 180
 181 GAGAGTGTGTGGTTTAAAAGGAGCGGCAGACTTTGAGTAAGACACTCCTTGCTGCAGAGT 240
 241 TGTCCAGTTCAGCTCCCACCGCCACCatgaccttcaacggctcctggaaaattgatcgca 300
   1                          M  T  F  N  G  S  W  K  I  D  R  N  12
 301 atgaaaactatgagaaattcatggaacaaatgggaattaacatggtgaagaggaagctgg 360
  13  E  N  Y  E  K  F  M  E  Q  M  G  I  N  M  V  K  R  K  L  A  32
 361 ctgctcacgacaacctcaagataaccattgaacagactggagacaagtttcatgtcaagg 420
  33  A  H  D  N  L  K  I  T  I  E  Q  T  G  D  K  F  H  V  K  E  52
 421 agagcagtaatttccgcactctggaaatagacttcaccctgggggtcaccttgagtaca 480
  53  S  S  N  F  R  T  L  E  I  D  F  T  L  G  V  T  F  E  Y  S  72
 481 gccttgcagatggaacagaactaacaggctcatggaccattgagggagacatgatgaagg 540
  73  L  A  D  G  T  E  L  T  G  S  W  T  I  E  G  D  M  M  K  G  92
 541 gggttttcatcagaaaggacaatggaaagcagctgacaacaaccagaatcattcaaggag 600
  93  V  F  I  R  K  D  N  G  K  Q  L  T  T  T  R  I  I  Q  G  D 112
 601 atgaactcgtacagagctacaactatgatggtgtggacgcaaagaggattttcaagaggg 660
 113  E  L  V  Q  S  Y  N  Y  D  G  V  D  A  K  R  I  F  K  R  G 132
 661 gttagACCACAAATGTTTGATTACAGGATTACATACAGTATTGTGATAAATCATTGACTT 720
       *
 721 ATACCTAATAACCTGTGAAAAGTGCACTTCTTGTAATGCCATATATTTGAATTGCATTGG 780
 781 ATTTTGATACTTGCAGTAATAAAGTGATACTGTAA                         815
```

Fig. 12.1 Predicted tertiary structure and nucleotide sequence and deduced amino acids of the intestinal fatty acid-binding protein (*I-FABP*) gene from golden pompano *Trachinotus ovatus* (Linnaeus 1758) (Lin et al. 2017). Cytosolic fatty-acid binding protein signature was underlined

EF-1α was used as the housekeeping gene and was also amplified. The full-length cDNA of I-FABP (GenBank accession: MF034871) spanned 815 bp, and a 399 bp open reading frame (ORF) encoding 132 amino acids (aa) polypeptide with a point of 6.13 theoretical isoelectric and a weight 15.24 kDa predicted molecule. The three-dimensional structure and nucleotide sequence of the I-FABP gene and the deduced amino acid sequence in the coding region are shown in Fig. 12.1. Blast results revealed that the I-FABP sequence of *T. ovatus* shared 67.94% identity with the I-FABP sequence from rats (PDB ID: 1ifc.1. A). I-FABP contained one beta sheet, two helixes in N-terminal amino acids, and ten antiparallel beta sheets forming a hydrophobic pocket. The amino acid sequence of the I-FABP gene from *T. ovatus* had high similarity and identity with *Lateolabrax japonicus* (97% and 87.9%, AOW69620.1) and *Larimichthys crocea* (93.2% and 85%, ALP43793.1). Similar to the FABP of other organisms, this special structure in I-FABP is essential for transporting lipid-soluble substances within cells like fatty acids (Andre et al. 2000; Hsu and Storch 1996; Venold et al. 2012).

Table 12.2 Identity and similarity of I-FABP between *T. ovatus* and other species homologue (Lin et al. 2017)

Species	Accession NO.	AA	Similarity (%)	Identity (%)
Trachinotus ovatus	Present study	132		
Lateolabrax japonicus	AOW69620.1	132	97	87.9
Larimichthys crocea	ALP43793.1	132	93.2	85
Oncorhynchus kisutch	XP_020352621.1	132	93.9	81.1
Danio rerio	AAF00925.1	132	90.2	82.6
Cyprinus carpio	ADF28554.1	132	90.2	80.3
Salmo salar	ACI66628.1	132	92.4	79.5
Ictalurus punctatus	NP_001187833.1	132	89.4	76.5
Columba livia	NP_001269737.1	132	86.4	75.8
Columba livia	NP_000125.2	132	82.6	65.2
Mus musculus	NP_032006.1	132	82.6	65.9
Rattus norvegicus	NP_032006.1	132	83.3	67.4

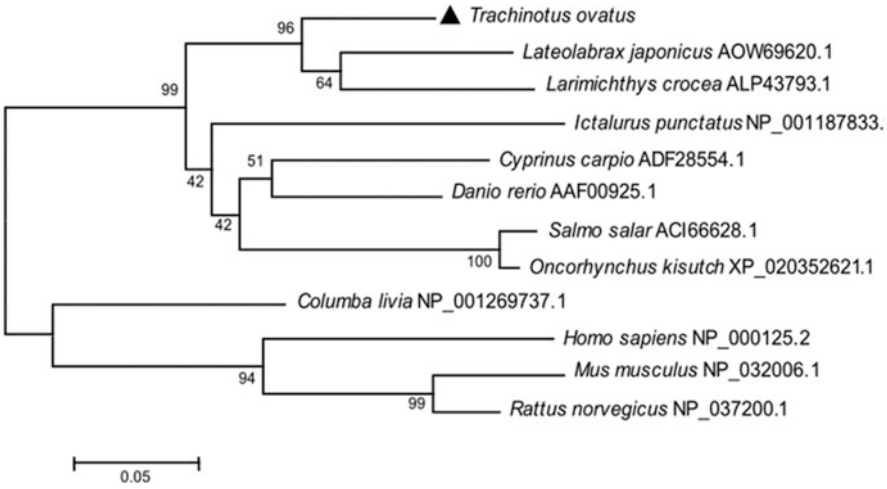

Fig. 12.2 Neighbor-joining tree based on phylogenetic analysis for amino acid sequences of the intestinal fatty acid-binding protein. The numbers represent the frequencies with which the tree topology was replicated after 1000 bootstrap iterations (Lin et al. 2017)

The amino acid sequences of golden pompano I-FABP genes were aligned with I-FABP amino acid sequences from other species (Table 12.2). The I-FABP amino acid sequence of golden pompano had higher sequence homology with I-FABP amino acid sequence in *Lateolabrax japonicus* (97% similarity and 87.9% identity, AOW69620.1) and *Larimichthys crocea* (93.2% similarity and 85% identity, ALP43793.1). Lower similarity (82.6–93.9%) and identity (65.2–82.6%) were shared with other organisms (Table 12.2). As shown in Fig. 12.2, the phylogenetic tree of hedgehogs comprises two main clusters, the I-FABPs from golden pompano, and other fish were clustered together, while the I-FABP from bird and mammal was

grouped (Fig. 12.2). Multiple polypeptide sequence alignment revealed the cytosolic fatty acid-binding protein signature, and all showed high identity and similarity (Fig. 12.3).

12.3 Ontogenetic Expression of the I-FABP Gene

Previous studies have reported the I-FABP gene expression pattern during embryogenesis and early developmental stage of zebra fish through in situ hybridization (Andre et al. 2000; Sharma et al. 2004). However, the expression level of I-FABP gene in the early developmental stage of larval fish has not been investigated yet. But some studies found that the expression of I-FABP gene significantly rises after hatching in terrestrial species such as pigeon, chick, and turkey (Ding and Lilburn 2002; Katongole and March 1980; Xie et al. 2013). On the 17th day of mouse embryonic development, the expression level of I-FABP gene increases sharply (Green et al. 1992). In golden pompano, the I-FABP gene expression remained at a low level during hatching but gently increased from 0 to 5 DPH. The I-FABP expression level suddenly increased and reached the maximum on 12 DPH (Fig. 12.4). These results indicate that the expression pattern is consistent with the development of the digestive tract, as the digestive system in larvae of *T. ovatus* is primitive at hatching, and a functional digestive system only appeared approximately 15 DPH (Ma et al. 2014). In addition, the increased expression of I-FABP after hatching may be related to the uptake of dietary fatty acids at the late developmental stage of *T. ovatus* larvae with the formation of a functional digestive tract.

Although FABPs were named according to the tissues where they were found, FABP can be widely expressed in animal tissues, but the expression is species-specific. For example, the I-FABP gene is only expressed in the intestine of human (Sweetser et al. 1987), but the expression of I-FABP gene can be detected in the intestine and brain of zebra fish (*Danio rerio*) (Sharma et al. 2004). In Atlantic salmon *Salmo salar*, the I-FABP gene expression can be detected in many tissues such as pyloric caeca, stomach, spleen, intestine, brain, and muscle (Venold et al. 2013). In golden pompano, the expression level of I-FABP gene in the intestine was higher than those in the eyes, heart, and muscle (Fig. 12.5). Moreover, the expression of I-FABP gene was also detected in the stomach of gold pompano larvae but was lower than that reported in Atlantic salmon (Venold et al. 2013). It is worthwhile to mention that no previous study found that the I-FABP gene can be expressed in the eyes of larval golden pompano. It may suggest that the function of this gene in the eye is related to the development of vision, but further studies are required to prove it.

