Study on the ontogenetic development of the digestive system of marine fish larvae for diet optimization

餌の最適化に向けた海産魚類仔魚の個体発生における消化機構の研究

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STUDY ON THE ONTOGENETIC DEVELOPMENT OF THE DIGESTIVE SYSTEM OF MARINE FISH LARVAE FOR DIET OPTIMIZATION

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ABSTRACT

Fish larvae hatch with a rudimentary digestive system, but demand a highly efficient enzymatic digestive machinery that is able to process the impressive amount of ingested nutrients required for supporting their high growth and development. This study aimed to investigate the early ontogeny of marine fish larvae through an integrative investigation to understand the general patterns of activity through the effect of feeding status, morphological changes, and gene expression on final enzymatic capacity. Three marine fish species were evaluated including red seabream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*) and Kawakawa tuna (*Euthynnus affinis*). The results showed that most of digestive enzymes (trypsin, chymotrypsin, lipase and amylase) were genetically programmed and activated at hatching. When the larvae begin exogenous feeding, the digestive tract gradually differentiates into specific-functional organs and digestive capacity improves with larval age. Energy metabolism (*cox, atp),* growth (*igf1)* and peptide regulatory factors (*cck, ny*) related genes were transcribed and translated at hatching to assist upon the onset of exogenous feeding. Detection of gastric glands and pepsin activity indicated the functionality of the stomach. The detectable point of gastric glands and pepsin varied in different fish species such as at 15 days after hatching (DAH) in *P. major*, 25 DAH in *P. olivaceus* and 10 DAH in *E. affinis*. However, the activation of functions of the gastric gland does not necessarily imply full functioning of the stomach and therefore weaning from live feed into formulated feed should be considered coinciding with the peak of pepsin activity. Dramatic drops of proteolytic and lipolytic enzyme activity could result in mass larvae mortality, these were observed at 15 to 20 DAH and 25–30 DAH in *P. major*, 20-30 DAH in *P. olivaceus* and 6 - 10 DAH in *E. affinis*. Delay in enzymatic precursor gene transcription and expression of different isoforms should be considered during the investigation.

Most fish larvae still rely on live feed as primary feed during their early ontogenetic stages. In this study, the feeding regime of rotifer *Brachionus plicatilis* species complex L-type enriched with *Chlorella vulgaris* containing DHA followed by *Artemia* nauplii showed efficient nutritional effects on the larvae. Larvae fed micro-diet responded positively to the digestion but failed to induce the secretion of digestive enzyme and therefore was found to be not effective in feed hydrolysis consequently leaving larvae malnourished. However, a co-feeding protocol (live fed and micro-diet) from 15 DAH accompanied with a 5 day co-feeding transitionary period could improve the growth and survival rate of *P. major* larvae. Investigation into novel uses of Shochu distillery by-product (SDBP) highlighted that it can be considered as a potential supplementary enrichment for live feed in fish larviculture, especially at 5 % and 10 % rotifer enrichment dosage. At 5 % and 10 % doses of SDBP showed significant improvement in nutritional qualities of enriched rotifers, especially protein, carbohydrate and HUFA. Larvae fed with rotifers enriched with salmon oil supplemented with SDBP also displayed enhanced enzymatic profiles and energy metabolism at early metamorphosis; which was reflected in improved larviculture performance of *P. olivaceus* larvae.

要旨

孵化直後の魚類仔魚は未発達な消化器系を有するが、摂餌によって効率的に栄養を獲得することが必要であり、その中で、消化酵素による食物の分解は高成長の到達・維持や発育を助けることに貢献している。本研究では海産魚類仔魚の初期発生における摂食状態がどのように形態変化、消化酵素の遺伝子発現および酵素活性に影響を及ぼすのかを統合的に解析した。本研究ではマダイ（*Pagrus major*）、ヒラメ（*Paralichthys olivaceus*）およびスマ（*Euthynnus affinis*）の 3 魚種を用いた。結果として、ほとんどの消化酵素（トリプシン、キモトリプシン、リパーゼおよびアミラーゼ）は孵化直後から遺伝子発現および活性が確認された。また、仔魚が摂餌を始めてから、消化管は徐々に機能的な構造を形成し、消化能力を持つようになることが明らかとなった。さらに、エネルギー代謝（*cox*、*atp*）、成長（*igf1*）および食欲（*cck*、*ny*）に関与する遺伝子は摂餌開始と同時に発現した。胃腺の形成やペプシンの活性は魚種ごとに異なっており、マダイは 15 日齢、ヒラメは 25 日齢、スマは 15 日齢に確認した。しかし、胃腺の機能活性化は必ずしも胃が完全に機能することを意味しないことが示唆された。したがって、ペプシン活性の変化を考慮して、生物餌料から配合飼料への転換を考える必要がある。タンパク質および脂質の分解酵素の活性の劇的な減少は仔魚の大量斃死を引き起こす。こうした大量斃死は、マダイでは 15～20 日齢、ヒラメは 25～30 日齢、スマは 6～10 日齢に観察された。今後は消化酵素の転写や異なるアイソフォームの遺伝子発現の遅れについての解析が必要である。

ほとんどの海産仔魚は個体発生での初期餌料として生物餌料に依存している。本研究では、まず初めに、DHA 高含有クロレラを給餌した L 型ワムシ（*Brachionus plicatilis*）を用い、その後、アルテミア *Artemia* ノープリウス幼生を給餌することに

より仔魚を効率良く育てられた。微粒子飼料により飼育した仔魚は、飼料を消化するような反応を示したが、消化酵素の分泌を十分に行っていなかった。結果として、栄養の分解が効率良く行われておらず、栄養不良の仔魚が見られた。一方、開口後生物餌料を給餌し始め、15 日齢から微粒子飼料と生物餌料を同時に与えることにより、マダイはその 5 日後から成長効率および生残率が良くなった。また、焼酎粕発酵物を用いた研究では、焼酎粕がワムシの栄養補強として役に立つ可能性が示唆された。特に、栄養強化剤重量の 5〜10%の焼酎粕発酵物を同時に投与することで、ワムシの栄養価が強化された。焼酎粕発酵物添加により強化されたワムシの成分を比較すると、粗タンパク質、炭水化物および高度不飽和脂肪酸が高かった。ヒラメ仔魚にサケ筋子乳化油および焼酎粕で強化したワムシを与えたところ、変態初期における酵素活性やエネルギー代謝が向上した。これにより、ヒラメ仔魚の飼育成績が良くなったことが示唆された。

LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
ARA	Arachidonic Acid
BSA	Bovine Serum Albumin
DAH	Day After Hatching
DHA	Docosahexaenoic Acid
DW	Dry Weight
EFA	Essential fatty acid
EPA	Eicosapentaenoic Acid
FAA	Free Amino Acid
HUFA	Highly-Unsaturated Fatty Acid
L-type	Large Type
SD	Standard Deviation
SDBP	Shochu distillery by product
SEM	Standard Error of the Mean

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Kagoshima University or other institutions.

TRAN NGUYEN DUY KHOA

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5. Khoa, T.N.D., Waqalevu, V., Honda, A., Matsui, H., Ishikawa, M., Shiozaki, K., Kotani, T**.** Enrichment effects of Shochu distillery by product on *Brachionus plicatilis* sp. rotifer and larviculture performance in the Japanese flounder (*Paralichthys olivaceus*). **(Submitted to Aquaculture).**

CHAPTER 1: General introduction

1.1 The ontogeny of digestive system of marine fish larvae:

Most newly hatched marine fish larvae are highlighted with a rudimentary digestive system lacking fully formed and functional organs (Zambonino-Infante and Cahu, 2001; Micale et al., 2006; Lazo et al., 2011). During the ontogenetic development, the fish larvae undergo major morphological and cellular changes; accordingly, larvae require a high efficient enzymatic digestive process to hydrolyze and absorb essential nutrients suited for supporting their rapid growth and development (Lazo et al., 2011; Yúfera et al., 2018). As a result of having an undeveloped digestive system, marine finfish larvae also have insufficient digestive capacity at hatching thus limiting them from certain types of feed (Zambonino- Infante and Cahu, 2001; Conceição et al., 2008; Rønnestad et al., 2013). Moreover, early larval developmental stages are an important period as factors such as starvation and subsequent mortality usually accompany it. Food deprivation after yolk reabsorption has been reported to be linked to abnormality in behavior and morphological development, alimentary tract deterioration and trunk musculature along with decreased feed utilization efficiency and feeding activity (Heming et al., 1982; Taylor and Freeberg, 1984; Rice et al., 1987; Gisbert et al., 2004). Therefore, a good understanding of the morphological and functional changes and nutritional requirement during early larval fish ontogeny is essential for the enhancement of larviculture techniques (Hamre et al., 2013; Qin, 2013).

In fish larvae, the digestive system develops via three main processes; functional secretion by the pancreas, emergence of the intestinal brush border membrane enzymes and complete development of the stomach function (Zambonino- Infante and Cahu, 2001; Yúfera, 2018). In which, the digestive enzymes of larval fish are genetically programed and are activated from hatching and that it is possible for larvae to establish digestive capacities during yolk stage and

at first feeding (Zambonino- Infante and Cahu, 2001; Conceição et al., 2008; Moguel-Hernández et al., 2016). The digestive tract gradually develops from a short and straight tube, often closed at both the mouth and anal ends in yolk-sac larvae, into a segmented and histologically differentiated tract in juvenile fish (Rønnestad et al., 2013). However, different fish species are equipped with various feeding structures and digestive mechanisms to hydrolyze and absorb essential nutrients (Moyle and Cech, 2000). The most critical issue is that early larval digestive enzymes are not fully functional thus leading to insufficient enzyme activity to aid in the effective digestion of formulated diets (Kolkovski et al., 1997; Langdon, 2003; Holt et al., 2011; Hamre et al., 2013). Therefore, the fish larvae still relies heavily on live feeds, which are known to contain gut neuropeptides and nutritional "growth" factors which enhance digestion ability (Liao et al., 2001; Kolkovski, 2001). Once the gastric glands of the stomach are differentiated and actively secreting hydrochloric acid and enzymes, a fully functional stomach and its associated acid pepsin-medicated digestion could process the digestion of formulated diets effectively (Hamre et al., 2013; Rønnestad et al., 2013). From this point, the weaning practices to replace live feed onto inert micro-diet could be considered.

Traditionally, the ontogeny of larval fish was illustrated throughout the histological structures and morphological changes of digestive organs; the activities of enzymes, hydrolyzed proteins, lipids, and carbohydrates. However, there is limited information available on functional enzymatic digestion in relation to histological-morphological changes, hydrolyzing capacities, feeding activity and the digestive function of marine fish larvae (Kolkovski, 2001; Hamre et al., 2013; Mata-Sotres et al., 2016; Yúfera, 2018). Recently, the uses of molecular biological approaches have complemented traditional methods in expressing the profiles of digestive enzyme precursors in fish (Murashita et al., 2014; Mata-Sotres et al., 2016; Moguel-Hernández et al., 2016) and thus providing insight into both temporal and spatial expression patterns of genes involved in the development and functionality of digestive

systems during early ontogeny. The hydrolysis of macronutrient compounds by digestive enzymes is programmed, activated, and modulated (Moguel-Hernández et al., 2016) and therefore the expression of mRNA involved codifications of different enzyme precursors is necessary to assess these activities (Yúfera et al., 2018).

Numerous studies have investigated the ontogenetic development of various larval fish species. However, these studies were conducted under different rearing conditions and feeding protocols and evaluated changes in the organization and functionality of the digestive system separately and their results are not directly comparable (Solovyev et al., 2016). Moreover, the morphological development does not always match the functionality (Gisbert et al., 2013). Therefore, it requires an investigation to understand the general patterns of activity through the effect of feeding status, morphological changes, and gene expression on final enzymatic capacity. A comprehensive analysis will permit a better understanding for diet optimization during early ontogenetic development and the essential regulation of the larval digestive functions according to morphological development.

1.2 The targeted fish species

Marine finfish aquaculture has undergone rapid development in the past three decades which has led to several substantial advances in larviculture and an increase in the reliability of supply of juvenile fish (Qin, 2013). Hatchery performance and efficiency were greatly enhanced through modifications in culture systems and nutrition regimes (Mahjou et al., 2013). However, there still remains major bottlenecks in high and irregular mortality, deformities, and nutritional at the onset of exogenous feeding (Planas and Cunha, 1999; Hamre et al., 2013). Therefore, good knowledge of age-dependent changes in larval digestive tract development, digestive physiological digestion, and nutritional requirement during larval stages could help in the optimization of diets and feeding regimes, improvement of marine fish larviculture and seed production quality (Holt, 2011; Srichanun et al., 2013; Hamre et al., 2013; Qin, 2013).

The red sea bream (*Pagrus major)* and Japanese flounder (*Paralichthys olivaceus)* are high value and targeted species for aquaculture and has been commercially produced in the coastal zone of Japan, Korea and China (Kikuchi and Takeuchi, 2002; Seikai, 2002; Hossain et al., 2018). On the other hand, Kawakawa tuna (*Euthynnus affinis*) has been considered as a novel target species for aquaculture in Japan due to its high market prices and fast growth (Yazawa et al., 2017; Manabe, 2019). As such, we decided to perform the integrative investigations on the larval ontogeny of the digestive system of these three important commercial fish species.

1.2.1 Red seabream (*Pagrus major*)

Figure 1. 1: Red seabream (*Pagrus major*)

(https://www.diark.org/diark/species_list/Pagrus_major)

The red sea bream (*P. major*) is high value and targeted species for aquaculture and stock enhancement in Japan with recent expansion into Korea and China (Hossain et al., 2018). As such, it has been intensively studied (Tanaka, 1971; Kohno et al., 1983; Moteki, 2002; Park et al., 2017) since it was initially studied by Harada (1969). In many previous studies, the external morphological developments have been reported in relation to larval behaviors (Fukuhara, 1969; Moteki, 2002; Park et al., 2017), swimming, and feeding functions (Kohno et al., 1983), digestive system (Tanaka, 1969; Tanaka, 1971; Tanaka, 1972) and transition from endogenous to exogenous nutritional sources (Moteki, 2002).

Fushimi (2001) also reported that technological developments of seed production in Japan has shifted its focus from quantity to quality, since health and adaptability of hatchery-raised seed are the most important factors determining the recovery rate. As a result, the developmental focus of stock enhancement technology for the red sea bream is on improving further the quality of the seeds in terms of health and adaptability (Basurco et al., 2011).

1.2.2 Japanese flounder (*Paralichthys olivaceus)*

Figure 1. 2: Japanese flounder (*Paralichthys olivaceus*)

*(*https://fishdb.sinica.edu.tw)

Japanese flounder (*P. olivaceus)* has been commercially produced in the coastal zone of Japan, Korea and China due its high market value (Kikuchi and Takeuchi, 2002; Seikai, 2002). This species was also remarked with high growth and feed conversion rate along with good adaption to water temperature variation (Seikai, 2002; Daniels and Watanabe, 2010). From the first success in artificial seed production in 1965 in Japan, rearing technologies were improved by the 1980's that suit mass production became stable with reliable supply of fingerlings for the aquaculture industry (Harada et al., 1966; Ijima et al., 1986; Seikai, 2002). Many studies have focused on the development of *P. olivaceus* larvae and its feed and nutritional requirements, larval morphological changes (Minami, 1982; Tomoda et al., 2006), digestive system development (Tanaka et al., 1996; Rønnestad et al., 2000; Kim et al., 2004), gene

expression and activity of digestive enzymes (Srivastava et al., 2002; Bolasina et al., 2006; Lee et al., 2015) and peptide and energy metabolism (Suzuki et al., 1999; Kurokawa et al., 2000; Lu et al., 2018). However, larval rearing techniques still heavily relied on live feeds which were considered high cost and contained associated pathogenic risks (Bai and Lee, 2010). Recently, the production of *P. olivaceus* was nearly saturated and faced several challenges such as lower market prices and demand, disease outbreaks, high production costs (Seikai, 2002). Critical issues such as nutritional demands, energy metabolism, and cannibalism still need further investigation (Suzuki et al., 1999; Dou et al., 2000; Lu et al., 2018). These bottleneck issues highlighted also the need to enhance production efficiency throughout its culture phase and improve innovations in culture technologies.

1.2.3 Kawakawa tuna (*Euthynnus affinis*)

Figure 1. 3 Kawakawa tuna *(Euthynnus affinis)*

(https://australianmuseum.net.au)

Kawakawa tuna (*Euthynnus affinis*) belongs to epipelagic tuna Scombridae family and are widely distributed in the tropical and subtropical waters of the Indo–Pacific region (Williamson, 1970; Chiou and Lee, 2004). Recently, *E. affinis* has been considered as a novel target species for aquaculture due to its high market prices and fast growth (Yazawa et al., 2017; Manabe, 2019). In Japan, some small scale cage culture of Kawakawa have been conducted with wild-caught seeds (Yazawa et al., 2017). However, the reliable supply of *E. affinis* juvenile from

local fishing boats was not achieved as the catch rate of juveniles were not high enough within Japans fishing waters. Therefore the need to develop artificial breeding approaches for stable mass seed production for the expansion of Kawakawa tuna farming arose (Yazawa et al., 2017; Amezawa et al., 2018; Yazawa et al., 2019). Some primary results in artificial breeding of *E. affinis* have been archived and these have mostly been achieved via the administration of a gonadotropin-releasing hormone analog (GnRHa) in recirculating culture systems (Yazawa et al., 2015; Yazawa et al., 2017; Amezawa et al., 2018; Yazawa et al., 2019). Since then, good progress in artificial breeding has been achieved, with high spawning and hatching index. As such, the development of larval rearing protocols subsequently required more attention as the demand for sufficient supply and high quality fingerlings for production grew. However, information on early larval development and rearing technology for this species is still very limited.

1.3 Purpose of study

Since current studies often highlight morphological organization and the functional development of the digestive system of marine fish larvae separately, there is limited data on digestive capacities and functional activities during larval stage development that looks at both simultaneously. This study looks to investigate the early ontogeny of larval fish through an integrative investigation to understand the general patterns of activity through the effect of feeding status, morphological changes, and gene expression on final enzymatic capacity. More specifically, the purpose of this study was to characterize the ontogeny of key digestive enzymes in marine fish larvae in relations to the histological morphology changes, feeding status, enzymatic activity and its related gene expression from hatching until juvenile stage. This comprehensive analysis will permit a better understanding for diet optimization during early ontogenetic development and the essential regulation of the larval digestive functions according to morphological development.

1.4 Objectives

This study specifically addressed the following:

1. To describe the early ontogenetic development, digestive enzymatic activity and related gene expression in red sea bream (*P. major*), Japanese flounder (*P. olivaceus)* and Kawakawa tuna (*E. affinis*).
2. To compare the early digestive enzyme activity and expression in red sea bream (*P. major*) fed on live feed and micro-diet.
3. To evaluate the effects of Shochu distillery by product enrichment on nutritional compositions of rotifer (*B. plicatilis*) and digestive enzymes, growth performance and survival of Japanese flounder (*P. olivaceus)* during larval stages.

CHAPTER 2: Early ontogenetic development, digestive enzymatic activity and gene expression in Red sea bream (*Pagrus major*)

Abstract

Red sea bream (*Pagrus major*) is an important aquaculture species in Japan. This study aimed to determine the ontogenetic development of the digestive system, enzyme activity and related gene expressions of red sea bream larvae from hatching to post-metamorphosis (40 days after hatching, DAH). The larvae were fed with the euryhaline rotifer *Brachionus plicatilis* species complex L-type enriched with *Chlorella vulgaris* containing DHA from 3-15 DAH followed by *Artemia* nauplii from 16-40 DAH. Post culture analysis included, histological analysis of the larval gut, enzyme assays (trypsin, chymotrypsin, lipase, pepsin) and real time PCR with larvae sampled from 1 to 40 DAH. At hatching, the larval digestive tract was undifferentiated and resembled a straight tube. There was low detection of enzyme activity and gene expression of trypsin, chymotrypsin, lipase, and amylase during yolk-sac stage, before mouth opened. At 3 DAH, the mouth opened and the larvae began to exogenous feeding followed by the differentiation of the digestive tract into buccopharyngeal capacity and intestine at 5 DAH. The pancreas, liver, and gallbladder were functional indicating the transition from endogenous to exogenous feeding. The enzyme activity and gene expression of trypsin, chymotrypsin, lipase, and amylase increased and fluctuated throughout the larval development, a pattern that is common among marine fish species. Pepsin activity and gene expression were not detected until 15 DAH and strongly increased from 20 DAH. Metamorphosis was complete by 40 DAH following the regular arranging of microvilli and the abundance of vacuoles in intestine.

2.1 Introduction

Nutrition of larval fish is an important factor for consideration as it influences survival and growth during the critical early larval stages of ontogeny (Kolkovski et al., 1993; Holt, 2011). Larval nutritive demands at first feeding also include a need for a highly efficient enzymatic digestive machinery which enable to process the feed required for supporting high growth and development upon reabsorption of the yolk sac (Conceiçãoet al., 1997; Yúfera, 2018). Studies by Holt (2011) and Solovyev et al. (2016) highlight that the yolk sac re-absorbance and exogenous feeding is a critical stage. Currently, seed production of marine fish relies on live feeds due to its many favorable characteristics that favor growth and enhancement of digestion (Watanabe et al., 1978; Kolkovski, 2001). Early larval developmental stages are an important period as factors such as starvation and subsequent mortality usually accompany it. Food deprivation after yolk reabsorption has been reported to be linked to abnormality in behavior and morphological development, alimentary tract deterioration and trunk musculature along with decreased feed utilization efficiency and feeding activity (Heming et al., 1982; Taylor and Freeberg, 1984; Rice et al., 1987; Gisbert et al., 2004). Therefore, good knowledge of age-dependent changes in larval digestive tract development, digestive physiological digestion, and nutritional requirement during larval stages could help in the optimization of diets and feeding regimes, improvement of marine fish larviculture and seed production quality (Holt, 2011; Srichanun et al., 2013; Hamre et al., 2013; Qin, 2013). Numerous scientific literature have described the activity of digestive enzymes of marine fish during ontogenetic development (e.g., Kolkovski, 2001; Hamre et al., 2013; Rønnestad et al., 2013; Mata-Sotres et al., 2016; Yúfera, 2018). The majority of these studies have traditionally illustrated the histological structures and morphological changes of digestive organs; the activities of enzymes, hydrolyzed proteins, lipids, and carbohydrates. However, there is limited information available on functional enzymatic digestion in relation to histological-morphological changes,

hydrolyzing capacities, feeding activity and the digestive function of marine fish larvae (Kolkovski, 2001; Hamre et al., 2013; Mata-Sotres et al., 2016; Yúfera, 2018).

Rønnestad et al. (2013) and Yúfera et al. (2018) highlighted that the digestive function of fish larvae is related to feeding activity and exogenous enzyme utilization aided by increasing digestion ability directly or the activation of zymogens already present in the gut of fish larvae. However, Solovyev et al. (2016) reported that some digestive parameters such as feeding status and gut contents were not always considered or assessed only in separate studies thus the difficulty in extrapolation of many specific processes obtained from model species (Hamre et al., 2013). Recently, the use of molecular biological approaches have complemented traditional methods in expressing the profiles of digestive enzyme precursors in fish (Murashita et al., 2014; Mata-Sotres et al., 2016; Moguel-Hernández et al., 2016) and thus providing insight into both temporal and spatial expression patterns of genes involved in the development and functionality of digestive systems during early ontogeny. The hydrolysis of macronutrient compounds by digestive enzymes is programmed, activated, and modulated (Moguel-Hernández et al., 2016) and therefore the expression of mRNA involved codifications of different enzyme precursors is necessary to assess these activities (Yúfera et al., 2018).

The red sea bream *Pargus major* is high value and targeted species for aquaculture and stock enhancement in Japan with recent expansion into Korea and China (Hossain et al., 2018). As such, it has been intensively studied (Tanaka, 1971b; Kohno et al., 1983; Moteki, 2002; Park et al., 2017) since it was initially studied by Harada (1969). In many previous studies, the external morphological developments have been reported in relation to larval behaviors (Fukuhara, 1969; Moteki, 2002; Park et al., 2017), swimming, and feeding functions (Kohno et al., 1983), digestive system (Tanaka, 1969; Tanaka, 1971; Tanaka, 1972) and transition from endogenous to exogenous nutritional sources (Moteki et al., 2001). However, these studies did not indicate the real digestive capacities and functional activities during larval stage

developments. Furthermore, limited information concerning structure-function relationships of the digestive tract and enzyme activities and the acquisition of digestive capacities investigated against morphological development of digestive organs have been still considered. As such, this study looks to investigate the early ontogeny of *P. major* through an integrative investigation to understand the general patterns of activity through the effect of feeding status, morphological changes, and gene expression on final enzymatic capacity. More specifically, the purpose of this study was to characterize the ontogeny of key digestive enzymes in *P. major* larvae in relations to the histological morphology changes, feeding status, enzymatic activity and its related gene expression from hatching until 40 days after hatching (DAH). This comprehensive analysis will permit a better understanding for diet optimization during early ontogenetic development and the essential regulation of the larval digestive functions according to morphological development.

2.2. Materials and methods

2.2.1 Fish larvae culture

P. major eggs were collected at the Fish Farming Center in Nagashima-cho, Kagoshima Prefecture, Japan. Fertilized eggs were delivered to the Kamoike On-shore Laboratory, Education and Research Centre for Marine Resources and Environment at Kagoshima University, Kagoshima Prefecture, Japan. A total of 10,000 eggs were stocked in 500L composite tanks with continuous aeration, 35 psu of salinity and temperature maintained at 20ºC using a titanium heater (Aqua Heat, Sunlight Supply Inc, Japan). Euryhaline rotifer *Brachionus plicatilis* species complex L-type Obama strain enriched with taurine diet (Aqua-Plus ET, Nisshin Marubeni Feed Co., Ltd., Tokyo, Japan) and freshwater *Chlorella vulgaris* containing docosahexaenoic acid (C22:6 *n*-3, DHA; Super Fresh Chlorella V-12, Chlorella Industry Co. Ltd., Tokyo, Japan) were introduced to the rearing tanks from 3-18 DAH at a density of 3-5 ind./mL. *Atermia* sp. nauplii (Great Salt Lake Artemia, Ogden, UT, USA) were

alternatively fed to larvae at 1-3 ind./mL from 15 DAH and switched to *Artemia* sp. only from 21 DAH. When the larvae started exogenous feeding, the water were continuously exchanged at 300 %/day via a drained vinyl pipe screened through a polyethylene mesh (0.2mm). Rotifer and *Artemia* density in rearing tanks were checked twice a day at 8 am and 3 pm and adjusted to 5 rotifers /mL and 3 *Artemia* nauplii /mL. *C. vulgaris* also was supplied after feeding at 3 mL/ 100 L of cultured volume to maintain green water condition.

Rotifer culture and nutritional enrichment

The methods of rotifer culture and enrichment were performed following Kotani et al. (2017). Rotifers were daily cultured in a continuous system at 20 ± 1 psu of salinity. Aeration was continuously supplied and temperature was maintained at 25 ºC by a heater. Freshwater chlorella *C. vulgaris* (Fresh Chlorella V-12, Chlorella Industry Co. Ltd., Tokyo, Japan) was automatically pumped into the culture tanks at a ratio of 20×10^3 cells/rotifer/day to maintain the continuous culture. Rotifers were enriched twice daily from 8am and from 5pm over a 24 h period with concentrated *C. vulgaris* containing DHA.

The commercial taurine diet was fed to rotifers at 0.06 g/ L of enriched volume. Taurine diet was only added once, at 5pm, for 15 h prior to feeding. After 24 h, the batch cultured rotifers were harvested and supplied to larvae.

2.2.2 Fish sampling

Sampling of larvae was conducted at 1, 3, 5, 10, 15, 20, 25, 35, 40 DAH. Samples were caught at two hours after feeding and the larvae were individually collected for different analysis in triplicates. For histological observation, 20 larvae of each time point were stored in Bouin's solution and kept at 4 ºC. For quantitative real time PCR, 135 larvae were collected (5 larvae x triplicate x 9 sampling points), rinsed in distilled water and submerged in the stabilization reagent (*RNA*later®, QIAGEN GmbH, Germany). The sample was stored overnight at 4 ºC to ensure that the solution penetrates the tissue. Thereafter the sample was

transferred to -20 ºC until further processing. For the enzyme assay, 135 larvae were collected (5 larvae x triplicate x 9 sampling points) and rinsed in distilled water, then placed in micro-tubes and stored at -80 ºC, samples of rotifer and *Artemia* also were collected (50 g of wet weight) to analyze the exogenous enzyme contribution. A total of 135 of fresh larvae (15 larvae x 9 sampling points) were used to measure body weight, standard length and the same number of larvae were sampled and reserved in 70 % ethanol throughout the experimental period gut content.

2.2.3 Histological analysis

After 24 h submersion in Buoin's solution, the samples were washed and reserved in 70 % alcohol. Subsequently, larvae were washed, dehydrated, cleared, and individually embedded in paraffin blocks. Sagittal sections (5 μm thick) were obtained using a rotary microtome (RM 2135, Leica, Nussloch, Germany), placed on glass slides, re-hydrated, and Hematoxylin–Eosin stained. Finally, the slide was permanently mounted (Entellan, EMD Millipore, Billerica, MA, USA) and examined under a light microscope (BX41, Olympus, Tokyo, Japan).

2.2.4 Quantitative real time PCR

Total RNA was extracted from pooled sample of five fishes in triplicate for each sampling time-point (5 larvae x triplicate x 9 sampling points). The larvae were homogenized in 1 mL of TRIzol™ reagent (Thermo Fisher Scientific, Invitrogen, USA) following the manufacturer's protocol. The RNA purity and concentration were checked using NanoDrop spectrophotometer (Thermo Scientific, Waltham city, Massachusetts, USA) ensured the OD260/280 of all samples ranged between 1.9 to 2.0. cDNA synthesis was obtained from 100 ng of RNA using iScript ™ cDNA Synthesis Kit (Bio-Rad Inc., Hercules, California, USA) following manufacturer's instructions in a final volume of 20 μL.

The expression level of mRNA for trypsin (*try*), chymotrypsin (*ctrb*), pepsin (*pep*), pancreatic lipase (*pl*) and amylase (*amy2α*) was determined by quantitative real time PCR (qRT-PCR). The primer sequences were listed in Table 2.1 (Kondo et al., 2017; Murashita et al., 2018). Each reaction contained 1 µL of cDNA, 0.4 µL of each primer, 0.4 µL ROX reference dye and 10 µL of qPCR Mix (KOD SYBR® qPCR Mix, Toyobo Co., LTD, Osaka, Japan) in a final volume of 20 µL. The analysis was conducted in technical triplicate using Applied Biosystems StepOne ™ (Thermo Fisher Scientific Inc, Waltham city, Massachusetts, USA) for 2 min at 98 °C; 40 cycles: 10 s at 98 °C and 10 s at 60 °C, 10 s at 68 °C. Melting curve stage was set for 15 s at 95 °C, 1 min at 60 °C and (gradually increased 0.3 °C) 15 s at 99 °C. The relative quantity of targeted gene transcripts was normalized and measured using the standard curve with diluted cDNAs. The expression levels of *β* - actin were stable during larval stages (until 40 DAH) and its geometric average expression was used as a normalization factor.

Table 2. 1 Primer sequences for Real time PCR

Gene	Primer forward	Primer reverse	Source
β-actin	GGCACTGCTGCCTCCTC	GCCAGGATGGAGCCTCC	Kondo et al., 2017
try	TCAGGTGTCTCTGAACTCTG	ACCTGAACACGAGACTTG	
pep	TGATCAAGGGAAAGACTGC	GTCAGCATCGTTTGTCATG	
ctrb	ACGGCGCCTGGACTCTGGTC	TTGGCGGCGAGGATCTGGTC	Murashita et al., 2018
amy2α	GCATGCCCGGTGGCACCTAC	GCCATCACCGCCAACATGGA	
pl	GCCACGATGCTGACAGGTTCACT	GCCACGATGCTGACAGGTTCACT	

Note: trypsin (*try*), chymotrypsin (*ctrb*), pepsin (*pep*), pancreatic lipase (*pl*) and amylase (*amy2α*)

2.2.5 Enzyme assay

For each sampling point, the crude enzyme extraction was made in triplicate, in which five individually larvae were transferred to 100 µL of ice-chilled homogenization buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5) (Bolasina and Yamashita, 2006), homogenized using pellet pestle cordless motor (Sigma-Aldrich, Saint Louis city, Missouri, USA). Thereafter the homogenate was mixed with 400 µL of homogenization buffer and coldly centrifuged for 30 min at 1700 x g. The supernatant was used for enzymatic assay analysis as crude enzyme. Bradford's method utilizing protein assay CBB solution (Nacalai tesque, Inc, Kyoto, Japan) was performed for protein content determination. 5000 rotifers and 500 *Artemia* nauplius were used for enzyme extraction. The same samples were lyophilized for 24 h and were used for protein content estimation.

2.2.5.1 Trypsin

The fluorometric assay for trypsin activity was performed according to Bolasina et al. (2006) using Z-$_L$-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA, C9521, Sigma-Aldrich) as the substrate. The substrate solution contained 50 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ-$_L$Arg-MCA. A total of 50 µL of crude enzyme was mixed with 500 µL substrate in a micro tube and incubated in a water bath at 30 °C for 30 min. The reaction was stopped by adding 100 µL of 30% acetic acid. For blank reading, the same pattern was prepared without the addition of acetic acid prior to mixing the substrate. Fluorescence was measured using a spectrofluorophotometer (F2000, Hitachi High-Technologies Corporation, Tokyo, Japan) and the difference in emissions at 440 nm (excitation 380 nm) was measured between the samples and the blanks. Trypsin activity was expressed in unit (U) in 30 min, as increase of emission per protein (U /µg protein).

2.2.5.2 Chymotrypsin

Chymotrypsin activity was assayed by following a modified method of Murashita et al. (2018) using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPFNA, Sigma-Aldrich) as a substrate. Each assay contained 240 µL of 100 mM Tris buffer containing 20 mM $CaCl_2$ (pH 8.5), 100 µL of 2.4 mM SAPFNA and 50 µL of crude enzyme. Production of *p*-nitroanilide (*p*NA) was measured by monitoring the increase in absorbance at 405 nm per minute for 7 min at 37 ºC using a spectrophotometer (U5100, Hitachi High-Technologies Corporation). One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in 1 min.

2.2.5.3 Pepsin

Pepsin activity was evaluated using of 2 % haemoglobin in 0.06 N HCl as substrate according to Natalia et al. (2004). In the test tube, 100 µL of enzyme extract was incubated with 500 µL of substrate for 10 min. The reaction was terminated using 1 mL of 5 % trichloroacetic acid and left for 5 min. The mixture was then centrifuged for 5 min at 12000 xg. Absorbance was recorded at 280 nm using a spectrophotometer (U5100, Hitachi High-Technologies Corporation). For blank reading, trichloroacetic acid was added to substrate prior to the addition of enzyme extract.

2.2.5.4 Lipase

Generally, neutral lipase is widely distributed along the digestive tract of larval fish, which hydrolyse the ester bonds of triacylglycerol to monoacylglycerol and free fatty acids (Rønnestad et al., 2013). Neutral lipase activity was determined based on protocol of Roberts (1985). The non-fluorescent substrate, 4-methylumbelliferyl butyrate (4 MUB) was used solubilizing in lecithin mixed micelles, is hydrolyzed to butyric acid and the highly fluorescent compound, 4-methylumbelliferone (4-MU). A 60 µL substrate solution (0.5 mM 4 MUB, 5

mM egg lecithin, 10 mM sodium taurocholate and 150 mM NaCl) was placed in tested tube with 20 µL of enzyme extract and 20 µL of Tris buffer (pH 7.5). Each assay was conducted simultaneously at both 4 ºC in an ice bath and at 37 ºC in a water bath for 10 min. A 3 mL cold (4 ºC) Tris buffer (1 M, pH 7.5) was added to quench the reaction. The change in fluorescence was recorded by a spectrofluorophotometer (F2000, Hitachi High-Technologies Corporation) utilizing emission setting at 450 nm (excitation 380 nm) and subtracting the reading of the 4 ºC incubated samples against the 37 ºC control samples for each assay. Fluorometer constants were calculated from standard stock solutions of 4-methylumbelliferone.

2.2.5.5 Amylase

Activity was assayed by following a modified protocol from Murashita et al. (2018) using 1 % starch solution as a substrate. The reaction mixture consisted of 50 µL of enzyme extract, 25 µL of the substrate solution and 25 µL 20mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl) followed by 60 min at 37 ºC of incubation. 50 µL dinitrosalicylic acid reagent (1 % dinitrosalicylic acid and 30 % sodium potassium tartrate in 0.4 M NaOH) was added into the mixture and incubation in boiling water for 5 min. The absorbance was recorded at 540 nm using a Hitachi U5100 spectrophotometer and the amount of maltose released was determined by reference to a standard curve. The activity was expressed in U, which was defined as the amount (µmol) of maltose released in 1 min.

2.2.6 Gut contents analysis

A total of 150 larvae (collected after starting exogenous feeding to the end of experimental time) were examined to constituents of larvae guts using a digital microscope (VHX- S90F, Keyence Corporation, Osaka, Japan) connected to an ultra-small, high-performance zoom lens (VH-Z20R, Keyence Corporation). The average number of preys for each stage was used to estimate the fraction of enzyme activity contributed by ingested prey.

2.2.7 Statistical analyses

The results were presented as a mean ± standard error of the mean (SEM) of triplicate. The level of gene expression and activity of digestive enzyme between different ages were analyzed by one-way ANOVA (SPSS 24.0, IBM, USA) applied Tukey's HSD (Honestly Significant Difference) test at α=0.05 significant level. The correlation between gene expression and enzyme activity of fish larva was performed by linear regression (α=0.05).

2.3. Results

2.3.1 Fish growth and survival rate of red sea bream larvae.

The larval growth in length and weight is shown in Fig. 2.1. The average size of newly hatched larvae was 2.43 ± 0.3 mm of body length and increased exponentially by following $y=2.4647e^{0.0362x}$ (R^2= 0.9638), reached to 11.45 ± 0.5 mm at 40 DAH. The initial wet weight was 0.010 ± 0.003g per 30 larvae, grew up to 2.5 ± 0.7g/fish after 40 days ($y = 0.001x^{2.1154}$, R^2= 0.924). The oral cavity opened and larvae started exogenous feeding at 3 DAH followed by the yolk sac completely absorbed at 5 DAH.

During the experimental periods, the ontogenetic development of larvae devised into four major stages (Fig. 2.2B). The first stage was endogenous nutritional stage, considered from hatching to mouth opening (0-3DAH). The second stage was from mouth open to yolk sac depletion considered as endo-exotrophic stage (3-5 DAH). The exogenous feeding stage recorded from 3 to 15 DAH followed by appearance of the gastric glands and pyloric appendages (15-40 DAH) which were remarked as Stage IV. Within 40 days, the larvae showed two critical stages (from 10 to 15 DAH and 20 to 30 DAH, respectively); whereby the survival rate dramatically dropped (Fig. 2.2A).