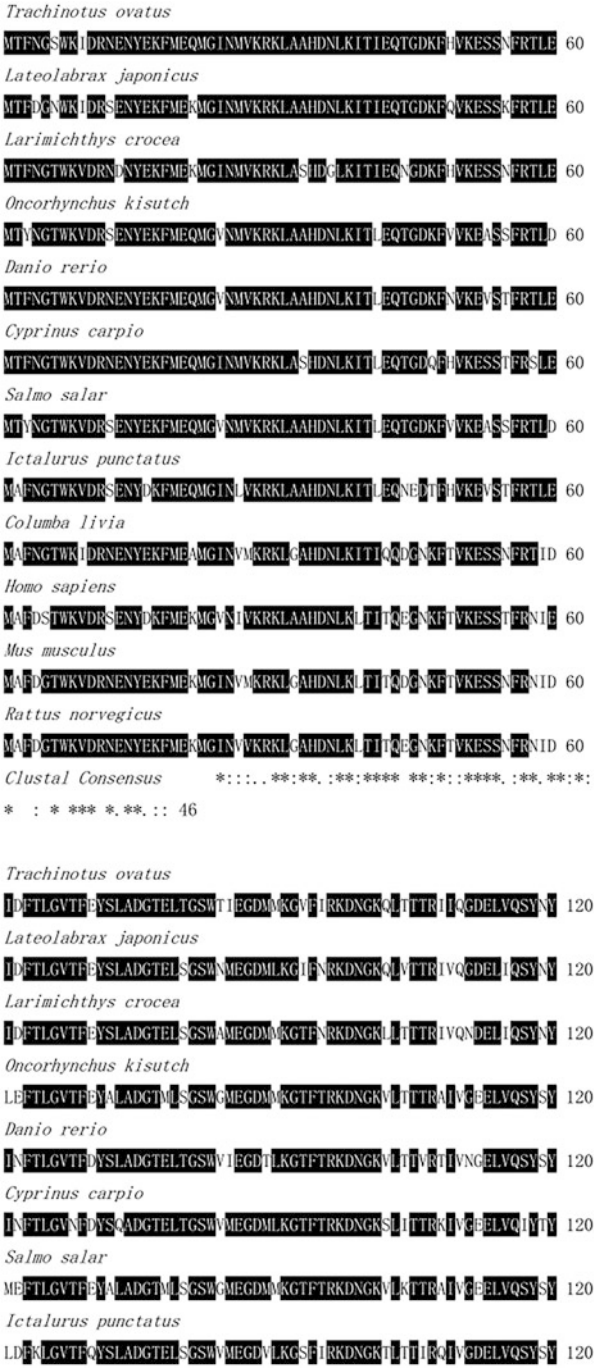

Fig. 12.3 Multiple sequence alignment of the deduced amino acid sequence of *T. ovatus* I-FABP with other known homologous I-FABP amino acid sequence (Lin et al. 2017)

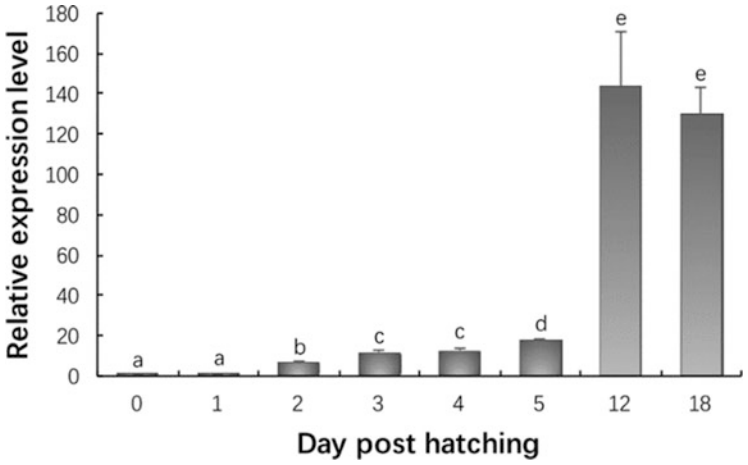

Fig. 12.4 Ontogenetic relative expression level of the *I-FABP* gene in larval *T. ovatus*. Data with different letters were significantly different ($P < 0.05$) (Lin et al. 2017)

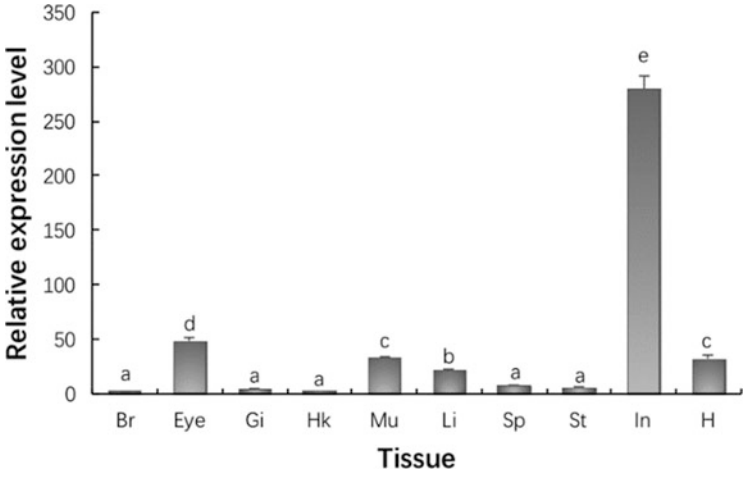

Fig. 12.5 Tissue relative expression level of *I-FABP* gene in larval *T. ovatus*. Data with different letters were significantly different ($P < 0.05$). Abbreviations: *Br* brain, *Gi* gill, *Hk* head kidney, *Mu* muscle, *Li* liver, *Sp* spleen, *St* stomach, *In* intestine, *H* heart (Lin et al. 2017)

12.4 Impact of Temperature and Nutrition on the Expression of I-FABP in Golden Pompano

Temperature is an essential environmental element in fish larvae development and can significantly impact fish feeding metabolism and behavior (Blaxter 1992; Bustos et al. 2007; Ma 2014). It has been proved that fatty acid metabolism and composition in fish can be regulated by environmental temperature (Farkas et al. 1980; Kemp and

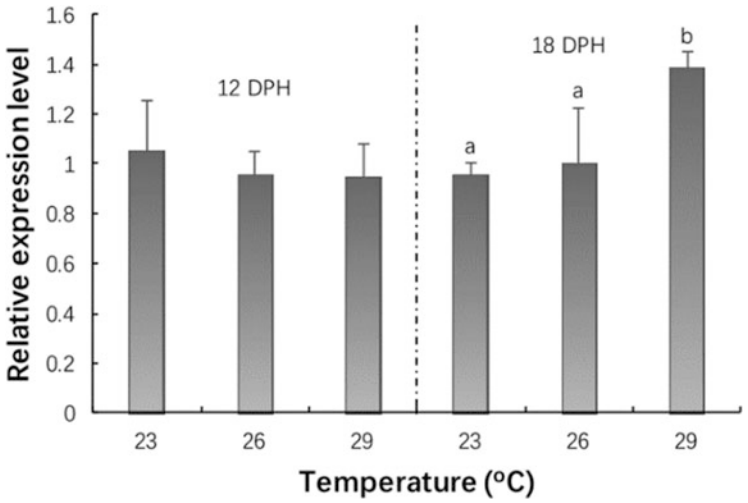

Fig. 12.6 Response of *I-FABP* gene to water temperature in larval *T. ovatus*. Data with different letters were significantly different ($P < 0.05$) (Lin et al. 2017)

Smith 1970; Skalli et al. 2006). I-FABP is a major fatty acid-binding protein in fish that plays an important role in fatty acid uptake (Storch and Thumser 2010). Nevertheless, during the early development of fish larvae, it is unclear if the I-FABP gene expression can be affected by temperature. In golden pompano, no significant difference was found in the expression level of I-FABP under temperature treatment on 12 DPH, but a significant difference was found on 18 DPH (Fig. 12.6). The results may reflect the developmental stage of the digestive system in *T. ovatus* larvae which did not appear functional in the stomach until 18 DPH.

Fatty acid-binding proteins can activate peroxisome-proliferating receptors and lead to upregulation of lipid-related genes (Lawrence et al. 2000; Tan et al. 2002). The expression level of I-FABP genes decreased when Atlantic salmon is fed with soybean meal (Venold et al. 2013). The inclusion of soybean meal in the diet can lead to inflammation and reduction in I-FABP gene expression. So, the low level of I-FABP gene expression may be a sign of the inability to bind to dietary fatty acids (Venold et al. 2013). In golden pompano, expression analysis showed that I-FABP gene was sensitive to the nutrition enhancement (Fig. 12.7). The maximum was found in the group fed with non-enriched *Artemia* nauplii, and the minimum I-FABP expression level was found in the group fed with enriched *Nannochloropsis*. This expression pattern of I-FABP gene seems to vary inversely as the content of polyunsaturated fatty acids in the diet. In this study, the content of polyunsaturated fatty acids in the *Nannochloropsis*-enriched diet is higher than that of the non-enriched diet (Yang et al. 2015). This may indicate that I-FABP gene expression in larval *T. ovatus* is negatively influenced by the total polyunsaturated fatty acid in the diet.

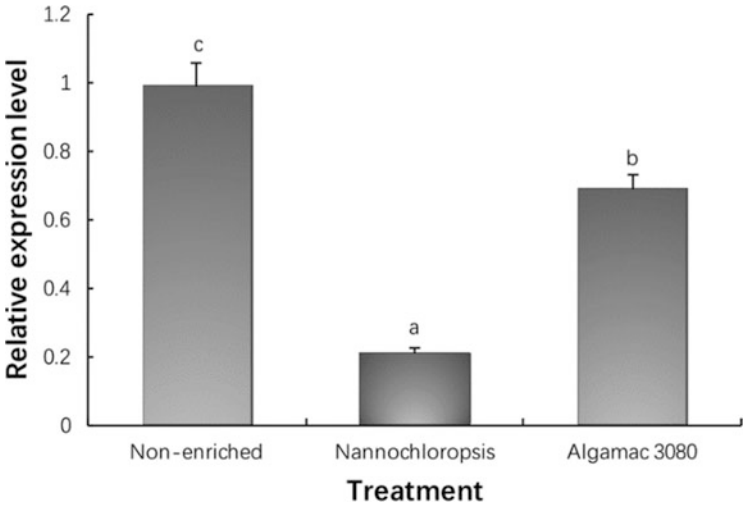

Fig. 12.7 Response of *I-FABP* gene relative expression level to nutrition manipulation in larval *T. ovatus*. Data with different letters were significantly different ($P < 0.05$) (Lin et al. 2017)

12.5 Conclusion

When the functional stomach formed on 18 DPH, the temperature and the content of fatty acid in the diet significantly affected the I-FABP gene expression level in larval *T. ovatus*. The time-dependent expression of I-FABP gene in fish larvae is of great significance for understanding the ontogenesis and growth of larval fish in early development. Monitoring the expression level of I-FABP gene in larval *T. ovatus* can be an effective indicator in the field and fish farm to quickly evaluate the effects of environmental conditions and nutrition on fish development.

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Chapter 13
Effects of Water Temperature and Nutritional Manipulation on the Expression of Liver-Type Fatty Acid-Binding Protein (L-FABP) Gene in Golden Pompano *Trachinotus ovatus* Larvae

Haijun Wei, Shengjie Zhou, and Mingjun Fu

Abstract The liver fatty acid-binding protein (L-FABP) is a 14-kDa cytoplasmic protein that has the function of binding long-chain fatty acids with high affinity. L-FABP cDNA was 604 bp in golden pompano, and its expression level varies from ages and tissues. Temperature and nutrition can significantly regulate the expression level of L-FABP in *Trachinotus ovatus*. On 12 and 18 days post hatch, the maximum expression appeared in fish larvae at 29 °C. The maximum expression of L-FABP was observed in fish fed with *Artemia* nauplii enriched with Algamac 3080, and the minimum expression was observed in fish fed with *Artemia* nauplii enriched with *Nannochloropsis*. This chapter addresses the expression of the L-FABP gene in *Trachinotus ovatus* larvae under different nutritional and environmental conditions. This study suggests that L-FABP can be used as a potential indicator to evaluate the digestive function of fish larvae during early development.

Keywords Liver-type fatty acid-binding protein · Nutrition · Temperature · *Trachinotus ovatus*

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_13

13.1 Introduction

The fatty acid-binding proteins (FABPs) belong to a multigene family with 14–16 kDa molecular mass, which could combine with fatty acids or other organic dissolved substances in eukaryotic organisms (Borchers et al. 1989, 1997; Kanda et al. 1989; Alvite et al. 2008). The length of FABPs was 126–137 amino acids, and it varies from species to species (Pelsers et al. 2005; Chen and Shi 2009). FABPs can protect cells from the cytotoxic effects of free fatty acids, target specific metabolic pathways, mediate the transport of free fatty acids, modify lipid metabolism enzymes, and participate in fatty acid signaling in the nucleus (Besnard et al. 2002; Storch and McDermott 2009; Lowe et al. 1987). According to the physiological characteristics of different tissues, different types of FABP fulfilled the specific functions (Banaszak et al. 1994; Veerkamp et al. 1991, 1993); therefore, FABPs have been named after the first mammalian tissue from which they were isolated, for instance, heart, adipose, myelin, intestine, and liver tissue.

Veerkamp and Maatman (1995) pointed out that a 14 kDa cytoplasmic protein, liver FABP (L-FABP), could bind long-chain fatty acids with high affinity. The complete primary structures of L-FABPs have been determined in some nonmammalian vertebrates, such as catfish, frogs, chick, and shark (Di Pietro et al. 1996; Schleicher and Santome 1996; Baba et al. 1999; Cecilian et al. 1994; Medzihradszky et al. 1992). Furthermore, mammalian L-FABPs, a small cytosolic protein in many tissues including kidney, liver, and small intestine, play an important character in intracellular fatty acid metabolism and trafficking (Her et al. 2003).

Due to rapid growth, strong suitability, and adaptability, *Trachinotus ovatus* has become a suitable species for culture (Ma et al. 2014). According to Storch and McDermott (2009), L-FABP can intervene the transport of free fatty acids to target specific metabolic pathways, and it can improve fingerling quality and our knowledge of the nutrition requirement and digestive ontogeny in fish larvae (Ma et al. 2012). Consequently, this chapter aims to discuss the L-FABP expression during the development in the first 18 DPH of golden pompano *T. ovatus* and the effects of temperature and nutritional manipulation on L-FABP gene expression. Such information will improve our understanding of the digestive organs of *T. ovatus* and provide potential indicators to evaluate digestive function during early development of fish larvae.

13.2 Cloning and Sequencing of L-FABP Gene cDNA

The Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used for designing the gene cloning primers (Table 13.1) based on the unpublished *T. ovatus* transcriptome sequences in our lab. The full length of L-FABP cDNA (GenBank accession No. MF034872) from *T. ovatus* was 604 bp, including a UTR of 154 bp, a 3′-UTR of 69 bp, and a 281 bp ORF encoding a 126 amino acids polypeptide with a

Table 13.1 Sequences of primers (Zhou et al. 2019)

Primers	Sequence (5′–3′)	Amplicon sizes (bp)
EF-1α-qF	CCCCTTGGTCGTTTTGCC	101
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA	
L-FABP-F	ATTGCGATGGGACCCC	539
L-FABP-R	TTAACTTCACTGCCAAGTT	
L-FABP-qF	CAAGGACATCAAGCCAATTACTG	100
L-FABP-qR	AATGGTAAAGGAATTGGTCACAG	

```
  1 GTTACTCATTACACATTGCGATGGGACCCCTTTGCCTTCCAGTATAAGAAGGTTTGGTAG  60
 61 CACATTCACATTCTCCACATTGTGTTGAGCTTCACACAGCTGTCTCAGCCTCCACTCCAC 120
121 TTTGGTGAAGGAGATCCCAGACCTTCTAGAGAAGatggacttcaatggaacatggcaggt 180
  1                                   M  D  F  N  G  T  W  Q  V   9
181 ttactctcaggagaattacgagtcgttcctcagggccatggaactcccagaagatgtcat 240
 10  Y  S  Q  E  N  Y  E  S  F  L  R  A  M  E  L  P  E  D  V  I  29
241 caagatggccaaggacatcaagccaattactgagatcaaacagagtggcaatgactttgt 300
 30  K  M  A  K  D  I  K  P  I  T  E  I  K  Q  S  G  N  D  F  V  49
301 tgtcacctccaagacccctggaaagtctgtgaccaattcctttaccattggtaaggaggc 360
 50  V  T  S  K  T  P  G  K  S  V  T  N  S  F  T  I  G  K  E  A  69
361 tgaaatcaccaccatggacggcaagaagctcaagtgcatcgtcaatctggagggtggcaa 420
 70  E  I  T  T  M  D  G  K  K  L  K  C  I  V  N  L  E  G  G  K  89
421 aatggtgtgcaagactggcaagttctgccacatccaagagctcaagggaggagagatggt 480
 90  M  V  C  K  T  G  K  F  C  H  I  Q  E  L  K  G  G  E  M  V  109
481 tgagacattgaccatgggctcaacaactctcgtcaggaagagcaaaaagatgtaaACTTG 540
110  E  T  L  T  M  G  S  T  T  L  V  R  K  S  K  K  M  *        126
541 GCAGTGAAGTTAACCAATGTCTAATAAAAGTGTTGCTTGATAACAAAAAAAAAAAAAAA 600
601 AAAA                                                         604
```

Fig. 13.1 Nucleotide sequence and deduced amino acid of *L-FABP* gene in *T. ovatus* (Zhou et al. 2019)

point of 8.73 theoretical isoelectric, and a weight 14.06 kDa predicted molecule (Fig. 13.1). The deduced protein sequence has the characteristics of cytoplasmic fatty acid-binding protein, as shown in the multiple sequence alignment, and this domain was found in all detected sequences (Fig. 13.2). Multiple sequence alignments showed that the L-FABP of *T. ovatus* was highly identical with other known orthologs (Fig. 13.2). The L-FABP sequence of *T. ovatus* ginseng is 76.61% identical to zebra fish liver bile acid-binding protein (PDB ID: 2qo4). Then, there are ten antiparallel β-sheets forming a hydrophobic pocket.