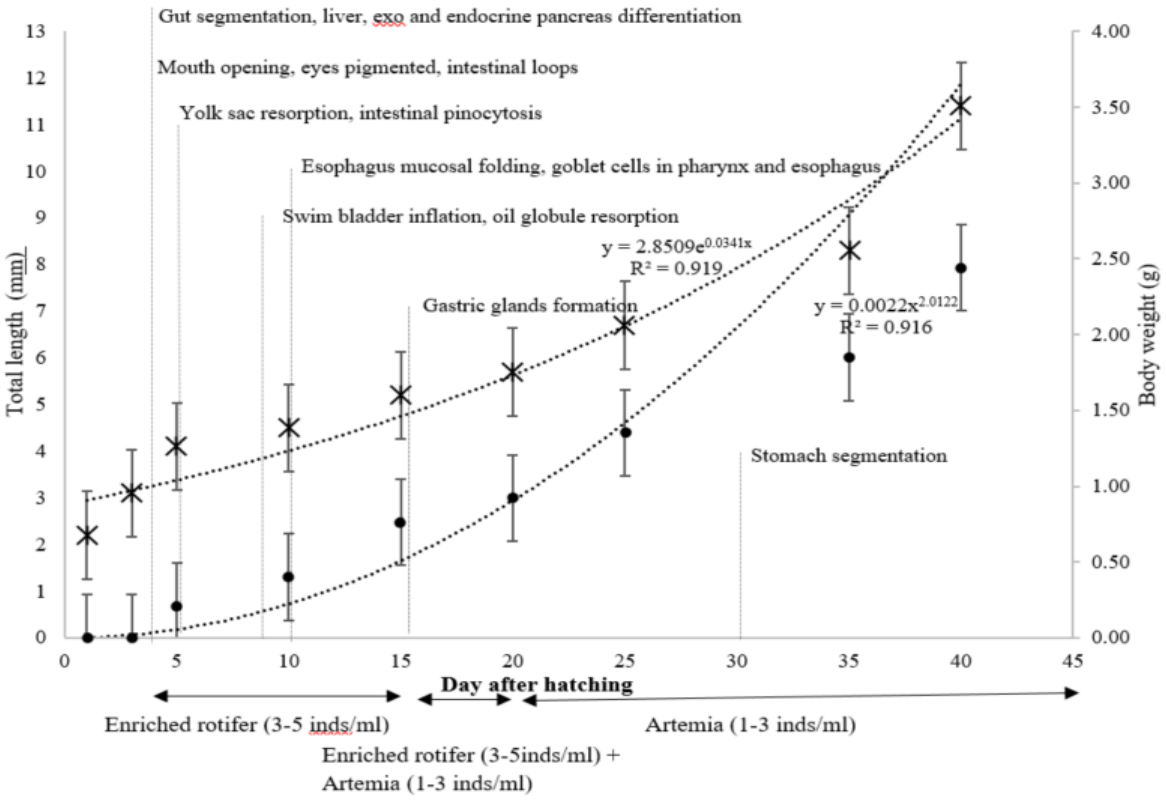


Figure 2. 1. Relationship of total length (mm), body weight (g), age post hatching (day) and major histological changes in digestive system from 1 to 40 DAH in red sea bream larvae.

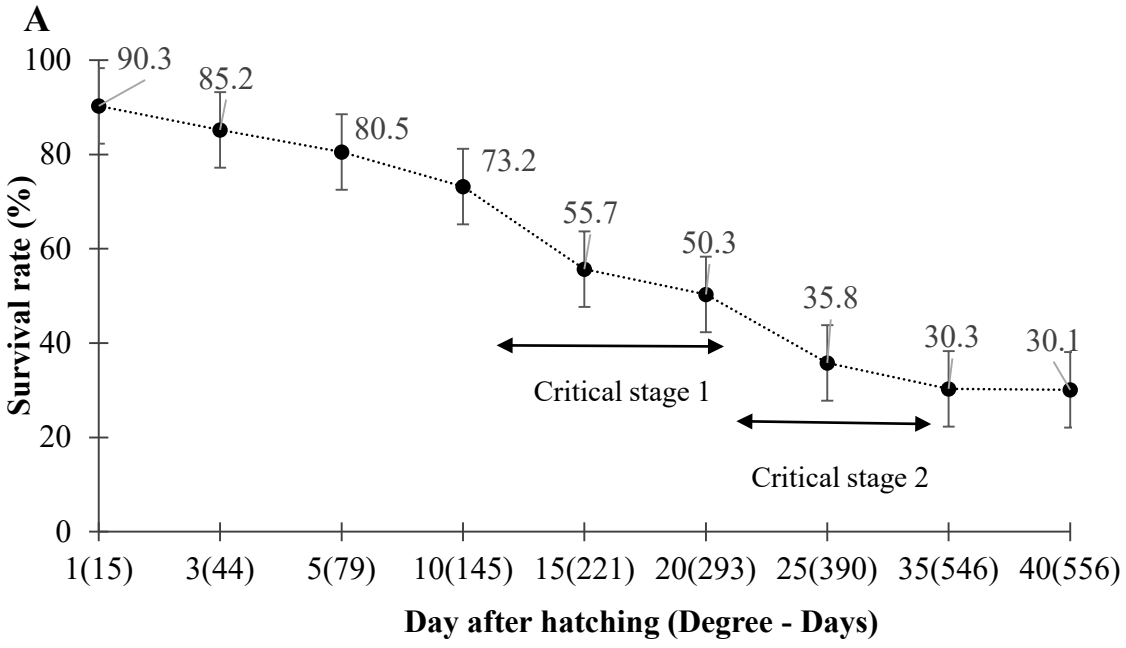
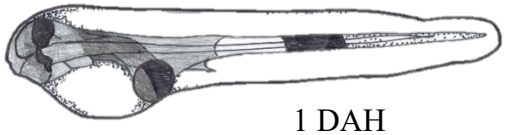
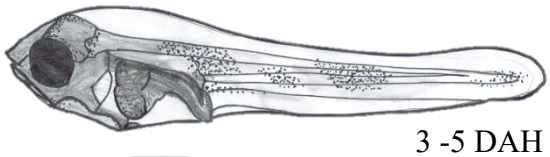


Figure 2. 2 The survival rates (A) and morphological changes (B) of red sea bream larvae during the ontogenetic development. The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

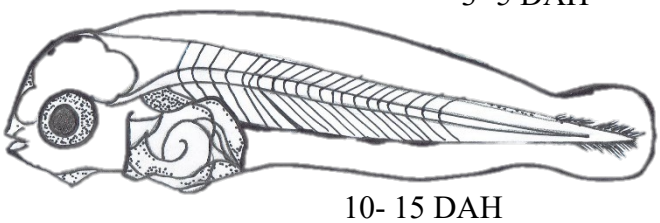
B



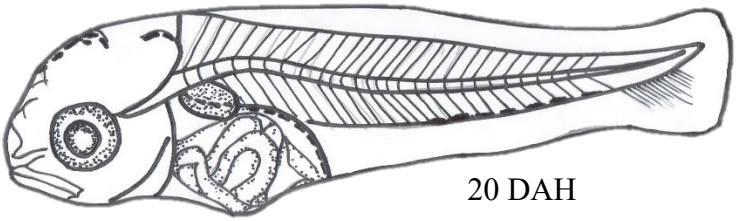
1 DAH



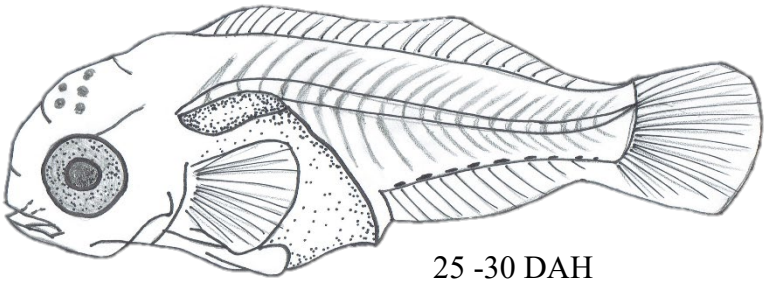
3 -5 DAH



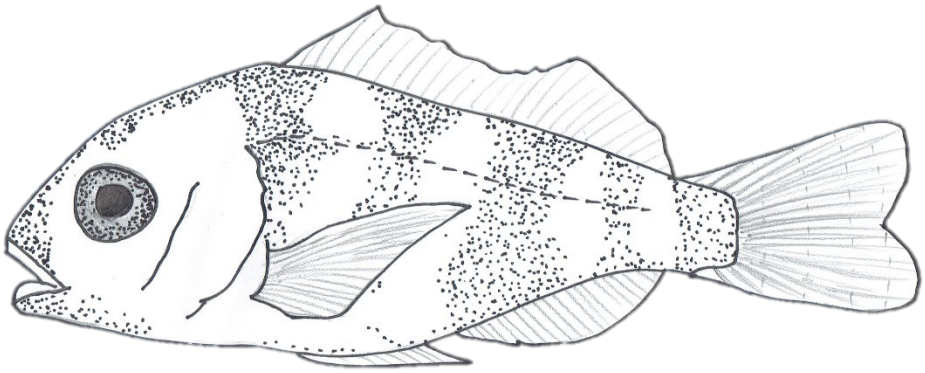
10- 15 DAH



20 DAH



25 -30 DAH



35 - 40 DAH

2.3.2 Histology of digestive tract development.

The newly hatched larvae were characterized by a large yolk sac at the front part of abdominal cavity (Fig 2.3A), and this yolk sac was completely absorbed at 5 DAH. At 3 DAH, the larvae started exogenous feeding, the mouth cavity and the anus opened, whereby the buccopharynx, oesophagus and intestine were differentiated and histologically recognized. The digestive tract was undifferentiated and displayed as a straight tube. The abdominal cavity relatively enlarged and the incipient liver and pancreas were observed (Fig. 2.3B). At 5 DAH, the incipient stomach appeared and the swim bladder was visible. The intestine folded and coiled, and was divided into two parts by an intestinal valve coinciding with the development of villi. However, the posterior intestine and liver did not show any lipid accumulation (Fig. 2.3C).

From 5 DAH to 15 DAH, the digestive system was completely grown in length. The oesophagus expanded and elongated followed by the oesophageal epithelium, which is usually considered as the stomach precursor, turning into simple columnar epithelium. The initial development of the stomach is indicated by the presence of mucous cells which contain acid glycoproteins and are visible along the stratified epithelium. The enlargement of the mucosal surface and the rapid increase in the density of mucous cells were observed from 12 DAH. The liver enlarged and it formed into a globular shape. Gastric glands were first detectable from 15 DAH, coinciding with the presence and increasing density of lipid inclusions in the hepatocytes (Fig. 2.3D). The gastric gland significantly developed in size and density in the cardiac and fundic sections at 25 DAH followed by thickening of muscle layers until 35 DAH. The number of goblet cells in the intestine increased gradually from 15 - 40 DAH (Fig. 2.3E and 2.3F).

At 35 DAH, the stomach was completely differentiated into a short cardiac segment connecting the stomach with the oesophagus. Besides the increase in size of the digestive structure and large lipid inclusions in the intestine and liver, there were no major morphological

changes in the histological structure of the digestive system observed until 40 DAH (Fig. 2.4 and 2.5).

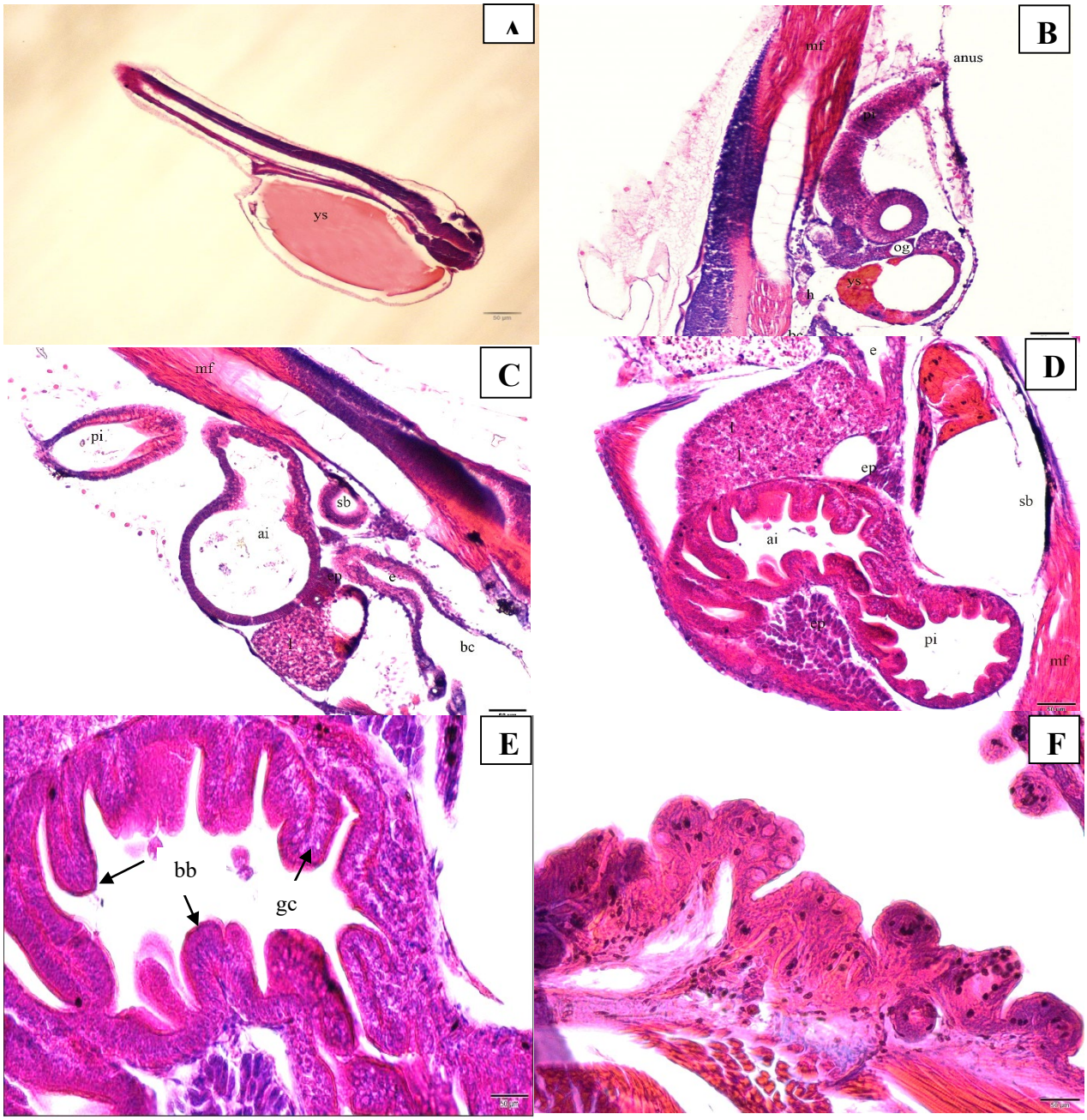


Figure 2. 3 Histological microsections of the digestive system in *P. major* larvae during development. A) Newly hatched larvae (1 DAH) with the yolk sac. B) 3 DAH larvae, mouth-opened with a straight digestive tract. C) 5 DAH with the stratified epithelium in oesophagus. D) 15 DAH with the appearance of gastric glands. E) 20 DAH a prominent anterior intestine and the folding of the oesophageal mucosa, appeared goblet cells and brush borders in intestine. F) 35 DAH with abundant vacuoles. Abbreviations: ys: yolk sac; bc: buccopharyngeal cavity; gc, goblet cell; ai: anterior intestine; e: oesophagus; pi: posterior intestine; mf= muscular fibers; bb: brush border; h: heart; sb: swim bladder; l: liver; p: pancreas; ep: exocrine pancreas; og: oil globule. Scale = 50 µm. Staining: Haematoxylin-Eosin.

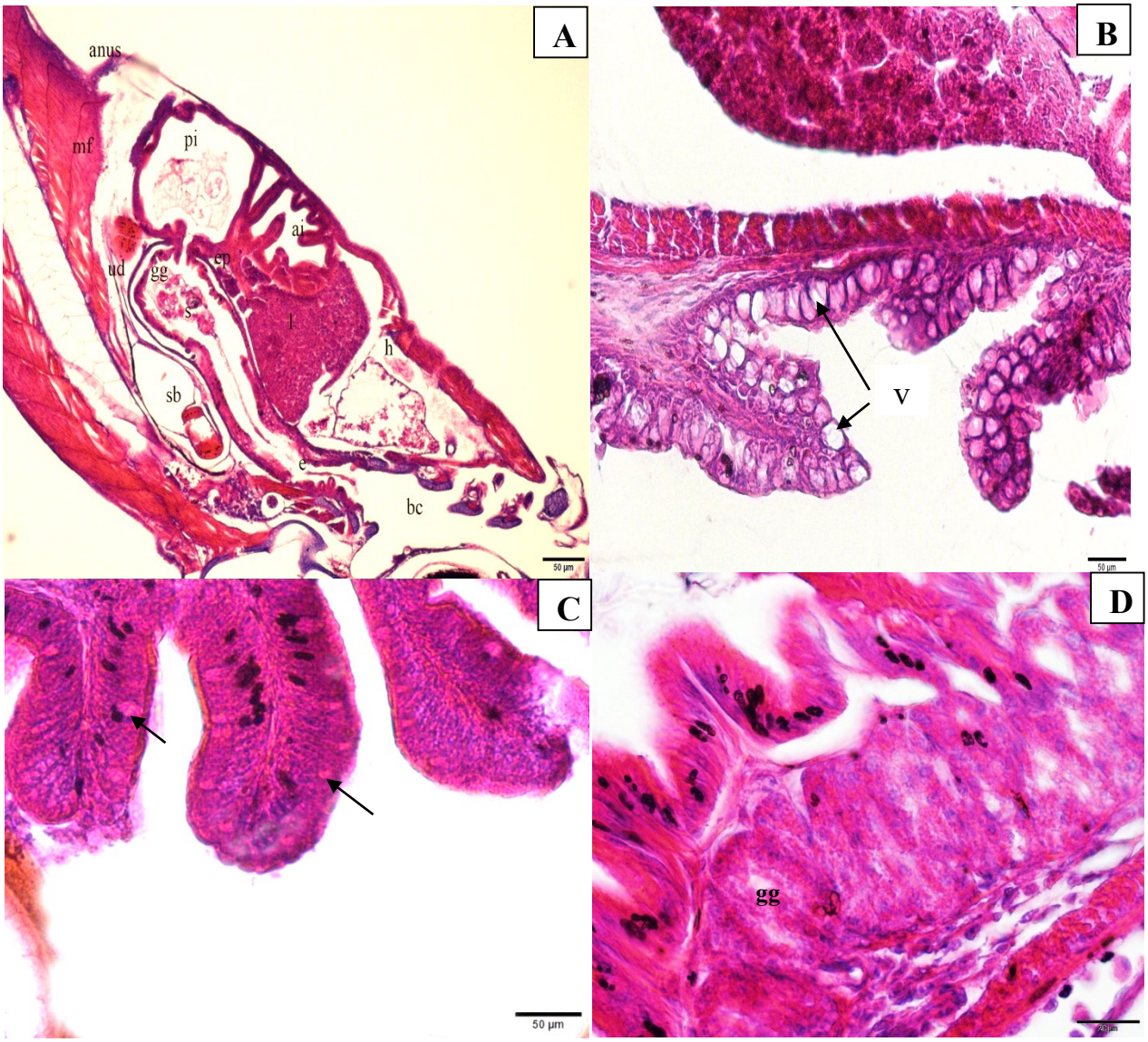


Figure 2. 4 Full function digestive system of *P. major* at 35 DAH. A) the stomach located between the oesophagus and anterior intestine is clearly visible. B) and C) digestive vacuoles. D) 40 DAH gastric glands (Scale =20 µm). Abbreviations: bc: buccopharyngeal cavity; gc, goblet cell; ai: anterior intestine; e: oesophagus; pi: posterior intestine; mf= muscular fibers; bb: brush border; h: heart; sb: swim bladder; l: liver; p: pancreas; ep: exocrine pancreas; og: oil globule; ud, urinary duct; v: vacuoles. Scale = 50 µm. Staining: Haematoxylin-Eosin.

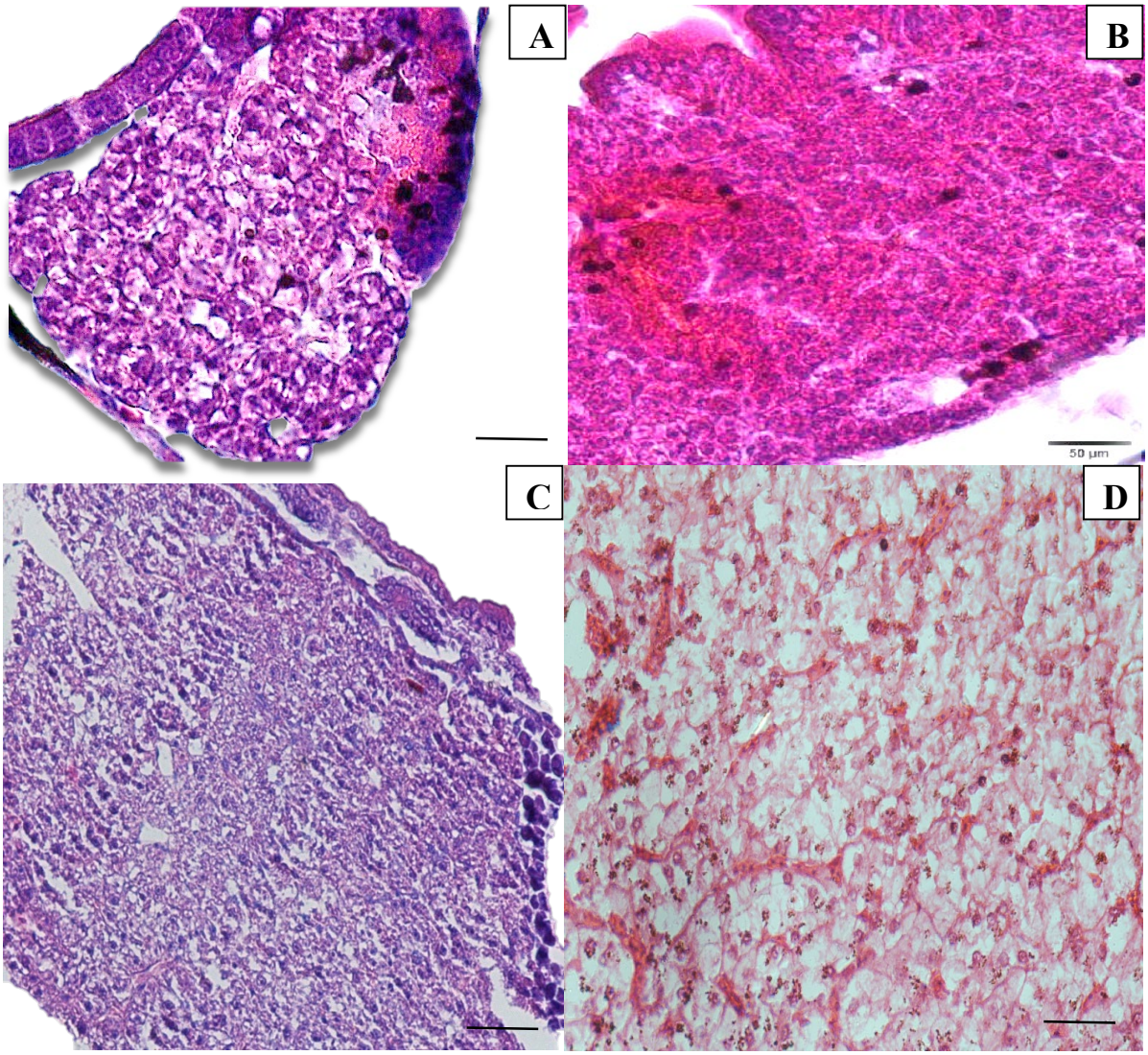
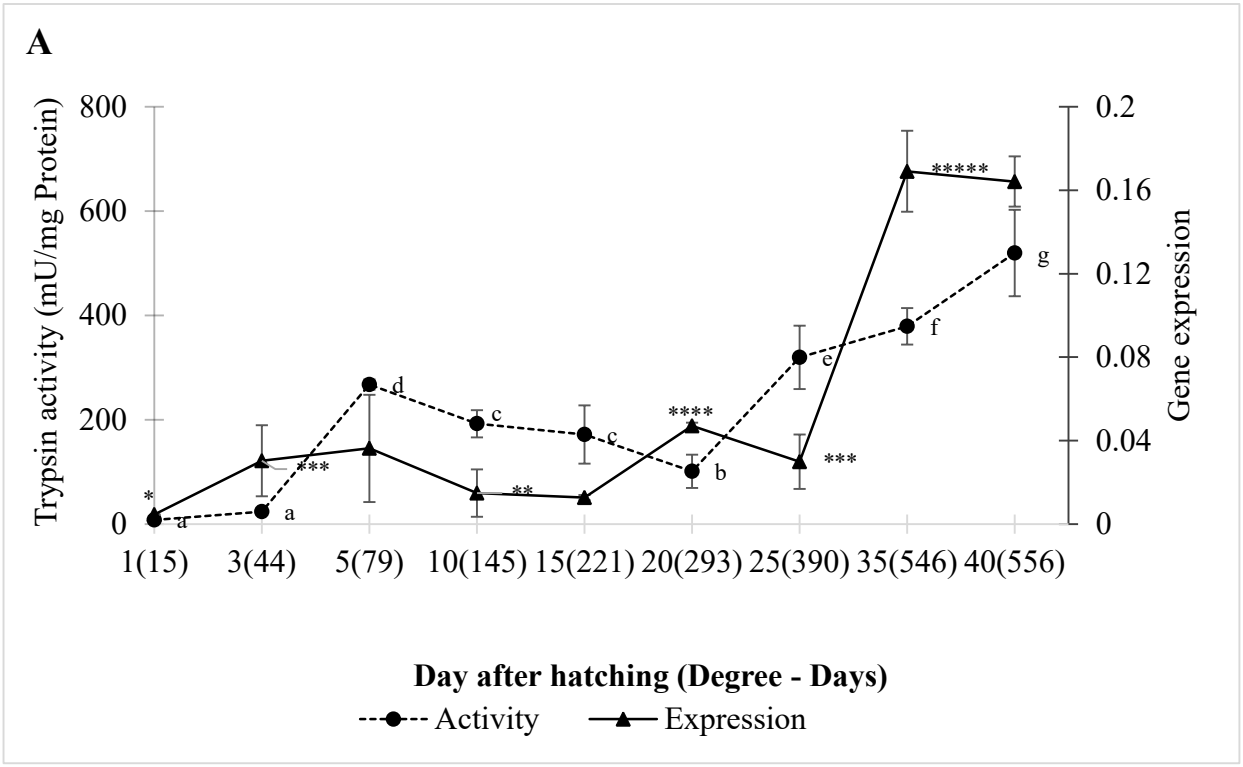


Figure 2. 5 Detail of the liver in *P. major* at different stages of development showing ontogenic differences in fat accumulation in hepatocytes. A) 5 – 10 DAH. B) 15 - 20 DAH. C) 25 - 30 DAH. D) 35 - 40 DAH. Scale = 50 µm. Staining: Haematoxylin-Eosin.

2.3.3 Gene expression and activity of digestive enzymes

Low trypsin activity was detected at hatching (8.13 ± 0.83 mU/mg protein) before it peaked at 5 DAH (267.44 ± 3.51 mU/mg protein), and fluctuated similarly to the expression expected at 15 to 20 DAH, whereby a significant decrease of activity was recorded (101.07 ± 3.75 mU/mg protein; $p < 0.05$). After 20 DAH, the trypsin activity tended to rise over the experimental period and made a peak at 40 DAH (519.54 ± 6.28 mU/mg protein). The *try* expression fluctuated considerably until 25 DAH, and showed a tendency to increase coinciding with larval development. The highest expression level was recorded at 35 DAH and remaining constant without a significant difference until 40 DAH ($p < 0.05$) (Fig. 2.6A). However, at 40 DAH the expression and activity of trypsin were significantly correlated ($R^2 = 0.6072$, $p = 0.009$) (Fig. 2.6B).

ctrb expression levels fluctuated considerably over the larval development (Fig. 2.7A). At hatching, *ctrb* expression was low and then significantly increased at the onset of first feeding (3 DAH) ($p < 0.005$). Subsequently, a high fluctuation was observed with 2 valley points at 5 and 10 DAH. After 10 DAH, the *ctrb* profile sharply increased until 35 DAH with minimal fluctuation ($p > 0.05$) before dramatically dropping at 40 DAH ($p < 0.05$). However, the *ctrb* expression was not significantly correlated to the chymotrypsin activity ($R^2 = 0.3386$, $p = 0.141$) (Fig. 2.7B). Low detection of chymotrypsin activity was detected at 1 DAH (0.53 ± 0.05 mU/mg protein) and at 3 DAH first feeding (0.57 ± 0.06 mU/mg protein). The activity gradually increased after 3 DAH and peaked at 25 DAH where it leveled until 35 DAH before dramatically dropping at 40 DAH ($p < 0.05$).



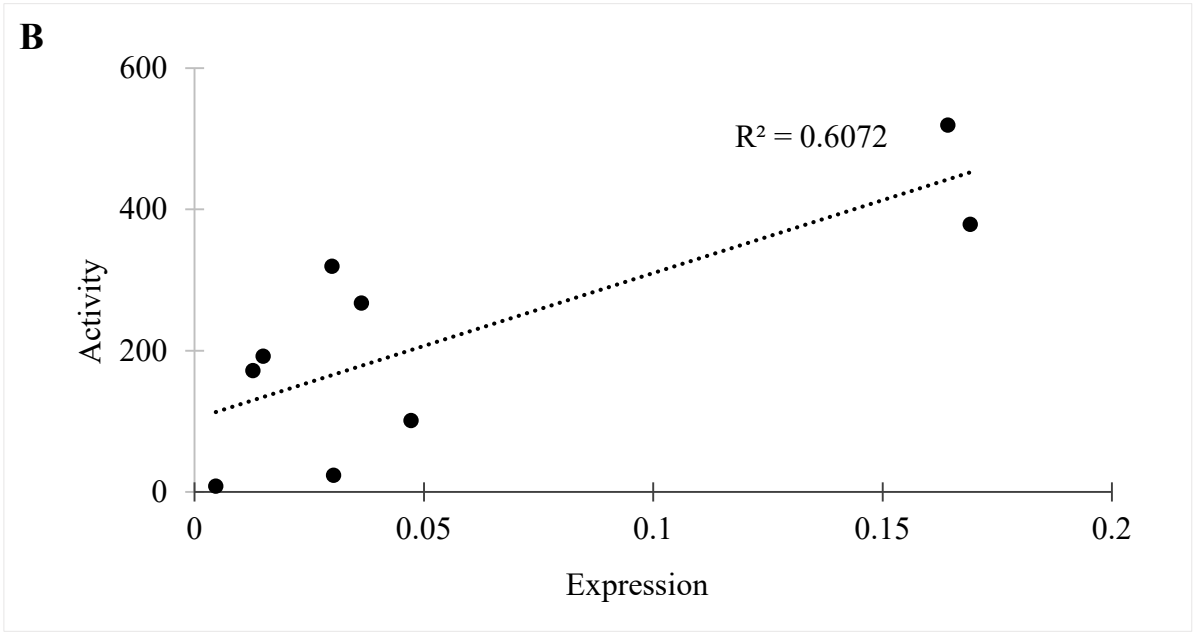


Figure 2. 6 Trypsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Dashed line and circle plot indicate trypsin activity. Straight line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Alphabetical letters indicate significant difference of trypsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d < e < f < g, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

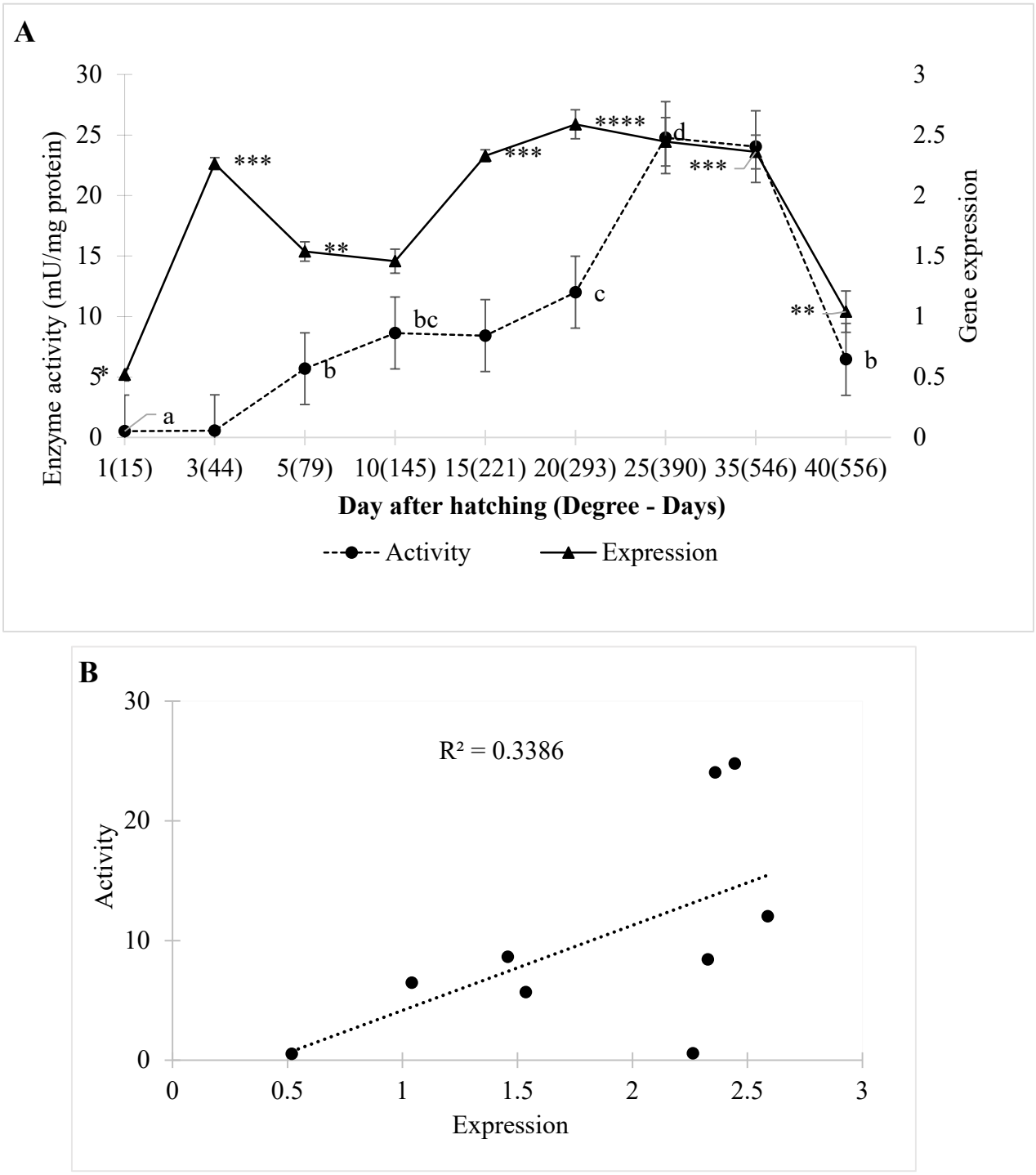


Figure 2. 7 Chymotrypsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Dashed line and circle plot indicate chymotrypsin activity. Straight line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Alphabetical letters indicate significant difference of chymotrypsin activity among developmental stages (ANOVA, Tukey's HDS test, a<b<c<d, $p<0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

pep expression was only detected at 10 DAH and its expression gradually increased over the experimental period (Fig. 2.8A). Expression levels remained low until 25 DAH, then sharply increased and reached a maximum at 40 DAH ($p < 0.05$). A similar pattern was noticed in the activity of pepsin with a significant correlation ($R^2 = 0.7779$, $p = 0.007$) (Fig. 2.8B). After 10 DAH (36.25 ± 4.21 mU/mg protein) the activity gradually increased until 20 DAH (85.22 ± 7.48 mU/mg protein) before dramatically increasing until 40 DAH (1244.04 ± 34.05 mU/mg protein).

pl expression showed a low value in newly hatched larvae before increasing gradually ($p < 0.05$). After first feeding, a slightly decrease in expression was observed until 10 DAH. Expression levels remained fairly constant without a significant variation until 25 DAH. Expression levels sharply increased and peaked at 40 DAH ($p < 0.05$) (Fig. 2.9A). On the other hand, the expression and activity of lipase showed a significant correlation ($R^2 = 0.6779$, $p = 0.005$) (Fig. 2.9B). Lipase activity rose from 14.10 ± 1.58 mU/mg protein (1 DAH) to 15.60 ± 1.44 mU/mg protein (3 DAH) before slightly increasing and remaining stable until 25 DAH. The lipase activity significantly peaked after 25 DAH and peaked at 40 DAH (15.97 ± 1.63 mU/mg protein; $p < 0.05$).

Low expression levels of *amy2α* in newly hatched larvae and at 3 DAH mouth opening were detected (Fig. 2.10A). *amy2α* expression remained low but continued to drop at 5 DAH, before sharply increasing at 10 DAH. Levels of *amy2α* expression then dramatically decreased at 15 DAH after the first peak before gradually increasing again at 25 DAH and then gradual decline until 40 DAH ($p < 0.05$). No significant correlation between expression and activity of amylase during larval development was recorded ($R^2 = 0.2181$, $p = 0.329$) (Fig. 2.10B). Amylolytic activity reached a peak at first feeding (0.85 ± 0.06 mU/mg protein), then continued with a generally decreasing fluctuating pattern with peaks at 3 DAH (0.85 ± 0.06 mU/mg

protein), 10 DAH (0.67 ± 0.02mU/mg protein) and 20 DAH (0.5 ± 0.02 mU/mg protein) before reaching an all time low level of 0.23 ± 0.02 mU/mg protein at 40 DAH ($p < 0.05$).