Table 13.2 shows the multiple sequence alignment of some known L-FABP family with the deduced amino acid sequences of L-FABP genes. The predicted amino acid sequence of L-FABP genes from *T. ovatus* had high identity and similarity with *Epinephelus coioides* (95.2% and 97.6%, ADG29164.1) and had different similarity (62.2–98.4%) and identity (40.9–84.1%) with other species (Table 13.2). Similar to the FABP of other species, the L-FABP in golden pompano can actively participate in the transport of fatty acids and other fat-soluble substances

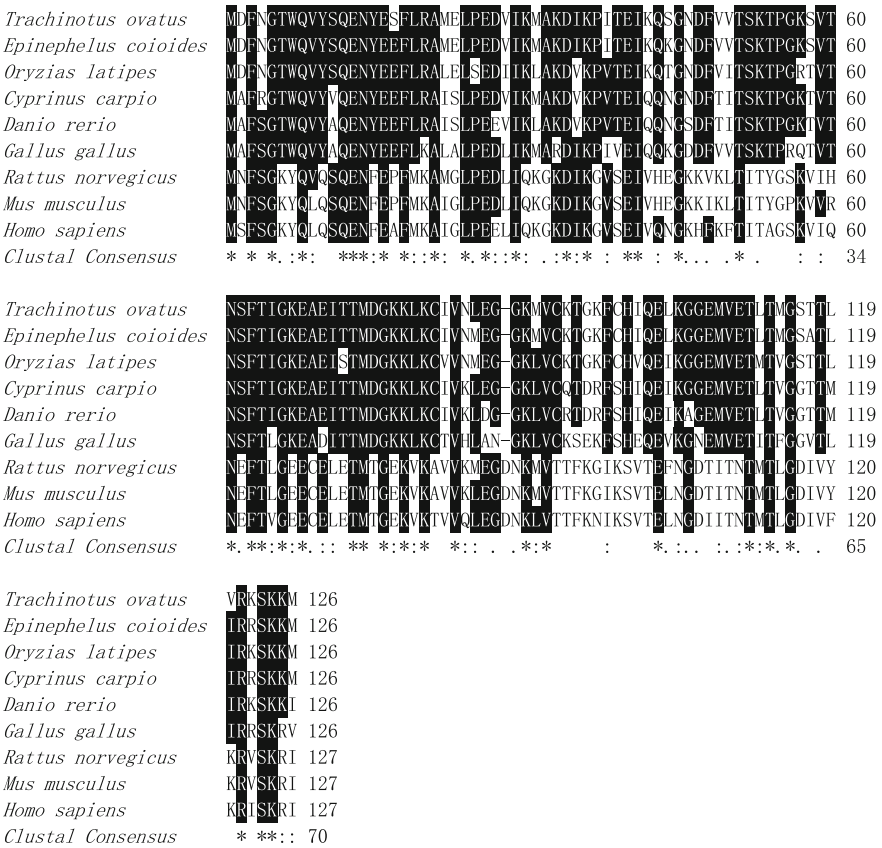

Fig. 13.2 Aligned of L-FABP with other known homologous H-FABP amino acid sequences in *T. ovatus* (Zhou et al. 2019)

Table 13.2 Multiple sequence alignment of L-FABP genes in golden pompano (Zhou et al. 2019)

Species	Accession NO.	AA	Similarity (%)	Identity (%)
Trachinotus ovatus	Present study	126	–	–
Epinephelus coioides	ADG29164.1	126	97.6	95.2
Oryzias latipes	XP_004078356.1	126	98.4	84.1
Cyprinus carpio	ACA64701.1	126	92.1	80.2
Danio rerio	NP_694492.1	126	92.9	76.2
Gallus gallus	NP_989965.1	126	87.3	70.6
Rattus norvegicus	NP_036688.1	127	62.2	40.9
Mus musculus	NP_059095.1	127	62.2	41.7
Homo sapiens	NP_001434.1	127	63.8	40.9

in cells, assigning fatty acids to different metabolic pathways (Hsu and Storch 1996; Andre et al. 2000; Venold et al. 2013; Storch and Corsico 2008).

13.3 Expression of L-FABP Genes in *T. ovatus*

On 18 DPH, the expression level of *T. ovatus* L-FABP gene in the heart, muscle, stomach, intestine, eye, spleen, head kidney, gill, and brain was similar and significantly lower than that in the liver ($P < 0.01$, Fig. 13.3). The expression level of L-FABP gene has been observed from the embryo stage to adult stage in zebra fish (Her et al. 2003). The study is rare on the expression level of L-FABP gene during early life of commercially cultured larval fish. During the embryogenesis of chicks and Japanese quails, a small amount of L-FABP mRNA is identified in the liver and intestinal tissues (Murai et al. 2009). The L-FABP gene expression level was low at hatching, but it continued to increase significantly from 0 DPH to 4 DPH (Fig. 13.3). The expression of L-FABP rapidly raised starting from 4 DPH and reached a high level and remained at a stable level until 18 DPH when the experiment was complete. Such expression pattern suggests that the *T. ovatus* L-FABP gene in larvae expressed before the digestive tract developed, as the digestive system of *T. ovatus* expressed before the development of the digestive tract, as the digestive system was immature at hatch, and a mature digestive system emerged around 15 DPH (Ma et al. 2014). Additionally, the upregulation of L-FABP expression may be connected with the uptake of dietary fatty acids after a fully mature digestive tract developed in *T. ovatus* larvae (Ma et al. 2014).

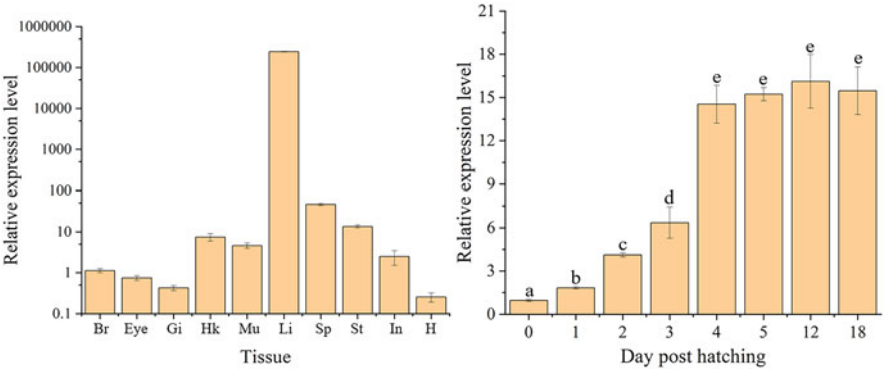

Fig. 13.3 Tissue and ontogenetic expression of L-FABP in *T. ovatus* larvae (Zhou et al. 2019)

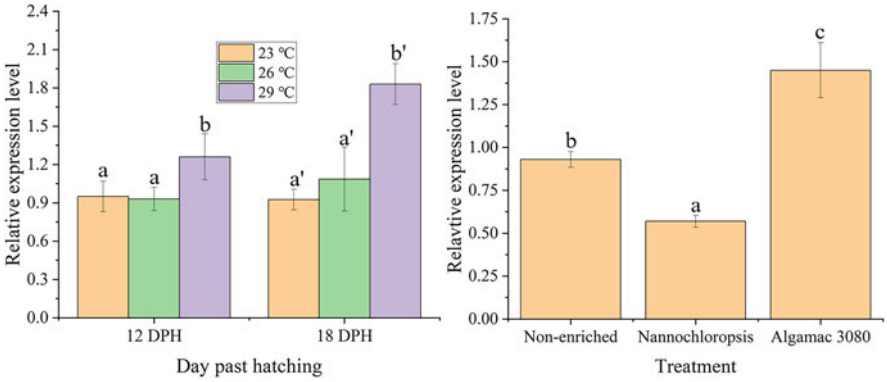

Fig. 13.4 Effects of temperature and nutrient enhancement on the expression of L-FABP gene in *T. ovatus* larvae (Zhou et al. 2019)

13.4 Temperature and Nutrient Enhancement Regulates the Expression of L-FABP Genes

Although genetic factors can control fish growth, fish development is also regulated by environmental parameters. As an essential environmental factor, the temperature can cause significant impact in fish metabolism and feeding activity (Ma et al. 2014), and water temperature can significantly affect the digestive function of fish larvae (Liu et al. 2017; Hevrøy et al. 2012). Fatty acid metabolism and fatty acid composition of fish can be regulated by temperature (Kemp and Smith 1970; Skalli et al. 2006; Farkas et al. 1980), but it is not clear whether temperature could impact the expression level of L-FABP gene in the early developmental stage of larval fish. In golden pompano, the expression level of the L-FABP gene has significant difference in different water temperatures on 12 and 18 DPH (Fig. 13.4). Compared to 12 DPH, a higher expression level of the L-FABP gene was noticed at 18 DPH, which may reveal the developmental process of the digestive tract in fish larvae, as the digestive system of *T. ovatus* seems to be more functional at 18 DPH (Ma et al. 2014).

FABP can affect gene regulation and activation of peroxisome proliferator-activated receptors, leading to the decline of the expression of lipid-related genes (Tan et al. 2002; Lawrence et al. 2000). Stimulation is not always the primary determinant as it may only stimulate the expression of the L-FABP gene slightly (Atsushi et al. 2009). In addition, it may be caused by the start of first feeding after yolk absorption, and the L-FABP gene expression level did not alter after incubation (Atsushi et al. 2009). In golden pompano, the L-FABP gene expression level had significant difference in different nutritional enhancement. The highest expression level of the L-FABP gene was observed in the Algamac 3080 treatment group, while the lowest expression level was found in the *Nannochloropsis* group (Fig. 13.4). This expression may have a parabolic relationship with the diet total saturated fatty

acid content. In the Algamac 3080 group, the high level of diet fatty acid content may facilitate the expression of the L-FABP gene in *T. ovatus* (Yang et al. 2015).

13.5 Conclusion

The expression of L-FABP gene in *T. ovatus* was significantly affected by temperature and nutrient treatments. The tissue-dependent and time-dependent expressions of the L-FABP gene in larval fish are essential for understanding the ontogeny and growth of fish during their early stage. The monitoring of L-FABP gene expressions in larval *T. ovatus* may serve as an effective indicator to assess the response of fish to the change of nutritional and environmental conditions during fish early development.

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Chapter 14
Follistatin-Related Protein Gene in Golden Pompano *Trachinotus ovatus* Larvae

Xingmei Huang, Mingjun Fu, and Weiming Jiang

Abstract The follistatin-related protein (FRSP), a member of the follistatin domain, has structural homology and functional similarity with follistatin. The FRSP gene plays many functions by binding with cellular regulatory proteins and transforming the growth factor in the β superfamily such as myostatin and activin. The *Trachinotus ovatus* FRSP gene full-length cDNA consists of 1500 bp for open reading frame of 311 amino acids with 34.55 kDa molecular weight. The FRSP gene expression in *Trachinotus ovatus* larvae varied significantly during ontogenetic stage and in different tissues. The highest expression of FRSP gene was observed in the liver, followed by the head kidney and eye, suggesting that the FRSP gene may be included in controlling the growth and function of these tissues. This chapter discusses the FRSP gene and its expression in golden pompano during ontogenetic development and supplies useful knowledge to investigate the functional mechanism of the FRSP gene in fish ontogeny for future studies.

Keywords Follistatin-related protein · Tissue expression · Temperature · Golden pompano *Trachinotus ovatus*

14.1 Introduction

The follistatin domain includes a large group of proteins with a highly conserved module of cysteine-rich sequence such as follistatin, follistatin-related protein (FSRP), secreted protein acidic, agrin, and Mac25 (Phillips and de Kretser 1998). Compared with other members of the follistatin domain, FSPR has higher structural

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_14

homology with follistatin. Both follistatin and FSRP of the follistatin domain have the property of modulating the activity of the TGF-TGF superfamily members (Lee and McPherron 2001; Hill et al. 2002). Moreover, FSRP has the characteristics of high affinity for activin and bone morphogenetic proteins (BMPs) binding protein similar to follistatin (Tsuchida et al. 2000; Tortoriello et al. 2001). Therefore, FSRP is deemed to be a functional homologue of folliclestatin (Sidis et al. 2002).