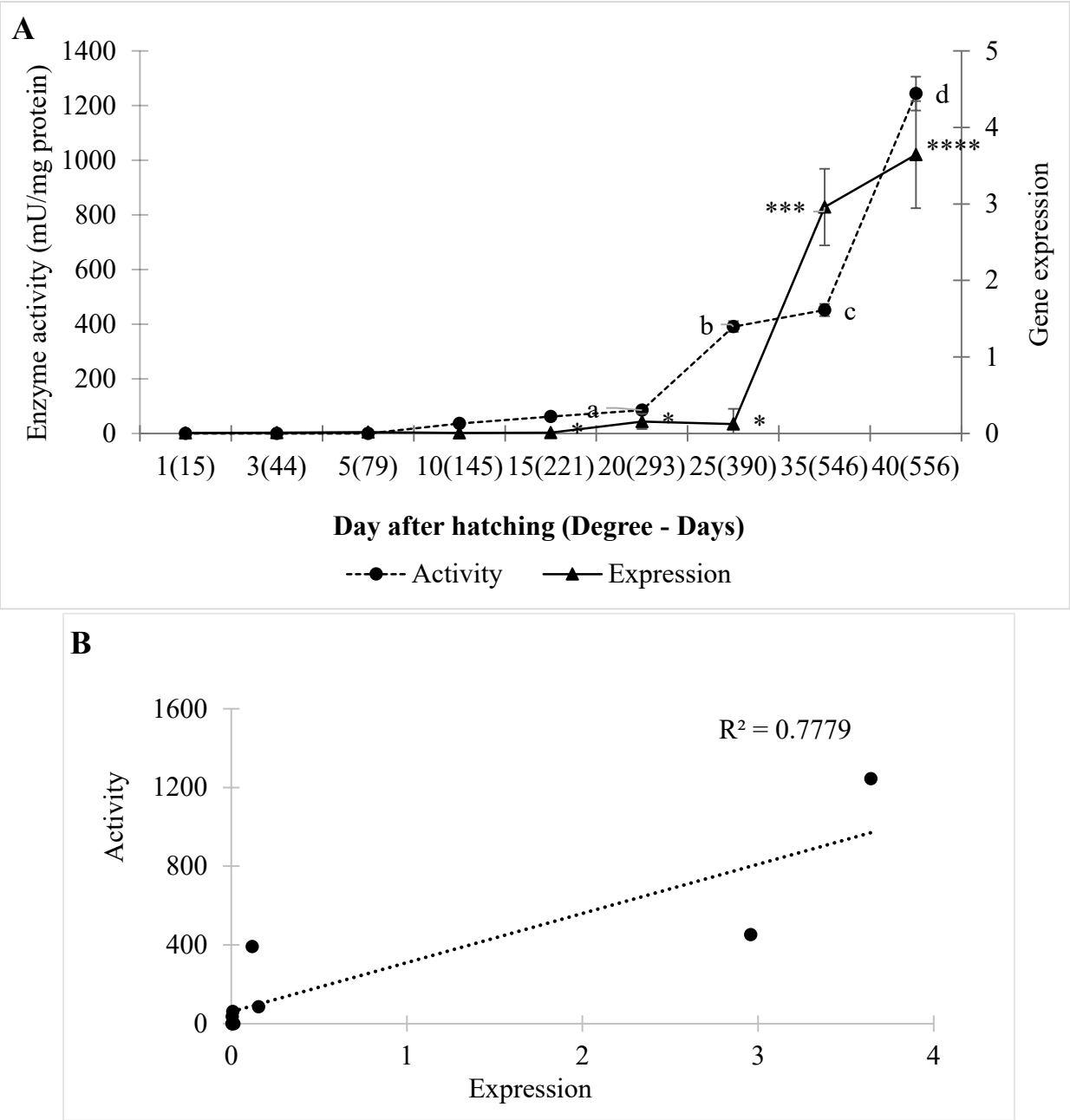
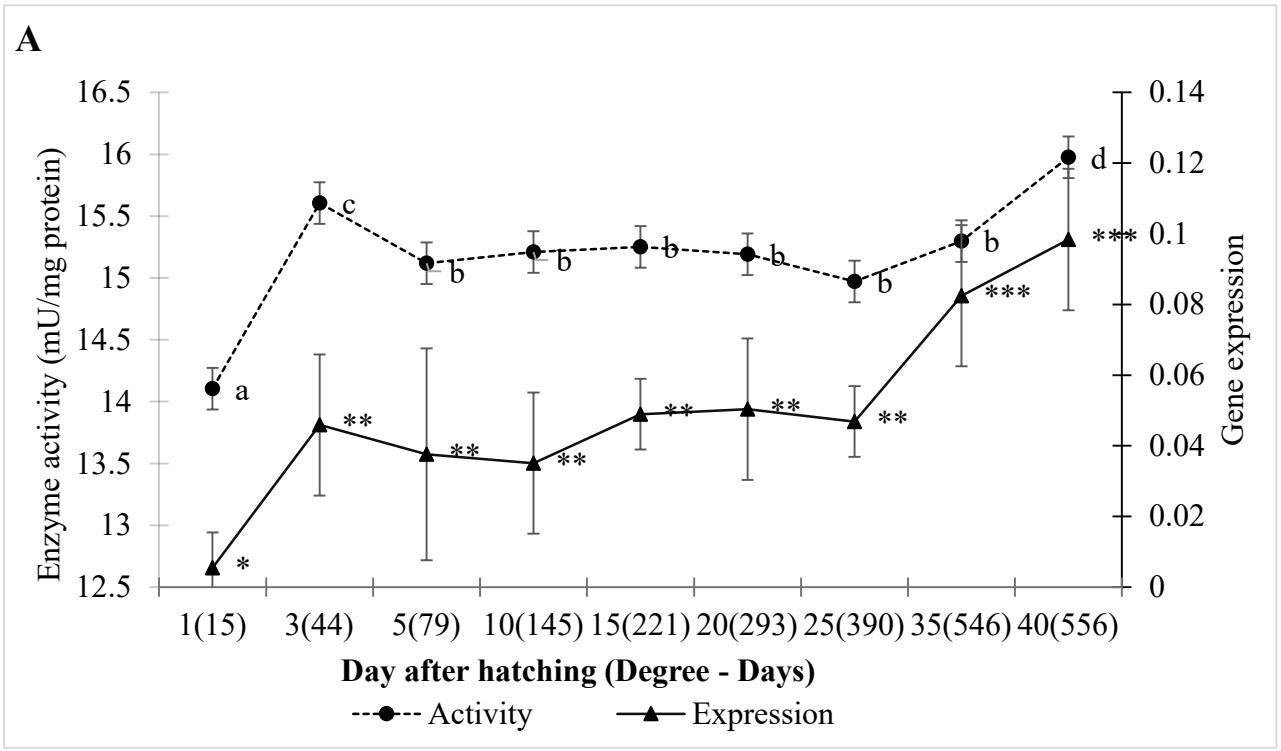


Figure 2. 8 Pepsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Dashed line and circle plot indicate pepsin activity. Straight line and triangle plot indicate gene expression. Delineated are regression line and R^2-values. Alphabetical letters indicate significant difference of pepsin activity among developmental stages (ANOVA, Tukey's HDS test, a<b<c<d, p<0.05). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).



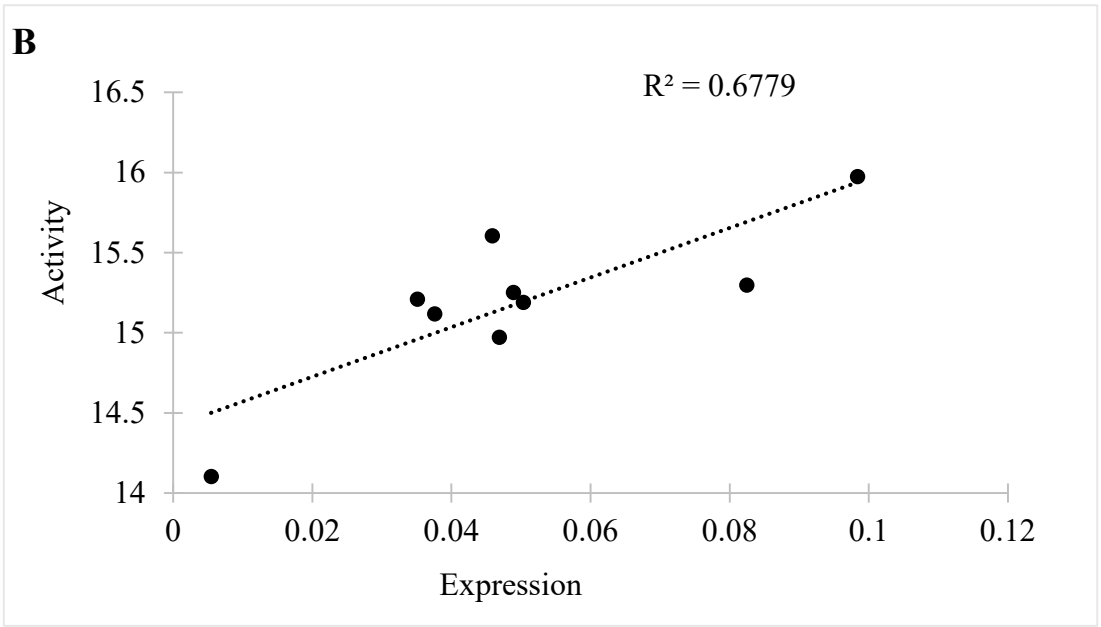
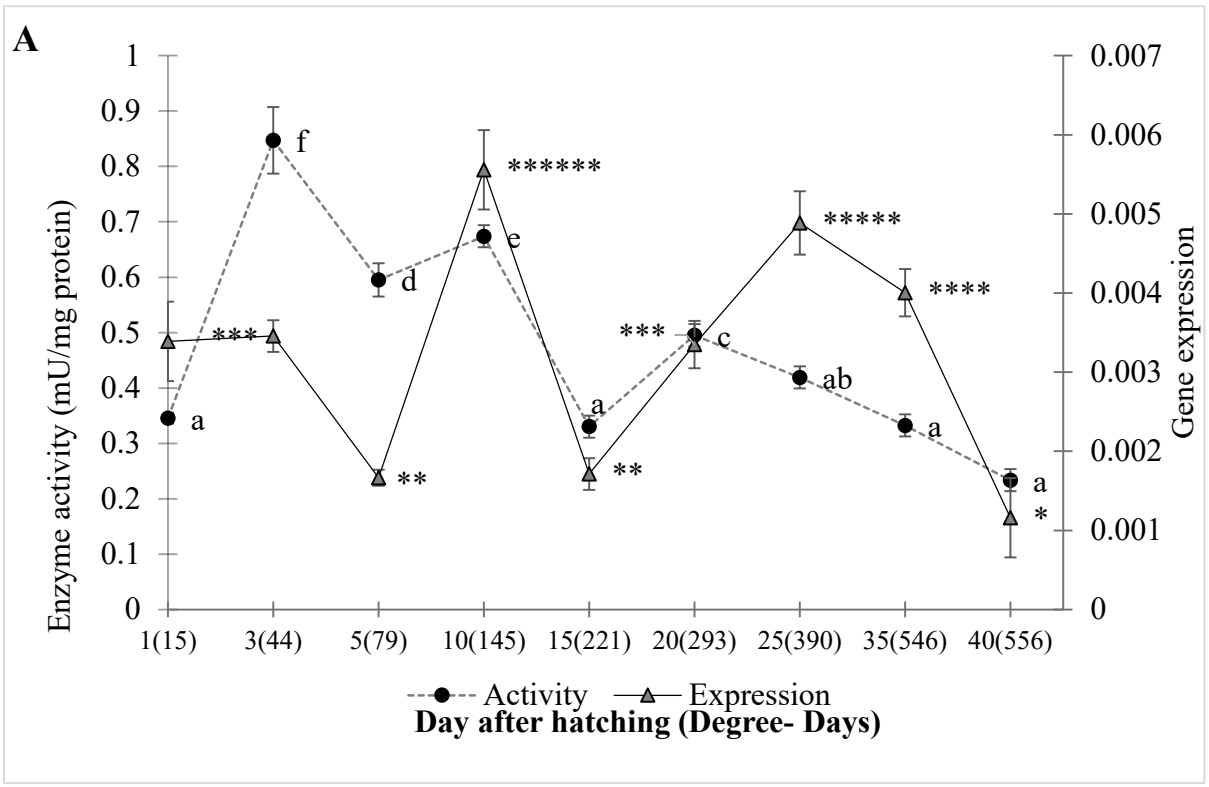


Figure 2. 9 Lipase expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Dashed line and circle plot indicate lipase activity. Straight line and triangle plot indicate gene expression. Delineated are regression line and R^2-values. Alphabetical letters indicate significant difference of lipase activity among developmental stages (ANOVA, Tukey's HDS test, a<b<c, $p<0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).



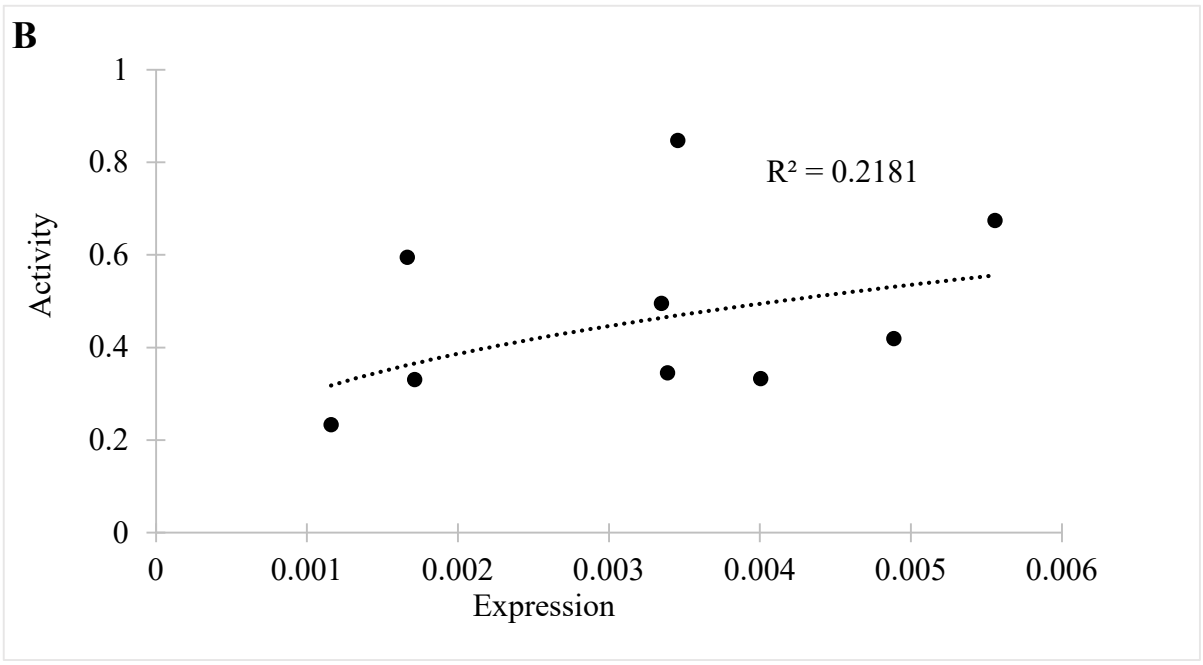


Figure 2. 10 Amylase expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Dashed line and circle plot indicate amylase activity. Straight line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Alphabetical letters indicate significant difference of amylase activity among developmental stages (ANOVA, Tukey's HDS test, a<b<c<d<e<f, $p<0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

2.3.4 Gut contents and exogenous enzyme contribution from prey to larval enzyme activities.

Larvae began exogenously feeding on rotifers at 3 DAH when its mouth opened (2.1 ± 0.5 rotifer/larvae), with gut content gradually increasing to 3.8 ± 0.7 rotifer/larvae at 5 DAH and 5.7 ± 1.2 rotifer/larva at 10 DAH. From 15 DAH to 20 DAH, larvae gradually fed both rotifer and *Artemia* with at 7.3 ± 1.4 rotifer/larva and 1.5 ± 0.5 *Artemia*/larva. At 25 DAH to 40 DAH, each larva sampled from 6.2 ± 1.5 *Artemia* nauplii within 2 hours (Fig. 2.11).

Generally, *Artemia* was observed to have higher enzyme activity than rotifers in all the enzymes assayed (Table 2.2). The contribution of exogenous enzyme (Fig. 2.12) from live feeds was observed to be not significant (less than 5%) for trypsin (0.18 – 1.2%), chymotrypsin (0.05 - 0.28%), pepsin (0.07 – 0.55%), lipase (0.03 – 1.16%) and amylase (0.03 – 3.25%).

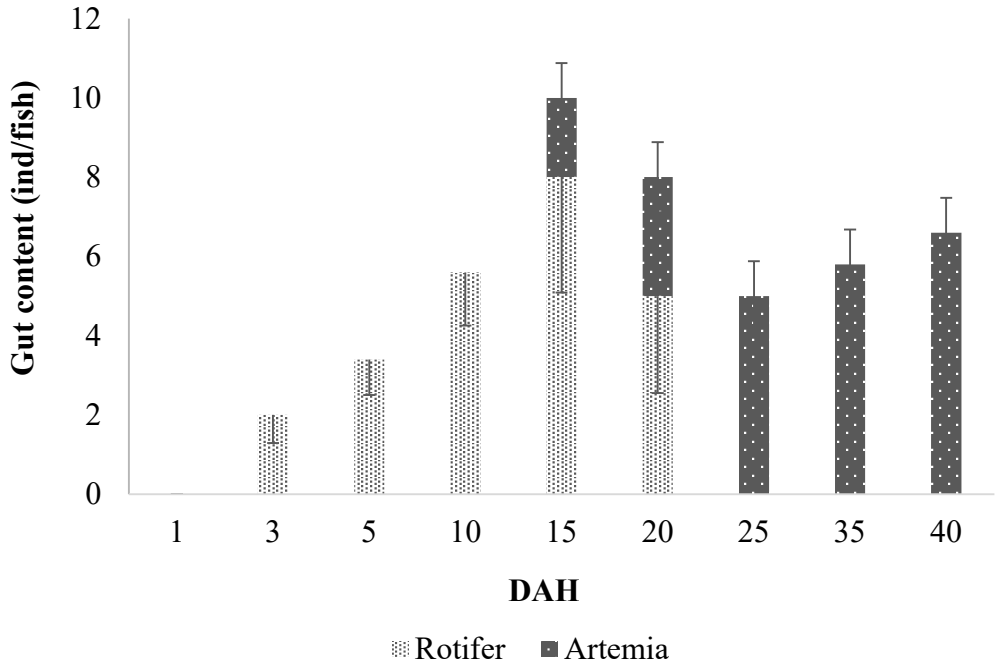


Figure 2. 11 Gut content of red seabream larvae (Mean ± SD) fed with enriched rotifer from 3DAH, alternative feeding with enriched Artemia and rotifer from 15 DAH and switched to Artemia only from 21 DAH. The minus error bars indicated SD of rotifer number, and the plus error bars indicated SD of *Artemia* number.

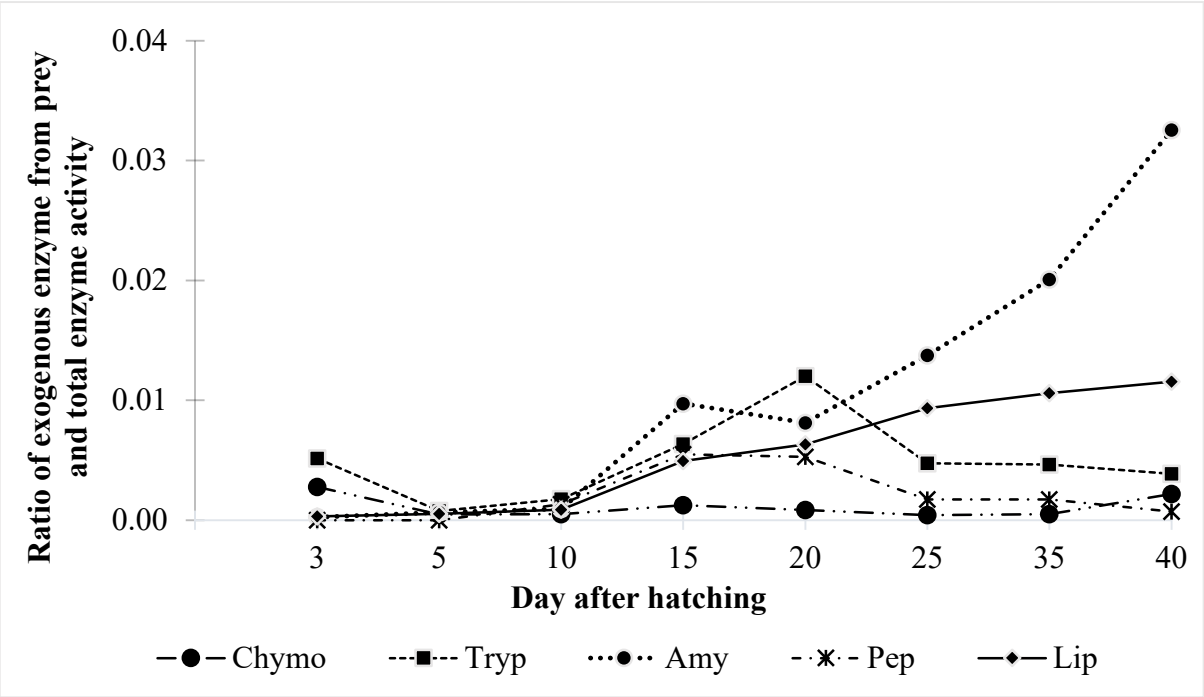


Figure 2. 12 The fraction of exogenous enzyme contributed by prey ingested and total enzyme activity of fish larvae during ontogenetic development.

Table 2. 2 Enzyme activities of preys (U/ind)

	Chymotrypsin	Trypsin	Amylase	Pepsin	Lipase
Enriched rotifer	0.00079 ± 0.00012	0.061 ± 0.008	0.00012 ± 0.00004	0.0086 ± 0.0005	0.0025 ± 0.0007
Artemia	0.0022 ± 0.0003	0.31 ± 0.01	0.0012 ± 0.0002	0.14 ± 0.07	0.028 ± 0.006

2.4 Discussion

The ontogenetic development of the digestive system in fish larvae provides valuable information and understanding of fish digestive physiology for diet optimization in mass marine fish larviculture (Zambonino- Infante and Cahu, 2001; Galaviz et al., 2015; Solovyev et al., 2016; Cui et al., 2017; Yúfera et al., 2018). Previous studies have highlighted that most marine larval fish hatch with a rudimentary digestive system (Micale et al., 2006; Moguel-Hernández et al., 2016) which then develops via three main processes; functional secretion by the pancreas, emergence of the intestinal brush border membrane enzymes and complete development of the stomach function (Zambonino- Infante and Cahu, 2001; Yúfera, 2018). In which, the digestive enzymes of larval fish are genetically programed and are activated from hatching and that it is possible for larvae to establish digestive capacities during yolk stage and at first feeding (Zambonino- Infante and Cahu, 2001; Conceição et al., 2008; Moguel-Hernández et al., 2016). Our results also concurred this hypothesis to be true too for *P. major,* as all the major digestive enzymes involved in the hydrolysis of proteins, lipids and carbohydrates were detected before mouth opening excepted pepsin presented at 15 DAH. Due to the lack of a functional stomach during early stages, the trypsin and chymotrypsin synthesized and secreted by the pancreas is mainly involved in protein hydrolysis. In addition, the trypsin and chymotrypsin activity relatively reflected the food presence and nutritional condition (Applebaum et al., 2001; Rønnestad et al., 2013; Mata-Sotres et al., 2016), in which trypsin became the most important alkaline protease in the early life stages of the fish larvae (Rønnestad et al., 2013). Therefore, the evaluation of trypsin and chymotrypsin profiles, could be applied as an indicator for nutritional status, as well as for evaluating effects of different feeding protocols and diets on fish larvae.

The mortality of larvae in the first week of development was 20 %, however this may not be necessarily due to physiological or digestive deficiencies during this ontogenetic stage.

Fish larvae undergo substantial changes in morphology and physiology, which are also reflected in their larval nutritional and physiological performance (Bolasina et al., 2006; Micale et al., 2006; Conceição et al., 2008; Solovyev et al., 2016). At 5 DAH, the activity of trypsin and chymotrypsin reached a peak coinciding with the oil globule reabsorption and complete reliance on exogenous feed (Fig. 2.3B). The trypsin activity then continued to decrease until 20 DAH, followed by a second peak at 35 DAH which coincided with the development of the digestive tract and accessory glands (Fig. 2.4B). Similar activity patterns were reported on many marine fish such as wrasse (*Labrus bergylta*; Hansen et al., 2013), golden pompano (*Trachinotus ovatus*; Ma et al., 2014)**,** rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2016)**,** meagre (*Argyrosomus regius*; Solovyev et al., 2016), crimson snapper (*Lutjanus erythopterus*; Cui et al., 2017). The *try* expression was strongly correlated to the activity (R^2 = 0.6072), in which correspondingly peaks were observed at 5 and 20 DAH (Fig. 2.6), then sharply expressed at 35-40 DAH. The delay in trypsin translation in *P. major* larvae was observed similarly in seabass *Lates calcarifer* (Srichanun et al., 2013) and *Lutjanus guttatus* (Moguel-Hernández et al., 2016). Additionally, the chymotrypsin activity gradually increased until 25 DAH, maintained high activity between 25-35 DAH then dramatically dropped by 40 DAH (Fig. 2.7). Contradictorily, the *ctrb* expression peaked at 3 DAH, and strongly fluctuated until 15 DAH (Fig. 2.7). From this stage, the expression was relatively constant until 35 DAH before dropping (Fig. 2.7). The correlation between expression and activity of chymotrypsin was not significant (R^2 = 0.3386). This is probably due to the fact that the isoform of chymotrypsin analyzed in this study was *ctrb 1,* and thus did not illustrate the full function of chymotrypsin. Therefore, highlighting that perhaps more analysis is needed on the different chymotrypsin isoforms. The weak correlation between activity and gene expression of chymotrypsin has been discussed previously in Japanese flounder (*Paralichthys olivaceus*; Lee et al., 2015) and spotted rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2016)

where with two chymotrypsinogen isoforms *ctrb 1* and *ctrb 2,* they found that only *ctrb 2* matched the pattern of enzyme activity.

Within the first 15 days after hatching, no detection of a morphologically distinct and functional stomach in red sea bream larvae was observed (Fig. 2.3A-C). Therefore, this suggests that digestion is mainly conducted by alkaline processes, without association of acid digestion. At 15 DAH, the larvae was in the transition period from larva to juvenile, pepsin was detected reflecting also the formation of the gastric glands and pyloric caeca, remarkably indicating the improvement of digestive function (Peña et al., 2003; Li et al., 2017). In this transition period, larvae were fed *Artemia* alternatively, however, the larval survival rate dramatically dropped (critical stage 1, Fig. 2.2). Takeuchi (2014) reported that, when larvae are fed *Artemia* only, mass mortality was observed and surviving larvae displayed lethargic behavior. However, the mass mortality experienced in this study may be due to the morphological changes during transition to post-larvae. According to Moteki (2002), several morphological development related to feeding behavior of *P. major* larvae occurred from 15 DAH such as the jaw, ceratobranchial teeth and premaxilla, allowed the larvae use grasping and sucking actions to feed on copepods. In the digestive system, the stomach epithelium turned into a structurally differentiated establishment of glands and gastric digestion detected (Diaz and Connes, 1997; Douglas et al., 1999; Rønnestad et al., 2013). Due to the development observed in the gut (Fig. 2.3), pepsin activity and expression increased from 15-40 DAH, with a significant correlation (R^2 = 0.7779). Similar findings were reported in greater amberjack *Seriola dumerili* (Wu et al., 2011); seabass *Lates calcarifer* (Srichanun et al., 2013)*;* golden pompano *Trachinotus ovatus* (Ma et al., 2014)*,* and bluefin tuna *Thunnus orientalis* (Murashita et al., 2014)*.* The combined digestive processes between pepsin and pancreatic enzyme resulted in higher efficiency of protein hydrolysis (Rønnestad et al., 2013). Therefore, the absence of pepsin enzyme activity during early stage could be considered as one of the reason for limited

digestibility of micro-diets. Additionally, the application of artificial feed as first feed for fish larvae remains challenges because the lack of denaturizing and proteolytic cleavage of food proteins by combined peptic and acid activity (Conceição & Tandler, 2018). Based on the aforementioned points, an integrated feeding regimes between live feed and micro diets could be considered for the young stage of marine fish larvae.

Amylase activity was detected at hatching and sharply increased at 3 DAH, then gradually decreased over time the period of the study (Fig. 2.10). Similar pattern has been documented in seabass *Lates calcarifer* (Srichanun et al., 2013), spotted rose snapper *Lutjanus guttatus* (Moguel-Hernández et al., 2016) and crimson snapper *Lutjanus erythopterus* (Cui et al., 2017). However, the *amy2α* expression strongly fluctuated during the first 25 days and was not significantly correlated to the enzymatic activity (R^2 = 0.2181). According to Moguel-Hernández et al. (2016), this often occurs due to diets change from rotifer to *Artemia*, which contains high levels of glycogen and thus glycolytic polymers like glycogen and starch could stimulate the expression and activity of amylase in fish larvae. These changes in amylase profile were consistent with the results on yellow croaker *Pseudosciaena crocea* (Ma et al., 2005) and totoaba *Totoaba macdonaldi* (Galaviz et al., 2015). The higher expression and activity of amylase in larvae fish compared to juvenile indicated a good transcriptional regulation of amylase synthesis during early ontogeny, and the juvenile turned into its carnivorous habits by reducing amylase activity.

During the endogenous nutritional stage, neutral lipase was detected and peaked at 3 DAH related to the digestion and absorption of nutrients derived from the yolk sac (Srichanun et al., 2013). The high patterns of lipase in this study is in line with the result from seabass *Lates calcarifer* (Srichanun et al., 2013), rose snapper *Lutjanus guttatus* (Moguel-Hernández et al., 2016) and gilthead sea bream *Sparus aurata* (Mata-Sotres et al., 2016). However, the decrease in activity and expression of *pl* at 25 DAH occurred from 25-30 DAH which also

coincided with a high mortality period (critical stage 2, Fig. 2.2). This could also indicate the high nutritional demand of fish larvae on phospholipids at this stage (Cahu et al., 2009; Srichanun et al., 2013; Moguel-Hernández et al., 2016). The intestinal digestive capacity was enhanced after 25 DAH indicated by the numerous microvilli formed, brush borders and abundant vacuoles and reflected by the high lipase activity and expression, these trends are known to be similar in other marine fish larvae as well (Srichanun et al., 2013; Mata-Sotres et al., 2016; Cui et al., 2017). Moreover, neutral lipase is not only distributed along the digestive tract of larval fish but also in the whole fish body (Chatzifotis et al., 2008; Sæle et al., 2010; Rønnestad et al., 2013). Because the marine fish larvae are very small at early stages, this study used whole larvae samples and applied an unspecific analytical method to measure the lipase activity, hence the activity of lipase was not a single specific activity in the digestive organs. The expression level of specific genes is more suitable to characterize the lipase ontogeny (Sæle et al., 2010; Rønnestad et al., 2013). Therefore, *pl* expression level might contribute to distinguishing pancreatic lipase. However, to assess the lipid digestion capacity, it required more studies to investigate the proportion of lipolytic activity in the digestive organs and non-digestive organs as well as the correlation between gene expression and enzymatic activity during the ontogenetic development of larval fish.

The contribution of exogenous enzymes from live preys have been investigated in some fish species (Oozeki and Bailey, 1995; Kurokawa et al., 1998; Bolasina et al., 2006; Rasdi et al., 2016; Loh and Ting, 2016). The fraction between exogenous enzyme and enzyme activity in fish larvae was high at first feeding, then reduced and remained at low levels during rotifer feeding (Fig. 2.12). From transition to *Artemia* which was contained higher exogenous enzymes, the fraction peaked from 15-20 DAH, then decreasing over time excepting amylase and lipase (Fig. 2.12). The results from the present study coincided with previous studies in that there were no significant contributions of exogenous enzymes from live feed to total

enzyme activity of fish larvae (Kurokawa et al., 1998; Lazo, Holt, & Arnold, 2000; Waqalevu et al., 2018). Previous studies suggest that live feeds could stimulate digestive enzyme secretions of fish digestive system by neurohormonal factors (Chan and Hale, 1992; Ruyet and Mugnier, 1993; Hamre et al., 2013) or related to an autolytic process in preys neutralized in the gut by the alkaline contents (Díaz et al., 1997). Further studies are necessary to assess the differences in the roles of exogenous enzymes to food ingestion during larval ontogenetic development.

The ontogenetic development of digestive system of *P. major* larvae was completed at 35 DAH. The evidence from histological, enzymatic and molecular analysis suggest that is possible for *P. major* larvae to switch to artificial feeds from 15 DAH as the gastric glands have formed by this time allowing for the activation of pepsin. After 25 DAH, the larvae turned into carnivorous habits with high requirement of phospholipids which when not met results in high mortality. Two critical stages of mass mortality were observed from 15-20 DAH and 25-30 DAH were related to the stomach's functionality as such these periods are to be highlighted as critical stages of development for this species. In addition, contribution from exogenous enzymes derived from live feed was not significant therefore alluding to their roles as a stimulant for the activation of zymogens and/or in an autolytic process during digestion as previously stated. Findings from this study could provide a better understanding of the morphological–functional changes during early ontogenetic development of the commercially important red sea bream.

CHAPTER 3: Comparative study on early digestive enzyme activity and expression in red sea bream (*Pagrus major*) fed on live feed and micro-diet

Abstract

The use of live feed to meet larval nutritional requirements has caused a bottleneck in marine fish larviculture whilst the use of micro-diets has had limited success in completely replacing live feeds. In this study, the variations in enzyme activity and its related gene expressions during early ontogenetic development of red sea bream (*Pagrus major*) were investigated. The experiment included four treatments: (i) using live feed (enriched L-type rotifers of the *Brachionus plicatilis* sp. complex) until 15 days after hatching (DAH) and gradually switching to *Artemia*; (ii) using micro-diet only; (iii) co-feeding diet (using both live feed and micro-diet) and, (iv) starvation (no food supplied). This study lasted 40 days and the activity and expression of digestive enzymes (trypsin (*try*), chymotrypsin (*ctrb*), pepsin (*pep*), amylase (*amy2α*), pancreatic lipase (*pl*), expression of energy metabolism (ATP synthase (*atp*), cytochrome-c-oxidase (*cox1*), cholecystokinin (*cck*) and growth (insulin like growth factor 1 (*igf1*)) were linked to larval biometrics. After 40 DAH, no significant difference was observed in growth (*igf1*), energy metabolism (*cox1, atp*) and survival rate between live feed and co-feeding diet treatments. However, there were significantly higher differences observed when these were compared to the starvation and micro-diet only treatments. The pancreatic enzymes and *cck* were regulated by dietary compositions and detectable from hatching and fluctuated throughout larval ontogeny. While *pep* was initially observed only after 15 DAH. Limits of larval survival and low enzyme activity were observed when the larvae were subjected to starvation for 10 days and were able to adapt to micro-diet only after 15 DAH accompanied with a 5-day co-feeding transitionary period.

3.1. Introduction

Marine finfish aquaculture has undergone rapid development in the past three decades. This has led to several substantial advances in larviculture and an increase in the reliability of supply of juvenile fish (Qin, 2013). Hatchery performance and efficiency were greatly enhanced through modifications in culture systems and nutrition regimes (Mahjou et al., 2013). However, techniques of marine finfish seed production still relies heavily on live feeds, which are known to contain gut neuropeptides and nutritional "growth" factors which enhance digestion ability (Liao et al., 2001; Kolkovski, 2001). The use of live feed has proven to have major bottlenecks for larviculture of many marine finfish and shellfish species (Coutteau et al., 1997; Bengtson, 2004) as it is generally and operationally expensive, unreliable, associated space requirements and laboriously intensive (Holt et al., 2011; Srichanun et al., 2013). Furthermore, it has been known that the risk of harmful pathogen infection is linked with the use of live feed (Lubzens and Zmora, 2004; Srichanun et al., 2013). Therefore, the development of micro-diets that are economical, palatable and provide essential nutrients for larval development is an important consideration. Numerous studies and weaning strategies have been conducted to replace live feed onto inert micro-diet to suit the expectation from commercial hatcheries (Kanazawa et al., 1989; Kolkovski et al., 1997b; Tang et al., 2010; Hauville et al., 2014; Moguel-Hernández et al., 2016), however these studies have highlighted that micro-diets have limited success in completely replacing live feeds and that it often leads to inadequate growth of larval fish (Lazo et al., 2000; Srichanun et al., 2013; Yúfera et al., 2018).

Previous studies have listed many reasons for difficulties in successful micro-diet applications for marine fish larvae during ontogenetic development. The most critical issue is that early larval digestive enzymes are not fully functional thus leading to insufficient enzyme activity to aid in the effective digestion of formulated diets (Kolkovski et al., 1997; Langdon,

2003; Holt et al., 2011; Hamre et al., 2013). Micro-diets are commercially composed of insoluble and limited amounts of soluble denatured proteins and carbohydrates (Langdon, 2003; Hamre et al., 2013). Therefore, the digestive efficiency of marine finfish larvae on these feeds is hard to evaluate (Koven et al., 2001; Langdon, 2003). In addition, the nutrient composition of artificial feeds might not match nutritional requirements of marine finfish larvae causing poor growth performance (Langdon, 2003; Hamre et al., 2013). Food detection by larvae is also an important consideration as fish larvae depend on prey movement (Cox and Pankhurst, 2000; Cahu and Zambonino-Infante, 2001). In addition, the fact that different species have various biological feeding behaviors and abiotic/biotic culture condition requirements adds another level of complexity to the ample challenges in feed commercialization (Kolkovski et al., 2009, Rønnestad et al., 2013). The lack of denaturizing and proteolytic cleavage of dietary protein by combined peptic and acid activity is also another challenge when using formulated feed for fish larvae at first feeding (Rønnestad et al., 2013; Rønnestad et al., 2018). It has been found that, the combined digestive processes between pepsin, an important digestive protease, and pancreatic enzyme activity has greater efficiency in protein hydrolysis as compared to when pepsin is not active (Rønnestad et al., 2018). Perhaps, the absence of a functional stomach and its associated acid pepsin-medicated digestion could lead to limited digestibility of protein diets during the ontogenetic development of fish larvae (Hamre et al., 2013; Rønnestad et al., 2013).

The advantages of co-feeding of live feed and artificial feed has been reported on many fish species (Kolkovski, 2001; Richard et al., 2015; Mata-Sotres et al., 2016). Feeding prey organisms for a short time to early stage larvae and gradually adding artificial feed is known to increase digestive activity and increase the larvae's ability to digest the dry diets (Kolkovski et al., 1997; Kolkovski, 2001) as live prey include certain neuro-hormonal factors which may stimulate digestive enzyme secretions in fish larvae (Chan and Hale, 1992; Ruyet and Mugnier,

1993; Hamre et al., 2013). Diets containing high energy levels, supplied as neutral lipid and phospholipid mixtures could promote larval growth (Cahu and Infante, 2001). The use of improved diets would sustain the production of constant high quality fingerlings in the hatchery (Cahu and Infante, 2001; Kolkovski, 2013; Takeuchi, 2014).

The red sea bream (*Pagrus major*) is considered as a commercially important fish for marine aquaculture in Asia-Pacific region (Murashita et al., 2014). In Japan, different larval rearing techniques have been developed for this species, but relies heavily on live feeds such as microalgae *Chlorella*, rotifer, copepods and *Artemia* within the first 40 days (Watanabe and Kiron, 1995; Tomoda et al., 2005). Historically, some survival was obtained in sea bream *Sparus aurata* (Fernández-Díaz and Yúfera, 1997) and *P. major* (Takeuchi et al., 1998) fed compound diet from mouth opening. These reports highlighted the issue of digestibility of micro diets in *P. major* at various stages of larval development and how to enhance its digestive processes. As particle size is an important consideration, micro-diet particle sizes of 200 μm and 310 μm (for larvae at 10 days after hatching (DAH) and 25 DAH) is known to be acceptable for *P. major* larvae at weaning time (Shirota, 1978; Kanazawa et al., 1989; Fukuhara, 1991).

This study aims to evaluate the effect of micro-diets on the ingestion, digestion, energy metabolism and growth performance of *P. major* larvae during early ontogenetic development through enzyme activity and gene expression profiles of targeted genes as a proxy in the hopes of optimizing the use of micro-diet as a feeding strategy using *P. major* as a model species.

3.2. Materials and methods

3.2.1 Larval rearing

Red seabream fertilized eggs were obtained from Ogata Suisan Co. Ltd., Kumamoto Prefecture, Japan. Fertilized eggs were delivered to the Kamoike On-shore Laboratory, Aquaculture Research Centre at Kagoshima University, Kagoshima Prefecture, Japan.

Experiments were conducted in triplicates with 4 experimental treatments: (1) live feed only (*Brachionus plicatilis* and *Artemia*), (2) micro-diet only, (3) co-feeding (live feed and micro-diet), and (4) a starvation treatment. A total of 2,000 eggs were stocked in 100 L transparent composite tanks with continuous aeration and 35 psu of salinity. Temperature in the rearing system was maintained at 18 ºC using a titanium heater (Nittokizai Corporation, Saitama, Japan) to heat water in a 500 L reservoir tank, which was then continuously supplied to the larval rearing tanks. Rotifers were added into the rearing tanks at 3 days after hatching (DAH) upon larval mouth opening until 18 DAH. Rotifer density in the rearing tanks were maintained at 5 ind./mL. The larvae were alternatively fed *Atermia* sp (Great Salt Lake Artemia, Ogden, UT, USA) nauplii at 3 ind./mL of density at 15-21 DAH before switching to its adult *Artemia* stages from 21 DAH. The density of rotifer and *Artemia* in cultured tanks were checked twice a day at 08 am and 03 pm. At the onset of exogenous feeding (3 DAH), a water exchange rate of 300 %/day was applied to maintain water quality in the rearing tanks and this was achieved via siphoning through a drain hose, screened through a 0.2 mm polyethylene mesh (Khoa et al., 2019). *Chlorella vulgaris* also was supplied directly into the larval cultured tanks from 3 DAH after feeding at 1.2×10^{10} cells/mL to sustain green water condition.

3.2.1.1 Primary rotifer culture and nutritional enrichment

The euryhaline rotifer were cultured and enriched following a method described by Kotani et al. (2017) and Khoa et al. (2019). L-type rotifer *B. plicatilis* were stock cultured in 200 L polycarbonate tanks at 20 psu of salinity. The system was continuously aerated with temperature maintained at 25 ± 1 ºC by a heater. Normal *C. vulgaris* (Chlorella Industry Co. Ltd., Tokyo, Japan) was automatically supplied to the rotifer tanks at a ratio of 20×10^3 cells/rotifer/day (Khoa et al., 2019). After harvesting from the continuous culture, rotifers were transferred to 5 L beakers placed in a heated water bath (27 ± 1 °C) for enrichment. Rotifers were fed twice daily (08 am, 05 pm) for 24 h with Super Fresh Chlorella-V12 (Chlorella

Industry Co. Ltd.) enriched with 45 % docosahexaenoic acid (C22:6 *n*-3; DHA). The needed volume of *C. vulgaris* was calculated following the formula:

$$C.vulgaris\ (mL) = \frac{(\text{stocking density (ind./mL)} \times 0.46 \times \text{culture volume (mL)})}{150}$$

Taurine (Aqua-Plus ET, Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) was introduced to rotifers at 0.06 g/L of enriched volume. Taurine enrichment was only added once (at 05 pm), 15 h before feeding. After 24 h of enrichment, the batch cultured rotifers were harvested and fed to larvae.

3.2.1.2 Micro-diet

LOVE LARVAE micro-diet ($N_1 \leq 200$ µm and 200 µm $\leq N_2 \leq 310$ µm, Hayashikane Sangyo Co., Ltd, Shimonoseki, Yamaguchi, Japan), which contained 48 % crude protein and 10 % crude fat, was used in this study (Table 3.2). In the co-feeding treatment, the larvae were fed live feed from mouth opening whilst the micro-diet and live feed were alternatively introduced to the larvae from 10 DAH and switched to micro-diet only from 15 DAH. Following protocols outlined by Kanazawa et al. (1989) the micro-diet was introduced into the culture tanks every 1 h. After weaning time, larvae were fed every 3 h (08 am, 11 am, 02 pm, 05 pm)

3.2.1.3 Weaning observation

The weaning and feed intake observations were undertaken following Hamre et al. (2019). In this study, fish larvae from 10 DAH were stocked in clear 30 L tanks (30 larvae for each tank in triplicate) and split into two separate treatments according to the two trialed feeds (micro-diet and *Artemia*). Larvae fed with micro-diets were fed rotifer and micro-diet alternatively for 5 days (from 10-14 DAH) before switching to micro-diet only (15 DAH). The control treatment composed of larvae that were fed live feed only in which larvae were fed

rotifer (from 3-15 DAH), followed by a combination of rotifer and *Artemia* (from 16-18 DAH) and *Artemia* only (from 18-40 DAH). An LED flashlight was used to exam the larvae for gut fullness. Based on visual observations, findings were put into three categories, full gut, partially full gut and empty gut. The larvae were then collected and constituents of the gut observed using a VHX- S90F digital microscope (Keyence Corporation, Osaka, Japan) connected to an VH-Z20R ultra-small, high-performance zoom lens (Keyence Corporation). The gut contents were used to evaluate the feed intake of fish larvae during weaning time.

3.2.2 Fish sampling

Fish larvae were sampled at hatching (day 1), first feeding (day 3), 5, 10, 15, 20, 25, 35, and 40 DAH. *P. major* larvae were separately collected at 2 h after the first morning feeding in triplicates. For quantitative real time PCR, larvae were collected (5 larvae x triplicate x 9 sampling points for each treatment), rinsed with deionized water and preserved in *RNA*later® Stabilization Reagent following the protocol suggested by the supplier (QIAGEN GmbH, Germany). For the enzymatic activity assay, larvae were sampled (5 larvae x triplicate x 9 sampling points for each treatment) and rinsed with distilled water, then kept frozen at -80 ºC. Fresh larvae were sampled (15 larvae x 9 sampling points for each treatment) throughout the experimental period to measure body weight, total length.

3.2.3 Quantitative real time PCR (qRT-PCR) analysis

For total RNA isolation, the larval pool (5 fishes of each replicate) for each sampling time-point was homogenized in 1 mL of TRIzol™ reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) in triplicate following the manufacturer's protocol. The RNA integrity was verified with 1 % agarose gel electrophoresis. The quality and concentration of RNA were determined by optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). The samples an absorption ratio ranged

between 1.9 to 2.0 was used for cDNA synthesis (100 ng of RNA) following the iScript ™ cDNA Synthesis protocol (Bio-Rad Inc., Hercules, CA, USA).

The expression levels of relative genes of digestive enzymes (*try, ctrb, pep, pl* and *amy2α*), Cholecystokinin (*cck*), growth (*igf1*), and energy metabolism (Cytochrome-C-Oxidase (*cox1*), Beta-F1-ATPase (*atp*)) were obtained by qRT-PCR. The primers used were shown in Table 3.1. In a 20 µL reaction, 1 µL of cDNA template, 0.4 µL of each primer, 10 µL of qPCR Mix, and 0.4 µL ROX reference dye (KOD SYBR® qPCR Mix, Toyobo Co. Ltd, Osaka, Japan) were used. The qRT-PCR was performed in triplicate using the Applied Biosystems StepOne ™ (Thermo Fisher Scientific Inc., USA). The thermal profile was 98 °C for 2 min; followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. The melting curve was generated from 60 °C to 99 °C with an increment of 0.3 °C and holding for 15 s. The β-actin expression levels among treatments were stable during the larval stages and its average expression was applied as a normalization factor. After standardization by β-actin, the mRNA levels were normalized and measured using the standard curve with diluted cDNAs (Khoa et al., 2019).

3.2.4 Enzyme assay

For enzyme extraction, pool larvae were homogenized in triplicate, each containing 5 larvae and 100 µL of ice cold buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5) (Bolasina et al., 2006; Khoa et al., 2019), using pellet pestle cordless motor (Sigma-Aldrich Inc., St. Louis, MO, USA). The pestle tip was rinsed with 400 µL of homogenization buffer, the tubes, containing 500 µL homogenate, were then centrifuged for 30 min at 1700 x g at 4 °C. The supernatant transferred into new 1.5 mL tubes and was used as the crude enzyme extract in protein content and enzymatic assay analysis later (stored at -80 ºC). Protein content was determined using the Bradford method (Bradford, 1976).

3.2.4.1 Trypsin

The Z-$_L$-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA, Sigma-Aldrich Inc., C9521) was used as the substrate to performed the fluorometric assay for trypsin activity according to Bolasina et al. (2006) and Khoa et al. (2019). Briefly, the reaction mixture included 500 µL of substrate (containing 50 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ-$_L$Arg-MCA) and 50 µL of crude enzyme was incubated for 30 min at 30 °C in a water bath, followed by adding 100 µL of 30% acetic acid for quenching. Blanks were similarly prepared but without adding acetic acid. Fluorescence measurements were conducted in a Hitachi F2000 spectrofluorophotometer (Tokyo, Japan). The samples were excited at 380 nm and emission was at 440 nm. Activity of trypsin was expressed in unit (mU) in 30 min, as percentage increase of emission per protein (mU mg protein^{-1}).

3.2.4.2 Chymotrypsin

The activity of chymotrypsin was measured according to Murashita et al. (2018) using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPFNA, Sigma-Aldrich Inc.) as a substrate. A 50 µL of crude enzyme was added to 240 µL of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$) and 100 µL 2.4 mM SAPFNA, then incubated for 7 min at 37 ºC. Production of *p*NA was measured at 405 nm using a Hitachi U5100 spectrophotometer. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in 1 min.

3.2.4.3 Pepsin

Pepsin activity was assayed according to Worthington (1993) and Natalia et al. (2004). Reaction was initiated by adding 100 µL of enzyme extract to 500 µL of substrate (2 % haemoglobin in 0.06 N HCl), then was incubated at 37 ºC (pH 2.5). After 10 minutes, the reaction was terminated by adding 1 mL of 5 % trichloroacetic acid and left for 5 min.

Following the centrifugation for 5 min at 12000 x g, the absorbance was read at 280 nm using a Hitachi U5100 spectrophotometer. For blank reading, the enzyme extract was added after trichloroacetic acid.

3.2.4.4 Lipase

Activity of neutral lipase was determined using 4-methylumbelliferyl butyrate (4 MUB) as a non-fluorescent substrate according to Roberts (1985) and Bolasina et al. (2006). Briefly, the reaction mixture was prepared by adding 20 µL of enzyme extract to 60 µL of substrate solution (consisting 0.5 mM 4 MUB, 5 mM egg lecithin, 10 mM sodium taurocholate and 150 mM NaCl) and 20 µL of Tris buffer (pH 7.5). Each assay was conducted simultaneously at both 4 ºC in an ice bath and at 37 ºC in a water bath for 10 min. The reaction was stopped by adding 3 mL of Tris buffer (4 ºC, 1 M, pH 7.5). The fluorescence measurements were conducted by Hitachi F2000 spectrofluorophotometer, in which the samples were excited at 380 nm and emission setting was at 450 nm, subtracted the reading of the 4 ºC incubated samples against the 37 ºC control samples for each assay. Fluorometer constants were calculated from standard stock solutions of 4-methylumbelliferone.

3.2.4.5 Amylase

Amylase activity was evaluated according to Murashita et al. (2018). A 1 % starch solution was used as a substrate. Briefly, a 50 µL of enzyme extract was added into 25 µL of 20 mM sodium phosphate buffer (containing 6.0 mM NaCl, pH 6.9) and 25 µL of the substrate solution, then the mixture was incubated by at 37 ºC at 60 min. Following the addition of 50 µL dinitrosalicylic acid reagent (1 % dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH) and incubation for 5 min in boiling water. Absorbance at 540 nm was read using a Hitachi U5100 spectrophotometer. The amount of maltose released from this assay was estimated from standard curve.

Table 3. 1 The primer sequences using for real time PCR

Gene	Primer forward	Primer reverse	Source	Efficiency (%)
β-actin	GGCACTGCTGCCTCCTC	GCCAGGATGGAGCCTCC	Kondo et al., 2017	98.3
try	TCAGGTGTCTCTGAACTCTG	ACCTGAACACGAGACTTG		97.1
pep	TGATCAAGGGAAAGACTGC	GTCAGCATCGTTTGTCATG		94.3
ctrb	ACGGCGCCTGGACTCTGGTC	TTGGCGGCGAGGATCTGGTC	Murashita et al., 2018	98.5
amy2α	GCATGCCCGGTGGCACCTAC	GCCATCACCGCCAACATGGA		95.3
pl	GCCACGATGCTGACAGGTTCACT	GCCACGATGCTGACAGGTTCACT		96.8
cox1	TGACTACTCCCCCCATCTTTCCTTC	GGGAGGATAAGAATGTAAACTTCTGG	Itoi et al., 2007	97.9
atp	TGTCTGCCCTGCTGGGTCGTATC	GTGGTGGCGTACAAGTGAGC		98.4
igf1	ACAGAATGTAGGGACGGAGCGAATGGAC	TTCGGACCATTGTTAGCCTCCTCTCTG	Aziri et al., 2016	98.1
cck	TCATCTCGTCCAGGAAAG	TTAGTCCCTGTCTGCTATCC	Kondo et al., 2017	97.5

In which: ***β-actin*** (AB252854); ***try***: trypsinogen (AB678427); ***pep***: pepsinogen (AB678431); ***ctrb***: chymotrypsinogen (AB678429); ***amy2α***: amylase (AB678421); ***pl***: pancreatic lipase (AB252856); ***cox1***: cytochrome-C-oxidase (AP002949); ***atp***: Beta-F1-ATPase (AB259835); ***igf1***: insulin-like growth factor 1 (EF688016); ***cck***: cholecystokinin (LC155429)

Table 3. 2 The nutritional composition of diets used for red seabream larvae.

	Enriched rotifer	*Artemia*	Microdiet (*)
Crude protein (%)	49.3	50.8	48
Crude lipid (%)	16.2	10.7	10
Carbohydrate (%)	14.2	14.1	-

(*): Hayashikane Sangyo Co Ltd, Shimonoseki, Yamaguchi Prefecture, Japan

3.2.5 Data analysis

Data were presented as a mean ± SEM (standard error of the mean) of triplicate. The results were subjected to one-way ANOVA (SPSS 24.0 for Windows, IBM, Armonk, NY, USA) applied Tukey's HSD (Honestly Significant Difference) test. All the differences were considered at α=0.05.

3.3 Results

3.3.1 Survival rate of fish larvae and growth performance

The survival rate was observed to reduce gradually in all treatments, with significant difference recorded between 10-15 DAH and 20-30 DAH. The highest survival rate was recorded in the live feed only treatment (30.5 ± 3.2 %), however, this was not significantly higher ($p < 0.05$) than the combined diet treatment (28.0 ± 1.2 %). Starved larvae survived until 10 DAH without being fed while those fed micro-diet only survived until 15 DAH (Fig. 3.1).

From the onset of exogenous feeding until 5 DAH, the starved larvae treatment and the micro-diet only treatment showed significantly lower ($p < 0.05$) total length (TL) (2.4 ± 0.3

mm for both treatments) compared to the live feed only treatment (3.1 ± 0.2 mm). The micro-diet could maintain the larvae until 15 DAH however with a lower growth performance. The live feed treatment and co-feeding treatment reached a significantly higher TL ($p < 0.05$) until the end of experimental period (Fig. 3.2). Fish larvae showed a sharp and significant increase in TL from 30 DAH and by 40 DAH the average size of larvae in both the live feed and co-feeding treatments were similar (11.4 ± 0.4 mm and 11.5 ± 0.2 mm) (Fig. 3.2).

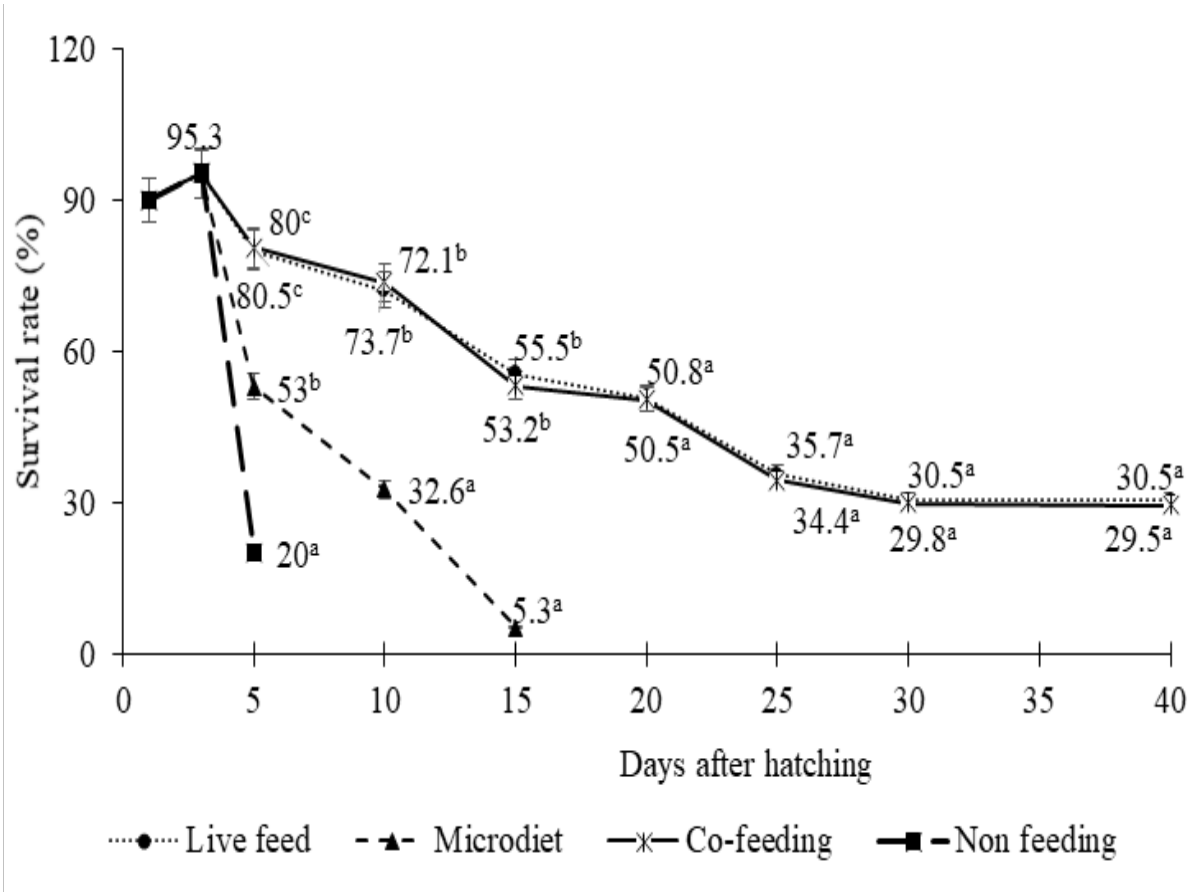


Figure 3. 1 The survival rates of red sea bream larvae among treatments during the ontogenetic development. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of survival rate among treatments at particular stages. (ANOVA, Tukey's HDS test, a < b< c, $p < 0.05$).

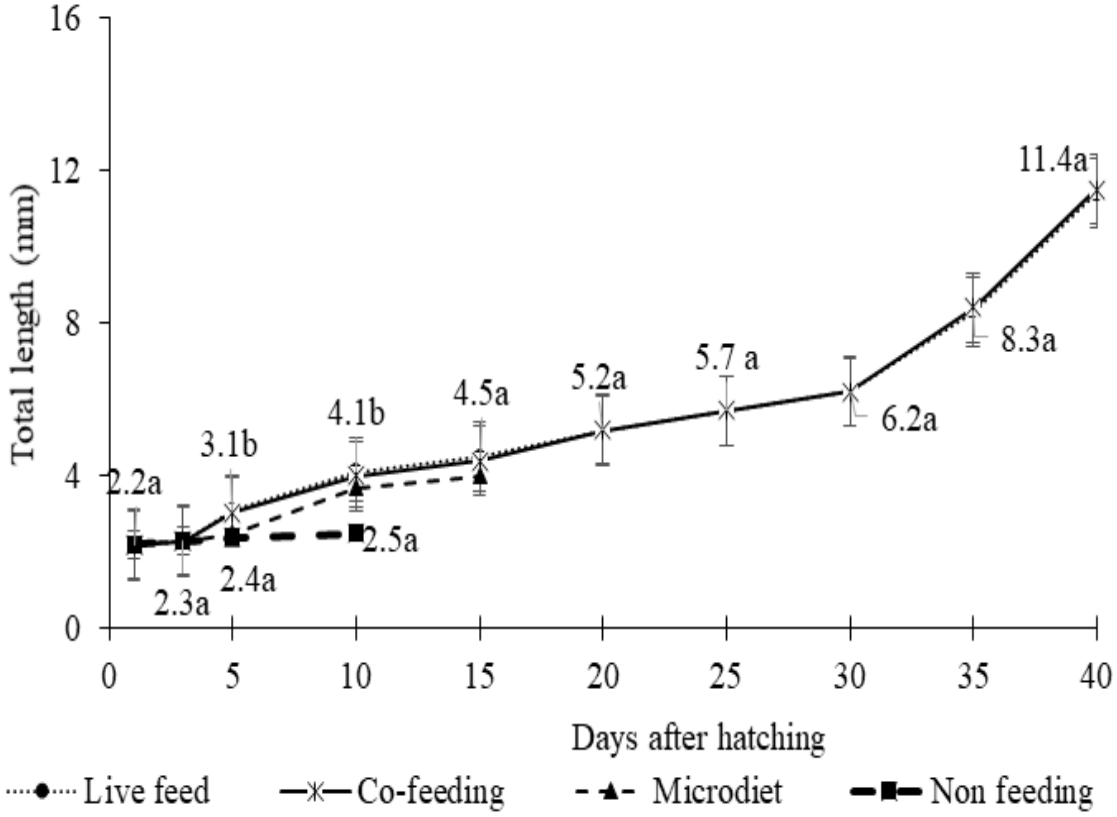


Figure 3. 2 The growth performance in total length of red sea bream larvae among treatments during the ontogenetic development. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of growth performance in total length among treatments at particular stages. (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

Table 3. 3 Gut fullness and contents in red seabream larvae fed live feed and microdiet during transition period and weaning time to other feed. The experiment was conducted in triplicate and lasted for 10 days. The data was presented in mean ± SD of full, partly full and empty gut of larvae. The grey background indicated the transition time from live feed (rotifer) onto microdiet/or Artemia.

Co-feeding (n=30 larvae/tank x triplicate = 90)						Live feed (n=30 larvae/tank x triplicate = 90)			
Days of experiment	**Full**	**Partly full**	**Empty**	**Number of prey (rotifer)**	**Microdiet**	**Full**	**Partly full**	**Number of prey (rotifer)**	***Artemia***
1	30 ± 0	0		5.3 ± 2.4	+	30 ± 0	0	6.3 ± 1.2	
2	30 ± 0	0		6.6 ± 1.3	+	30 ± 0	0	7.2 ± 1.5	
3	30 ± 0	0		6.3 ± 1.1	++	30 ± 0	0	8.5 ± 2.5	
4	30 ± 0	0		6.3 ± 1.6	++	30 ± 0	0	8.2 ± 1.5	
5	30 ± 0	0		7.1 ± 2.4	+++	30 ± 0	0	5.3 ± 1.3	2.3 ± 1.3
6	4.7 ± 0.6	14.0 ± 1.0	11.3 ± 1.5			30 ± 0	0	4.3 ± 1.1	2.5 ± 1.1
7	6.3 ± 0.6	17.7 ± 1.2	6.0 ± 1.0			30 ± 0	0	3.8 ± 1.5	3.5 ± 1.5
8*	11.3 ± 1.5	15.3 ± 1.2	0			28 ± 0	2 ± 0		4.1 ± 1.1
9*	19.3 ± 0.6	5.3 ± 0.6	0			28 ± 0	1 ± 0		4.6 ± 1.5
10*	23.3 ± 0.6	0	0			28 ± 0			5.3 ± 1.7

(*): The number of larvae was less due to the mortality

- Co-feeding: Day 1 – Day 7 (n=90), Day 8 (n = 80), Day 9 (n = 74), Day 10 (n = 70)

- Live feed: Day 1 – Day 8 (n = 90), Day 9 (n = 87), Day 10 (n = 84)

3.3.2 Weaning observation

During the transition and weaning onto the new feed, *P. major* larvae adapted to *Artemia* better than micro-diet. For the first 2 days of weaning, 93 % of the larvae were recorded with a full gut of *Artemia* compared to only 13-20 % of larvae with full guts in the micro-diet treatment. 46 - 60% larvae were observed with a partly full gut and 20 – 40% larvae were empty gut in larvae fed micro-diet for the first 2 days. From the third day of weaning, all larvae fed *Artemia* was observed with full gut, while larvae took a longer time (5 days) to reach 100 % larvae full gut with micro-diet only treatment. It was observed that generally, *P. major* larvae required approximately 5 days for transition period when changing its diet from live feed to inert feed (Table 3.3). After the weaning period, the mortality of larvae weaned onto *Atermia* was only 6.7 % and significantly lower than the micro-diet treatment had (22.2 ± 1.9 %).

3.3.3 Activity and expression of digestive enzymes

3.3.3.1 Trypsin

Low levels of trypsin activity (106.1 ± 10.5 mU mg protein^{-1}) and expression were detected at hatching (Fig. 3.3). At the onset of exogenous feeding at 3 DAH until 10 DAH, trypsin activity was observed to be similar in the starvation treatment and micro-diet treatment. In contrast, the live feed treatment and co-feeding treatment showed a significantly higher activity that peaked at 5 DAH ($p < 0.05$, 192.4 ± 23.4 mU mg protein^{-1} and 193.4 ± 32.5 mU mg protein^{-1}) followed by a gradual reduction until 20 DAH in the live feed only treatment. From 10 to 20 DAH, the surviving co-feeding larvae remained and displayed enhanced trypsin activity until 35 DAH. During this period, considerable difference between co-feeding and live feed treatment was recorded between 20-25 DAH ($p < 0.05$). After 25 DAH, trypsin activity in the live feed treatment and co-feeding treatment sharply increased, however the co-feeding

treatment achieved significantly higher tryptic activity by 40 DAH ($p < 0.05$, 701.9 ± 40.3 mU mg protein^{-1}; Fig. 3.3A).

Similar patterns were observed in trypsin expression (Fig. 3.3B) with the exception between 5-10 DAH, where *try* (trypsin) expression in the micro-diet treatment was significant higher than the live feed treatment ($p < 0.05$). The micro-diet treatment showed a complex fluctuation of *try* expression with two peaks at 5 DAH and 15 DAH with a dramatic drop in expression at 10 DAH. Starved larvae were observed to have a significant increase in *try* expression at 10 DAH prior to all larvae dying. Remarkably, the *try* expression in both live feed only and co-feeding treatments dramatically reduced from 20 DAH to 25 DAH, before fluctuating in similar fashion until 40 DAH.

3.3.3.2 Chymotrypsin

The activity of chymotrypsin (Fig. 3.4) in all treatments was low at hatching (0.54 ± 0.01 mU mg protein^{-1}) and at mouth opening (3 DAH, 0.56 ± 0.02 mU mg protein^{-1}), and then gradually increased until 5 DAH (ranged from 0.53 to 0.57 mU mg protein^{-1}). At 10 DAH, the live feed only treatment was observed significantly increase ($p < 0.05$) and it had a higher chymotrypsin activity compared to the other treatments at 20 DAH (8.4 ± 0.5 mU mg protein^{-1}). In this treatment, chymotrypsin activity increased gradually until 35 DAH (24.8 ± 1.1 mU mg protein^{-1}) before a dramatic decrease at 40 DAH in the live feed treatment (6.5 ± 0.7 mU mg protein^{-1}). Compared to the other treatments, larvae fed co-feeding displayed stable chymotrypsin activity during the duration of the experimental period (Fig. 3.4A).

The gene expression of *ctrb* was not significantly correlated (R^2 = 0.0385 - 0.3386) to its enzymatic activity, especially within the first 5 days after hatching. Larvae fed live feed only showed a peak of *ctrb* expression at 3 DAH, and the expression subsequently remained at high levels until 35 DAH. On the other hand, the co-feeding treatment showed a low and stable

expression level until 35 DAH before dramatically dropping at 40 DAH (Fig. 3.4B). It was observed that the *ctrb* expression level in the live feed only treatment was significantly higher compared to the micro-diet only treatment ($p < 0.05$).

3.3.3.3 Pepsin

The *pep* expression and enzymatic activity were only detected after 15 DAH (ranged between 36.3 - 51.1 mU mg protein^{-1} among the treatments) and gradually increased over the experimental period (Fig. 3.5). From close to zero at 15-20 DAH, the pepsin activity sharply increased from 20 DAH and peaked at 40 DAH. The co-feeding treatment (1735.4 ± 360.5 mU mg protein^{-1}) showed a significantly higher pepsin activity compared to the live feed only treatment ($p < 0.05$, 1244.2 ± 145.4 mU mg protein^{-1}; Fig. 3.5A).

With a significant correlation with pepsin enzymatic activity ($R^2 = 0.7374 – 0.9063$), the expression of *pep* was observed to have similar patterns of fluctuation as its enzymatic activity. There were no significant differences in *pep* expression observed within the first 35 days between live feed only and co-feeding treatments. However, the co-feeding treatment reached a significantly higher level at 40 DAH ($p < 0.05$; Fig. 3.5B).

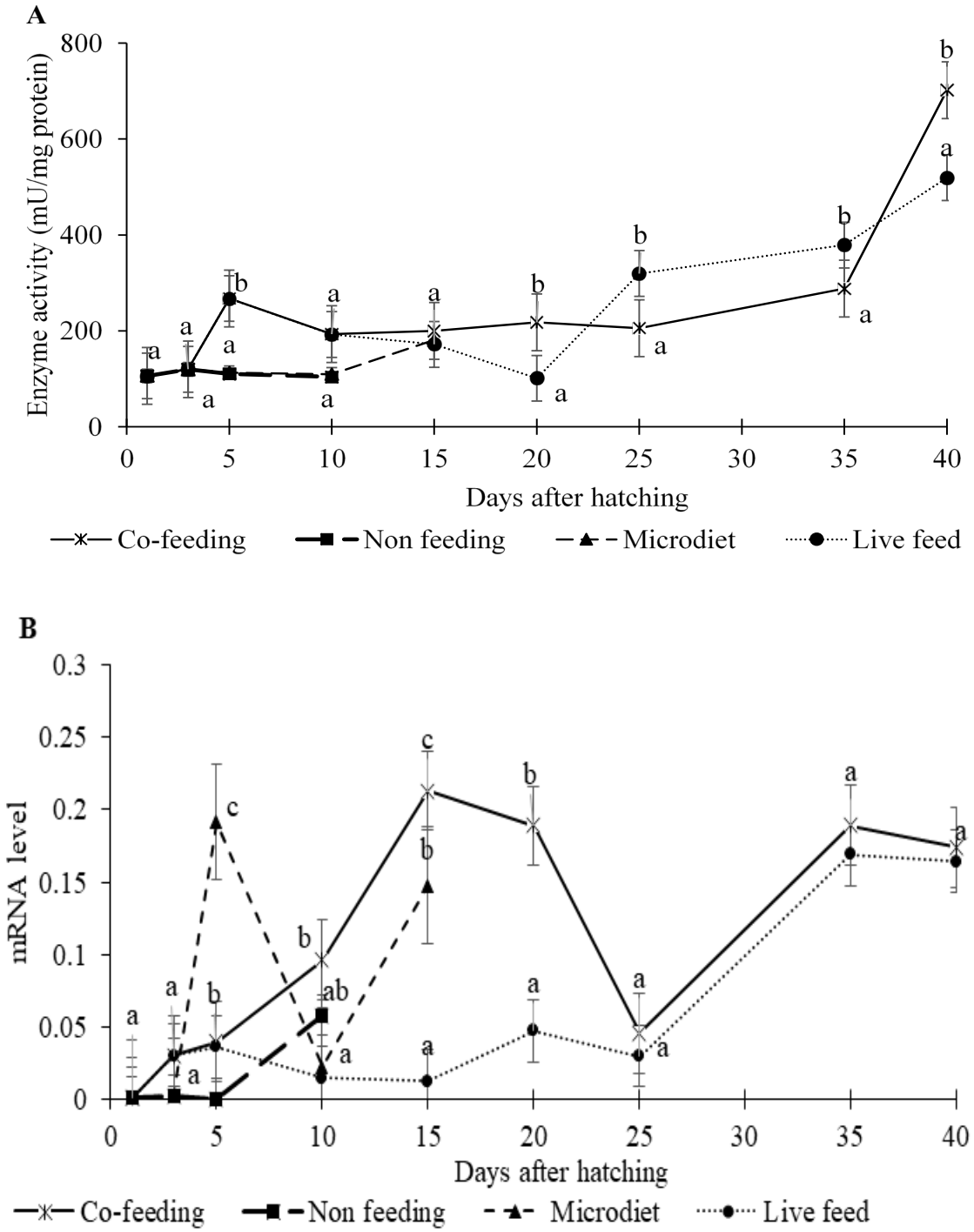


Figure 3. 3 Trypsin activity (A) and *try* expression (B) during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of trypsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

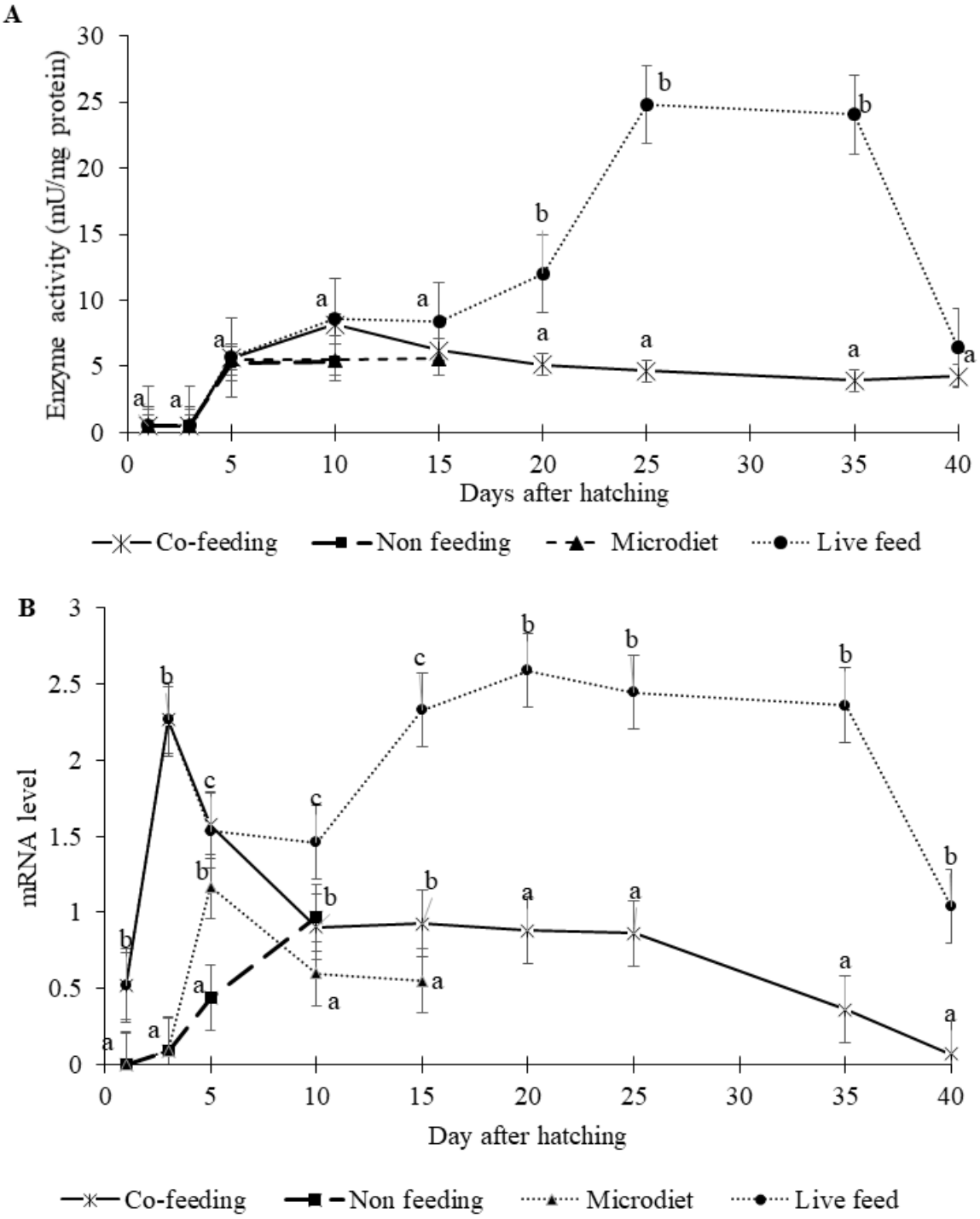


Figure 3. 4 Chymotrypsin activity (A) and *ctrb* expression (B) during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of chymotrypsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

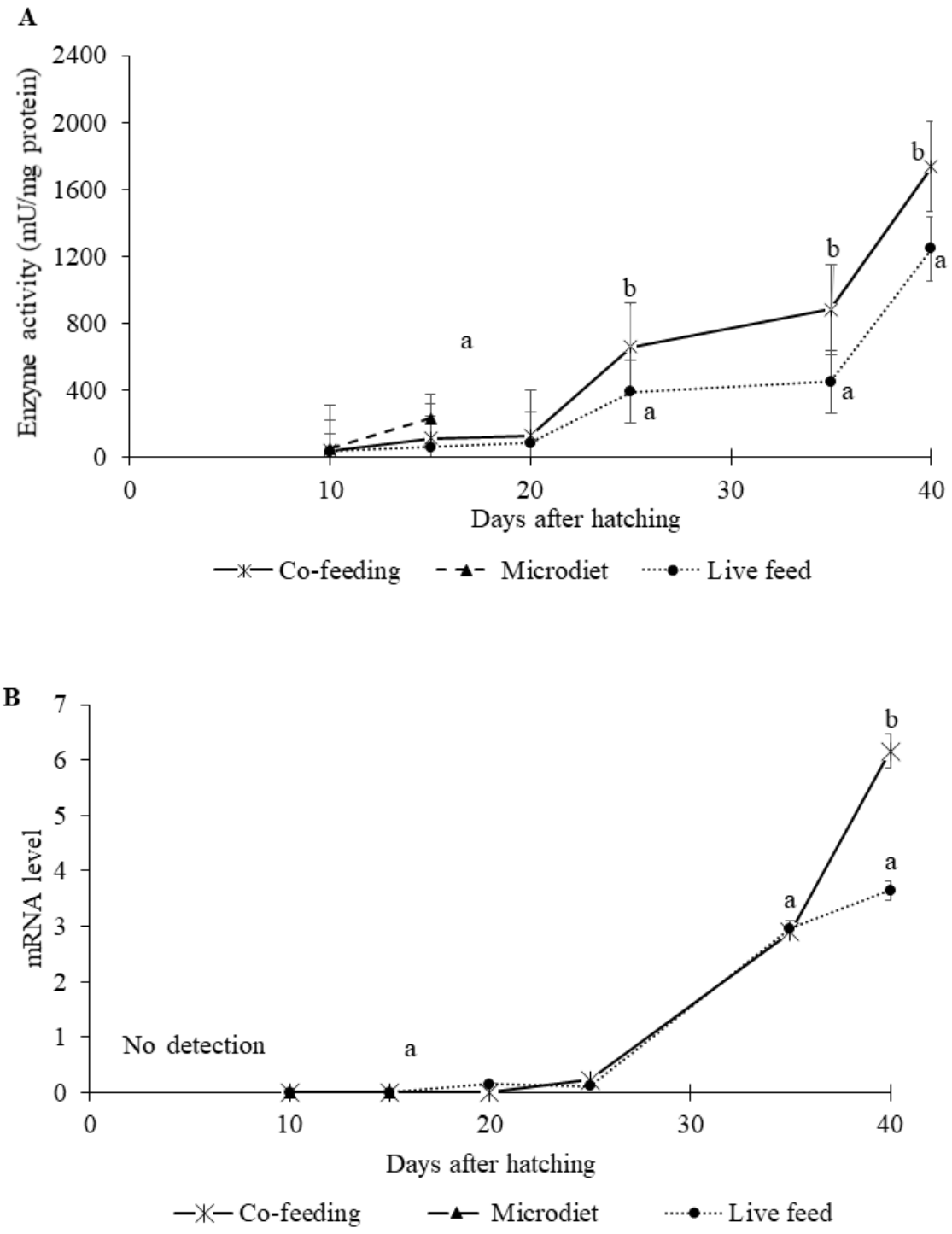


Figure 3. 5 Pepsin activity (A) and *pep* expression (B) during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of pepsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b, $p < 0.05$).