In terms of disease prevention and control, FRSP has the functions of regulating osteoarthritis and arthritis (Tanaka et al. 2003; Kawabata et al. 2004), inhibiting the growth of some cancer cells (Sumitomo et al. 2000; Chan et al. 2009) and protecting myocardial cells from hypoxia and apoptosis (Oshima et al. 2008). In chicks, the FSRP gene not only controls neural induction and mesodermal dorsalization (Patel et al. 1996) but also regulates signal pathways by BMPs or related ligands (Towers et al. 1999). During mouse heart development, FSRP acts as a negative adjuster of active protein family member (Takehara-Kasamatsu et al. 2007). In clinical applications, functional hematopoietic progenitors are controlled by the reciprocity between FSRP and fibronectin (Maguer-Satta et al. 2003, 2006), and FSRP induces differentiation of immature progenitor cells, which is clinically relevant to treatment strategies and hematopoietic regulation (Maguer-Satta and Rimokh 2004). Studies have shown that FSRP contributes to the bone formation by preventing hematopoietic precursor and osteoclast differentiation (Bartholin et al. 2005), activates Toll-like receptor 4 (TLR4), and motivates innate immune responses (Murakami et al. 2012).

FSRP gene has clinical significance, and it can regulate the formation of organs and tissues. In the early stages of development, FSRP gene can be expressed in the total organs (Patel et al. 1996; Adams et al. 2007), but in the later development, the FSRP expression is mainly restricted in mesenchymal tissues (Adams et al. 2007). In the process of erythropoiesis, follistatin and FSRP expression are negatively correlated, because follistatin is expressed in mature erythrocytes while FSRP is expressed in immature erythrocytes (Maguer-Satta et al. 2001). The FSRP was cloned from a mouse as a TGF-β-inducing protein for the first time (Shibanuma et al. 1993). Currently, the FSRP gene is mainly understood through research on the human (Tortoriello et al. 2001), chick (Towers et al. 1999), mouse (Kawabata et al. 2004), and tick (Zhou et al. 2006), but there is extremely finite knowledge about research in fish.

Fish are more diversified in phylogeny than other vertebrates, and they are also the major protein source for human consumption. The myogenesis inhibitory activity of the FRSP gene can promote the growth and regeneration of Chinese perch *Siniperca chuatsi* muscle (Chu et al. 2016), which shows the significance of FRSP in aquaculture. In the early developmental stages of larval, FRSP acts a pivotal part in the differentiation and development of organs (Patel et al. 1996; Adams et al. 2007). However, there is no research and details on the function of FSRP in the development of larval fish. The essential to the growth and survival of fish in juvenile stages depends on the differentiation and development of organs experienced by fish larvae in the early stages of development. Temperature is a foremost exogenous factor affecting ontogenesis, metabolism, and survival of fish larvae.

Evaluating the expression of FSRP in fish larvae at different temperatures helps understand the function of exogenous factors in FRSP expression. The purpose of this study was to investigate the characteristics of the FRSP gene and its expression level in the early developmental stage of *Trachinotus ovatus*.

14.2 Cloning and Sequencing of Golden Pompano FSRP Gene cDNA

At present, there are some gaps in the information about FRSP genes in fish. The National Biotechnology Information Database only has available sequences for Atlantic salmon (*Salmo salar*), zebra fish (*Danio rerio*), and Chinese perch (*Siniperca chuatsi*). According to the transcriptome sequence of golden pompano obtained by our experiments (Illumina HiSeq2000, annotated, KOG, kegg, and Swissprot), we used Primer 5.0 to design (Table 14.1) gene cloning primers. The full-length cDNA of the FSRP gene (GenBank accession number: KY307810) of the golden pompano was 1500 bp, the ORF was 936 bp, it encodes 311 amino acids (Fig. 14.1), the protein molecular weight was about 34 kDa (ExPASy), and the theoretical isoelectric point (p*I*) was 4.92 (ExPASy). The derived FSRP gene amino acid sequence contains multiple domains or motifs: signal peptide (1–19aa), Kazal domain (51L–E98), and EF-hand calcium-binding domain (191L–P226) (Hassan et al. 2020). The Kazal domain contains six cysteines, which form three disulfide bonds (C52–C82, C56–C75, and C64–C96) (PSIPRED Workbench). The three-level structure (SWISS-MODEL) of the FSRP gene is shown in Fig. 14.2. The relatively long cDNA of the FSRP gene was found in Chinese perch (Chu et al. 2016) and clawed frog (Okabayashi et al. 1999), which were 311 amino acids encoded by 2466 bp and 299 amino acids encoded by 2637, respectively. In comparison, the cDNAs of the FSRP genes of rats (Zwijsen et al. 1994) and ticks (Tian et al. 2009) are relatively short, with 306 amino acids encoded by 1307 bp and 289 amino acids encoded by 814 bp, respectively. The protein molecular weights of golden pompano, Chinese perch, rat, human, and tick are 34 kDa, 50.0 kDa (Chu et al. 2016), 50–55 kDa (Zwijsen et al. 1994), 55–75 kDa (Tanaka et al. 1998), and 56 kDa (Zhou et al. 2006), respectively. These differences indicate that there is distinct in the cDNA size between the FRSP genes of different species.

Table 14.1 Sequences of primers of *Trachinotus ovatus* (Hassan et al. 2020)

Primers	Sequence (5′–3′)
FSRP-F	TCCAGACCAGCTCCCA
FSRP-R	GTGACTTCCACATTCCAA
0FSRP	GGCAGCAATGGAAAGACCTAC
FSRP	CCATCGGGGACAACCTCA
EF-1α-qF	CCCCTTGGTCGTTTTGCC
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA

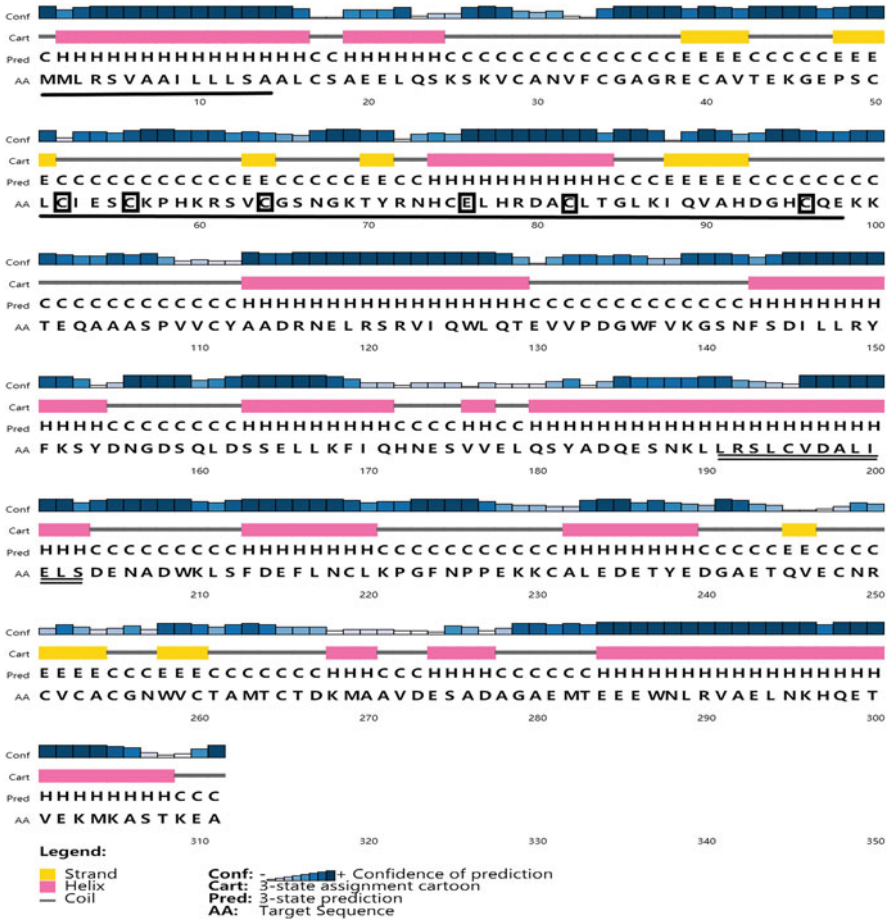

Fig. 14.1 Amino acid sequence of FSRP gene from *T. ovatus* (Linnaeus 1758) (Hassan et al. 2020). Note: black single underline: signal peptide (1M–A19); Kazal domain (51L–E98): contains six boxed cysteines; double underlines: EF-hand calcium-binding domain (191L–P226)

The FRSP domain of golden pompano is similar to that of human with signal peptide, EF-hand calcium-binding domain, and Kazal domain, but there is an extra domain in front of the Kazal domain of FRSP of human (Tsuchida et al. 2000). Compared with other domains in *T. ovatus*, the function of Kazal domain is most crucial during the early stage of fish development (Chaly et al. 2014; Sylva et al. 2013). The Kazal domain, also known as the follicle-like domain (Patthy and Nikolics 1993), not only inhibits C-terminal dimers from binding to receptors (Hill et al. 2002) but also recognizes the binding pockets of members of the TGF superfamily, which contains Kazal domain proteins. For instance, the Kazal domain in mice can bind to myostatin (Hill et al. 2002) and activin (Tsuchida et al. 2000), confirming that the FRSP of *T. ovatus* has a role in controlling the activities of TGF-β superfamily members.

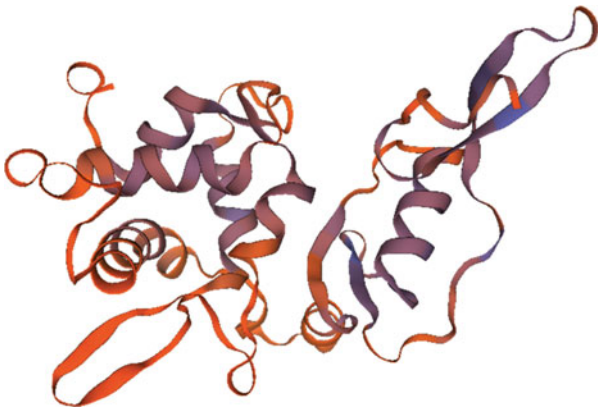

Fig. 14.2 The three-level structure of *T. ovatus* FSRP gene (Hassan et al. 2020)

14.3 Multiple Sequence Alignments and Phylogenetic Analysis of FSRP Gene

The multi-sequence alignment results of amino acid sequences derived by FSRP in different species (Hassan et al. 2020). The results showed that the amino acid sequences of *T. ovatus* FSRP had the closest homology and similarity with Chinese perch *Siniperca chuatsi* (96.1% and 98.4%) and yellow croaker *Larimichthys crocea* (94.5% and 97.1%), but the homology and similarity with brown rat *Rattus norvegicus* were distant (68.4% and 80.4%, respectively). The phylogenetic tree of the FSRP consists of two major clusters: (1) the mammal cluster and (2) the fish cluster (Hassan et al. 2020). The FSRP amino acid sequence of fish was inferred from the developmental tree results to contain signal peptides and Kazal domains, and the FSRP had six conserved cysteines and three disulfide bonds in all species.

14.4 Relative Expression of the FRSP Gene During Ontogeny

It has been reported that the FRSP gene expression begins at the gastrula stage, increased progressively in the blastocyst stage and the ganglion stage, and kept at stable level in the tail-bud stage during the embryonic development of *Xenopus* (Okabayashi et al. 1999). In golden pompano, the expression level of the FRSP gene increased with the development of larvae from 0 DPH to 3 DPH (Fig. 14.3). On 4 DPH, the FRSP gene expression level in golden pompano decreased significantly and maintained at a steady level until 18 DPH. This difference may be caused by the nutrition transaction of fish where nutrient supplement of fish larvae transferred from endogenous nutrition to exogenous food intake. The FRSP gene expression pattern for nutrient intake was associated with digestive function over time in *T. ovatus* (Ma et al. 2014). The activity of digestive enzymes (e.g., amylase, trypsin, and

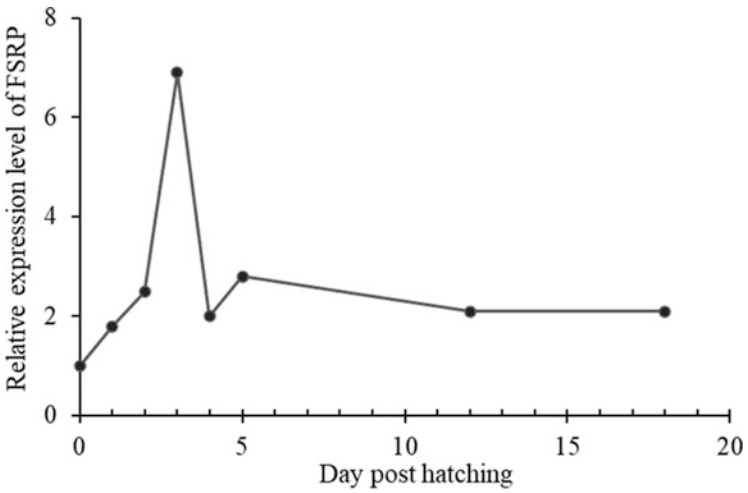

Fig. 14.3 Relative expression levels of mRNA of follistatin-related protein gene during larvae development in *T. ovatus* (Hassan et al. 2020)

lipase) was similar to the expression of FRSP gene and upregulation with the start of the first feeding on 3 DPH and maintained a steady level until 15 DPH when fish formed gastric glands.