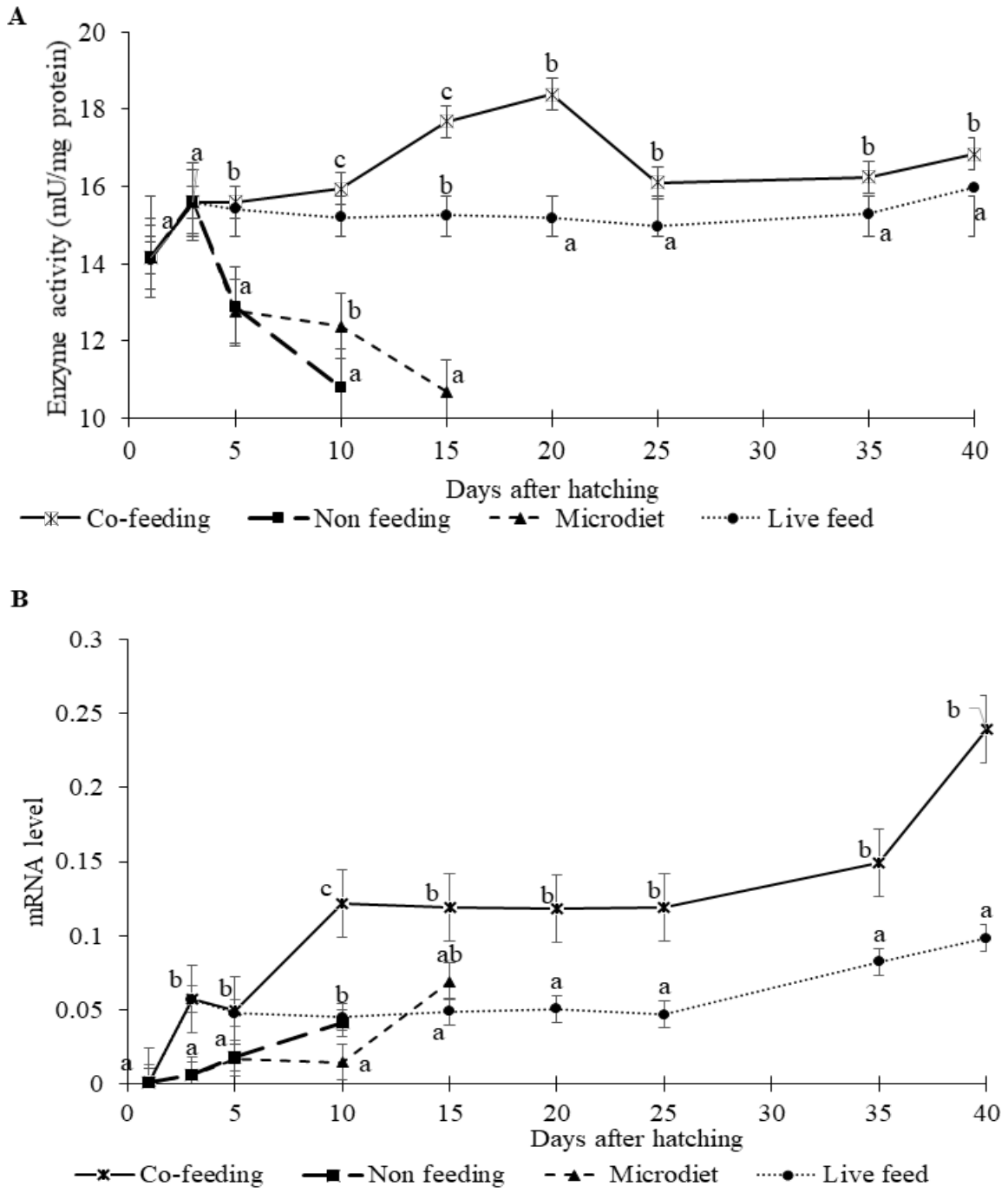


Figure 3. 6 Neutral lipase activity (A) and *pl* expression (B) during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of neutral lipase activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

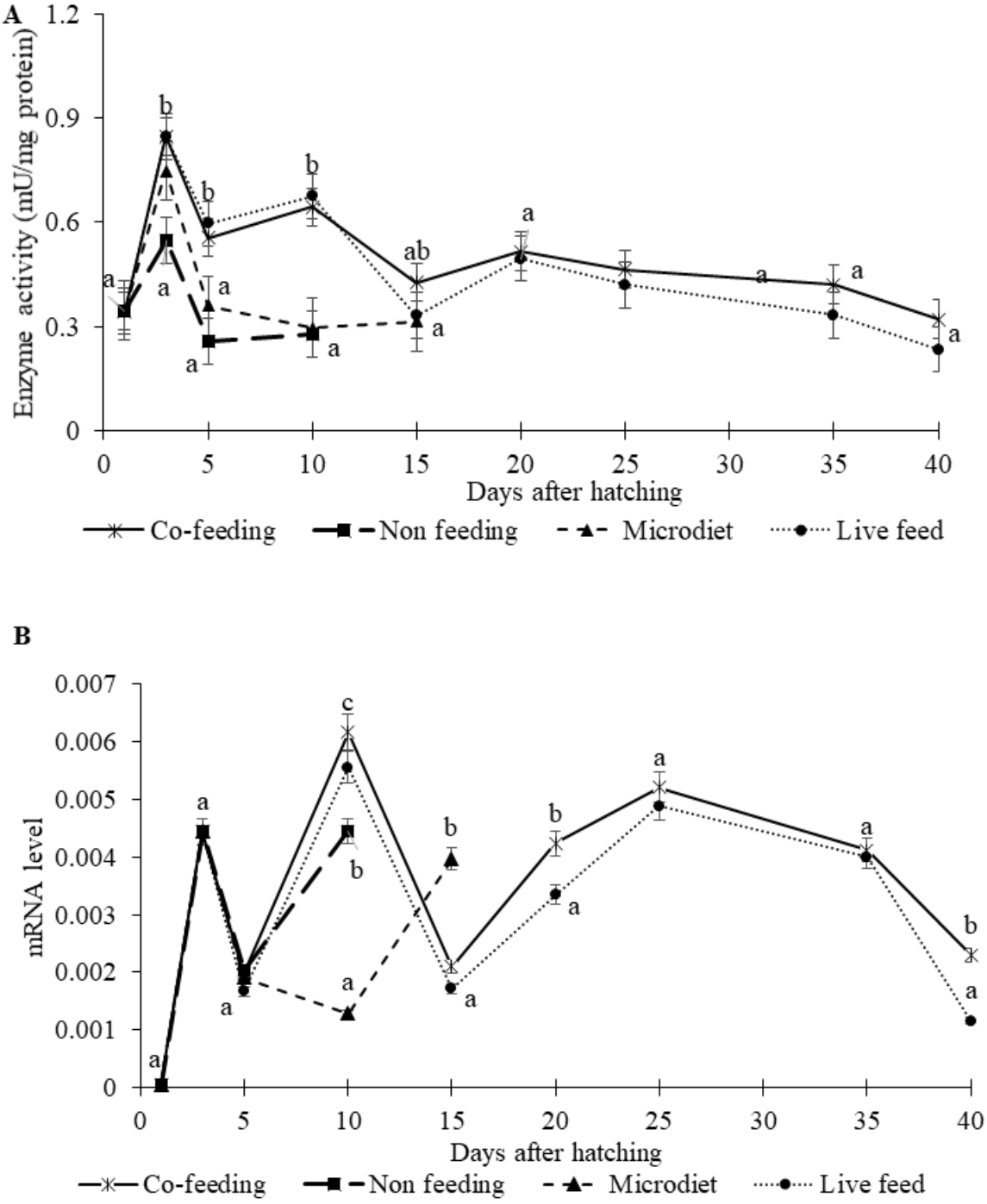


Figure 3. 7 Amylase activity (A) and *amy2α* expression (B) during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of amylase activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

3.3.3.4 Lipase

At mouth opening (3 DAH), neutral lipase activity in live feed treatment and co-feeding treatment gradually increased whilst a dramatic drop in activity was observed in the micro-diet only treatment and non-feeding treatment (Fig. 3.6). The co-feeding treatment (14.2 – 18.4 mU mg protein^{-1}) and live feed only treatment (14.1 – 16.0 mU mg protein^{-1}) displayed a significantly higher activity compared to the micro-diet only and starvation treatment ($p < 0.05$, 14.1 – 10.7 mU mg protein^{-1}, 14.1 – 10.8 mU mg protein^{-1}, respectively). The highest activity of neutral lipase was recorded in the co-feeding treatment at 20 DAH and in the live feed treatment at 40 DAH (Fig. 3.6A; 18.4 ± 1. mU mg protein^{-1}, 16.8 ± 1.6 mU mg protein^{-1}, respectively).

In contrast, the expression of *pl* gradually increased in all treatments (Fig. 3.6B). The larvae in the starvation treatment and micro-diet treatment had very low levels of expression that slightly increased at 10 DAH and 15 DAH. On the other hand, live feed treatment was observed to have stable expression levels until 25 DAH before a dramatic increase peaking at 40 DAH. The highest expression level and significant difference compared to other treatments ($p < 0.05$) was observed in the co-feeding treatment at 10 DAH after which its expression levels remained relatively constant until 25 DAH before sharply increasing and peaking at 40 DAH.

3.3.3.5 Amylase

Similar to the other enzymes investigated in this study, the gene expression (*amy2α*) and activity of amylase were detectable at hatching (Fig. 3.7). Amylase showed a decreasing trend throughout the experimental period. Amylase activity was low level at hatching (0.3 - 0.35 mU mg protein^{-1} among treatments), peaking at 3 DAH (0.554± 0.003 mU mg protein^{-1} in co-feeding treatment and 0.595 ± 0.04 mU mg protein^{-1} in live feed treatment). Amylase activity continued to decrease with larval age. Larvae fed live feed only and co-feeding

treatment maintained a significantly higher level of activity until 35 DAH ($p < 0.05$). Three peaks in activity were observed (3, 10 and 20 DAH) with fluctuations between each peak with a generally decreasing trend over the experimental period (Fig. 3.7A).

Similar to the enzymatic activity, the expression of *amy2α* tended to decrease with larval age after mouth opening at 3 DAH (Fig. 3.7B). Nevertheless, the expression of *amy2α* in the micro-diet only treatment displayed statistically higher expression ($p < 0.05$) with the exception of a dramatic drop observed at 15 DAH. Notably, the starvation treatment and micro-diet treatment displayed a sharp increase of *amy2α* expression immediately prior to mass mortality at 10 DAH and 15 DAH.

3.3.4 Energy metabolism, appetite regulator and growth expression

Cox1 expression levels in all treatments showed a moderated increase within the first 5 days after hatching. Larvae in the starvation treatment displayed consistent decrease in expression levels until all fish died at 10 DAH. Larvae fed micro-diet (including co-feeding treatment) showed constant expression from 10-25 DAH. As the micro-diet treatment ended at 15 DAH, a stable expression level was recorded in co-feeding treatment until 25 DAH, whilst the live feed treatment peaked at 15 DAH (0.123 ± 0.003) and 25 DAH (0.060 ± 0.001). *cox1* expression dramatically decreased at 35 DAH in both live feed only and co-feeding treatment with significantly higher level of expression ($p < 0.05$) in the co-feeding treatment (0.043 ± 0.002) by the end of the experiment (Fig. 3.8).

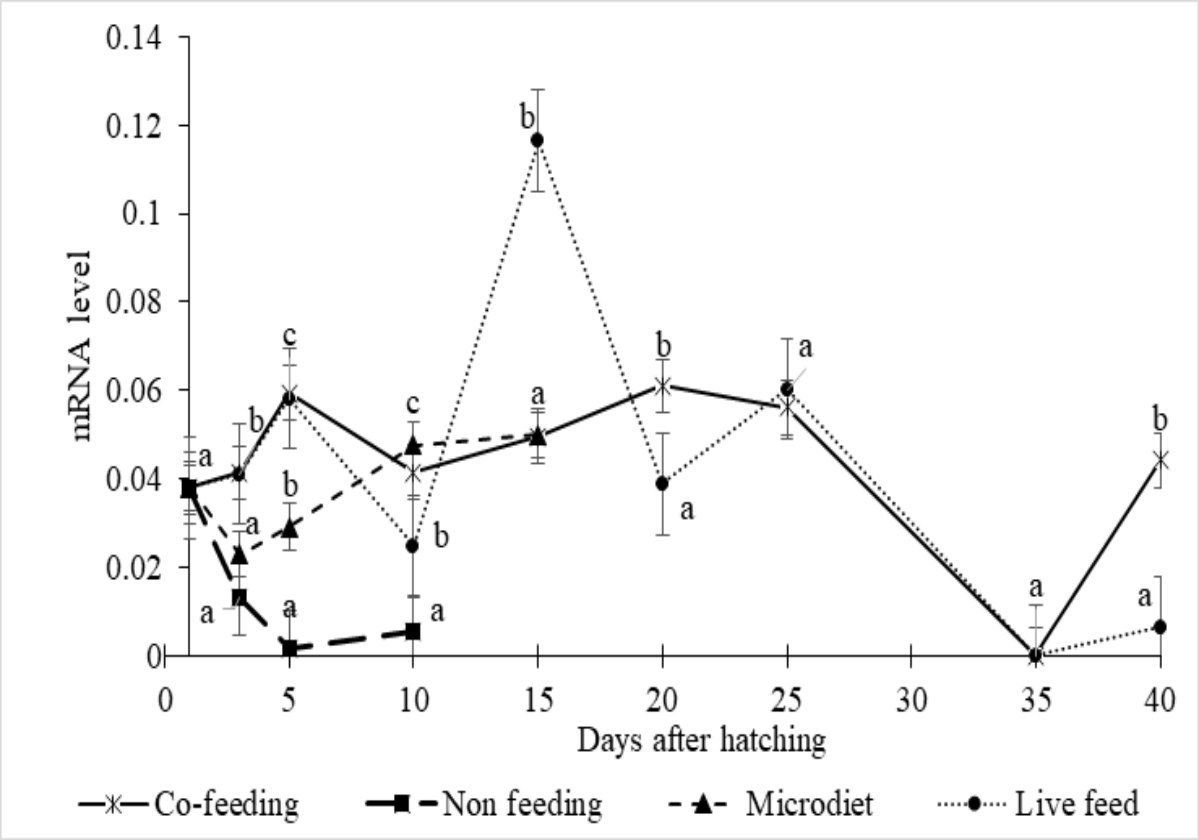


Figure 3. 8 *cox1* expression during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate Microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of cox1 expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

The *atp* expression levels displayed similar patterns with *cox1* (Fig. 3.9). The co-feeding treatment showed a stable expression of *atp* until 25 DAH, before bottoming out at 35 DAH and recovering at 40 DAH. *atp* expression in larvae fed live feed only were observed to fluctuate throughout the study with major peaks at 5, 15 and 25 DAH. Thereafter the expression decreased critically over the experimental period (reduced 99 % at 35 DAH and 86.2 % at 40 DAH). The expression of *atp* in the micro-diet treatment stabilized until 15 DAH, however it failed to maintain the fish survival rate (Fig. 3.9). However, the expression of *cox1* and *atp* in the micro-diet treatment was significantly higher than in the starvation treatment ($p < 0.05$).

Larvae fed live feed only or the combined diet from 5 DAH did not show any significant change in expression of *cox1* and *atp* gene (Fig. 3.8 and Fig. 3.9).

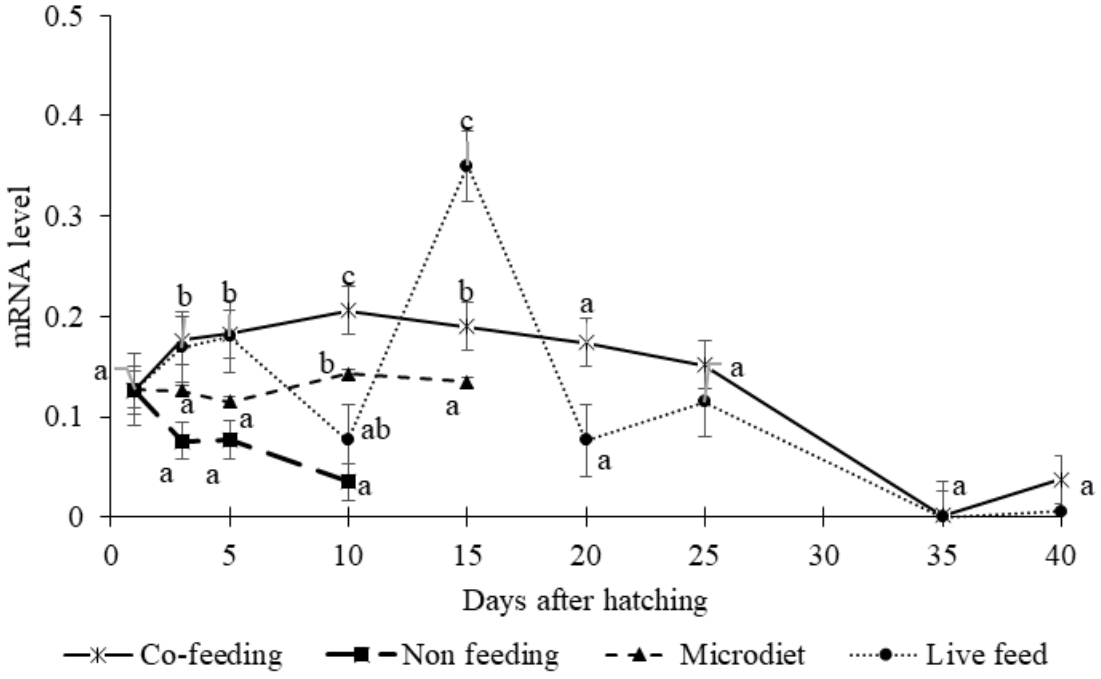


Figure 3. 9 *atp* expression during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of atp expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

The *cck* expression was low in the first 3 days after hatching (ranged from 0.14 to 0.15), before sharply increasing in all treatments (Fig. 3.10). However, the live feed only treatment and co-feeding treatment continuously increased and peaked at 10 DAH (0.28 ± 0.01 and 0.25 ± 0.01). The starvation treatment and micro-diet treatment showed dramatic drops in *cck* expression until all larvae died at 10 and 15 DAH. After 10 DAH, *cck* expression in the co-feeding treatment gradually decreased and stabilized at (0.14 - 0.18) from 20 - 40 DAH. In contrast, larvae fed live feed only displayed a strong fluctuation in *cck* expression, with a

dramatic decline from 15 DAH (0.25 ± 0.02) before recovering in expression levels at 25 DAH (0.06 ± 0.05) until the end of the experimental period with 0.10 ± 0.02 at 40 DAH (Fig 3.10).

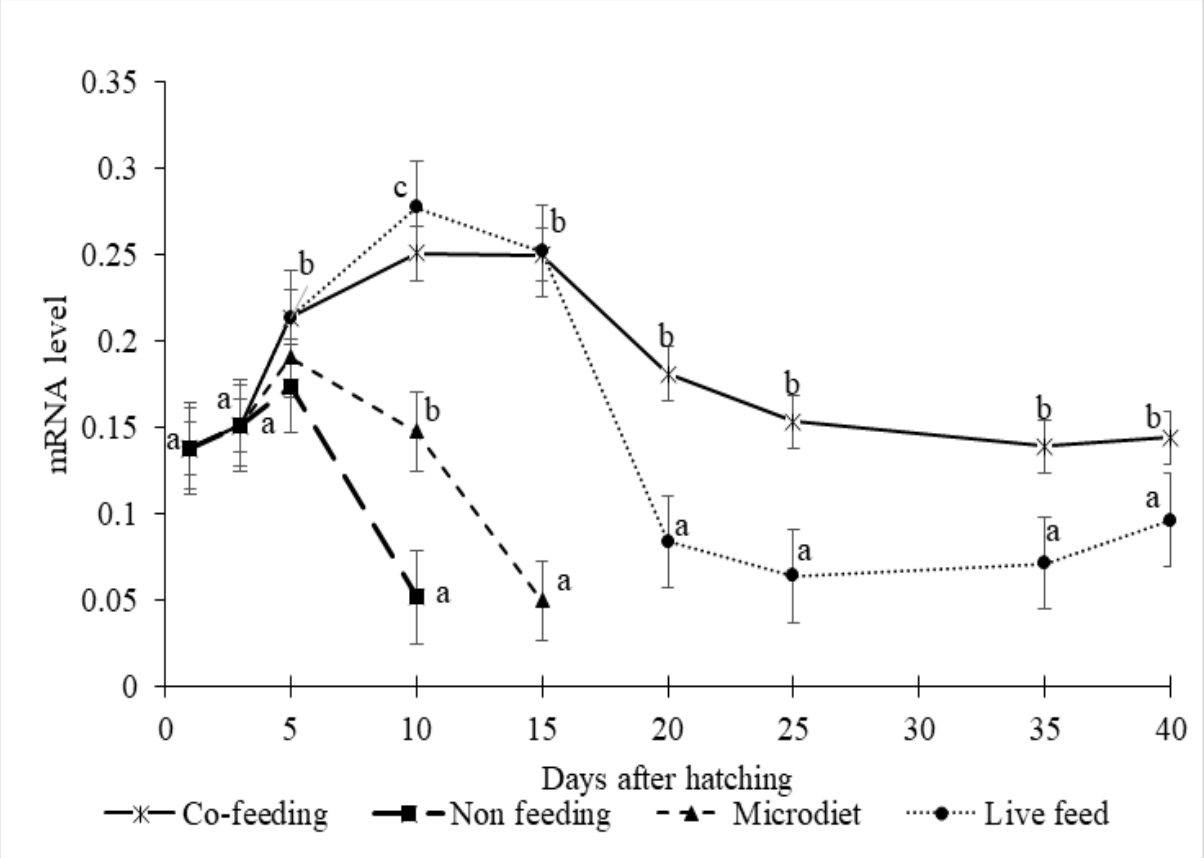


Figure 3. 10 *cck* expression during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of cck expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

The expression of *igf1* gene in all treatments drastically increased from hatching (0.0049 ± 0.0003) until the larvae were able to start exogenous feeding at 3 DAH (0.0354 ± 0.002), The starvation treatment and micro-diet only treatment then returned to initial expression levels observed at hatching (0.002 - 0.004) (Fig. 3.11) and remained low until 10 DAH and 15 DAH when all the larvae in both treatments died. On the other hand, the live feed only treatment and co-feeding treatment showed a stable and significantly higher expression

from 5-10 DAH ($p < 0.05$). Over the next 20 days *igf1* gene expression level in both the live feed only treatment and co-feeding treatment sharply increased and reached a peak (0.121 ± 0.003) at 35 DAH. However, *igf1* expression were observed to decrease in both treatments (live feed and co-feeding) by 40 DAH with no significant differences recorded between live feed only treatment and the co-feeding treatment.

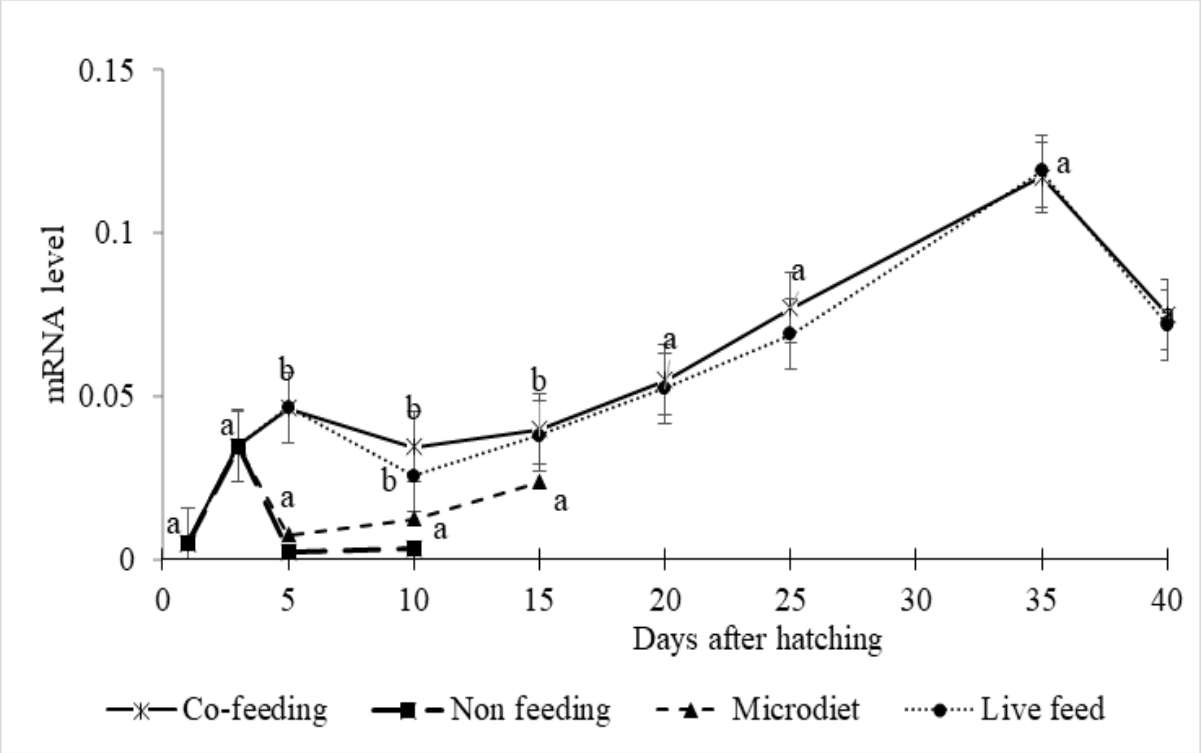


Figure 3. 11 *igf1* expression during larval development under different diets. The data was presented as mean ± SEM. Dashed line and circle plot indicate live feed. Dashed line and triangle plot indicate microdiet. Straight line and asterisk plot indicate co-feeding. Bold - dashed line and square plot indicate non-feeding. Alphabetical letters indicate significant difference of igf1 expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, p < 0.05).

4. Discussion

Fish larvae nutrition is species specific and fluctuates at different stages across ontogenetic development due to changes in morphological and physiological characteristics (Holt et al., 2011; Politis et al., 2018). As such, different fish species are equipped with various feeding structures and digestive mechanisms to hydrolyze and absorb essential nutrients suited for their appropriate development (Moyle and Cech, 2000). Most larval marine finfish possess a rudimentary digestive system at hatching, in which digestive enzymes are genetically initiated and activated before the transition from the yolk-based endogenous nutrition to exogenous feeding (Micale et al., 2006; Moguel-Hernández et al., 2016). As a result of having an undeveloped digestive system, marine finfish larvae also have insufficient digestive capacity at hatching thus limiting them from certain types of feed (Zambonino Infante and Cahu, 2001, Conceição et al., 2008; Rønnestad et al., 2013). In this study, *P. major* larvae survived only 10 days when starved, as is to be expected, but surprisingly lasted only 5 days later at 15 DAH when fed micro-diet only. In contrast, co-feeding of micro-diet and live feed for *P. major* larvae improved the digestibility of micro-diet showing non-significant difference in larval growth performance and survival rate, compared to larvae fed live feed only. Kanazawa et al. (1989) reported the successful weaning from live feed onto micro-diet in *P. major* from 10 DAH. They hypothesized that the success of co-feeding protocol possibly involved the ingestion facilitation of micro-diet by live feed via synergistic visual and chemical stimulation, habituating the larvae to feed on inert micro-diet and allowing for an earlier weaning to formulated feed (Cañavate and Fernández-Díaz, 1999; Rosenlund et al., 1997). Furthermore, numerous nutritional factors from live feed have been highlighted that could facilitate the stimulation of pancreatic enzyme secretions and endocrine responses, all of which play an important role digestive system maturation (Kolkovski et al., 1997; Koven et al., 2001; Kolkovski, 2013). As larval fish develop its hunting abilities, its reliance on visual and chemical stimulation will decrease due

to the development of visual acuity, neutral and olfactory capacity as the larvae ages (Blaxter, 1968; Kolkovski et al., 1997; Rønnestad et al., 2013). As such, any transition period between the use of live feed and inert feed may need to be extended with periods of co-feeding to ensure effective change to inert feed (Cañavate and Fernández-Díaz, 1999). This study conducted co-feeding from 5 DAH to condition the larvae to prey on micro-diet. This was followed by the complete removal of live prey by 10 DAH. Our results showed no significant difference in survival rate and growth when compared to live feed treatment (Fig. 3.1). Contrarily, larvae fed only micro-diet was recorded with a significantly lower survival rate and growth than the co-feeding treatment and the live feed only treatment (Fig. 3.1, $p < 0.05$). One of the challenges of weaning into formulated diets is how to maintain good larval survival and growth rates, as larvae require a high efficiency of protein hydrolysis in order to digest micro-diets (Rønnestad et al., 2013). The absence of pepsin in early larval stage is considered as one of the critical reasons behind the limitations of digestive capacity of micro-diets (Rønnestad et al., 2013; Khoa et al., 2019). The survival and growth rates achieved in this study suggested that it was reasonable to assume that *P. major* larvae can physiologically accept inert micro-diet feed in its early ontogenetic stages when introduced together with live feed, especially between 10 DAH to 14 DAH. Our results also show that *P. major* larvae can grow on a feeding regiment that consists of micro-diet only however survival is much lower due to the inability to digest complex proteins in the micro-diet. Our results highlight that live feed play an important role in stimulating digestion process during the first feeding for the proper development of larval fish (Fig. 3.1 - 3.7 and 3.11). As such, a feeding strategy for *P. major* consisting only of micro-diet is likely fail due to its incapacity to meet the nutritional requirements needed to maintain adequate growth performance. Live feed production is expensive and laborious therefore weaning fish as early as possible is a major economical challenge in aquaculture to reduce production cost (Parma and Bonaldo, 2013). These were also reported on other fish species

such as sea bass *Dicentrarchus labrax* (Cahu and Infante, 1994), red drum *Sciaenops ocellatus* (Lazo et al., 2000), common sole *Solea solea* (Parma et al., 2013), spotted rose snapper *Lutjanus guttatus* (Moguel-Hernández et al., 2016), Atlantic halibut *Hippoglossus hippoglossus* (Hamre et al., 2019); whereby the total replacement of live feed by micro-diets at early stages resulted in a consistent decrease of growth performance and delayed metamorphosis of larvae. In Sparidae fishes, previous studies have suggested that commercial micro-diets could be applied when the functionality of stomach and pepsin activity were detectable (Moyano et al., 1996; Suzer et al., 2006, 2007 ; Sánchez-Amaya et al., 2007; Khoa et al. 2019). Studies on weaning protocol for *P. major* larvae by Kanazawa et al. (1989) highlighted formulated diets containing high essential amino acid (EAA) or Vitamin C + phospholipid supplementary are suggested at 10 DAH. Whilst a similar study by (Ren et al., 2010) highlighted that 26 DAH was the optimum timing for weaning onto micro-diets. However, this is based on the first detection of pepsin activity and stomach function as reported by Gisbert et al. (2013) and Khoa et al. (2019). It is reasonable to suggest that following a co-feeding protocol from 10 DAH and switching to micro-diet only from 15 DAH is the most suitable weaning strategy for *P. major* larvae.

Particle size is reported as an important factor which affects growth and size dispersal of fish larvae (Parma and Bonaldo, 2013). According to previous studies by Jobling (1994) and Parma and Bonaldo (2013), small size of particles are easier to capture, handle, ingest and digest however there is lower energy content while it was the inverse for larger size particles. The application of excessively large prey or food particles is also known to lead to a rapid increase of size disparity among the cohort as larger particles benefit essentially the larger larvae (Azaza et al., 2010; Parma and Bonaldo, 2013). These issues are reported similarly and systematically when the larval fish was transited from a prey type to new feed types, especially when weaning fish onto micro-diets containing high energy but with a higher difficulty in

acceptance or digestion as compared to live feed (Kubitza and Lovshin, 1999; Ruyet and Mugnier, 1993). From observations in our study, it is evident that *P. major* larvae could recognize and effectively catch the particle size of the micro-diet (200 and 310 μm) used in this study (Table 3.3). The amount of ingested micro-diet was not studied in the present study, however, gut fullness was used as a proxy following protocols similarly undertaken in a study by Hamre et al. (2019). Information on the correct evaluation of age or size of fish larvae at weaning could help to enhance the growth, survival of larvae and reduce cannibalism (Francis and Bengtson, 1999; Kubitza and Lovshin, 1999; Kestemont et al., 2007; Ljubobratović et al., 2015). Therefore, further studies are required to evaluate new weaning protocols at early stages for *P. major* larvae with appropriate nutritional quality and physical properties.

After hatching, the transcription and enzymatic activity of all target enzyme species in this study were detected (except Pepsin which was detected from 15 DAH). The ontogenetic development of the digestive system of *P. major* was described in detail of Khoa et al. (2019) and our results concurred with the established hypothesis derived from previous studies that the digestive system of larval fish are genetically preprogramed and activated from hatching, in order to maximize the digestive capacity (Moguel-Hernández et al., 2016; Khoa at al., 2019). Due to the rudimentary digestive system and lack of stomach function in early stage *P. major* (Micale et al., 2006; Moguel-Hernández et al., 2016; Khoa et al., 2019), protein hydrolysis is mainly conducted by trypsin and chymotrypsin. During the weaning time (10 – 20 DAH), no significant difference was observed in expression and activity of trypsin, chymotrypsin and pepsin between live feed and co-feeding treatments (Fig. 3.3, Fig. 3.4 and Fig. 3.5). However, it was observed that *try* expression in co-feeding larvae was significantly higher than the other treatments. This possibly alludes to some adaptability to the diet by the larvae as previous studies have highlighted that trypsin activity is mainly influenced by the protein content and amino acid profile of diets (Grendell and Rothman, 1981; Tseng et al., 1982; Péres et al., 1998).

The *try* expression level in co-feeding larvae peaked at 10 DAH and remained high. On the other hand, by the end of the study, the enzymatic activity in co-feeding treatment was similar to live feed treatment, which also suggests the possibility that the characteristics of protein modulates the transcription of mRNA of protease as highlighted by Péres et al. (1998) and Moguel-Hernández et al. (2016). Other studies have also highlighted that differences observed in *try, ctrb, pep* expression levels between co-feeding and live feed only treatment could be caused by the dietary protein property (Nguyen et al., 2011; Moguel-Hernández et al., 2016). Furthermore, a higher level of protease is required to digest micro-diets depending on the different peptide molecular weight distribution of the dietary protein fraction (Holt et al., 2011; Skalli et al., 2014; Moguel-Hernández et al., 2016; Engrola et al., 2007). Increase of protease activity at weaning were similarly recorded in *Senegalese senegalensis* (Engrola et al., 2007), *Sciaenops ocellatus* (Kolkovski, 2001)*; Lutjanus guttatus* (Skalli et al., 2014) and *Totoaba macdonaldi* (Galaviz et al., 2015). For any conflicting trends, such as that observed in in cobia which was attributed to food ingestion rates (Nguyen et al., 2011), further studies will need to be conducted on effects of different nutritional compositions and different ratios of protein and carbohydrate on the expression and enzymatic activity in larvae.

The expression and activity of lipase in co-feeding treatment in this study was observed to be significantly higher than the live feed treatment (Fig. 3.6). Previous studies have documented that diet composition affects the synthesis and secretion of neutral lipase and phospholipase in marine fish species, both of which play a major role in lipid digestion in fish (Hoehne-Reitanet al., 2003; Murashita et al., 2015). Micro-diets containing fish meal have been reported to strongly stimulate the synthesis and secretion of the pancreatic digestive enzymes including increased lipase expression and activity in *P. major* (Murashita et al., 2015). Our results showed good growth rates, high survival rates, enzymatic expression and activity of lipolytic enzymes in the co-feeding treatment and therefore it can be assumed that larvae

adapted to the weaning protocol from 10 DAH. In contrast, if larvae had not adapted to the weaning protocol, low feed ingestion would have been observed which would have been reflected by poor growth and enzymatic activities in larvae as highlighted in a study by Hoehne-Reitanet et al. (2003). Further, in this study, the neutral lipase was extracted from whole fish larvae and applied an unspecific analytical method to measure the lipase activity. Therefore, the lipase activity was not indicated the specific lipase activity in the digestive organs because the neutral lipase is not only distributed along the digestive tract of larval fish but also in the whole fish body (Chatzifotis et al., 2008; Sæle et al., 2010; Rønnestad et al., 2013). According to Sæle et al. (2010) and Rønnestad et al. (2013), the expression level of specific genes is more suitable to characterize the lipase ontogeny, hence the pancreatic lipase could be evaluated through the *pl* expression. The proportion of lipase in digestive organs and non-digestive organs of fish larvae need to investigate in further studies to understand the real lipid digestion capacity of *P. major* larvae.

Amylase expression and activity in *P. major* larvae decreased throughout this study as the larvae gradually changed to carnivorous feeding. Other marine finfish species with carnivorous feeding habits have also been highlighted to showcase this enzymatic behavior (Moguel-Hernández et al., 2016; Yúfera et al., 2018; Khoa et al., 2019). Despite this trend, the expression and activity of amylase in larvae fed micro-diet was observed to be significantly higher than those fed live feed only. Cahu et al. (2004) suggested that carbohydrate (especially starch) concentration and nature in micro-diet and live feed (rotifer and *Artemia*) differed which can result in the variability of amylase expression and activity. This was observed in seabass larvae (*Dicentrarchus labrax*) whereby amylase activity and expression were higher in larvae fed a higher carbohydrate ratio (Péres et al., 1998; Cahu and Zambonino-Infante, 2001). Moreover, the high expression of *amy2α* in non-feeding and fed only micro-diet larvae maintained until the larvae died completely and this predisposition was similarly reported on

Dicentrarchus labrax larvae in malnourished or food restricted conditions whereby the larvae tend to use carbohydrate as a nutritional substrate (Zambonino-Infante et al., 1996; Péres et al., 1998). Accordingly, the fish age, diet compositions, and diet quantity affect the molecular controlling mechanism of carbohydrase. Hence, amylase of fish can be modulated by adjusting the diet composition {Formatting Citation}. It is also suggested more investigation on the effect of different concentration and nature of carbohydrate on the feeding responses of fish larvae for dietary ingredient development purposes. The data in this study also showed that the levels of enzymatic activity were not exactly corresponded to the gene expression; it might due to the different expression of various isoforms within the same gene or a delay in the translation time (Srichanun et al., 2013; Moguel-Hernández et al., 2016; Khoa et al., 2019).

Cholecystokinin **(***cck*) is a peptide which plays a major role in the feeding regulation that influences digestion and activation of satiety signals (Raybould, 2007; Bertucci et al., 2019). It is one of the most important regulators of pancreatic digestive enzyme secretion of teleosts (Tillner et al., 2013). *cck* expression and production in fish were investigated on some marine finfish species including Japanese flounder *Paralichthys olivaceus* (Kurokawa et al., 2000), Atlantic halibut *Hippoglossus hippoglossus* (Kamisaka et al., 2001; Rojas-García and Rønnestad, 2002), bluefin tuna *Thunnus thynnus* (Kaji et al., 2002), seabass *D. labrax* (Cahu et al., 2004), yellowtail *Seriola quinqueradiata* (Murashita et al., 2006), rose snapper *Lutjanus guttatus* (Moguel-Hernández et al., 2016), European eel *Anguilla anguilla* (Politis et al., 2018). In this study, *cck* levels were detected at hatching and peaked at 5 DAH before gradually decreasing from 15 DAH (Fig. 3.10). We also observed that *try* levels were low from 5 DAH and sharply increased from 15 DAH (Fig. 3.3). These results support the hypothesis that trypsin degrades *cck* releasing factors which was reported on seabass *Dicentrarchus labrax* larvae by Cahu et al. (2004). The mechanisms controlling cholecystokinin secretion is complex with signaling interrelationships from the gut and brain, both controlling food intake and digestion

(Raybould, 2007; Moguel-Hernández et al., 2016), but in this study we only focused on the expression of *cck* and key digestive enzymes during the ontogenetic development of larval fish. Expression of *cck* in both live feed and co-feeding treatment tended to reduce after 15 DAH, but the level of *cck* in co-feeding larvae remained significantly higher than the live feed treatment (Fig. 3.10). Similar decreases in *cck* levels were also observed in rose snapper *Lutjanus guttatus* (Moguel-Hernández et al., 2016) and Atlantic cod *Gadus morhua* (Kortner et al., 2011). According to Kortner et al. (2011), *cck* mostly is expressed in brain during larave development, suggesting that reduction of *cck* expression is possibly caused by a reduction of proportion of brain tissue with respect to the whole larval fish body (Cahu et al., 2004; Moguel-Hernández et al., 2016). Higher levels of *try* and *cck* in co-feeding larvae at weaning also suggests that *cck* levels, which are regulated by level and nature of protein content combined with intraluminal proteolytic activity, play an important role in fish digestive physiology (Cahu et al., 2004). Moreover, Polakof et al. (2011) suggested that *cck* also play an important role in the communication axis between brain and gut through the signals of nutrient, hormones and neural pathways. In which, the *cck* level in the digestive system releases the satisfied signals to the brain. The present study showed that *cck* and trypsin have been proposed to be preprogramed early in fish larvae and regulated the exogenous feeding. Accordingly, *cck* expression levels strongly increased when trypsin activity remained at low levels (1-20 DAH), thereafter, the *cck* decreased significantly from 20 DAH when trypsin activity peaked to high levels (Fig. 3.3 and Fig. 3.10). The reversed trend in levels of trypsin and *cck* suggested that hormones and neuronal factors in gut including *cck* played a potentiating role in regulation of digestion and this role changed through the larval development. Similarly, Tillner et al. (2013, 2014) and Navarro-guillén et al. (2017) suggested a negative feedback mechanism between *cck* and trypsin in *Solea senegalensis* and *G. morhua* larvae . Nevertheless, the present study used whole body fishes to analyze *cck* expression which might include both expression of *cck* in

brain and gastrointestinal tract; and only one sample during the day time, therefore could not indicate the regulatory loop occurs between these two digestive players.

Genes related to energy metabolism (*cox1* and *atp)* in *P. major* were expressed in a similar pattern throughout this study. *cox1* and *atp* levels dropped at 10 DAH, 20 DAH and 35 DAH coinciding with the critical stages of metamorphosis of *P. major* larvae (Khoa et al., 2019). These results could help to explain the high mortality of rea sea bream larvae within these stages. Moreover, larvae fed micro-diet only showed a more intensive expression than the starved larvae as they could survive after 15 DAH even as the gut were observed to be full (Fig. 3.8, Fig. 3.9 and Table 3.3). Politis et al. (2018) reported similarly on marine eel (*A. anguilla*), where larvae succeeded in ingesting the feed, however mass mortality still occurred and they postulated that malnutrition was the cause. From the relationship between low digestive enzyme activity and low efficiency in growth and energy metabolism, it is reasonable to assume that the micro-diet only treatment did not provide for the appropriate nutritional requirements needed to sustain growth and survival of *P. major* larvae during critical stages of early development. Co-feeding of live feed and micro-diet achieved high enzyme activity and stable energy metabolism in *P. major* larvae (from Fig. 3.3 to Fig. 3.10). Furthermore, no significant differences in growth performance and survival rate were observed between co-feeding and micro-diet treatments indicating that larvae successfully adapted to the micro-diet (Fig. 1, Fig. 2 and Fig. 11). Studies on *cox1, atp* and energy metabolism response in fish larvae coupled with the effects of dietary nutrition are very limited. Further studies are recommended to evaluate more genes involved in digestion and energy metabolism as indicators for the nutrient status of fish.

igf1 expression was observed in several tissues which involved a key role to promote cellular proliferation and differentiation in vertebrates (Duguay et al., 1992; Plisetskaya, 1993; Koppang et al., 1998; Bertucci et al., 2019). *igf1* is considered to be associated with growth,

metabolism and development of some finfish species (Reinecke et al., 2005; Azizi et al., 2016; Bertucci et al., 2019). *igf1* was suggested as the most promising biomarker to evaluate the growth in fish relevant to feeding regimen, diet composition and protein sources (Picha et al., 2008). When *P. major* larvae were weaned from live feed (rotifer) onto *Artemia* or micro-diet and we observed that even though the diet compositions changed (Table 3.3), the expression of *igf1* increased (Fig. 3.11). Moreover, Marti-Palanca et al. (1996) reported that seabream fed high protein - low lipid diet archived greater growth and *igf1* level than low protein - high lipid. We also observed that the *igf1* level increased until 35 DAH with no significant differences observed between live feed and co-feeding treatment (Fig. 3.11). Considering growth performance, survival rate and enzymatic response in this study, these results indicate that larvae could adapt to the weaning well without any reduction in growth and survival rates. Further studies are needed to evaluate the *igf1* level in larvae fed different diet compositions and protein sources for assessing growth biomarkers in *P. major* larvae.

Gene expression for enzymes and enzyme activity which related to digestive processes, energy metabolism, appetite and growth of *P. major* larvae were observed to have few significant differences between larvae fed live feed only and those that were co-fed live feed and micro-diet. There were also no significant differences in growth performance and survival rate. Larvae fed micro-diet responded positively to the digestion but failed to induce the secretion of digestive enzyme and therefore not effective in hydrolysis of the feed leaving larvae malnourished. *P. major* larvae were able to adapt to micro-diet only from 15 DAH with a 5 days co-feeding transition. This study also suggests that some sensitive indicator genes such as *try*, *cox1* and *atp* could demonstrate the feeding responses of *P. major* larvae during ontogenetic development while the response of *igf1* and *cck* were regulated by diet composition. Findings from this research could contribute to developing appropriate feeding strategies to

enhance growth and survival rates in hatchery practices of *P. major* larvae and there are hopes that parallels can be drawn from our results for other commercially important fish species.

CHAPTER 4: An integrative description of the digestive system morphology and function of Japanese flounder (*Paralichthys olivaceus*) during early ontogenetic development.

Abstract

A good understanding of the morphological and functional changes during early larval fish ontogeny is essential for the enhancement of larviculture techniques. This study applied an integrative approach to describe the ontogenetic development of the Japanese flounder (*Paralichthys olivaceus*) emphasizing the changes in histology and related functions of its digestive system. Changes in histological structures of the digestive tract, expression and activity of digestive enzymes (trypsin, chymotrypsin, lipase, pepsin, amylase), expression of energy metabolism (ATP synthase (*atp*), cytochrome-c-oxidase (*cox*), regulatory factors (neuropeptide Y (*npy*), cholecystokinin (*cck*)) and growth (insulin like growth factor 1 (*igf1*), growth hormone (GH)) were investigated and linked to larval biometrics. The larvae absorbed nutrients from its yolk upon hatching until mouth opening (3 DAH), before beginning its exogenous nutritional phase. The digestive tract differentiations were histologically observed at 5 DAH. The results showed that the targeted enzymes were genetically programmed and activated from hatching, except pepsin which was detected at 25 DAH coinciding with visible development of the gastric glands. The larval stages (3-5 DAH) were marked with high mortality at exogenous feeding transition and also at the onset of metamorphosis (20-30 DAH). Energy metabolism, growth and peptide regulatory factor related genes were transcribed and translated at hatching to assist upon the onset of exogenous feeding. Less feeding and growth delay were noticed between 20-30 DAH. The digestive system was fully developed by 35 DAH and larvae transited to settlement life.

4.1 Introduction

The Japanese flounder (*Paralichthys olivaceus)* has been commercially produced in the coastal zone of Japan, Korea and China due its high market value (Kikuchi and Takeuchi, 2002; Seikai, 2002). This species was also remarked with high growth and feed conversion rate along with good adaption to water temperature variation (Seikai, 2002; Daniels and Watanabe, 2010). From the first success in artificial seed production in 1965 in Japan, rearing technologies were improved by the 1980's that suit mass production became stable with reliable supply of fingerlings for the aquaculture industry (Harada et al., 1966; Ijima et al., 1986; Seikai, 2002). However, larval rearing techniques still heavily relied on live feeds which were considered high cost and contained associated pathogenic risks (Bai and Lee, 2010). Recently, the production of *P. olivaceus* was nearly saturated and faced several challenges such as lower market prices and demand, disease outbreaks, high production costs (Seikai, 2002). This bottleneck highlighted also the need to enhance production efficiency throughout its culture phase and improve innovations in culture technologies.

Many studies have focused on the development of *P. olivaceus* larvae and its feed and nutritional requirements, larval morphological changes (Minami, 1982; Tomoda et al., 2006), digestive system development (Tanaka et al., 1996; Rønnestad et al., 2000; Kim et al., 2004), gene expression and activity of digestive enzymes (Srivastava et al., 2002; Bolasina et al., 2006; Lee et al., 2015) and peptide and energy metabolism (Suzuki et al., 1999; Kurokawa et al., 2000; Lu et al., 2018). However, many of the aforementioned studies were conducted separately with different rearing conditions or feeding diets and therefore could not illustrate the real digestive capacity of the larvae when exposed to uniform variables (Rønnestad et al., 2013; Solovyev et al., 2016). The larviculture protocols for *P. olivaceus* normally achieved high survival rates (Seikai, 2002; Bai and Lee, 2010; Seikai et al., 2010). However, critical issues such as nutritional demands, energy metabolism, and cannibalism still need further

investigation (Suzuki et al., 1999; Dou et al., 2000; Lu et al., 2018). These concerns require an overall and uniform approach in order to understand the nutritional physiology of larval fish, especially on the relationships between morphological changes and funtions of the digestive system.

Many larval fishes, including *P. olivaceus*, hatch with a very rudimentary digestive system, then gradually develop a short and straight tube before it eventually develops into specific organs (Gisbert et al., 2004; Rønnestad et al., 2013). During the stages when the digestive system undergoes, the larvae are variably able to digest certain feed (Zambonino-Infante et al., 2008; Mahjoub et al., 2013). Therefore, nutrition is also an important consideration along with the organization and functionality of the digestive systems at various stages (Lazo et al., 2011). Numerous studies have been conducted to investigate the changes in morphological and histological structures, quantify and characterize the digestive enzymes biochemically (Yúfera et al., 2018). However, these traditional approaches showed limitations that could not illustrate full functions of the associated digestive progresses (Lazo et al., 2011; Srichanun et al., 2013; Khoa et al., 2019). Besides, the sampling time, assay conditions (temperature, pH), other factors such as sensitivity and specificity of the substrates also impact the real enzymatic activities of larvae (Yúfera et al., 2018). Recently, the advances in molecular biological tools have provided a complementary and essential understanding of the structure and function of genes involved in the digestive enzyme encoding (Yúfera et al., 2018). They could also assist in the investigation of digestive enzyme precursor codifications, complexity and overlap of regulatory mechanisms involved in ontogenetic development of the digestive system in *P. olivaceus* (Zambonino-Infante et al., 2008; Yúfera et al., 2018). Thus, this study aims to illustrate the ontogenetic development of *P. olivaceus* digestive system through an integrative investigation to access relations of morphological and histological changes, feeding status, expression of related genes and final digestive enzymatic activity of *P. olivaceus* larvae from

hatching to 40 days after hatching (DAH). These comprehensive descriptions will provide a better understanding of the ontogenetic development of the digestive system *P. olivaceus* and further knowledge around aspects of diet optimization and rearing practices.

4.2 Materials and methods

4.2.1 Fish larvae rearing

Fertilized eggs of Japanese flounder were bought from Hayato Aquaculture Station, MBC Kaihatsu Co. Ltd., Kirishima, Kagoshima Prefecture, Japan. And they were transferred to the Kinko-Bay On-shore Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Eggs were stocked in 500 L composite tank at a density of 20 eggs /L and at a salinity of 35 psu. The rearing tank was continuously aerated and maintained at a temperature of 15 ºC using a platinum heater. The rearing protocol was applied following a protocol described by Khoa et al. (2019). From first feeding (3 DAH) to 18 DAH, larvae were fed rotifer *Brachionus plicatilis* species complex L-type enriched with *Chlorella vulgaris* containing docosahexaenoic acid (C22:6 *n*-3, DHA; Super Fresh Chlorella V-12, Chlorella Industry Co. Ltd., Tokyo, Japan) and Taurine enrichment diet (Aqua-Plus ET, Nisshin Marubeni Feed Co., Ltd., Tokyo, Japan) at 3–5 rotifers /mL of rotifer density. From 15 DAH, larvae were subsequently fed *Artemia* nauplii at 1-3 nauplii /mL of density of *Artemia* hatched from diapausing eggs (Great Salt Lake Artemia, Ogden, USA), and switched to *Artemia* nauplii only from 18 DAH. The protocols for rotifer culture and enrichment were described in detail by Kotani et al. (2017) and Khoa et al. (2019). Flow rate of water exchange was continuously set at 300 %/day from 3 DAH, through a mesh (0.2 mm). The green water rearing condition was obtained by adding *C. vulgaris* at 3 mL/100 L of rearing volume.

4.2.2 Fish sampling

Specimens were randomly collected at 1, 3, 5, 10, 15, 20, 25, 30, 35 and 40 DAH. Twenty larvae from each point time were randomly sampled and stored in Buoin's solution at 4 ºC to process for histological observation. For quantitative real time PCR, 150 larvae were rinsed with distilled water before kept in *RNA*later® stabilization reagent (QIAGEN GmbH, Germany). Those samples were stored overnight at 4 ºC, thereafter transferred to -20 ºC for subsequence molecular analysis. For digestive enzyme activity, 150 larvae were collected and washed with distilled water, then immediately frozen at -80 ºC pending for enzyme determination. In addition, every sampling day, 15 fresh larvae were caught for growth determination (length and weight), and 20 larvae were fixed with 70 % ethanol for gut content examination. Larvae was examined under a VHX- S90F digital microscope with high performance zoom lens (Keyence Corporation, Osaka, Japan) to estimate the constituents of larval guts.

4.2.3 Histological observation

The samples were submerged in Buoin's solution for 24 h, then washed and stored in 70 % alcohol. Following this, the process was continued with dehydration in a graded series of ethanol, and embedding in paraffin block. The serial sagittal sections (5 µm) were achieved using a Leica microtome (RM 2135, Nussloch, Germany) and placed on glass slides. The slides were stained with Haematoxylin and Eosin protocol. After staining, the slides were covered by a coverslip and permanently mounted. The histological preparations were observed under a light microscope (BX41, Olympus, Tokyo, Japan).

4.2.4 Quantitative real time PCR

Pool samples of five larvae were used to isolate total RNA, in triplicate for each point time. Fish larvae were homogenized with 1 mL of TRIzol^{TM} reagent (Thermo Fisher Scientific,

Invitrogen, USA) applied the manufacture's protocol. The quality of RNA was tested using a NanoDrop (Thermo Scientific, Waltham city, Massachusetts, USA) with a range of OD260/280 within 1.9 to 2.0. Total RNA (100 ng) from each sample was reverse-transcribed using ReverTra Ace® qPCR RT Master Mix with gDNA remover (Toyobo Co., LTD, Osaka, Japan) following the manufacture's protocol in a final volume of 10 µL.

The quantitative real time PCR was carried out to determine the expression level of related genes for digestive enzymes (trypsin 3 (*try3*), chymotrypsin 2 (*ctrb2*), pepsin (*pep*), pancreatic lipase (*pl*), amylase (*amy2α*), intestinal fatty acid-binding protein (I - FABP 1)), energy metabolism (Cytochrome-C-Oxidase (*cox*), ATPase-pk (*atp*)), cholecystokinin (*cck*), neuropeptide Y (*npy*), growth (insulin-like growth factor 1 (*igf1*), growth hormone (*GH*)). The primer sequences were listed in Table 4.1. Each sample was performed in triplicate in a Biorad CFX connect^{TM} Real time system (Bio-Rad Laboratories, Inc., Japan). PCR reaction consisted 1 µL of cDNA template, 0.4 µL of each primer, 10 µL of qPCR Mix, and 0.4 µL ROX reference dye (KOD SYBR® qPCR Mix, Toyobo Co. Ltd, Osaka, Japan) in a final 20 µL reaction. The thermal profile was 98 °C for 2 min; followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. The melting curve was generated from 60 °C to 95 °C with an increment of 0.3 °C and holding for 15 s. The expression levels of targeted genes were normalized to the expression of β-actin and elongation factor-1 (*ef1*) and measured using the standard curve with diluted cDNAs.

4.2.5 Enzyme assay

Pool samples of five larvae was homogenized in 100 µL of ice cold buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5) to extract crude enzyme (Bolasina et al., 2006) using a pellet pestle cordless motor (Sigma-Aldrich Inc., St. Louis, MO, USA). After homogenization, the pestle tip was rinsed with 400 µL of homogenization buffer, thereafter, the tubes containing 500 µL homogenate were centrifuged for 30 min at 1,700 x g at 4 °C. The

supernatant was transferred into new 1.5 mL tubes and was used as the crude enzyme extract in protein content and enzymatic assay analysis later, stored at -80 ºC. The extraction was conducted in triplicate for each sampling point. Bradford method (Bradford, 1976) was applied to determine the protein content of samples.

4.2.5.1 Trypsin

The activity of trypsin was measured using Z-L-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA, Sigma-Aldrich Inc., C9521) as a substrate (Bolasina et al., 2006). The reaction contained 500 µL of substrate (containing 50 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ- L Arg-MCA) and 50 µL of crude enzyme was incubated for 30 min at 30 °C in a water bath, followed by adding 100 µL of 30 % acetic acid for quenching. The fluorescence was read using a spectrofluorophotometer (F2000, Hitachi High-Tech Corporation, Tokyo, Japan) at 380 nm of excitation and 440 nm of emission. The activity of trypsin was expressed in unit (mU) in 30 min, as percentage increase of emission per protein (mU mg protein −1).

4.2.5.2 Chymotrypsin

Chymotrypsin activity was determined using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPFNA, Sigma-Aldrich Inc.) as a substrate (Murashita et al., 2018). Briefly, the reaction mixture was prepared by adding 50 µL of crude enzyme to 240 µL of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$) and 100 µL 2.4 mM SAPFNA, followed by incubation for 7 min at 37 ºC. Production of *p*NA was measured at 405 nm using a spectrophotometer (U5100, Hitachi High-Tech Corporation). One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in 1 min.

Table 4. 1 The sequence of primers for qRT-PCR

Gene	Primer forward	Primer reverse	Source
try3	TATGAGTGCACGCCCTACTC	GTTCTCACAGTCCCTCTCAGAC	Lee et al., 2015
ctrb2	ACTACACCGGCTTCCACTTC	GAACACCTTGCCAACCTTCATG	
pep	TGAGTCCATGACTAATGATGCT	CACCTGATTGGCCACAGAG	
I-FABP 1	GGAAAGTCGACCGCAATGAG	CACTCTGCGTGTTACTGCTTC	
pl	ATGGGAGAAGAAAATATCTTATTTTTGA	TACCGTCCAGCCATGTATCAC	
amy2α	CACTCTTCATGTGGAAGCTGGTTC	CCATAGTTCTCAATGTTGCCACTGC	Moguel-Hernández et al., 2016
cox	AAATCAACGACCCCCACCTC	CGGGGATTGCTCAAGAGTGT	Lu et al., 2018
atp	GTCAAAGAAGTCGGCTCGGA	GTCAGCTGCCTTACGGATGA	
β-actin	GGAATCCACGAGACCACCTACA	CTGCTTGCTGATCCACATCTGC	
igf1	GCCACACCCTCTCACTACTGCT	GCCTCTCTCTCCACACACAAAC	Zheng et al., 2012
cck	TGCTGCAAGCTTCAACGC CC	TTGATGACACCGACAGCCAC	Suzuki et al., 1999
ef1-α	TGCTGCAAGCTTCAACGCCC	TTGATGACACCGACAGCCAC	Srivastava et al., 2002
npy	CCAAATACTACTCAGCCCTG	GACCGTGTCCAATGGTTAAG	Kurokawa and Suzuki, 2002
GH	CAGTTGGAGGATCAACGTCTTCT	CTGCCTTGTGTCTCGTGTTTG	Li et al., 2017

Noted: ***try3***: trypsinogen 3; ***ctrb2***: chymotrypsinogen 2; ***pep***: pepsinogen; ***pl***: pancreatic lipase; ***amy2α***: amylase; ***cox***: Cytochrome-C-Oxidase; ***atp***: ATPase-pk; **I-FABP1**: intestinal fatty acid-binding protein 1; ***cck***: cholecystokinin; ***igf1***: insulin-like growth factor 1; **GH**: growth hormone; ***npy***: neuropeptide Y; **β-actin**: beta actin; ***ef1***: elongation factor-1

4.2.5.3 Pepsin

The activity of pepsin was assayed using 2 % haemoglobin in 0.06 N HCl as a substrate (Worthington, 1993; Natalia et al., 2004). A total of 100 µL of enzyme extract was mixed with 500 µL of substrate, then was incubated at 37 ºC for 10 minutes. The reaction was terminated by adding 1 mL of 5 % trichloroacetic acid and left for 5 min. Following the centrifugation for 5 min at 12000 x g, the absorbance was read at 280 nm using a spectrophotometer. For blank reading, the enzyme extract was added after trichloroacetic acid.

4.2.5.4 Lipase

The activity of lipase was determined using *p*-nitrophenyl myristate (PNPM, Sigma-Aldrich Inc.) as a substrate (Albro et al., 1985). The reaction was prepared by adding 450 µL of 100 mM Tris–HCl buffer (pH 8), 100 µL of 3.5 mM PNPM (containing 0.5 % Triton X-100, Nakarai, Osaka, Japan) and 50 µL of enzyme extract, then incubated for 7 minutes at 37 ºC. The production of *p*-nitrophenol (*p*NP) was measured by a spectrophotometer at 405 nm.

4.2.5.6 Amylase

Amylase activity was evaluated using 1 % starch solution as a substrate (Murashita et al., 2018). A total of 50 µL of enzyme extract was mixed with 25 µL of 20mM sodium phosphate buffer (containing 6.0 mM NaCl, pH 6.9) and 25 µL of the substrate solution, followed by incubation at 37 ºC at 60 min. Subsequently, 50 µL dinitrosalicylic acid reagent (1 % dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH) was added and incubated for 5 min in boiling water. Absorbance at 540 nm was read using a spectrophotometer. The amount of maltose released from this assay was estimated from standard curve.

4.2.6 Statistical analysis

The results were presented as a mean ± SEM (standard error of the mean) of triplicate. The data were analyzed by one-way ANOVA (SPSS 24.0 for Windows, IBM, Armonk, NY, USA)

applied Tukey's HSD (Honestly Significant Difference) test with a significance level of $p < 0.05$. The linear regression was performed to estimate the correlation between gene expression and enzyme activity of larval fish (α=0.05).

4.3 Results

4.3.1 Growth performance, morphological change, gut content, and survival rate

Growth in length and weight of Japanese flounder larvae is presented in Fig. 4.1. During the experimental time, the larvae significantly grew in length from 3.90 ± 0.23 mm to 11.53 ± 0.57 mm following y = 0.1991x + 3.5686 (R^2= 0.9928). The wet weight was exponentially increased by following $y = 0.485e^{0.1066x}$ (R^2= 0.912), reached to 24.2 ± 0.3 mg at 40 DAH compared to an initial weight of 0.2 ± 0.02 mg per 30 larvae.

The morphological change and survival rate of larvae were shown in Fig. 4.2. The larvae hatched out with a large yolk and oil globule (1 DAH), mouth opened and established exogenous feeding at 3 DAH (2.3 ± 1.3 rotifer/larvae)**.** The yolk and oil globule completely depleted at 5 DAH coining to a gradually increasing of gut content (4.5 ± 1.5 rotifer/larvae). The larvae were able to also ingest *Artemia* nauplii from 15 DAH (10.3 ± 3.3 rotifer and 2.3 ± 1.3 *Artemia*/larvae) and completely switched to *Artemia* from 18 to 20 DAH (4.5 ± 1.5 *Artemia*/larvae). The metamorphosis was observed from 23 DAH and completed at 35 DAH whereby the two eyes overlapped. The larvae changed to the settling stage and consumed 5-8 *Artemia*/larvae within 2h (Fig. 4.2A and B). High mortality was recorded at first feeding (3 – 5 DAH), pre-metamorphosis and metamorphosis stage (from 20–35 DAH), accounting up to 40–50 % of total mortality during the experimental time. After 40 DAH, the larvae achieved 26.5 ± 3.8 % of survival rate (Fig. 4.2A).

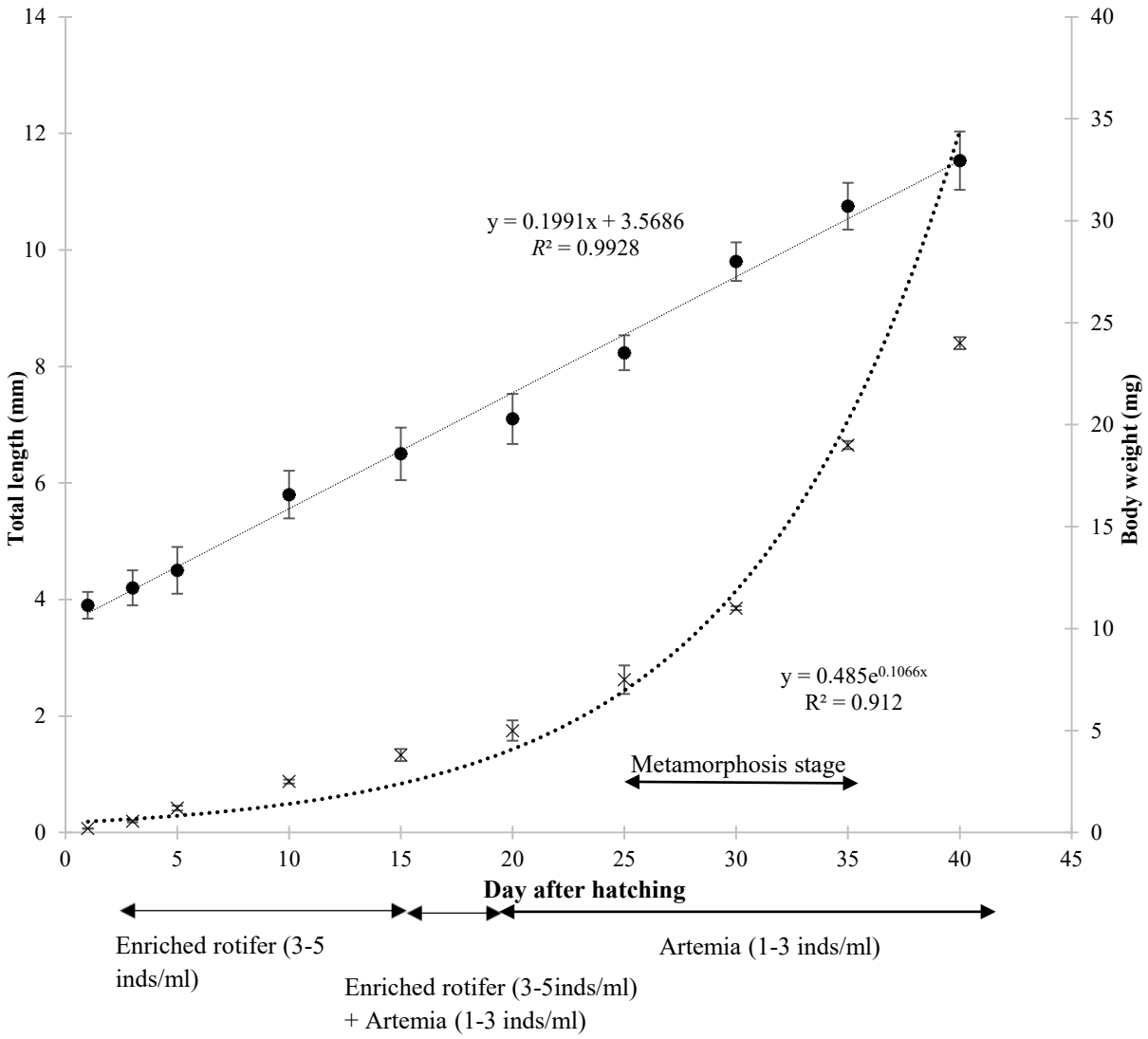


Figure 4. 1 Relationship of total length (mm), body weight (g), age post hatching (day) and major histological changes in digestive system from 1 to 40 DAH in Japanese flounder larvae.

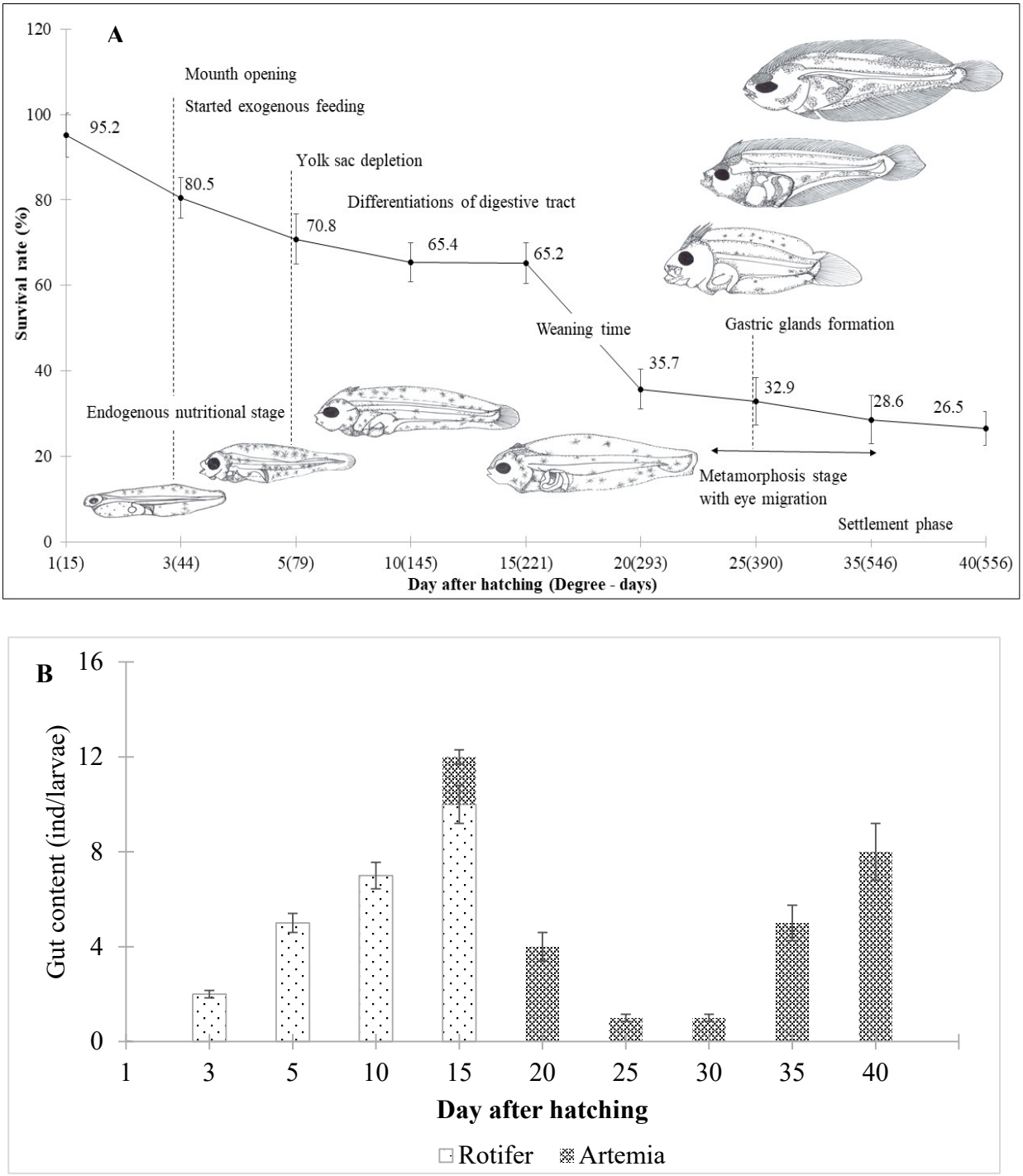


Figure 4. 2 The survival rates, morphological changes(A) and gut contents (B) of Japanese flounder larvae during the ontogenetic development. The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

4.3.2 Histological features of digestive tract

The larvae had a large yolk and elliptic oil globule during its endotrophic stage (0–2 DAH, Fig. 3A). The mouth and anus opened at 3 DAH while the digestive tract included a straight and undifferentiated tube (Fig. 4.3B). The yolk resorption and oil globule depletion completed at 5 DAH when the liver, oesophagus, anterior intestine, posterior intestine, and pancreatic tissue could be histologically visible (Fig. 4.3C). The oesophagus elongated with prominent longitudinal folds during the exogenous feeding transition. From 10-15 DAH the digestive tract differentiated into oesophagus, incipient stomach, and anterior, middle, and posterior intestine. The intestine loop and the intestinal mucosa folds were histologically observed with a surrounding external circular layer of muscle cells (Fig. 4.3D, E). The mucosa increased folds and intestine coils mostly presented in the abdominal area. The stomach was formed and increased in size with several epithelial folds. The mucous cells rapidly increased in density coinciding with the enlargement of mucosal surface at 20 DAH. However, the gastric glands were not detected during this period (Fig. 3.3F and 3.4A). The presence of a large number of goblet cell was observed in the epithelium lining the oesophageal lumen (Fig. 4.4E).

The first developing of gastric glands in columnar epithelium cells was recorded at 25 DAH indicated the function of stomach organ, and increased in number with the growth of larvae (Fig. 4.4B and C). The mucosa of the stomach fold up during the development and microvilli presented in cardiac and pyloric region with abundance of mucous. The liver started differentiation early from 5 DAH by enlarging and increasing the accumulation of lipids (Fig. 4.5). The digestive system completely developed from 35 DAH and the fish transformed into the settlement phase of its life cycle.

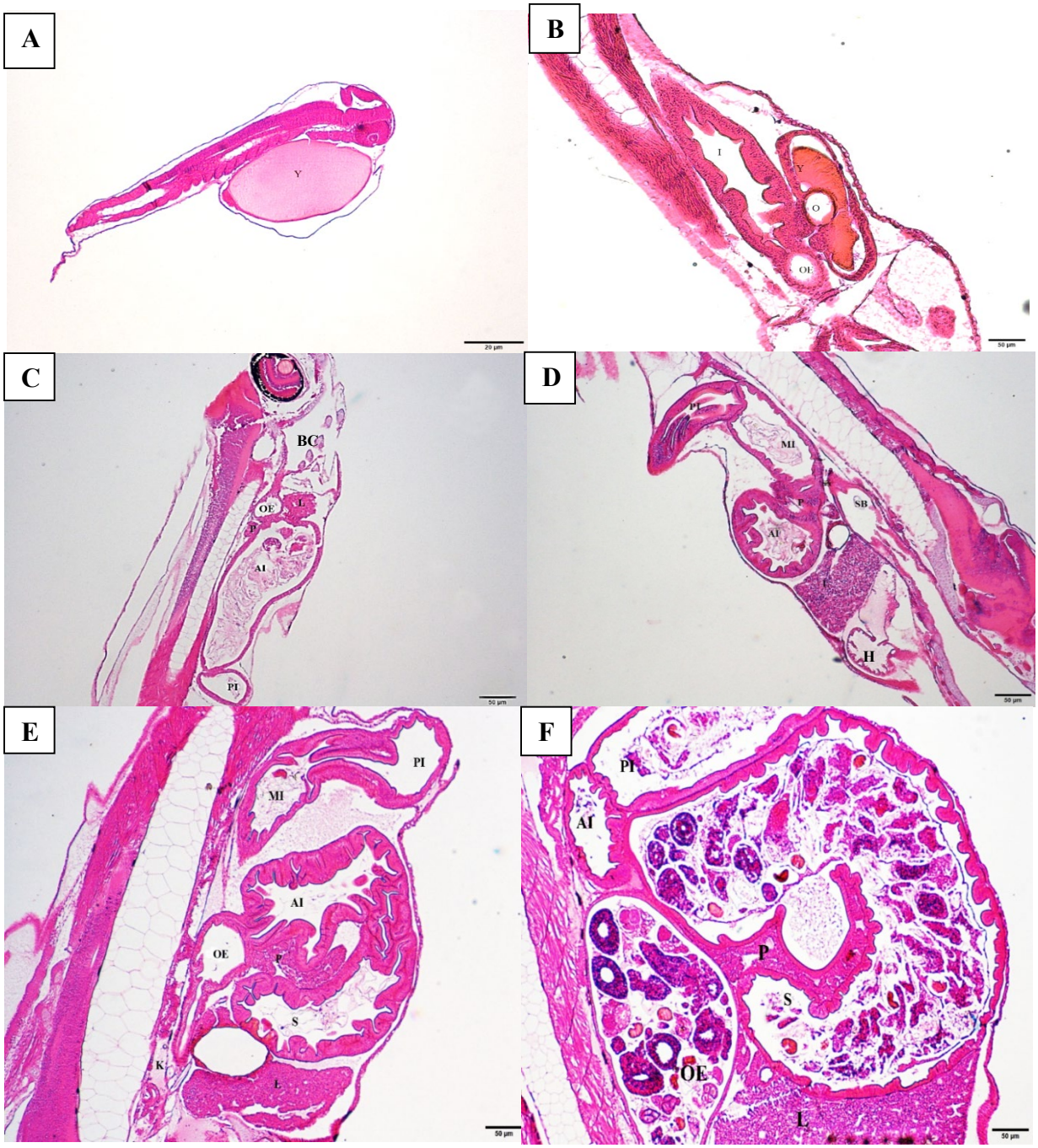


Figure 4. 3 Histological microsections of the digestive system in *P. olivaceus* larvae during development. A) Newly hatched larvae (1 DAH) with the yolk sac. B) 3 DAH larvae, mouth-opened with a straight digestive tract, Oil globule (O) remains. C) 5 DAH with the stratified epithelium in oesophagus. D) 10 DAH with the appearance of anterior intestine, middle intestine, liver, pancreas, posterior intestine, swimbladder. E) 15 DAH a prominent anterior intestine and the folding of the oesophageal mucosa. F) 25 DAH appeared gastric glands, goblet cells and brush borders in intestine. Abbreviations: Y: yolk sac; BC: buccopharyngeal cavity; AI: anterior intestine; MI: middle intestine; OE: oesophagus; PI: posterior intestine; H: heart; SB: swim bladder; L: liver; P: pancreas; O: oil globule; K: kidney; S: stomach. Scale = 50 µm. Staining: Haematoxylin-Eosin.