14.5 Relative Expression of FRSP Gene During Temperature Challenge

Ambient temperature cannot affect the FRSP gene expression in larval *T. ovatus* ($P > 0.05$, Fig. 14.4). The expression levels of FRSP gene at 23, 26, and 29 °C were not significantly different on both 12 DPH and 18 DPH. Although the temperature is an essential factor affecting the metabolic and physiological activities of larval fish, FRSP gene expression levels in golden pompano were similar under different temperature conditions, indicating that FRSP gene expression is not affected by rearing temperature, and the influence of exogenous factors on FRSP gene expression is limited to some extent. Since this is the first attempt to explore the effect of different temperatures on FRSP gene expression in fish, it is necessary to conduct further investigation on testing the effect of exogenous factors on expression of FRSP gene.

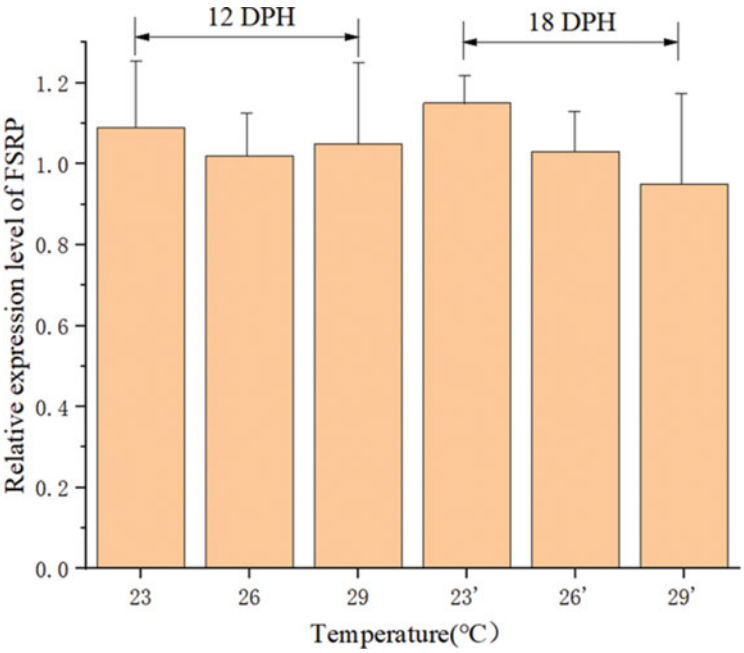

Fig. 14.4 Relative expression levels of mRNA of FSRP gene of *T. ovatus* larvae at different temperatures on 12 DPH and 18 DPH. The expression of FSRP was not significantly affected by the rearing temperatures ($P > 0.05$) (Hassan et al. 2020)

14.6 Relative Expression of FRSP Gene in Different Tissues

The mRNA expression level of FRSP gene was specific between tissues and species. In the mouse, the FRSP gene was highly expressed in the heart, lungs, kidneys, and testes, while it was less expressed in the thyroid, skeletal muscle, and saliva (Tsuchida et al. 2000). The FRSP gene expression was discovered in all the organs of the larval *T. ovatus*, but the expression level of the FRSP gene diverges between different tissues. In the current study, the expression level of FRSP gene was higher in the liver, head kidney, and eyes but lower in the kidney and muscle, indicating that the gene may be related to the growth and function of these tissues (Fig. 14.5). However, the FRSP gene expression level is high in the muscle of Chinese perch (Chu et al. 2016) and has been indicated to be participated in bone development. These findings may suggest that the function of the FRSP gene may vary between species.

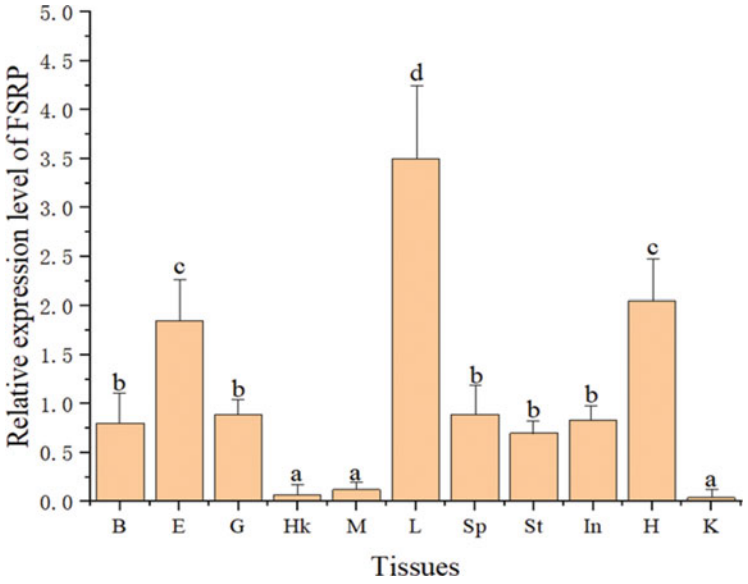

Fig. 14.5 Relative expression levels of mRNA of the FSRP gene in different tissues of *T. ovatus*. Different letters indicate significant differences in expression levels ($P < 0.05$) in different tissues (Hassan et al. 2020). Each bar represents mean ± SD of three replicates. Abbreviation: *B* brain, *E* eye, *G* gill, *Hk* head kidney, *M* muscle, *L* liver, *Sp* spleen, *St* stomach, *In* intestine, *H* heart, *K* kidney

14.7 Conclusion

The full-length cDNA cloning results of the FRSP gene from larval *T. ovatus* added to a new understanding of this gene's variation in the osteichthyes. The Kazal domain of FRSP may be closely relevant to the activity of TGF-β superfamily members, and the high expression of the FRSP gene in the liver, head kidney, and eyes suggests that the FRSP gene may participate in the regulation of the growth and function of these tissues.

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Chapter 15
Functional Feed Additives to the Diet of Golden Pompano *Trachinotus ovatus* Juveniles

Chuanpeng Zhou, Heizhao Lin, Zhong Huang, Jun Wang, Yun Wang, and Wei Yu

Abstract Golden pompano *Trachinotus ovatus* is an economically important warm-water farmed marine fish species. For the past few years, as *T. ovatus*-intensive aquaculture expanded and culture density raised, diseases have happened more repeatedly, causing many economic losses. In order to improve survival, several chemotherapeutic agents, vaccines, and antibiotics as well as some immunostimulants have been used to prevent bacterial, viral, fungal, and parasitic diseases at many hatcheries and fish farms. Therefore, the optimum type and supplementation level of functional additives are essential to the growth performance of *T. ovatus* juveniles in aquaculture. In this chapter, we review the functional feed additives (soybean isoflavones and marine red yeast *Rhodotorula mucilaginosa*) and their effects on the growth performance, nonspecific immune response, and disease resistance of juvenile *T. ovatus*.

Keywords Juvenile *Trachinotus ovatus* · Functional additives · Feed · Marine red yeast · Soybean isoflavones

15.1 Introduction

Golden pompano *Trachinotus ovatus* is an economically important warm-water marine fish species farmed in south coast of China and Southeast Asia (Sun et al. 2014; Zheng et al. 2014). As intensive aquaculture expanded and culture density

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_15

increased, diseases happened more repeatedly, especially from May to October, causing many economic losses (Xia et al. 2012; Zhang et al. 2014a, b). Fish under intensive culture conditions are more susceptible to pathogen infection than wild fish. The environmental stress caused by discharge of farms into estuaries and bays and the stress caused by high rearing density are factors that make animals more sensitive to pathogens (Bilen et al. 2011). For reduce mortality, several fish farms and hatcheries use many chemotherapeutic agents, vaccines, and antibiotics, as well as some immunostimulants, to prevent bacterial, viral, fungal, and parasitic diseases (Dügenci et al. 2003). However, the drug applications listed above are quite expensive in intensive culture. In addition, they may cause adverse effects like pollution, bioaccumulation, and antibiotic resistance, which can be transferred to wild and human pathogenic microorganisms, thereby posing a threat to human health and sociopolitical and environmental issues (Harikrishnan et al. 2011).

Immunostimulants increase resistance to infectious disease by enhancing nonspecific defense mechanisms. Consequently, the response duration is very short because there is no memory component (Sakai 1999). Since immunostimulants are considered safe and effective against various pathogens, the use of immunostimulants in fish culture to enhance immunity and disease resistance has received considerable attention (Sakai 1999; Harikrishnan et al. 2011). Immunostimulants can be applied via injection, oral administration, or bathing (Sakai 1999; Yin et al. 2006; Jeney and Anderson 1993a, b). Although intraperitoneal injection has been verified to be the most speedy and effective way of administration, incorporation in the diet is considered as most suitable for fish culture, as this method is non-stressful (Siwicki et al. 1994; Esteban et al. 2001). Several immunostimulants, such as levamisole (Siwicki et al. 1990; Jeney and Anderson 1993a, b), chitosan (Siwicki et al. 1994), yeast (Siwicki et al. 1994), glucan (Jorgensen and Robertsen 1995), lipopolysaccharide (Solem et al. 1995), growth hormone (Sakai et al. 1995, 1996), glucan plus vitamin C (Verlhac et al. 1996), yeast RNA (Sakai et al. 2001), and zeranol (Keles et al. 2002), have been administered as feed additives to modulate nonspecific immunity of fish, such as *Ictalurus punctatus* Rafinesque, *Cyprinus carpio* L., *Salmo salar* L., and *Oncorhynchus mykiss*. In this chapter, we aim to evaluate the role of dietary additives of soybean isoflavones and marine red yeast *Rhodotorula mucilaginosa* as an immunostimulant to promote growth performance and enhance the nonspecific immune ability and production of golden pompano.

15.2 The Effects of Dietary Soybean Isoflavones (SI) in the Feed of Juvenile Golden Pompano

Isoflavones are a class of molecules called flavonoids, whose basic structural unit is composed of two benzene rings connected by a heterocyclic pyran ring and belongs to a large family of polyphenols (Barnes et al. 2011; Chen et al. 2011). Because

isoflavones are structurally similar to natural estrogen, they can exert a variety of estrogen-like biological effects in animals (Ng et al. 2006). In mammals, isoflavones have a wide range of biological activities including antioxidant effects (Jiang et al. 2007), antiestrogenic effects (Cassidy et al. 1995), anticancer effects (Dijsselbloem et al. 2004), anti-inflammatory effects (Verdrengh et al. 2003; Hämäläinen et al. 2007), enzyme-inhibitory effects (Yamashita et al. 1990), cardioprotective effects (Anthony et al. 1996), and antifungal effect (Naim et al. 1974).

15.2.1 Growth

Soybean isoflavones (SI), which are a plant chemical with estrogenic activity, can weakly bind to estrogen receptors, causing competition between natural estrogens and isoflavones (Turan 2006; Kelly et al. 1993). However, Martin et al. (1978) suggested that isoflavones may act as anti-estrogens in the presence of high levels of endogenous estrogens (Martin et al. 1978). Because isoflavones may have hormone-like functions, they may affect animal growth. The effects of soybean isoflavones on growth performance are somewhat variable. For example, supplemental SI can increase the growth rate in barrows (Cook 1998), African catfish *Clarias gariepinus* (Burchell 1822) (Turan and Akyurt 2005), tilapia *Oreochromis aureus* (Yu et al. 2006) and tilapia *Oreochromis aureus* (Turan 2006). Zhou et al. (2015) found that with the increase of dietary SI, the growth performance of *T. ovatus* was significantly improved when dietary SI level is up to 40 mg kg^{-1}, indicating that dietary SI might promote the growth of fish at a suitable dose.

15.2.2 Nonspecific Immune Response

Proteins are the most important compound in the serum, with albumin and globulin being the major serum proteins (Kumar et al. 2005; Jha et al. 2007). The hemolymph protein content is used as an immune parameter to indicate whether or not a fish is healthy (De Smet and Blust 2001). The complement system is the major humoral component of the innate immune responses and plays an essential role in alerting the host immune system of the presence of potential pathogens as well as their clearance (Muller-Eberhard 1988). The complement system is initiated by one or a combination of three pathways, namely, the classical, lectin, and alternative. All three pathways merge at a common amplification step involving C3 and proceed through a terminal pathway that leads to the formation of a membrane attack complex, which can directly lyse pathogenic cells (Boshra et al. 2006). The results in the previous study on *T. ovatus* showed that the total plasma protein and C3 content were significantly increased by feeding the dose of SI at 40 mg kg^{-1} feed (Zhou et al. 2015). However, these immune parameters were not further increased by feeding the fish with the diet supplemented with SI at the level of 60 and 80 mg kg^{-1} feed,

suggesting that high supplementation, especially the dose of 40 mg kg^{-1} feed is optimal for the increase of plasma total protein and C3 content. To date, there is no exact explanation on how SI works to increase plasma total protein and C3 content in fish. Further studies are necessary to explore the mechanism of the effects of SI on plasma total protein and C3 content of fish.

The innate immune system of fish is considered to be the first line of defense against a broad spectrum of pathogens and is more important for fish as compared with mammals. Lysozyme level or activity is an essential index of innate immunity of fish and is ubiquitous in its distribution among living organisms (Saurabh and Sahoo 2008). The plasma LYZ activity of *T. ovatus* increased with dietary SI level, increasing from 0 to 40 mg kg^{-1} SI, with no significant differences among the treatments with over 40 mg kg^{-1} SI (Zhou et al. 2015). Similarly, a large number of immunostimulants have been reported to increase serum lysozyme levels in fish that may be due to either an increase in the number of phagocytes secreting lysozyme or due to an increase in the amount of lysozyme synthesized per cell (Kumari and Sahoo 2006).

Respiratory bursts are produced by phagocytes in order to attack invasive pathogens during phagocytosis and have been widely used to evaluate the defense ability against pathogens. However, the excessive accumulation of reactive oxygen intermediates (ROIs) is extremely toxic to host cells (Dalmo et al. 1997). It is clear that the SI has an enhancing effect on respiratory burst activity of golden pompano (Zhou et al. 2015). Data from this study showed that compared with the control, the 40 mg kg^{-1} SO group significantly increased respiratory burst activity, and the other groups supplemented with 20, 60, and 80 mg kg^{-1} SI had a tendency of an increase in respiratory burst activity. A previous study has demonstrated that dietary administration of immunostimulant (such as emodin) significantly affected respiratory burst activity of Wuchang bream (Zhang et al. 2014a, b). These findings suggest that long-term feeding of the proper immunostimulant (SI) supplementation can maintain the activation of phagocytic cells throughout the experimental period and is fundamental in achieving disease resistance.

15.2.3 Hepatic Antioxidant Status and the Expression of Hepatic Gene HSP70

The nonspecific defense mechanisms of fish include neutrophil activation, production of peroxidase and oxidative free radicals, and initiation of other inflammatory factors (Ainsworth et al. 1991). The stress response might also impact factors such as total antioxidation capacity and levels of glutathione, catalase, SOD, and various peroxidases (Liu et al. 2010; Itou et al. 1996). The SI has exhibited antioxidant effects both in vitro and in vivo (Chen et al. 2011). Isoflavone-supplemented diets can reduce lipid peroxidation and F2-isoprostane levels, a biomarker of lipid peroxidation, in humans (Wiseman et al. 2000). Disilvestro et al. (2005) reported that the

SI in capsules could elevate erythrocyte superoxide dismutase in human (Disilvestro et al. 2005). Previous studies showed that SI supplemented at the dose of 20 mg kg^{-1} feed significantly increased the SOD activity in *L. vannamei* when compared with other treatments (Chen et al. 2011), and the hepatic antioxidative capacity was strongly increased by dietary SI in tilapia (*Oreochromis aureus* Steindachner) (Yu et al. 2006). Cai and Wei (1996) suggested that dietary genistein enhances the activities of antioxidant enzymes (SOD, CAT, glutathione reductase, and glutathione peroxidase) in various organs in mice, which may be a mechanism of genistein's chemopreventive action (Cai and Wei 1996). Consistent with these studies, Zhou et al. observed that compared to the control the treatments supplemented with 40 mg kg^{-1} SI increased activities of hepatic antioxidant enzymes (SOD, T-AOC, CAT) whereas malondialdehyde content was reduced (Zhou et al. 2015). The present results imply that SI can improve antioxidative status, inhibit free radical formation, and reduce the harm of lipidic superoxide in juvenile golden pompano.

Heat shock proteins (HSPs), also known as stress proteins and extrinsic chaperones, are a suite of highly conserved proteins of varying molecular weight (*c.* 16–100 kDa) produced in all cellular organisms when they are exposed to stress (Roberts et al. 2010). HSP70 is mainly involved in stress protection, improving cell survival and raising tolerance to environmental stressors or harm (Basu et al. 2002). Thus, HSP70 has been widely used as a bioindicator of stress. HSP70 is induced by heat and chemical shocks in fish, like in mammals (Gornati et al. 2004). A number of studies have shown that Chinese herbs enhance the expression of HSP70 in Wuchang bream (Liu et al. 2012) and white shrimp (Lei and Zeng 2008), and higher dietary carbohydrate increases the expression level of HSP70 (Zhou et al. 2013). Moreover, HSP70 mRNA of broilers was also changed positively by the dietary genistein (Kamboh et al. 2013). Similarly, the relative level of hepatic HSP70 mRNA of *T. ovatus* increased with increasing dietary SI levels up to 40 mg kg^{-1} and thereafter levelled off, indicating that dietary SI could enhance the expression of HSP70 (Zhou et al. 2015).

15.2.4 Challenge Test

Currently, because the methodology to comprehensively investigate immunity and disease resistance of fish is still limited, a useful biomarker for disease resistance of fish is difficult to identify. Therefore, bacterial challenge tests have often been used as a final indicator of fish health status after nutrition trials (Lin et al. 2012; Jin et al. 2013). Vibriosis is caused by *V. harveyi*, a halophilic gram-negative bacterium that is known to cause disease to fish, shrimp, and shellfish either in the culture system or in the wild aquatic environment (Sharma et al. 2012; Austin and Zhang 2006). A previous study showed that dietary SI showed an improved survival rate of *L. vannamei* against an intramuscular challenge with *V. alginolyticus* (Chen et al. 2011). This agrees well with the results of other workers (Huang et al. 2005), who found SI-supplemented diets improved survival rate against an intramuscular

injecting with *V. parahaemolyticus*. Zhou et al. (2015) found that dietary SI showed an increased survival rate of *T. ovatus* against challenge with *Vibrio harveyi* (Zhou et al. 2015). Therefore, SI showed positive effects on preventing golden pompano against *Vibrio harveyi* infection.

15.3 Effects of Dietary Additive of Marine Red Yeast *Rhodotorula mucilaginosa* on Golden Pompano

Yeast can affect nonspecific immunity and protection against furunculosis in rainbow trout (Siwicki et al. 1994). Yeast may improve fish health as antagonists to pathogens and by immunostimulation (Andlid et al. 1995). Rorstad et al. (1993) also reported that yeast glucan showed an adjuvant effect when included in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.). Nakano et al. (1999) observed that red yeast had a reducing effect on oxidized oil-induced oxidative stress in rainbow trout (*Oncorhynchus mykiss*). Xia et al. (2013) observed that marine red yeast *Rhodotorula mucilaginosa* could promote the growth and immunity of *Litopenaeus vannamei*.

15.3.1 Growth

The marine red yeast has been widely used for its potential beneficial effect in aquaculture (Yang et al. 2010; Zhang et al. 2013; Sun et al. 2015). Zhou et al. (2016) found that the WG and SGR of *T. ovatus* fed with 1‰ *R. mucilaginosa* diet were higher than those of control group. Previous studies also showed that compared to the control group, weight gain (WG) and specific growth rate (SGR) of *Litopenaeus vannamei* with fed *R. paludigenum* supplementation increased significantly (Yang et al. 2010; Scholz et al. 1999). Zhang observed that addition of 1 g kg^{-1} *Rhodotorula benthica* into brown fish meal can significantly improve feeding rate, protein efficiency rate, and growth performance of turbot, a similar growth level to white fish meal (Zhang et al. 2013). Tovar-Ramírez et al. (2004) found that final mean weight of sea bass larvae in the group fed with 1.1% of marine yeast *D. hansenii* CBS8339 was twice as that of the other groups. These results suggested that the marine red yeast produces many bioactive substances, such as protein, amino acids, fatty acid, polysaccharide, and carotenoids, which could promote the growth of aquatic animals.

15.3.2 Serum Biochemical and Immune Parameters

LYZ is a bactericidal peptide, which is an important component of the immune defense of marine fish species (Liu et al. 2012). It is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus provides some protection against pathologic infection (Lie et al. 1989; Hauge et al. 2002). The AKP is an important component of lysosomal enzymes that originate from hemocytes to destroy extracellular binvadersQ (Cheng and Rodirick 1975). Therefore, phagocytic competence and AKP activity are related to the quantity and quality of hemocytes. The activities of the immunity active factors (serum LYZ and AKP) in the serum of the lady crab *Charybdis japonica* have different degrees of the enhancement in 48 h after infection with the polysaccharide of oceanic red yeast (Sun et al. 2015; Wang et al. 2011). Similarly, compared to the control, the *R. mucilaginosa* diets significantly increased serum LYZ and AKP activities of juvenile *T. ovatus* (Zhou et al. 2016). Nitric oxide produced by NOS is associated with diverse actions in neurotransmission, vascular systems, and immunity, including antimicrobial and antiviral activities by inhibiting DNA as well as protein and lipid synthesis (Bredt and Snyder 1994; Karupiah et al. 1993; Howe et al. 2002; Lepoivre et al. 1990). Compared to other treatments, the group supplemented with 2‰ *R. mucilaginosa* significantly increased serum NOS activity (Zhou et al. 2016). This agrees well with the finding of Zhang et al. (2011), who found that the shrimp (*Penaeus japonicus*) fed the diet with both *Bacillus* probiotics and IMO (T3) produced significantly higher immune parameters (LYZ activity and NOS activities) than the control group. These results suggested that oceanic red yeast has immune stimulation to some extent.

15.3.3 Hepatic Antioxidative Status

The increase in free radical content may lead to an increase in lipid peroxidation content and lipid peroxidation injury in fish (Liu et al. 2012). The breakdown of hepatic lipid peroxide yields large amounts of aldehydes, alcohols, and hydrocarbons such as MDA, a strongly toxic chemical. The antioxidant enzyme system plays a prominent role in resisting lipid oxide damage (Holmblad and Soderhall 1999; Lopes et al. 2001). Dietary supplementation with marine red yeast can significantly enhance antioxidant activity in aquatic animals (Bon et al. 1997; Li and zhang 2004). A previous study showed the SOD activity of hepatopancreases from *L. vannamei* in groups fed with the live yeast diet and the dry yeast diet was significantly higher than that in the control group, whereas no statistical difference was found in MDA content of hepatopancreases (Yang et al. 2010). Zhou et al. (2016) found that compared to the control, the groups supplemented with 2–4‰ *R. mucilaginosa* increased hepatic SOD activity, whereas the 2–5‰ *R. mucilaginosa* treatment groups decreased hepatic MDA content, especially in the 4‰ *R. mucilaginosa*

group. Taken together, our results suggest that the supplementation with *R. mucilaginosa* reduces the potential for oxidative damage in *T. ovatus*.

15.3.4 *Effect of* R. mucilaginosa *on Survival in* T. ovatus

Currently, bacterial challenge test has often been used as a final indicator of fish health after nutrition trials (Zhou et al. 2015). Vibriosis is caused by *V. harveyi*, a halophilic gram-negative bacterium causing disease in fish, shrimp, and shellfish (Sharma et al. 2012; Austin and Zhang 2006). A previous study showed that supplementation of red yeast could make the red yeast colonize in the intestine of fish larvae, which could affect the growth of the larvae and accelerate the maturity of the digestive system to improve survival rates (Gatesoupe 2007). The survival rate of the mice in the group fed with the diet supplemented with the astaxanthin produced by red yeast was higher than that of the control group (Bennedsen et al. 1990). Similarly, dietary *R. mucilaginosa* showed an increased survival rate of *T. ovatus* against challenge with *V. harveyi* (Zhou et al. 2016).

15.3.5 *The Effect of* R. mucilaginosa *on Hemolymph Complement 3 and Complement 4 of* T. ovatus *After* V. harveyi *Infection*

The complement system is the major humoral component of the innate immune responses and thus plays an essential role in alerting the host immune system of the presence of potential pathogens as well as their clearance, which is initiated by one or a combination of three pathways, namely, the classical, alternative, and lectin (Holland and Lambris 2002; Zhou et al. 2014). The complement C3 is the central component of the complement system, being activated into its respective cleavage products C3a and C3b through one of the three pathways (Boshra et al. 2006). The complement C4 plays an integral role in the activation of the classical and lectin pathways (Boshra et al. 2006). A previous study showed that after challenge by *Aeromonas veronii*, there was no significant difference in complement 3 among all groups (Yu et al. 2014). However, compared to the control group prior to infection, the serum C3 level significantly increased in the group supplemented with *R. mucilaginosa*; compared to the control after infection, the 0.1–0.3‰ *R. mucilaginosa* groups significantly increased serum C4 levels (Zhou et al. 2016).

15.4 Conclusion

In conclusion, dietary soybean isoflavones and *R. mucilaginosa* supplementation, as an immunostimulant, could promote growth performance and enhance the nonspecific immune ability and production of golden pompano in aquaculture.