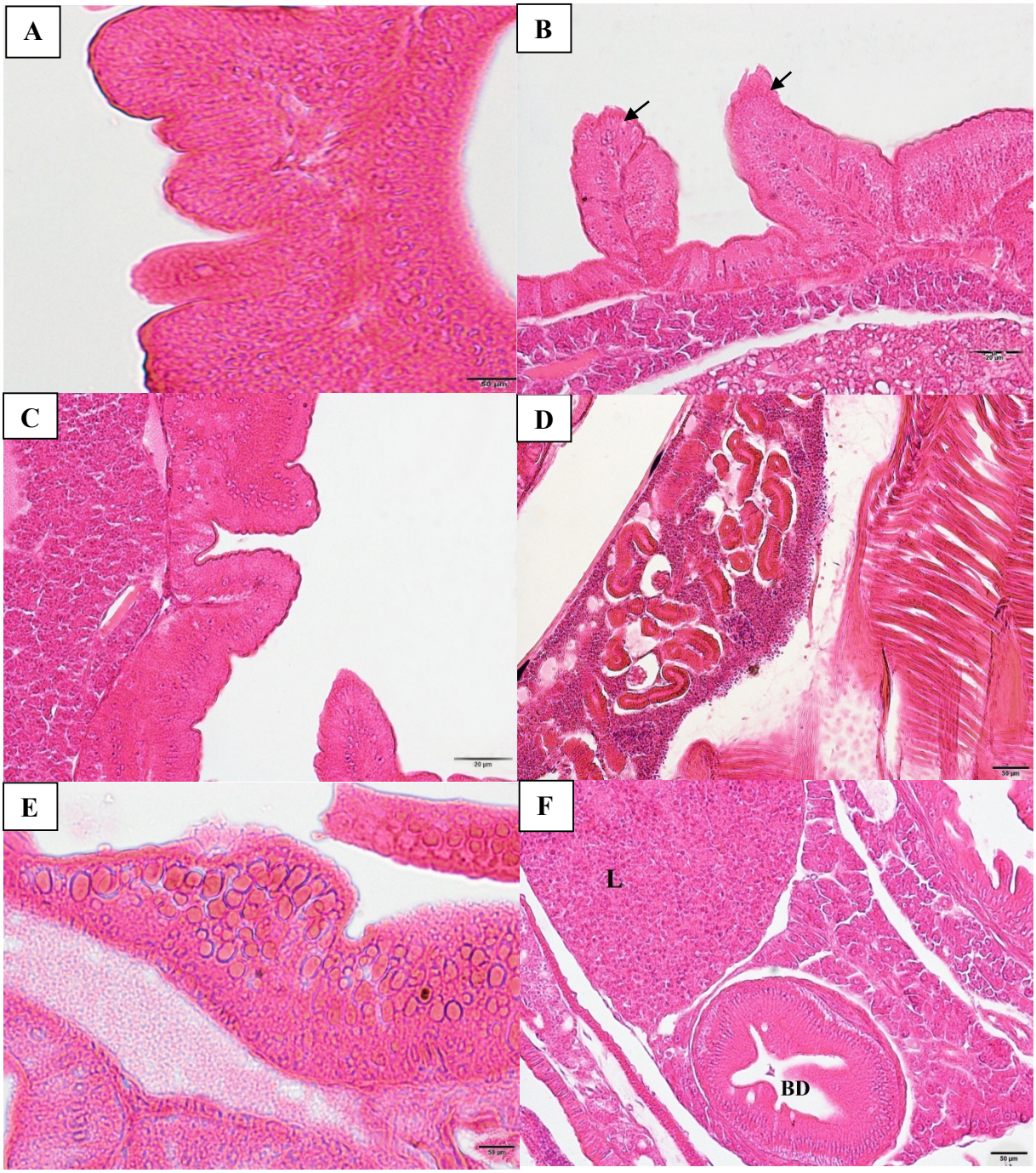


Figure 4. 4 Full function digestive system of P. olivaceus from 20 - 40 DAH. A) the epithelium without gastric glands at 20 DAH. B) stomach function and the gastric glands are clearly visible at 25 DAH. C) Abundance of gastric glands at 35 DAH (Scale =20 µm). D) 40 DAH kidney. E) digestive vacuoles. F: bile duct. Abbreviations: K: kidney; BD: bile duct. Scale = 50 µm. Staining: Haematoxylin-Eosin.

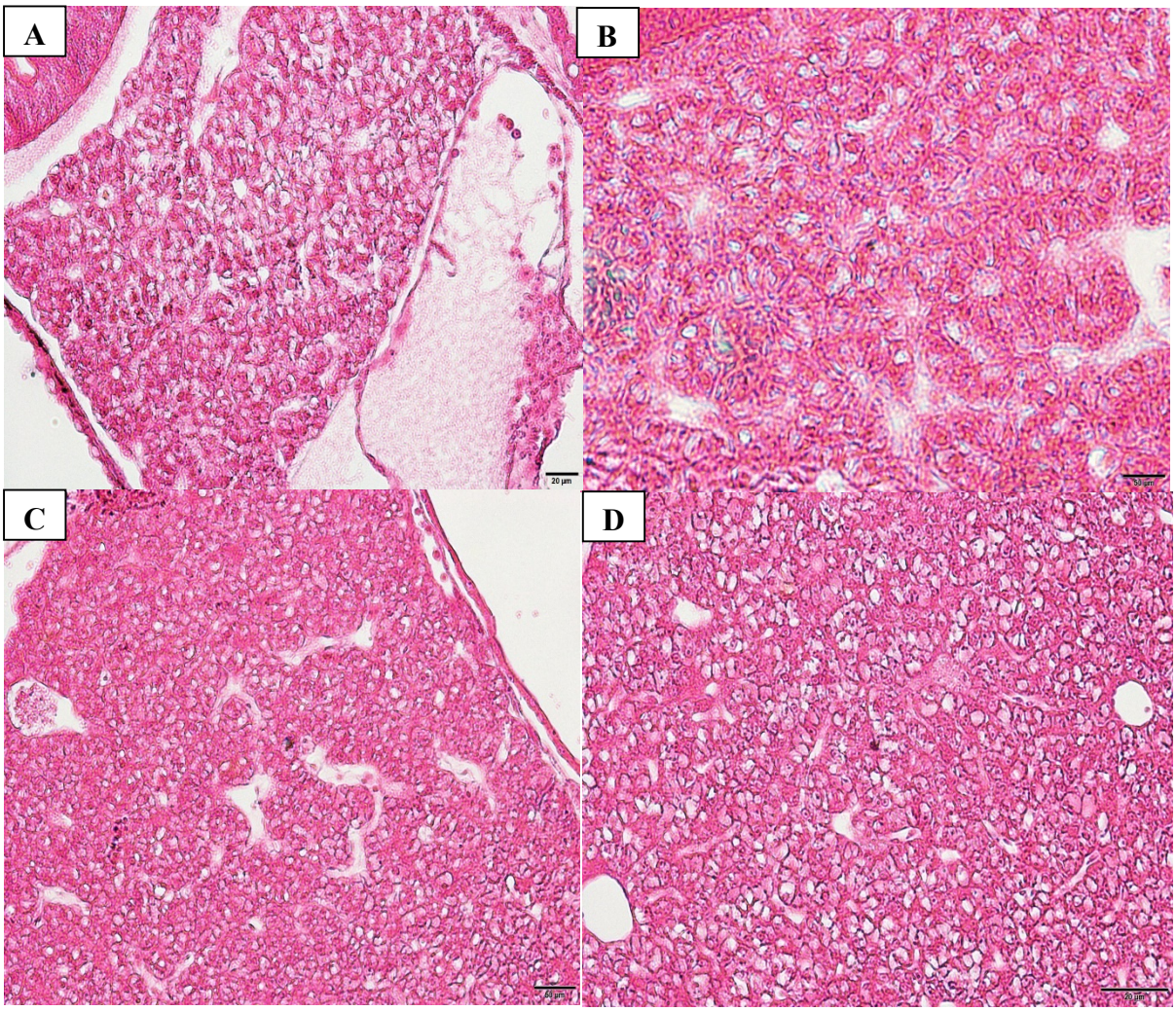


Figure 4. 5 Detail of the liver in *P. olivaceus* at different stages of development showing ontogenic differences in fat accumulation in hepatocytes. A) 5 – 10 DAH. B) 15 - 20 DAH. C) 25 - 30 DAH. D) 35 - 40 DAH. Scale = 20 µm. Staining: Haematoxylin-Eosin.

4.3.3 Enzymatic activity and expression of digestive enzymes.

4.3.3.1 Trypsin

The changes in expression and activity of trypsin were shown in Fig. 4.6. Trypsin was detectable from hatching at a very low activity (0.12 ± 0.01 mU/mg protein), then gradually increased and peaked at 15–20 DAH (0.41 - 0.45 mU/mg protein; $p < 0.05$). From 25 DAH, the trypsin activity tended to decrease and recover to the initial level, which was similar to levels at first feeding. Expression of *try3* showed a sharp increase after hatching until 20 DAH.

A critical drop of *try3* expression was observed during the metamorphosis stage (25–30 DAH) with a valley point at 30 DAH ($p < 0.05$), followed by a slight increase from 35 DAH (Fig. 4.6A; $p > 0.05$). Moreover, the expression and activity of trypsin was significantly correlated during the larval development ($R^2 = 0.7248$, $p = 0.02$; Fig. 4.6B).

4.3.3.2 Chymotrypsin

The pattern of expression and activity of chymotrypsin complicatedly fluctuated during the larval stages, and no significant correlation between expression and enzymatic activity was recorded ($R^2 = 0.074$, $p = 0.447$; Fig. 4.7B). In which, the chymotrypsin activity at hatching was at low level (0.1 - 0.15 mU/mg protein) and remarkably increased from 3 DAH with a peak at 5 DAH (0.3 ± 0.04 mU/mg protein; $p < 0.05$). Chymotrypsin activity remained at significantly higher levels until 20 DAH (0.18 - 0.3 mU/mg protein; $p < 0.05$) and then progressively decreased after 20 DAH, remaining stable without statistical difference until 40 DAH ($p > 0.05$; Fig. 4.7A). However, the expression of *ctrb* was not followed the enzymatic activity within the first 20 days, whereby high *ctrb3* expression levels were observed from 10 to 20 DAH with a peak at 20 DAH.

4.3.3.3 Pepsin

No detection in expression and enzymatic activity of pepsin was recorded from hatching until 20 DAH (Fig. 4.8). The first detection of pepsin was at 20 DAH but was nearly zero levels (0.017 ± 0.005 mU/mg protein), followed by a sharp increase tendency in both expression and activity throughout the development of fish larvae. In which, the highest pepsin activity was measured at 40 DAH (34.2 ± 3.2 mU/mg protein; Fig. 4.8A). On the other hand, the expression and activity of pepsin showed a significant correlation ($R^2 = 0.9075$, $p = 0.0001$; Fig. 4.8B).

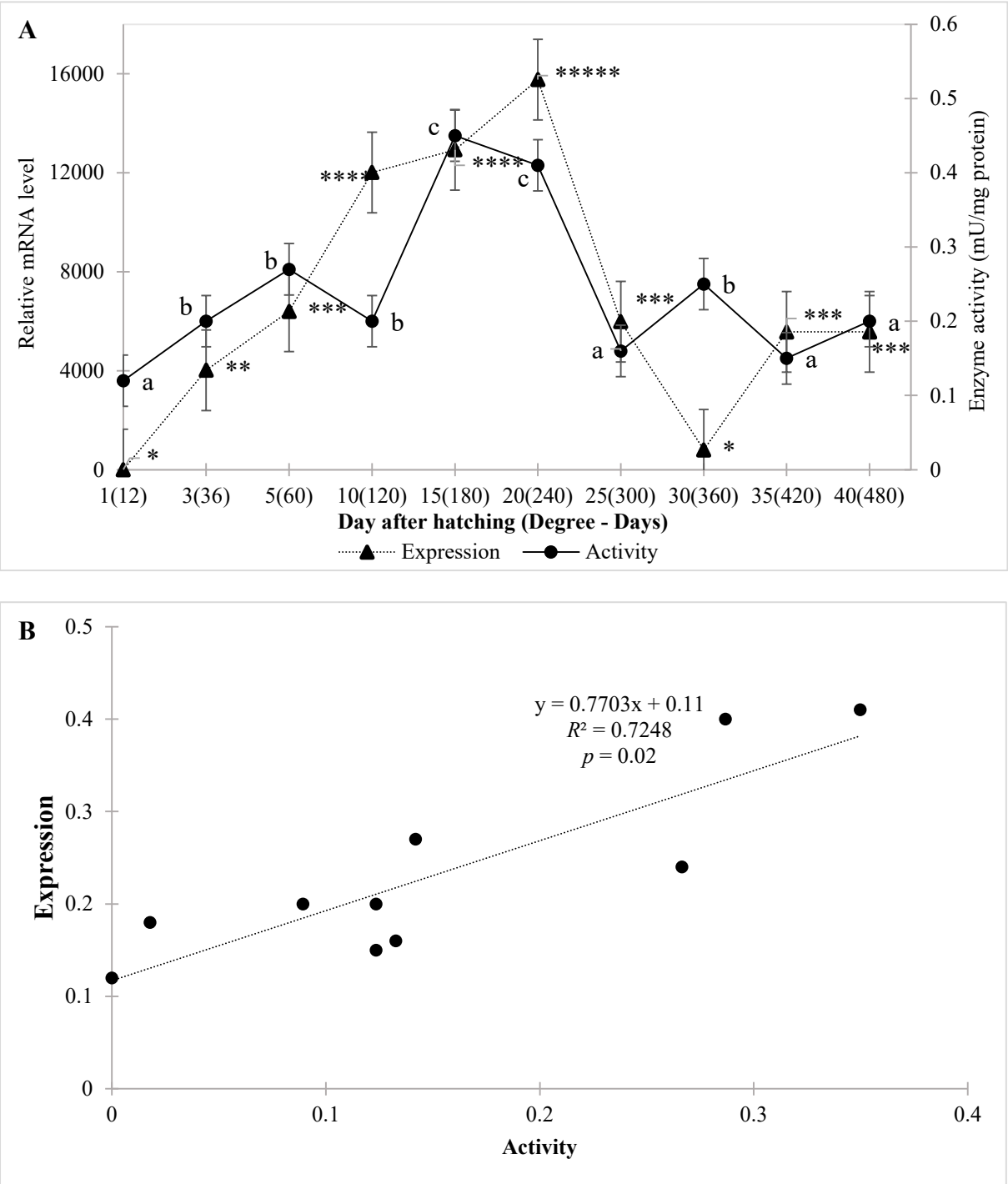


Figure 4. 6 Trypsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate trypsin activity. Dashed line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *try3* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of trypsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

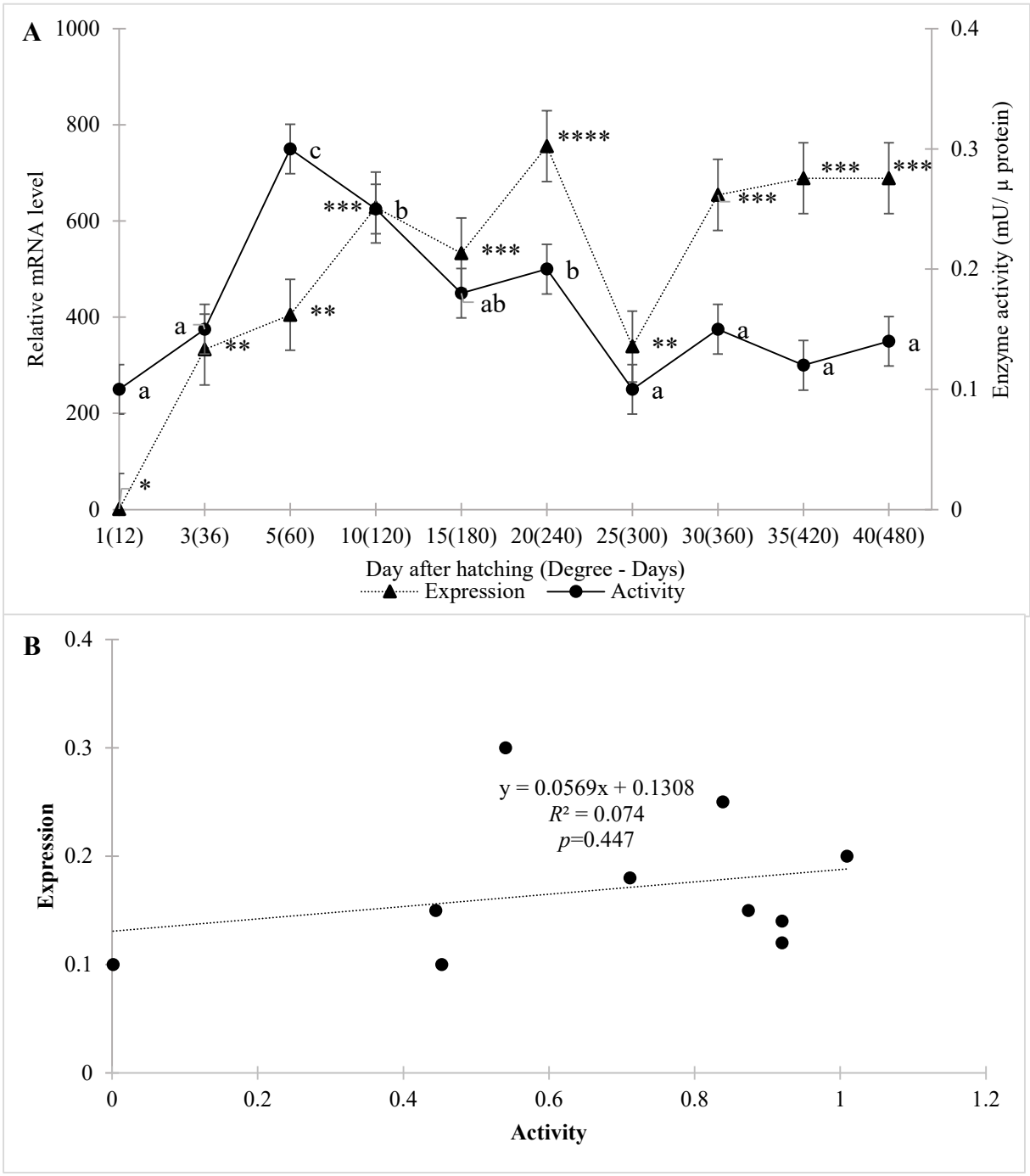


Figure 4. 7 Chymotrypsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate chymotrypsin activity. Dashed line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *ctrb2* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of chymotrypsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

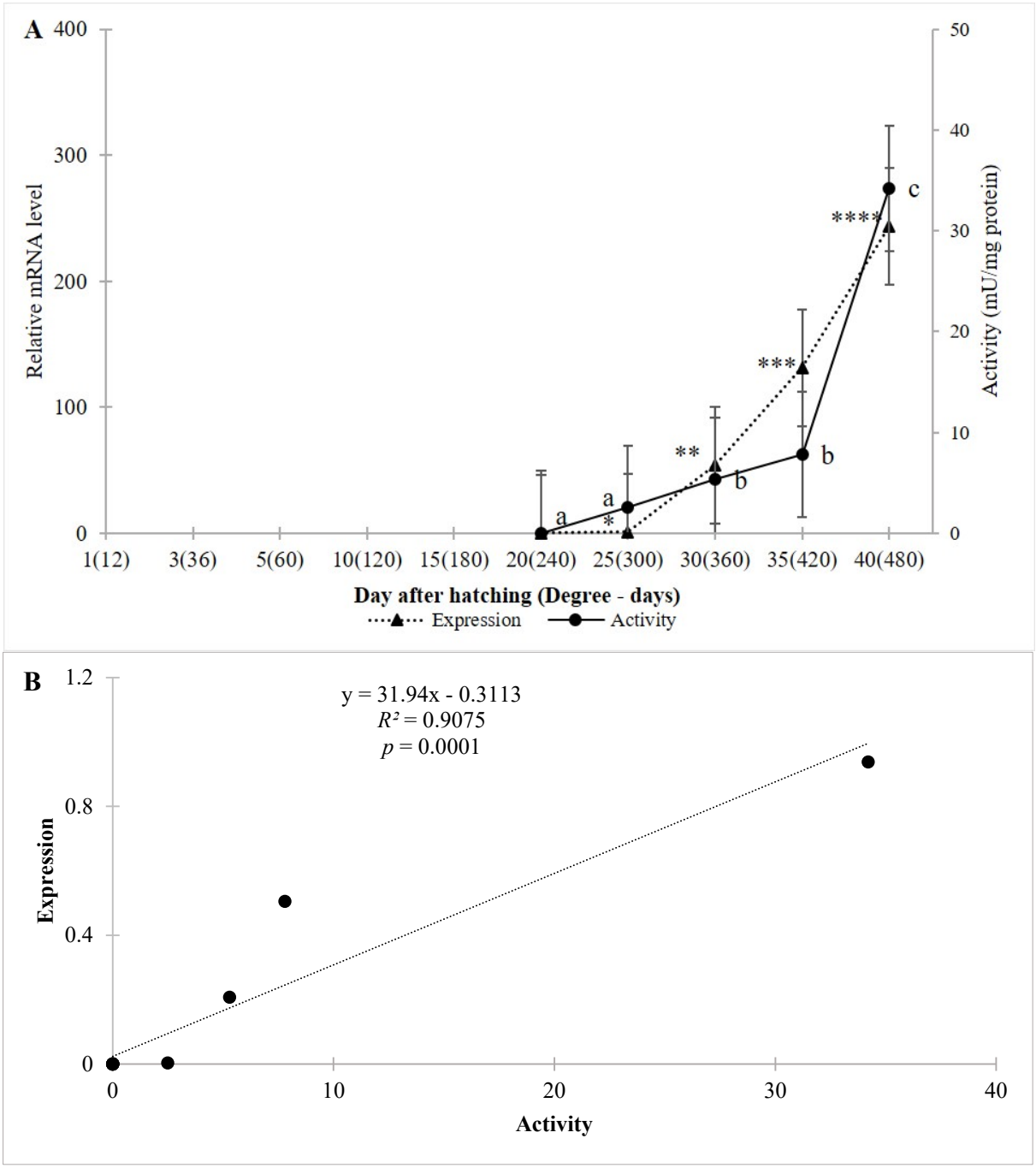


Figure 4. 8 Pepsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate pepsin activity. Dashed line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *pep* at 20 DAH were used as reference samples. Alphabetical letters indicate significant difference of pepsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

4.3.3.4 Lipase and expression of intestinal fatty acid – binding protein (I-FABP 1)

The lipase activity was detected at hatching with a low level (3.1 ± 0.6 mU/mg protein; Fig. 4.9). When the larvae started the exogenous feeding, the activity of lipase strongly increased throughout the larval development with a peak at 40 DAH ($p < 0.05$; increasing from 4.5 to 12.8 mU/mg protein; Fig. 4.9A). In which, no significant changes in lipase was observed from first feeding to 20 DAH (4.5 - 6.7 mU/mg protein). However, the expression levels of *pl* were not significantly correlated to the BAL activity ($R^2 = 0.2033$, $p = 0.191$). Especially, the expression of *pl* made two peaks at 3 DAH and 20 DAH whereas the larvae were transited to endo-exotrophic stage and weaned from rotifer to *Artemia*, respectively ($p < 0.05$; Fig. 4.9B).

Besides, the expression of intestinal fatty acid – binding protein also presented an increasing tendency during larval development excepted at 5 and 10 DAH. In which, the highest expression level was noticed from 30 to 40 DAH after a significant decline at 5 – 10 DAH (Fig. 4.10).

4.3.3.5 Amylase

Amylase activity and expression of *amy2α* were detected from hatching and fluctuated complicatedly throughout the development of larvae (Fig. 4.11). Levels of amylase activity remained low until 15 DAH (0.5 – 1.2 mU/mg protein) before sharply increasing with a peak at 25 DAH (12.1 ± 1.3 mU/mg protein). Thereafter, dramatic declines of amylase activity were observed over until 40 DAH (3.5 ± 0.4 mU/mg protein; $p < 0.05$). Contradictorily, *amy2α* expression remained at high levels from first feeding until 25 DAH before dropping and recovering to the initial expression as at hatching (Fig. 11A). Moreover, *amy2α* expression was not significantly correlated to enzymatic activity ($R^2 = 0.0292$, $p = 0.637$; Fig. 4.11B).

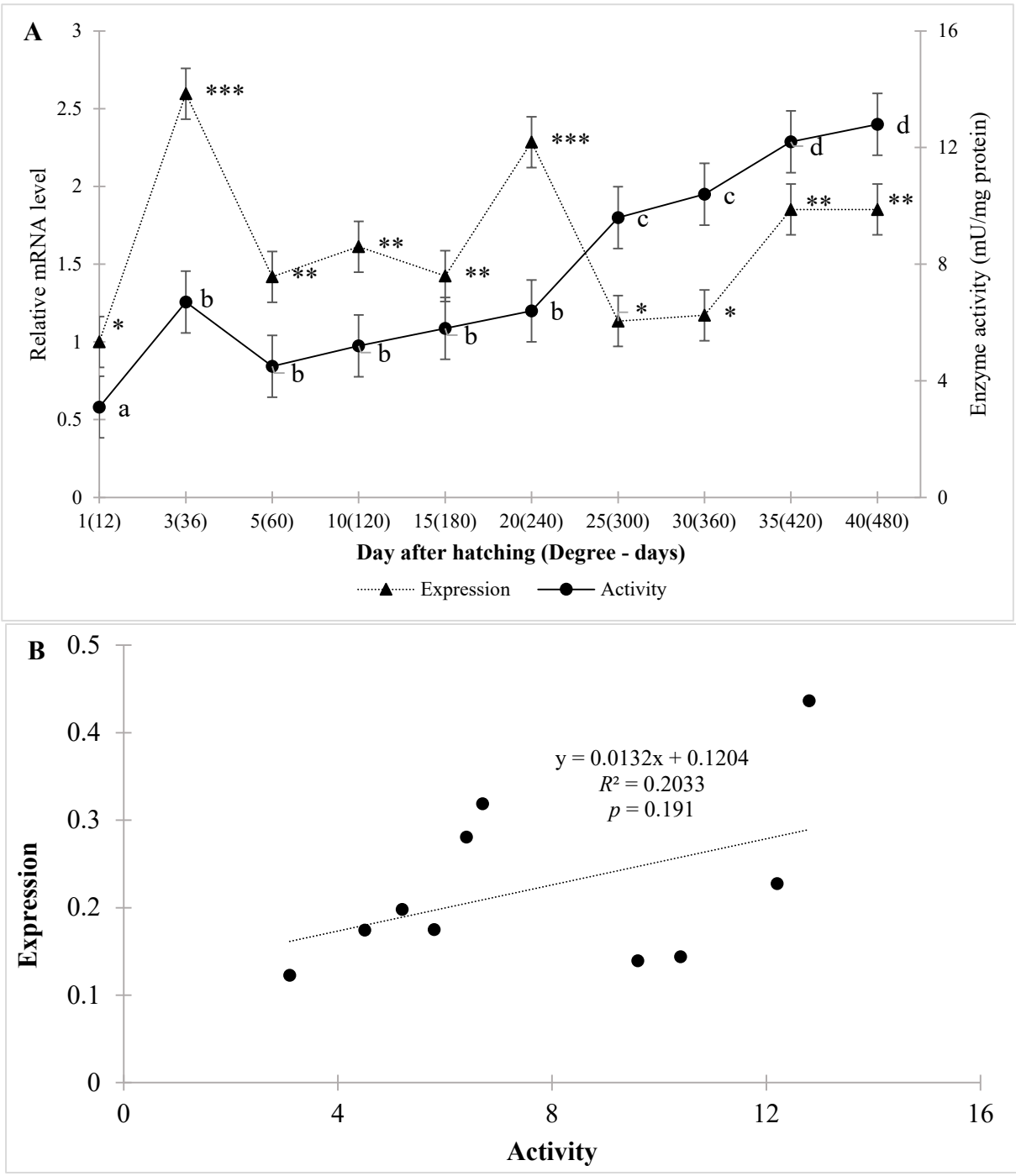


Figure 4. 9 Lipase expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate lipase activity. Dashed line and triangle plot indicate gene expression. Delineated are regression line and R^2-values. Expression of *pl* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of lipase activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

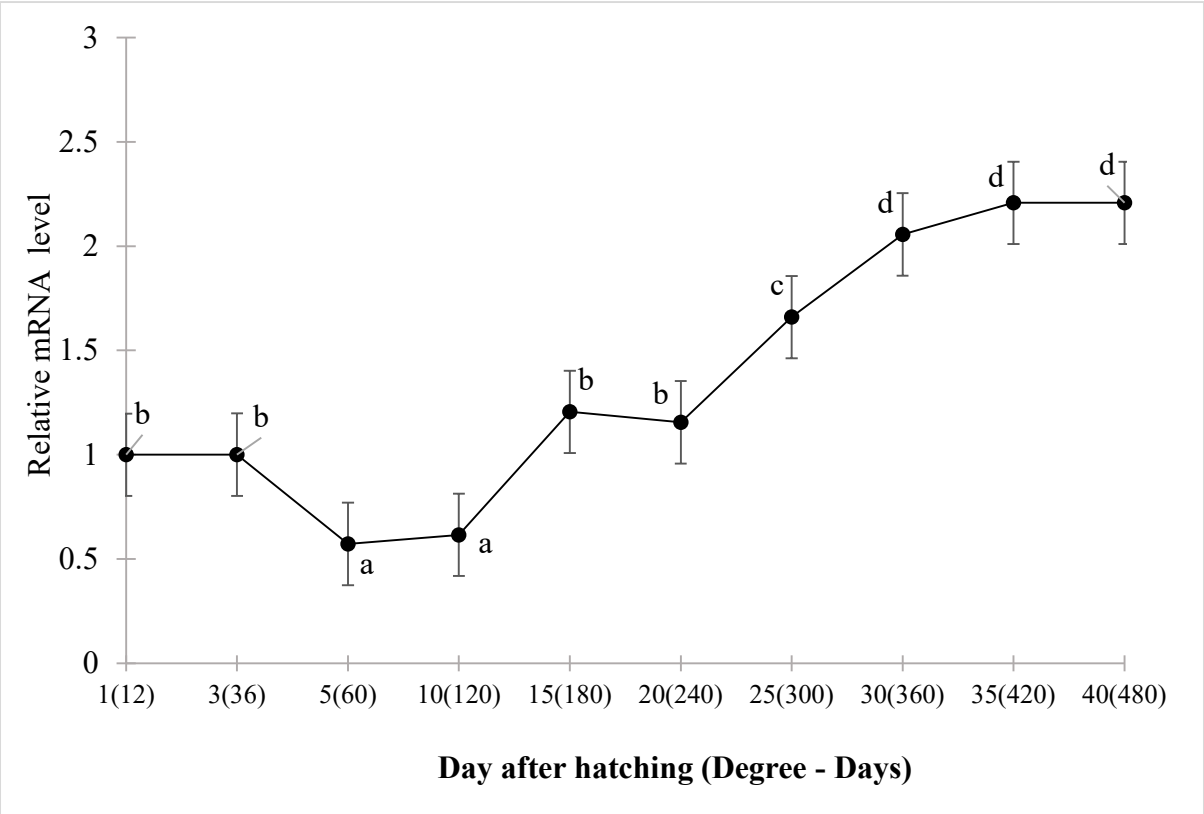


Figure 4. 10 I-FABP 1 expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of I-FABP 1 expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

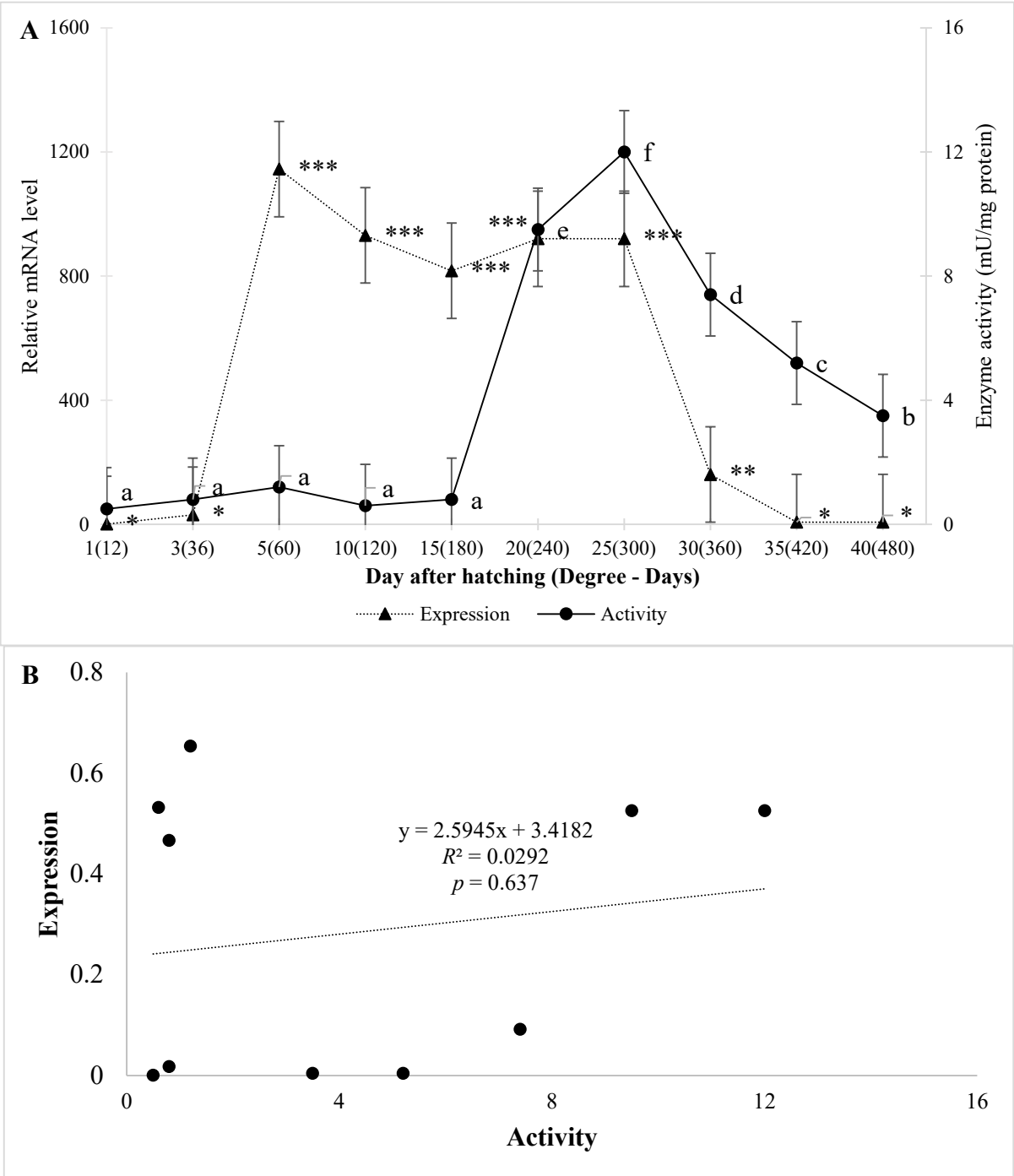


Figure 4. 11 Amylase expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate amylase activity. Dashed line and triangle plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *amy2α* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of amylase activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d < e < f, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

4.3.4 Energy metabolism related gene expression

Expression levels of *cox* during the ontogenic development of *P. olivaceus* showed a progressive increase (Fig. 4.12). At hatching, *cox* expression was at low level and significantly peaked when the larvae started endo-exotrophic stage (3-5 DAH; $p < 0.05$). However, the expression recovered to the initial level as at hatching and remained relatively constant until 25 DAH. From 30 DAH, *cox* expression increased continuously and significantly until 40 DAH ($p < 0.05$).

ATPase-pk expression presented a different pattern compared to *cox.* In which, the expression of *atp* was very low from hatching until 20 DAH. A remarkable increase at 25 DAH was noted ($p < 0.05$), but could not maintain due to a statistical drop in *atp* expression at 30 DAH. After the metamorphosis, the expression of *atp* was sharply raised until the end of the experimental time ($p < 0.05$; Fig. 4.13).

4.3.5. Growth related gene expression

igf1 expression of *P. olivaceus* drastically increased from hatching until the first feeding (3DAH). A dramatic drop in *igf1* expression occurred at 5 DAH ($p < 0.05$) and remained relatively constant until 20 DAH. However, a significant growth was remarked during the metamorphosis stage whereas the *igf1* expression sharply increased until 40 DAH ($p < 0.05$; Fig. 4.14).

On the other hand, expression of GH gene was very low at endogenous nutritional stage (0-3 DAH), but increased intensively thereafter and peaked 5 DAH ($p < 0.05$). The ontogeny was continued with a dramatic decrease of GH expression until 35 DAH. After the metamorphosis, the expression level of GH considerably recovered (Fig. 4.15).