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Chapter 16
The Intestine Microbiota Community and Enzyme Activity in *Trachinotus ovatus* After Short-Time Antibiotic Bath Administration

Xing Zheng, Siqi Lin, Zhifeng Gu, and Zhenhua Ma

Abstract The control of microbiota is essential for the prevention of bacterial and fungal diseases in aquaculture. Antibiotic is often used as an effective strategy for health management in fish farming. This chapter reviews and updates the recent research outcomes in preventing and treating bacterial infections in golden pompano *Trachinotus ovatus*. A short-time antibiotic bath administration was used with 5 mg enrofloxacin/L for 24 h. The results indicate that 5 mg/L enrofloxacin bath administration for 24 h did not induce mortality and affect the gut bacterial richness of golden pompano, but dramatically reduced pathogen bacteria. Furthermore, the short-time antibiotic bath administration is unlikely to result in a dysfunction of the anti-oxidative system or a digestive system disorder. Thus, 5 mg/L enrofloxacin bath administration is safe to prevent bacterial diseases in *T. ovatus* farming. This chapter sheds light on bacterial disease prevention and treatment to optimize the use of enrofloxacin in the *T. ovatus* farming to improve health management in the aquaculture of this economically important fish species.

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Z. Ma et al. (eds.), *Ontogenetic development of pompano Trachinotus ovatus*,
https://doi.org/10.1007/978-981-19-1712-7_16

Keywords *Trachinotus ovatus* · Short-time antibiotic bath administration · Gut microbiota · Biochemical enzyme activity

16.1 Introduction

It is well known that the control of microbiota is essential in aquaculture as bacterial and fungal diseases are a severe challenge to aquaculture enterprises (Buchmann 2015). There is a need to develop effective methods for disease control. Furthermore, a hatchery operation is different from the grow-out facility in general, and juvenile fish would be disinfected before being pooled into reared tanks or cement pools.

Golden pompano (*Trachinotus ovatus*) is an economically important warm-water marine fish species (25–32 °C), widely distributed in the tropical and temperate seas of China, Japan, Australia, and other countries. In recent years, it has become a popular cultured species in the Asia-Pacific region for its fast growth and high flesh quality (Li et al. 2006; Ma et al. 2014, 2016). However, high stocking density and low water quality can make fish susceptible to microbial and parasitic infections. There are severe economic losses in *T. ovatus* due to disease outbreak in the last decade (Guo et al. 2018; Harikrishnan et al. 2011; Kumari and Sahoo 2006). Vibriosis, viral necrosis, and cryptocaryon are the primary diseases in *T. ovatus* farming (Guo et al. 2018; Xia et al. 2012). Thus, there is a need to identify a method to control infectious diseases successfully.

To prevent fish disease outbreak, antibiotics, vaccines, chemical medicine, and immunostimulants have been widely used in aquaculture. Particularly, antibiotics and chemicals have traditionally been used to control pathogens in hatcheries (Rico and Van den Brink 2014). Furthermore, the method of treatment is vital to develop a cost-effective management strategy to mitigate microbial infections. Oral administration with feed, direct injection, and immersion in antibiotic bath solutions are commonly used for fish health management (Fang et al. 2018). The advantage and disadvantage of different methods are listed in Table 16.1. The addition of antibiotics in fish feed is the most common application method, but the infected fish often have a reduced appetite making oral uptake less efficient. Antibiotic injections are a

Table 16.1 Advantage and disadvantage of different modes for antibiotics administration (Armstrong et al. 2005; Haya et al. 2005)

Administration modes	Advantage	Disadvantage
Oral with feed	Time-saving; cost-effective	Less efficient due to low appetite
Intramuscular/intraperitoneal injection	Direct and efficient Less antibiotic used and losses	High labor costs
Bath administration	Easy to use and control	Antibiotics need to be physically removed or destroyed before discharge; stressful to the fish

direct and efficient way to administer medicine, but it incurs a high labor cost. Bath administration is the most convenient way and ease of use and effective for bacterial infected skin diseases, but antibiotics in the solution need to be physically removed or destroyed before discharge (Armstrong et al. 2005; Haya et al. 2005).

However, previous studies have indicated that antibiotics and administration modes to control fish diseases can stress the host. It may induce drug-resistant pathogens, suppress aquatic animals' immune system, and change intestinal bacteria community composition (Cabello et al. 2013; Xu et al. 2018).

This chapter reviews and updates the recent research outcomes in preventing and treating bacterial infections in *T. ovatus* farming. Enrofloxacin bath administration was chosen under 5 mg/L for 24-h, and this dose has been used to treat bacterial diseases by farmers. This chapter aims to provide fundamental knowledge and improve the health management for golden pompano farming.

16.2 Changes of Digestive Enzyme Activity in the Stomach After Enrofloxacin Bath Administration

The digestive system in marine fish is likely to be affected by reactive oxygen species (ROS) induced by environmental stress to disturb normal physiological function (Deng et al. 2010). The activity of digestive enzymes (amylase, pepsin, trypsin, and lipase) is used to indicate the digestive processes and nutritional condition of fish (Abolfathi et al. 2012). Various digestive enzymes are involved in digestive and absorptive processes. Evidence has suggested that the availability of digestive enzymes is essential for fish growth and development and is also essential to cope with the stress from the environment (Yufera et al. 2000; Yufera and Darias 2007).

According to Lin et al. (2019), the significant changes of specific enzyme activities (e.g., pepsin and trypsin) were not observed in *T. ovatus*' stomach ($P > 0.05$, Fig. 16.1). The specific activity of pepsin was 34.58 ± 19.96 U/g protein after 5 mg/L enrofloxacin bath administration for 24-h and was 38.89 ± 14.48 U/g protein in control (Fig. 16.1a). The trypsin-specific activity was 0.83 ± 0.24 U/mg protein after 5 mg/L enrofloxacin bath administration for 24 h but was 1.16 ± 0.04 U/mg protein in control (Fig. 16.1b). This study indicates that 5 mg/L enrofloxacin bath administration for 24 h is unlikely to cause a disorder of the digestive system.

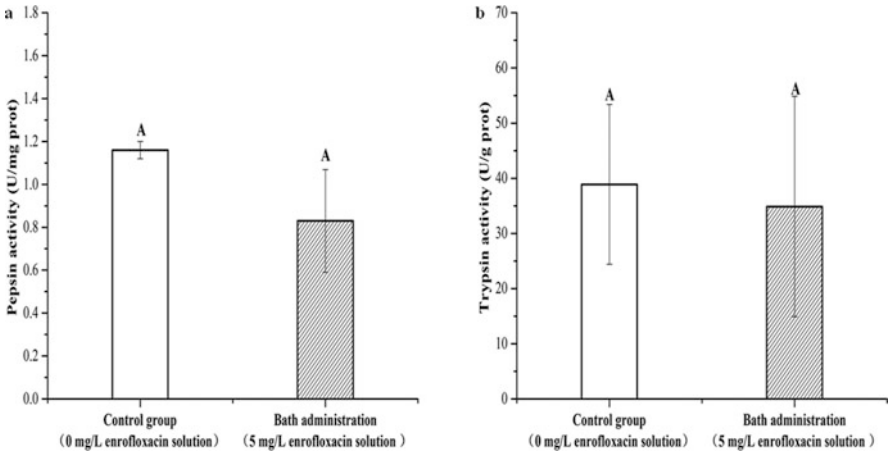

Fig. 16.1 The total activity of pepsin (**a**) and trypsin (**b**) in *Trachinoutus ovatus* stomach after 5 mg/L enrofloxacin bath administration for 24-h. Fish in the natural condition from recirculating system was used as control. (Reproduced from Lin et al. (2019), mean ± SD ($n = 10$) with the same superscript letter are not significantly different (increase the letter size of the *x*-axis label))

16.3 Changes of Antioxidant Enzyme Activity in the Liver After Enrofloxacin Bath Administration

Reactive oxygen species (ROS) normally increases when animals are subjected to stress, and this process will induce oxidative stress. To copy with ROS stress, physiological responses normally happen, especially the antioxidant defense system (Martínez-Álvarez et al. 2005). The antioxidant system protects cells by maintaining ROS at low levels and attenuating damages related to their high reactivity. All organisms have their own cellular antioxidant defense system, comprising both enzymatic and nonenzymatic components. Antioxidant enzyme activities are usually used as potential indicators of oxidative stress in fish (Lesser 2006; Xu et al. 2014), consisting of alkaline phosphatase (AKP), superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione peroxidase (GPX), which provide cellular defense against endogenous and exogenous ROS (Winston 1991).

The liver tissue is the major metabolic organ assisting in digestion by secreting enzymes that break down fats and storage carbohydrates and plays a vital role in digestion, metabolism, immunity, and the storage of nutrients inside the body. All other metabolic pathways depend upon the efficiency of liver for their energy supply (Satyaparmeshwar et al. 2006). Thus, the function of the liver is important to evaluate the antioxidant defense systems.

According to Lin et al. (2019), the significant change of antioxidant enzyme activities was not observed in the liver of *T. ovatus* ($P > 0.05$, Fig. 16.2), including AKP, POD, SOD, GPX, and CAT. The results suggest that enrofloxacin bath

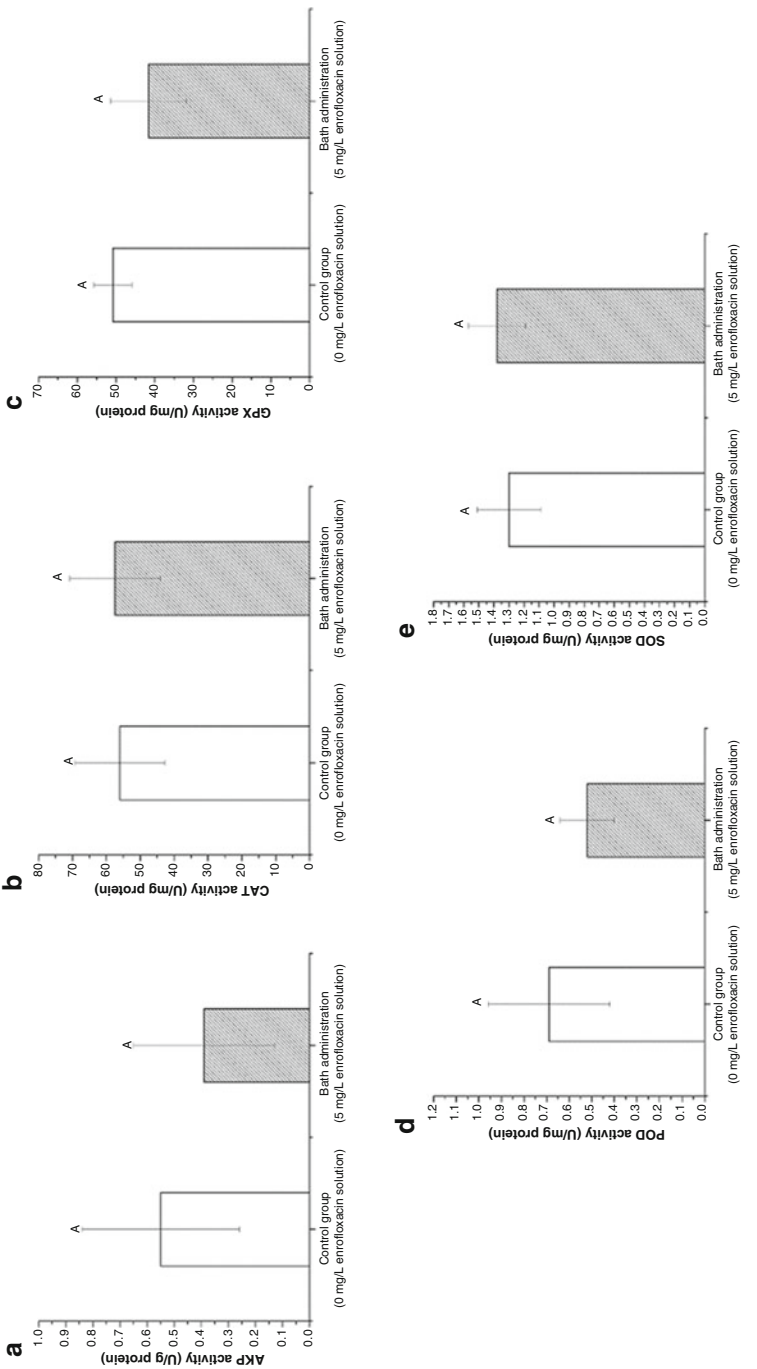

Fig. 16.2 Antioxidant enzyme activity of *Trachinotus ovatus* liver after 5 mg/L enrofloxain bath administration. (Reproduced from Lin et al. (2019), mean ± SD ($n = 10$). (**a**) Alkaline phosphatase (AKP) activity, (**b**) catalase (CAT) activity, (**c**) glutathione peroxidase (GPX) activity, (**d**) peroxidase (POD) activity, and (**e**) superoxide dismutase (SOD) activity

administration under 5 mg/L for 24 h did increase the production of reactive oxygen species or cause dysfunction of the anti-oxidative system in the liver.

To reduce the damage of ROS, O_2^- are dismutated by SOD to H_2O_2 which is reduced to water and molecular oxygen by CAT or is neutralized by GPX that catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols using glutathione (GSH) as a source of reducing equivalent (Verlecar et al. 2007). CAT provides the first line of defense to clean up ROS, while GPX is involved in detoxification of hydroperoxides (Farombi et al. 2007). Phosphatases remove phosphate groups from the substrates by hydrolyzing phosphoric acid monoesters into phosphate ions and molecules with free hydroxyl groups. ACP and AKP are two important phosphatases in marine organisms, participating in the degradation of foreign protein, carbohydrate, and lipid, as well as immune regulation, ion secretion, and other important physiological functions (Foss et al. 2009; Liu et al. 2004). ACP is used as a marker for detecting lysosomes within cell fractions and is also a reliable tool for assessing environmental pollution (Blasco et al. 1993; Mazorra et al. 2002; Rajalakshmi and Mohandas 2005). AKP is an intrinsic plasma membrane enzyme in the cell membranes (Blasco et al. 1993; Jing et al. 2006; Mazorra et al. 2002).

16.4 Changes of Gut Bacterial Diversity, Evenness, and Community Composition

The gut is the home of trillions of microbial cells known as gut microbiota (Zhang et al. 2016). The gut microbiota, which comprises a diverse and vast population of microorganisms, plays critical functions in host nutrient and physiology (Ray et al. 2012; Tremaroli and Bäckhed 2012). The gut microbiota composition and interactions affect energy extraction efficiency and are essential in metabolism and immunity (Moore et al. 2011; Tremaroli and Bäckhed 2012). In comparison to mammals, the gut microbial composition in fish is more likely to be affected by the environment, such as diet (De Filippo et al. 2010), drug (Zwolinska-Wcislo et al. 2011), and stress (Galley et al. 2014; Xia et al. 2014). An altered microbiota in the gut can change host immune function and increase disease risk (Morgan et al. 2012). Evidence has demonstrated that antibiotic exposure, including oral, intramuscular, or bath administration, would stress the treated animals and change the gut bacteria community composition, diversity, and evenness (Cabello et al. 2013; Xu et al. 2018; Zhou et al. 2018). Antibiotic exposure can adversely affect the health of the host. Therefore, maintaining a functional and steady gut microbiota is vital to the host.

The fish microbiome with rich biodiversity can predictably react to the changing gut condition (Hennersdorf et al. 2016). In *T. ovatus* gut bacteria community, the community richness (estimators by ACE and Chao1) was not significantly affected by 5 mg/L enrofloxacin bath administration ($P > 0.05$, Fig. 16.3a, b), but the Shannon index for diversity decreased significantly from 4.00 ± 0.12 to

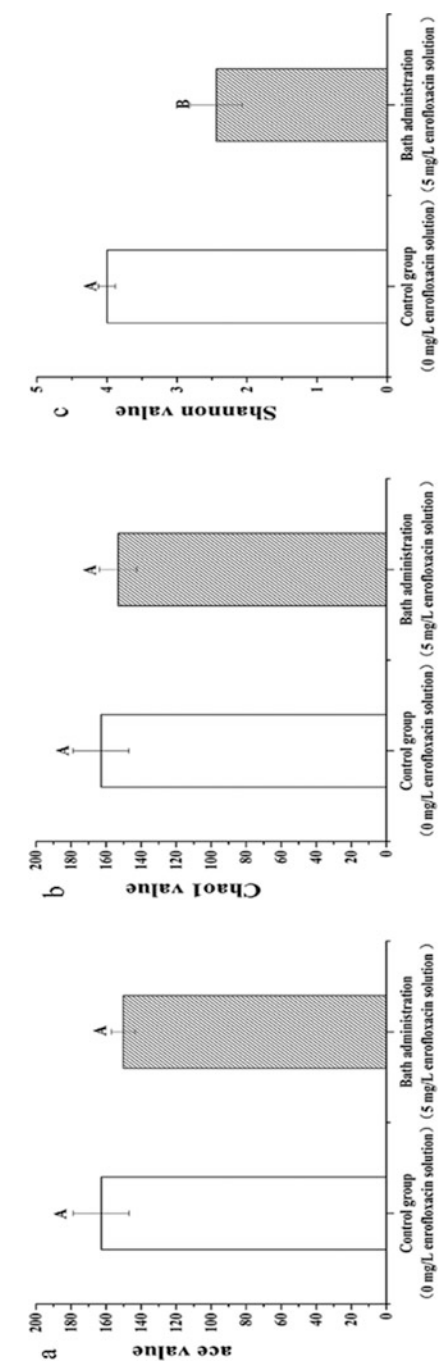

Fig. 16.3 The gut bacterial community richness (ACE and Chao1) and diversity (Shannon) in *Trachinotus ovatus* after 5 mg/L enrofloxacin bath administration. (**a**) ACE estimator. (**b**) Chao1 estimator. (**c**) Shannon estimator. Mean ± SD ($n = 5$) with the different superscript letter are significantly different

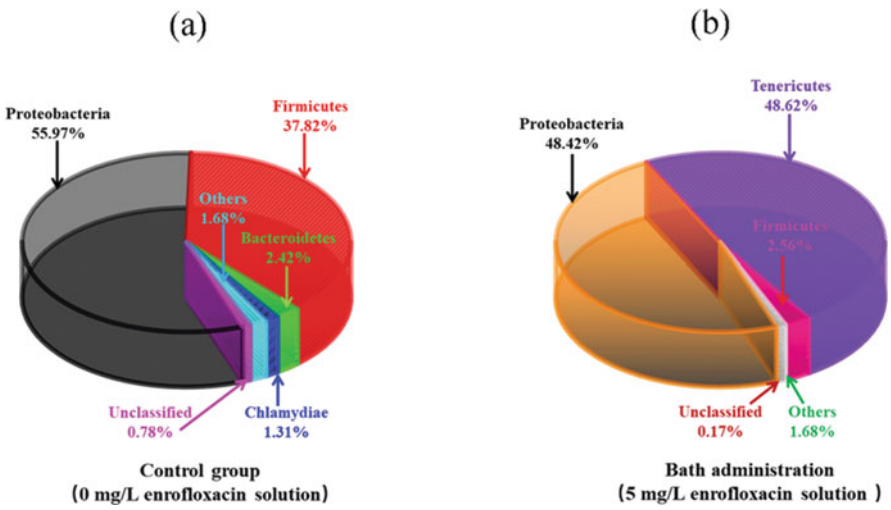

Fig. 16.4 The gut bacteria communities in *Trachinotus ovatus* at phyla level. "Others" meant the sum of bacteria relative abundance were less than 1%. $N = 5$. (**a**) Control group and (**b**) bath administration

2.44 ± 0.37 ($P < 0.05$, Fig. 16.3c). Alpha diversity shows the richness of *T. ovatus* gut bacteria was not affected significantly by the administration of low concentrations of enrofloxacin. Similarly, a previous study has also demonstrated that the gut microbiota of aquatic animals is not affected significantly by the environment (Zhang et al. 2016). In contrast, diversity was negatively affected. Bacterial diversity or composition may be more susceptible to low administration concentrations of enrofloxacin, which is consistent with the results of zebra fish gut bacteria composition after oxytetracycline exposure (Zhou et al. 2018).

The composition of the gut bacterial community varies with a unique core microbiome in each specific host species. Firmicutes and Bacteroidetes are the most dominant phyla in mammals (Qin et al. 2010), whereas Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Bacteroidetes are the major phyla in the intestine of carnivorous marine fish (Rückert et al. 2008). Our research found a total of 12 phyla that were detected in the gut bacterial community of *T. ovatus*. Proteobacteria, Tenericutes, and Firmicutes were the most common phyla (Fig. 16.4). The result is similar to the study of the woody forage effect on golden pompano intestinal bacteria diversity (Chen et al. 2018). This finding is also in agreement with the studies of the Atlantic salmon parr (Dehler et al. 2017), rainbow trout (Lyons et al. 2015), and East African cichlid (Baldo et al. 2015). It is speculated that Proteobacteria and Firmicutes are the common gut microbes in fish and play an important role in intestinal function. Proteobacteria could catabolize feedstuff components (Jumpertz et al. 2011), and Firmicutes may be involved in energy resorption (Komaroff 2017) and have demonstrated probiotic properties in fish (Bøgwald and Dalmo 2014). We found that the relative abundance of Proteobacteria was not

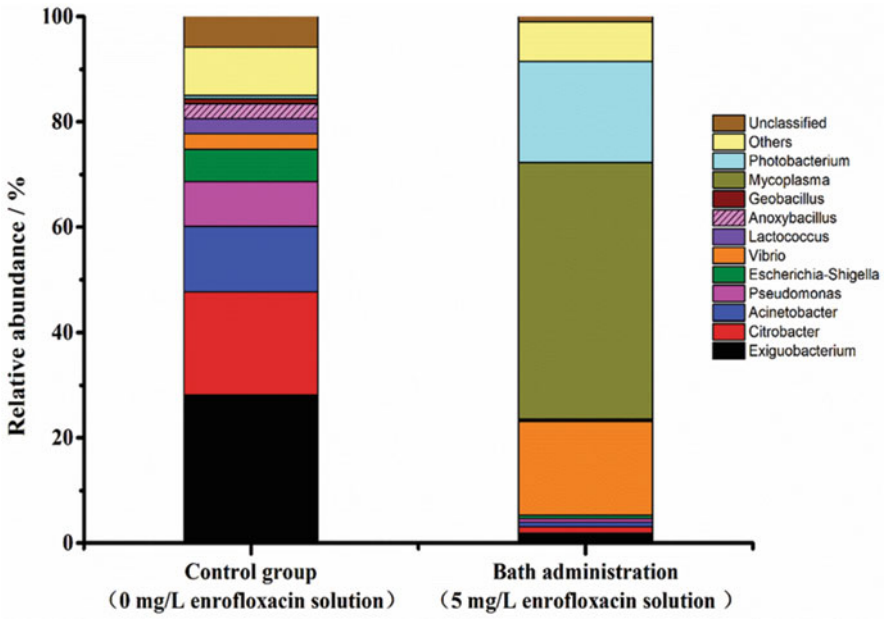

Fig. 16.5 The intestinal bacteria communities in *Trachinotus ovatus* intestine at the genus level. "Others" meant the sum of bacteria relative abundance was less than 1%. $N = 5$

significantly affected by the dose of 5 mg/L enrofloxacin bath administration for 24 h. It is consistent with the zebra fish results with sulfamethoxazole bath (Zhou et al. 2018), indicating that a short-term enrofloxacin bath administration has no significant effect on Proteobacteria.

Microbial identification is meaningful only when microbiota can be classified at the genus or species concerning animal husbandry (Petrosino et al. 2009). A total of 84 genera were detected in the *T. ovatus* gut microbiota composition from the control group (0 mg/L enrofloxacin solution) in our research, including *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas*, and *Escherichia-Shigella* as the dominant genera. It is similar to previous results, showing that *Aeromonas*, *Vibrio*, *Micrococus*, *Alteromonas*, and *Acinetobacter* are the main genera in marine fish (Blanch et al. 1997; Newman et al. 2011). Moreover, 33 genera were detected in the *T. ovatus* gut microbiota from the treatment of 5 mg/L enrofloxacin bath administration, including the dominant genera of *Mycoplasma*, *Photobacterium*, *Vibrio*, and *Desulfovibrio* (Fig. 16.5). *Escherichia-Shigella* and *Vibrio* were conditional pathogens (Hao et al. 2017; Tan et al. 2014). Our result found the relative abundance of *Escherichia-Shigella* was significantly decreased after bathing with 5 mg/L enrofloxacin, indicating 5 mg/L enrofloxacin bath administration for 24 h is useful to control the quantity of conditional pathogen in the *T. ovatus* gut microbiota composition.

Antibiotics can cause adverse effects on animal physiology by affecting host tissues or confusing commensal microbiota (Morgun et al. 2015). A disturbance in gut microbial community can lead to changes in the microbial diversity and abundance of certain bacteria, resulting in beneficial or harmful effects in fish (Gómez and Balcázar 2008). The altered microbiota in the intestine can lead to the change of the host immune functions and increase the risk of disease infection (Morgan et al. 2012). Antibiotic treatment can stress the treated animal, and the animal physiological response may occur, depending on the strength and duration of stress. The results indicate that the short-term enrofloxacin bath under a low concentration can help control the number of conditional pathogens, without significantly affecting the intestinal bacteria richness. It is useful for golden pompano farming and health management to prevent and treat bacterial diseases without significantly changing gut microbial community.

16.5 Conclusion

Golden pompano *T. ovatus* have harbored specific and core intestine microbiota, including the dominant phyla, Proteobacteria and Firmicutes, and the dominant genera, *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, *Pseudomonas*, and *Escherichia-Shigella*, in a circulating aquaculture system. Short-time antibiotic bath administration (5 mg/L enrofloxacin bath for 24 h) did not induce mortality and affect the gut bacterial richness of golden pompano, but reduced the diversity, where the conditional pathogen declined dramatically. Furthermore, a short-time antibiotic bath is unlikely to result in dysfunction of the anti-oxidative system or disorder of the digestive system. Thus, the dose of 5 mg/L enrofloxacin bath may be a safe way to prevent bacterial diseases in *T. ovatus* in aquaculture.

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