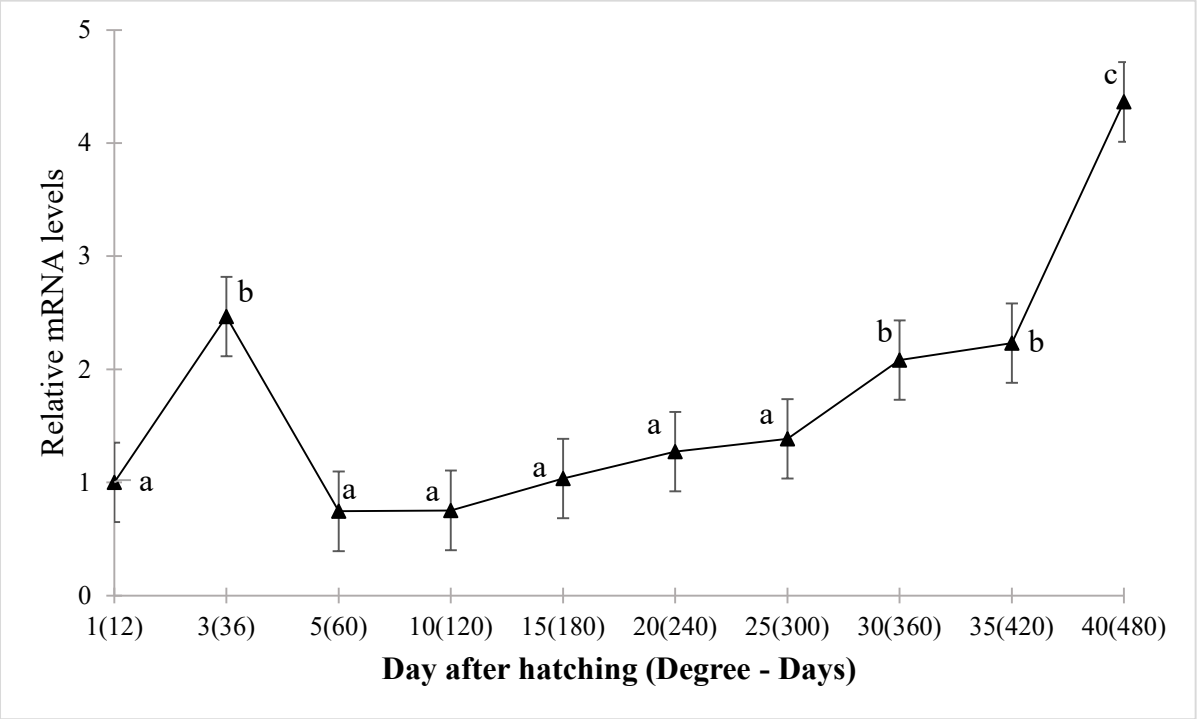


Figure 4. 12 *cox* expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of *cox* expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

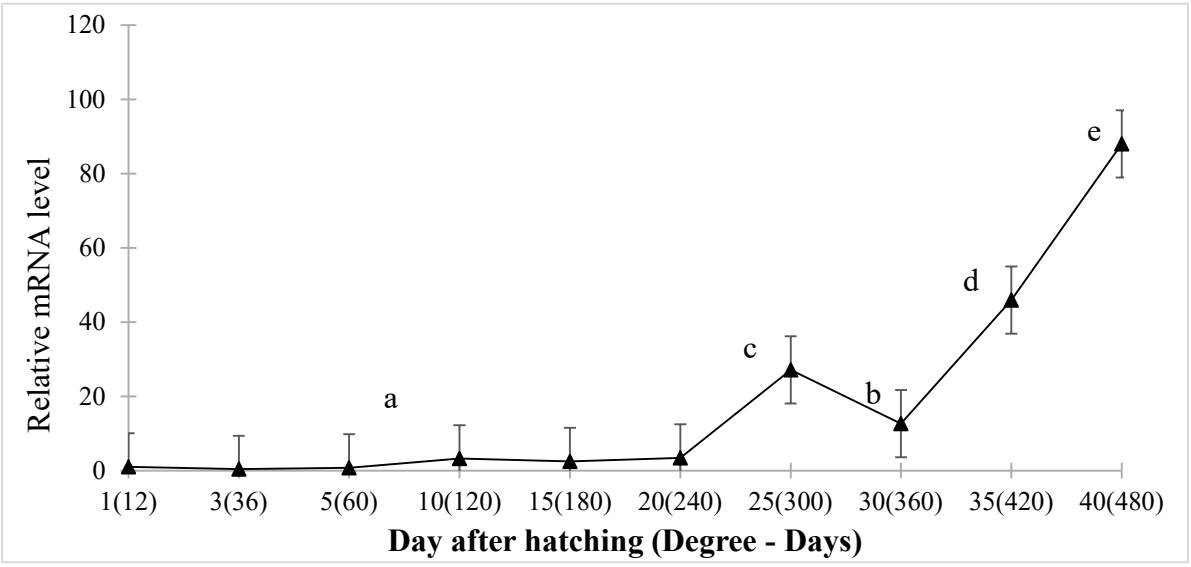


Figure 4. 13 *atp* expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of *atp* expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d < e, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

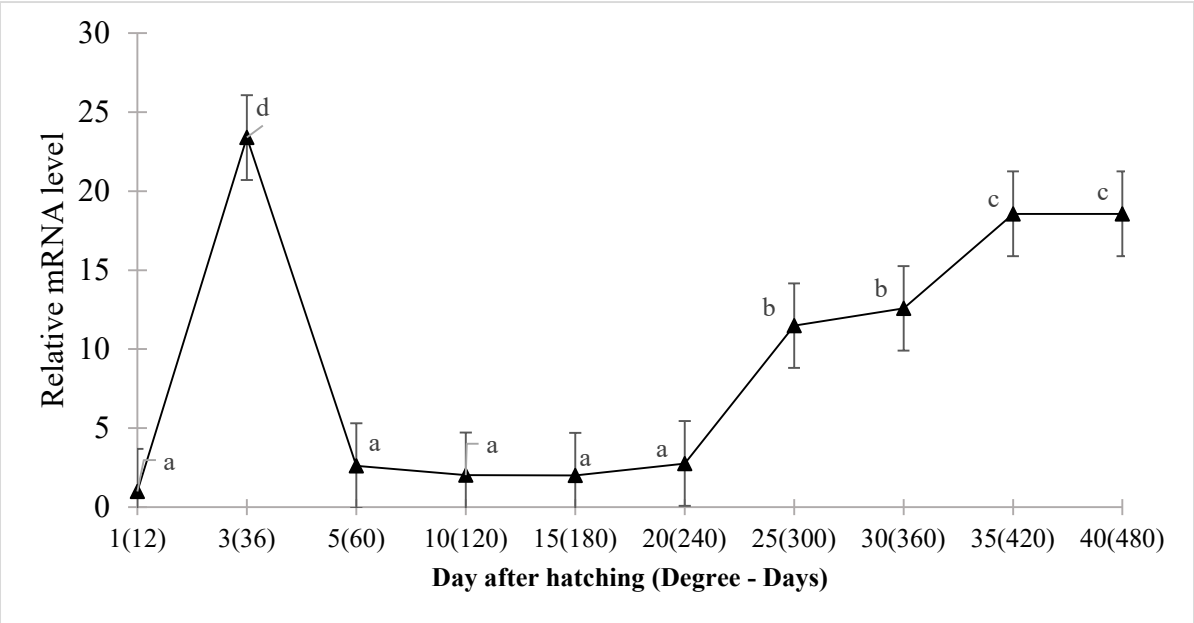


Figure 4. 14 *igf1* expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of *igf1* expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

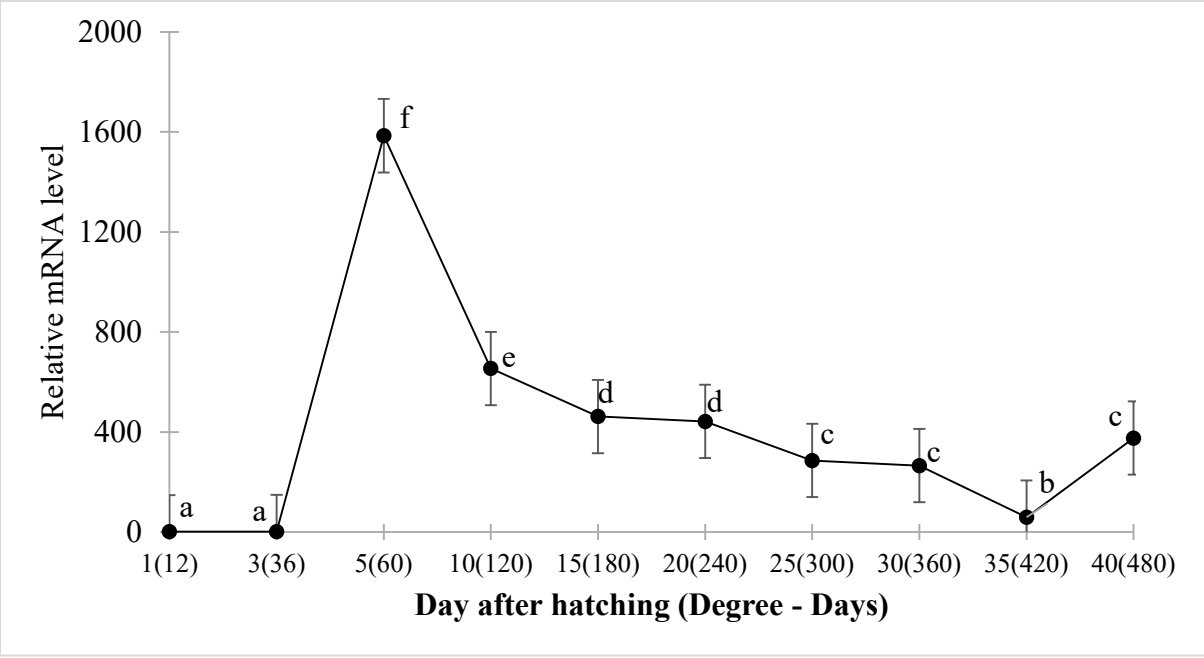


Figure 4. 15 GH expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of GH expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d < e < f, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

4.3.6 Appetite regulator related gene expression

Expression of *cck* gene was low and not significantly different changes from hatching until 25 DAH (Fig. 4.16). The expression level of *cck* strongly increased at 30 DAH ($p < 0.05$) followed by a decline at 35 DAH. After the metamorphosis, the *cck* expression rapidly increased over 40 DAH ($p < 0.05$).

On the contrary, *npy* gene showed a complexed fluctuation during the ontogenic development of *P. olivaceus* larvae (Fig. 4.17). During endogenous nutritional stage (0 – 3 DAH) and after metamorphosis (35 - 40 DAH), *npy* expression was low and no significant change was observed. From the first feeding, the expression levels of *npy* sharply increased but was not stable. In which, three peaks of *npy* expression were recorded at 5, 15 and 30 DAH followed by a dramatic drop at 35 DAH.

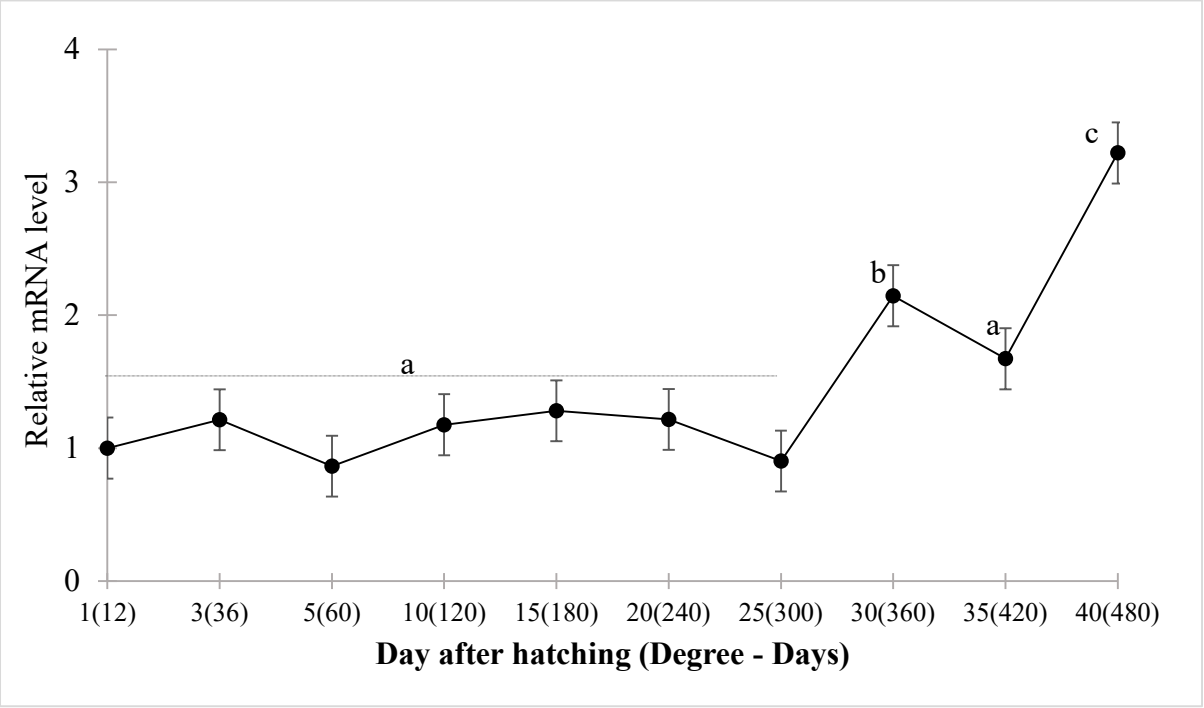


Figure 4. 16 *cck* expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of *cck* expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

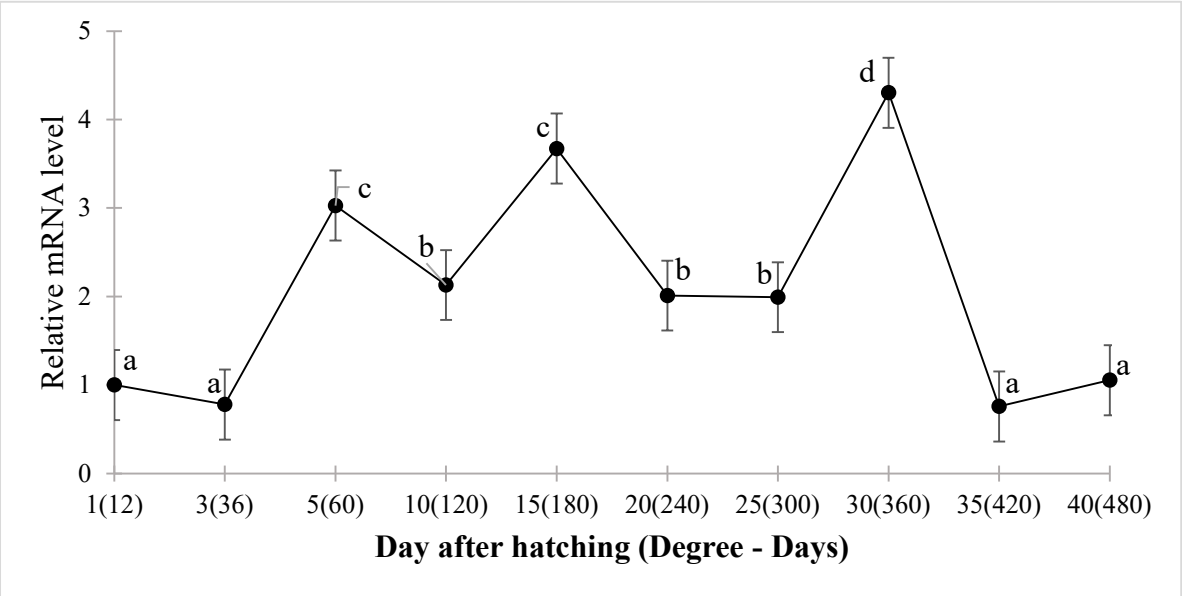


Figure 4. 17 *npy* expression during larval development under different diets. The data was presented as mean ± SEM. Alphabetical letters indicate significant difference of *npy* expression among developmental stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

4.4 Discussion

Knowledge on the ontogenetic development of fish digestive system during the larval stages is an invaluable tool in the optimization of feeding protocols and the improvement of mass seed production, whereas enhancing the survival rate, growth performance of larval fish (Gisbert et al., 2004; Lazo et al., 2011; Moguel-Hernández et al., 2016; Khoa et al., 2019; Khoa et al., 2020). This study illustrated the ontogenetic development of *P. olivaceus* through an integrated approach with observations of morphological-histological changes of the larval digestive system as well as the molecular-metabolic responses.

Despite low detections, the enzymatic activity of trypsin, chymotrypsin, lipase and amylase were detected as early as hatching. Pepsin, however, was only detectable for the first time at 20-25 DAH as a reflection of the development and functioning of the gastric glands at metamorphosis. Similar results were observed in some studies (Bolasina et al., 2006; Lee et al., 2015; Waqalevu et al., 2019). However a conflicting finding was reported by Tanaka et al.

(1996) where pepsin was detected at 5 DAH. This differing pepsin detection could be due to the amount of samples used during the enzyme assay (0.5 g of larval sample compared to 5 larvae in this study) which was elaborated in a study by Yúfera et al. (2018). Furthermore, it is more reasonable to consider that the error came from the sampling amount, pepsin could come from the contribution of live preys because gastric glands was firstly observed at the onset of metamorphosis (Tanaka et al., 1996). Evidence from histological observation, expression and activity of digestive enzymes from this study clearly demonstrated that the first functional stomach and pepsin activity appeared at the onset of metamorphosis (20-25 DAH). This was also supported in the study findings of Lee et al. (2015). Generally, the enzymes involved in hydrolysis of protein, lipid, amylase are genetically programmed and activated at hatching, with the exception of pepsin at 20-25 DAH coinciding with gastric gland visibility. Similar findings have been reported for several marine fish species such as white seabass (*Atractoscion nobilis*, Galaviz et al., 2011), Asian seabass (*Lates calcarifer*, Srichanun et al., 2013), golden pompano (*Trachinotus ovatus*; Ma et al., 2014)**,** rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2016)**,** meagre (*Argyrosomus regius*; Solovyev et al., 2016), crimson snapper (*Lutjanus erythopterus*; Cui et al., 2017), red seabream (*Pagrus major,* Khoa et al., 2019). However, due to a rudimentary digestive system at hatching, most of marine fish larvae possess insufficient digestive enzyme capacity to handle compound diets. Consequently, the marine larviculture still rely on the live feed at early stages (Zambonino-Infante et al., 2008; Qin, 2013, Rønnestad et al., 2013; Yúfera et al., 2018).

Trypsin and chymotrypsin are mainly involved with the hydrolysis of proteins by alkaline digestion until stomach function with pepsin activity (Rønnestad et al., 2013; Mata-Sotres et al., 2016; Yúfera et al., 2018). Trypsin and chymotrypsin data in the present study agreed with this hypothesis, with activity strongly increasing at first feeding (3-15 DAH; Fig. 4.6) but was reduced dramatically upon activation of pepsin at 25 DAH (Fig. 4.6, 4.7 and 4.8). Rønnestad

et al. (2013) and Khoa et al. (2019) highlighted that trypsin was the most important alkaline protease in fish larvae during its early stages and that it also reflects food presence for enzyme substrate activation and the nutritional status of *P. olivaceus* larvae. As such, it might be applied as a bio-indicator for evaluation of the nutritional response of larvae within the first 25 days post hatch. This is an important consideration as fish larvae require highly efficient digestive enzymes to facilitate rapid growth and ontogenetic development upon completion of their endogenous nutritional stage (Conceição and Tandler, 2018; Yúfera, 2018; Khoa et al., 2019).

According to Lee et al. (2015), the precursor of *try* and *ctrb* gene of *P. olivaceus* includes different isoforms and each isoform might display different expression profiles at a similar larval stage. Some delays in the translation of enzymatic precursors in this study were also observed (Fig. 4.6 – 4.9) and could possibly explain the weak correlations between expression and activity of digestive enzymes in this study. This issue has been similarly reported by Srichanun et al. (2013) on seabass, rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2016) and Khoa et al. (2019, 2020) on *Pagrus major*. Therefore, to illustrate the real digestive capacity of fish larvae through related gene expressions, it required the investigation of the isoform expression profiles in a correlation to enzymatic activity.

During early ontogeny*, P. olivaceus* larvae pass through major morphological changes (Minami, 1982) and survival rates was also observed to reduce accordingly (Fig. 4.2). This study recorded two critical ontogenetic stages at 3-5 DAH and 20-35 DAH, whereby the larval survival rate dramatically dropped (from 80.5 % to 70.8 % and from 65.2 % to 28.6 %, respectively). The high mortalities of *P. olivaceus* larvae within these periods have been mentioned by Ground et al. (1989) and Hossain et al. (2003) due to the transition from endogenous to exogenous nutrition stage (3 - 5 DAH), early phase of metamorphosis and settlement (20-35 DAH). In which, Hossain et al. (2003) suggested that a lack of energy reserves accumulated during the pre-metamorphosis might have led to a shortage of stored

energy for metamorphosis. Besides, the high mortality during the first week of larval development may not be necessarily due to physiological or digestive deficiencies because the nutritional and physiological performance of fish larvae were displayed through substantial metamorphoses in morphology and physiology (Conceição et al., 2008; Conceição and Tandler, 2018; Khoa et al., 2019).

One of the most important points is the detection of pepsin and stomach functions to determine the weaning time from the live feeds to micro-diets (Parma and Bonaldo, 2013). In Pleuronectiformes fishes, previous studies reported that there were possible to change to formulated inert diets before metamorphosis (20-30 DAH) without significant effect on survival, however, weaning after eye migration resulted in a better growth (Rosenlund et al., 1997; Hamre et al., 2001; Faulk and Holt, 2009; Parma and Bonaldo, 2013). Wang et al. (2004) applied micro-diets and co-feeding for *P. olivaceus* larvae from day 3-10 DAH, but the larval survival was limited compared to live feed. The lack of pepsin and acid digestive association have been previously discussed to be important reasons in insufficient digestive capacity of micro-diets by larval fishes (Conceição and Tandler, 2018). Therefore, pepsin in combination with other pancreatic enzymes could assist in the efficient processing and hydrolysis of proteins (Rønnestad et al., 2013). Based on our results, it can be reasonably stated that weaning onto micro-diets of *P. olivaceus* larvae can begin from day 20 DAH, however, to secure the survival and growth rate, co-feeding protocols of live feed and micro-diet should also be considered (Hamre et al., 2013; Khoa et al., 2020).

Lypolytic enzymes in *P. olivaceus* were detected at hatching, which are vital in assisting the larvae digest lipids. In which, bile salt activated lipase secreted from the pancreas was considered the most important lipase in teleosts (Gjellesvik et al., 1992; Hoehne-Reitan et al., 2001; Murray et al., 2003). In this study, the activity of lipase gradually increased but remained low until 20 DAH and strongly increased from 25 DAH (Fig. 4.9A). Additionally, Bolasina et

al. (2006) reported that neutral lipase in whole body fish of *P. olivaceus* larvae sharply increased at pre-metamorphosis stage (12-20 DAH), followed by a decline of neutral lipase during metamorphosis (20-35 DAH). Accordingly, neutral lipase level was related to the lipid metabolism reserve accumulated during pre-metamorphosis and the larvae would mobilize this energy source for morphological alterations (Martínez et al., 1999; Yúfera et al., 1999; Bolasina et al., 2006). Our results suggest that the high mortality observed between 20-35 DAH may possibly be related to a shortage of energy reserves. Low pancreatic lipase and lipid metabolism at pre-metamorphosis could have possibly led to the lack of sufficient energy provision at metamorphosis; a similar hypothesis was postulated by Hossain et al. (2003). As such, further studies are required on the effects of high energy or lipid diets suitable for *P. olivaceus* larvae prior to metamorphosis at 20 DAH.

In this study, lipase and I-FABP were synthesized before hatching to assist the nutrition absorbance from the yolk. Thereafter, the transcript levels of *pl* and I-FABP intensified at pre-metamorphosis to reserve energy. The developmental progress was continued with a sharply increase in lipase activity and I-FABP levels until the metamorphosis completed (35 DAH) and remained constantly over the experimental time (Fig 4.9A and Fig. 4.10). Lipids and amino acids are considered to be the main energy source during endogenous nutritional stages of fish larvae (Watanabe, 1982; Finn et al., 1991; Zhu et al., 2003). In which, *n*-3 highly unsaturated fatty acids (HUFA) are indispensable for improved fish survival and growth (Lee et al., 2015; Rønnestad et al., 2000). Additionally, I-FABP played a crucial role in free amino acid uptake in the fish intestine (Her et al., 2003; Esteves et al., 2016). Our trends for lipase and I-FABP levels were similar as reported of Srivastava et al. (2002) and Lee et al. (2015), except after the metamorphosis period. This difference might be due to differences between live feed and micro-diet feeding strategy used in each respective study. Moreover, the similarity in the correlated fluctuation patterns of lipase and I-FABP was also remarked.

Amylase displayed complex fluctuation patterns in both expression and enzymatic activity. According to Tanaka et al. (1996) and Li et al. (2013), amylase activity of *P. olivaceus* larvae is normally low at hatching and gradually increases until pre-metamorphosis, with some marine fish species reaching peak activity at this stage (Zambonino Infante and Cahu, 2007). A strong peak of amylase activity could be due to re-feeding after the cessation of feeding (Tanaka et al., 1996; Bolasina et al., 2006; Li et al., 2013). However, the activity of pancreatic enzymes including amylase in this study dramatically dropped after settlement (30-35 DAH) and juvenile turned into its carnivorous habits (Fig. 4.11A). Non-feeding and reducing pattern of pancreatic enzymes during metamorphosis have also been reported in flat fish larvae (Tanaka et al., 1996; Gwak et al., 1999; Kolkovski, 2001), therefore, the feeding protocols should be based on the digestibility of feeds for larval fish according to its developmental stages to avoid mortality.

The expression of related genes to energy metabolism showed different sensitive expression profiles reflective to the nutritional status of fish. In which, *cox* gene expression was more sensitive to feeding diet change than *atp* gene. Accordingly, *cox* expression level peaked when the larvae started the exogenous feeding and slightly increased when larvae fed *Artemia* (Fig. 12) while *atp* level remained low constantly for the first 20 DAH (Fig. 4.13). This trend suggests that the *cox* gene is mainly involved in energy metabolism during the first 20 DAH of *P. olivaceus*, thereafter, the two genes play different roles during the metamorphosis stage. From 20–25 DAH, *atp* expression strongly increased which indicates an increase in the stored energy consumption while *cox* levels increased to keep energy status stable until the end of metamorphosis. A similar mechanism of *P. olivaceus* stocked in cold temperature conditions was reported by Lu et al. (2018). After metamorphosis, larvae fed strongly again led to peaks of enzyme activities, and the expression levels of *atp* and *cox* strongly recovered to provide energy for fish performance (Jesper et al., 1996; Gjevre and Næss, 1996; Dalziel et al., 2006).

The growth hormone (GH) and insulin-like growth factor (*igf1*) have been considered as promising indicators in evaluating fish instantaneous growth (Picha et al., 2008; Fuentes et al., 2013; Pérez-Sánchez et al., 2018). In the present study, *igf1* expression sharply peaked from hatching to 3 DAH while GH expression levels were low during this stage. The reserve trends in expression of GH and *igf1* has been described for many species (Davey et al., 2001; Inagaki et al., 2008; Fuentes et al., 2013), in which, GH was transcribed and translated at early stages of fish development to regulate growth and metabolism, especially *igf1* (Davey et al., 2001; Bertucci et al., 2019). GH peaked at 5 DAH coinciding with a drop of *igf1*, highlighting that these factors balance each other to maintain stable larval growth rate until 20 DAH (Fig. 4.14 and 4.15). Additionally, a delay in growth was noticed between 25-30 DAH through expression levels of GH and *igf1*. These results molecularly supported reports on growth delay in *P. olivaceus* (Bolasina et al., 2006; Lee et al., 2015), common sole (*Solea solea*) (Parma et al., 2013) during metamorphosis. Growth performance of larval fish was affected by several internal and external factors (Bertucci et al., 2019); besides the specific growth rate in body length and weight of fish; GH and *igf1* could be applied as possible markers for evaluation of growth rate. Such considerations will reduce the need for lengthy and costly grow-out trials.

The present study did not focus in detail on the complex regulations among peptide, gut contents and digestive enzyme levels but to understand its fluctuations throughout ontogeny alongside key digestive enzymes trends. *cck* is expressed in both the brain and intestinal organs (Suzuki et al., 1999), whilst *npy* expression is only detectable in the brain and retina of *P. olivaceus* (Kurokawa and Suzuki, 2002). Functions of *cck* and *npy* has been detected in fish larvae at hatching and is known to be regulated during exogenous feeding (Kurokawa and Suzuki, 2002; Polakof et al., 2011; Khoa et al., 2020). In this study, *cck* expression remained at low levels during the first 25 DAH and increased after metamorphosis, and *npy* expression pattern was relatively similar to *try* (Fig. 4.6, 4.16 and 4.17). According to Tillner et al. (2013),

cck plays a catalytic role in the regulation of digestion of fish with a negative feedback mechanism to trypsin. The results in this study were in line with this suggestion. However, the levels of *npy* was different to *cck,* but relatively mirrors *try* pattern. For this aspect, Kurokawa and Suzuki (2002) reported that Japanese flounder larvae has three main neuropeptide Y (NPY)-related peptides that control several physiological functions in fish brain, including maturation and food intake (Peng et al., 1990; Silverstein et al., 1998). Peptide Y has a dual role as a neuropeptide and a digestive hormone while NYP and PYY mainly expressed in brain (Kurokawa and Suzuki, 2002). The limitations of this study were using pooled - whole fish samples of RNA extraction and collecting samples once a day - point, hence, the data could not explain the peptide regulation through the signals of nutrients, hormones and neural pathways. Further detailed studies are recommended in order to better address these functions and understand the complex regulations between each peptide in accordance with physiological development and digestive enzymes.

P. olivaceus larvae took 35 days to complete ontogenetic development of its digestive system, thereafter transitioning to settlement stage. The function of stomach and pepsin were detectable at 25 DAH through histological, molecular and biochemical approach. The expression and activities of targeted digestive enzymes were age-dependent and fluctuated with remarkable drops at metamorphosis. High mortality occurred during the transition of exogenous feeding and metamorphosis. mRNA of genes related to energy metabolism, growth and peptide were transcribed and translated at the early stage to assist and regulate exogenous feeding and growth performance of fish larvae. Findings from this study provide a more holistic insight into morphological–functional changes during early ontogenetic development of Japanese flounder larvae for the optimization of feeding and rearing protocols to minimize feed-related and metamorphosis-related mortalities.

CHAPTER 5: Enrichment effects of Shochu distillery by product on *Brachionus plicatilis* sp. rotifer and larviculture performance in the Japanese flounder (*Paralichthys olivaceus*).

Abstract

The effective use of distillery by-products of the shochu sweet potato has long been considered as a rich source of protein, fiber, amino acids, fatty acids, probiotics with a great potential as a functional feed for animals. This study applied Shochu distillery by-product (SDBP) and salmon roe emulsion oil for rotifer enrichment to evaluate its effects on the: (i) nutritional composition of rotifers and (ii) feeding responses of *Paralichthys olivaceus* larvae when fed with enriched rotifers. The salmon roe oil enrichment was set as a control diet and SDBP was supplemented at different doses (1, 5 and 10 % based on the salmon roe oil amount). The results showed that salmon roe oil enriched rotifer supplemented with SDBP at 5 and 10 % significantly enhanced protein, carbohydrate, and HUFA content. Similarly, the activity and expression profiles of targeted enzymes including trypsin, chymotrypsin, lipase and amylase effectively responded at early and middle-metamorphosis of the larvae to 5 and 10 % enrichment regiment. These two treatments were also found to be better supported in energy metabolism evidenced by *cox* and *atp* expression in the larvae. Cessation of feeding and delay in growth during the metamorphic process was also molecularly demonstrated. Larvae suffered low survival (24.5 – 25.3 %) in control and 1 % supplementation of SDBP, but significantly improved at 5 and 10 % (36.4 - 38.7%). However, the growth performance of larval fish was not remarkable after 40 days of culture. The results of this study revealed that SDBP could be considered as a potential supplementary enrichment for live feed in fish larviculture, especially at 5 and 10 % rotifer enrichment dosage.

5.1 Introduction

Nutrition during early stages of fish larvae is an important consideration in optimizing rearing protocols for marine fish larviculture (Planas and Cunha, 1999; Hamre et al., 2013; Conceição and Tandler, 2018). Due to major changes in morphology and functions of the digestive system during ontogeny, marine fish larvae are very vulnerable and require certain biotic and abiotic conditions to ensure survival and proper development (Hamre et al., 2013; Rønnestad et al., 2013). Fish larvae require feeds that provide high energy, essential fatty acids, and protein that are appropriate in proper size, shape and easily palatable (Ozkizilcik, 2001; Takeuchi, 2014). Live feed such as rotifers and *Artemia* are usually the most common primary feed in marine larviculture due to their acceptable price and quality, possibility for mass culture and manipulation of its nutritional qualities (Sorgeloos et al., 2001; Dhert et al., 2001; Kotani et al., 2009; Waqalevu et al., 2019). However, several micronutrients such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), vitamins and minerals are still insufficient in rotifer and *Artemia* and have been observed to be unable to fulfill nutritional requirements of marine fish larvae, leading to high mortality and low growth rates (Hamre et al., 2008; Dhaneesh and Ajith Kumar, 2017). Various live feed enrichment methods have been developed to optimize nutrient compositions and enhance growth and survival rates of fish larvae. As such, many commercial enrichment media products for live feed have demonstrated the enrichment of protein, lipids and amino acid (Kotani, 2017; Eryalcin, 2018; Waqalevu et al., 2019), vitamins (Dhert et al., 2001; Srivastava et al., 2011; Negm et al., 2013), minerals (Matsumoto et al., 2009; Nordgreen et al., 2013) or probiotic (Patra and Mohamed, 2003; Jamali et al., 2015).

Moreover, the digestive ability, survival rate, and growth performance of fish larvae indicate the acceptance of larvae on the enriched live feed (Cahu et al., 2003; Waqalevu et al., 2019). In which, the biochemical quantification of digestive enzymes was previously applied as an effective method to access the digestive capacity of fish larvae (Lazo et al., 2010; Yúfera

et al., 2018) and the gene expression and activity of digestive enzymes were important indicators to evaluate the nutritional condition of fish (Lazo et al., 2010; Yúfera et al., 2018). Besides, insulin-like growth factor (IGF) and growth hormone (GH) levels have been considered as promising indicators in evaluating fish instantaneous growth (Picha et al., 2008; Fuentes et al., 2013; Pérez-Sánchez et al., 2018). Therefore, the combination of biochemical and molecular approaches could provide insights on the regulation mechanisms and digestive functions of fish as well as their changes under different nutrient factors.

Sweet potato has been reported as a rich source of dietary fiber, vitamins, phenolic compounds, antioxidants and carotenes (Fanta and Neela, 2019; Zhang et al., 2019b) and has since been widely applied as animal feed (Campbell et al., 2017). In Kagoshima prefecture, Japan, sweet potatoes are the main ingredient in the production of Shochu liquor. After Shochu distillery processing, large amounts of sweet potato distillery waste are discharged which can create a serious environmental problem (Mahfudz et al., 1996; Nakano et al., 2010). According to Nakano et al. (2010) and Campbell et al. (2017), the fermentation of sweet potato with rice and beneficial microbes could enhanced protein, fiber, amino acids and fatty acids levels in feed giving it a great potential as probiotic enriched and functional feed. Several studies have been conducted to reuse sweet potato distillery waste as feed for animals and fish (Mahfudz et al., 1996; Ohtsuka et al., 1997; Ultana et al., 2013; Zhang et al., 2019a; Zhang et al., 2019b; Fanta and Neela, 2019). Ultana et al. (2013) suggested that the waste product after Shochu distillation could be used a supplemented feed to enhance the growth rate of juvenile red seabream (*Pagrus major*). This is a potential functional compound feed source for aquatic animals however, to date there is little known literature and application in the aquaculture industry on the uses of Shochu distillation waste, especially in larval rearing.

Japanese flounder (*Paralichthys olivaceus)* has been commercially produced in the coastal zone of Japan, Korea and China due to its high market value (Kikuchi and Takeuchi,

2002; Seikai, 2002). The larval rearing techniques of this species still heavily relies on live feeds (Bai and Lee, 2010) using various commercial enriched media consisting of DHA-enriched *Chlorella vulgaris,* salmon roe oil and taurine (Chen et al., 2005; Lee et al., 2015; Waqalevu et al., 2019). According to Waqalevu et al. (2019), rotifer enriched with salmon roe oil and DHA-enriched *C. vulgaris* showed high content of high unsaturated fatty acid (HUFA) and eicosapentaenoic acid (EPA), however, the population and egg bearing ratio of rotifer declined after enrichment. Furthermore, *P. olivaceus* larvae fed with this enriched rotifer showed negative responses in survival, growth and enzyme activity due to too high a lipid content, short retention period of nutrients and poor water quality (Dhert et al., 2001). In this study, the Shochu distillery by-product (SDBP) was applied as a supplement along with salmon roe oil emulsion for rotifer enrichment in the larviculture of *P. olivaceus*. The purpose of this study was to evaluate the nutritional composition of *Brachionus plicatilis* sp. complex rotifer enriched with SDBP and salmon roe oil and to assess its impacts on feeding response and performance of *P. olivaceus* larvae to determine the appropriate doses of SDBP enrichment appropriate for *P. olivaceus* larvae at early stages.

5.2 Materials and methods

5.2.1 Shochu distillery by-product (SDBP) preparation

The SDBP was provided by Shochu liquor factory (Kagoshima Prefecture, Japan). The waste was centrifuged at 3500 x g for 10 min to remove the supernatant. Thereafter, the waste was dried using a freeze dryer (EYELA FDU-1200, Tokyo Rika Kikai Co., Ltd, Japan) for 24 h at 15 kPa absolute reduced pressure. The dried SDBP was in powder form and stored at -80 ºC prior to experimental use.

5.2.2 Experiment design

This experiment included four rotifer enrichment treatments using salmon roe emulsion

oil and SDBP; (Control) Salmon roe emulsion oil only; (S1) Salmon roe emulsion oil + 1 % SDBP; (S5) Salmon roe emulsion oil + 5 % SDBP and (S10) Salmon roe emulsion oil + 10 % SDBP. The dosage of salmon roe oil used was 0.03 g/L of enriched volume as per manufacturers recommendations and this dosage was similar in all treatments. The addition of SDBP was calculated based on percentages of the dosage of emulsion oil. Each larval rearing treatment was replicated 3 times and all culture conditions maintained uniformly. Water quality parameters (pH, salinity, dissolved oxygen and temperature) were monitored twice daily before feeding.

5.2.3 Rotifer culture and enrichment

L-type *Brachionus plicatilis* sp. complex rotifer Obama strain was stock cultured in a continuous system at 20 ± 1 psu of salinity using fresh water chlorella *C. vulgaris* (Fresh Chlorella V-12, Chlorella Industry Co. Ltd., Tokyo, Japan) following a protocol described by Kotani et al. (2009) and Khoa et al. (2019). For enrichment, rotifers were enriched in 5 L beaker with trial dosages of salmon roe emulsion oil (MarineTech Co., Ltd., Japan) and SDBP. The 5 L enrichment cultures were enriched twice (0800, 1700) and were harvested 24 h after inoculation before being collected and washed carefully with fresh water before feeding.

5.2.4 Fish larvae rearing

Japanese flounder fertilized eggs were bought from MBC Kaihatsu Co. Ltd., in Kirishima, Kagoshima Prefecture, Japan and transferred to the Aquaculture Research Center at Kagoshima University, Japan. A total of 2,000 eggs were stocked in 100 L transparent composite tanks with continuous aeration and 35 psu of salinity. Temperature in the rearing system was maintained at 15 ºC using a titanium heater (Nittokizai Corporation, Saitama, Japan) to heat water in a 500 L reservoir tank, which was then continuously supplied to the larval rearing tanks. Rotifers were added into the rearing tanks at 3 days after hatching (DAH) upon larval

mouth opening until 18 DAH. Rotifer density in the rearing tanks were maintained at 5 ind./mL. The larvae were alternatively fed *Atermia* sp (Great Salt Lake Artemia, Ogden, UT, USA) nauplii at 3 ind./mL of density at 18-21 DAH before switching to adult *Artemia* from 21 DAH. The density of rotifer and *Artemia* in the cultured tanks were checked twice a day at 0800 and 1500. At the onset of exogenous feeding (3 DAH), a water exchange rate of 300 %/day was applied to maintain water quality in the rearing tanks and this was achieved via siphoning through a drain hose, screened through a 0.2 mm polyethylene mesh (Khoa et al., 2019).

5.2.5 Sample collection

The fish sampling was conducted at 1, 3, 5, 10, 15, 20, 25, 30, 35 and 40 DAH. Larvae were randomly collected at 2 h after feeding in triplicates for different analyses. The samples were rinsed in distilled water, then preserved in *RNA*later® solution (QIAGEN GmbH, Germany) overnight at 4 ºC and stored at -20 ºC for gene expression quantification. For enzyme activity, larval samples were stored at -80 ºC after washing. At the end of the experiment, larvae were collected and preserved at -80 ºC for the biochemical compositions (crude protein, crude carbohydrate, lipid, fatty acids and free amino acids). The Shochu waste, enriched rotifer and *Artemia* samples were also analyzed for the above-mentioned biochemical parameters. In addition, 15 larvae were also sampled from each treatment to measure total length, body weight and 15 larvae were fixed with 70 % alcohol for gut content analysis (Supplementary 5.3).

5.2.6 Quantitative real time PCR

Total RNA extraction was taken from a pooled sample of 5 larvae in triplicates using TRIzol™ reagent (Thermo Fisher Scientific, Invitrogen, USA) following the manufacturer's protocol. The RNA integrity was verified with 1% agarose gel electrophoresis. The quality and concentration of RNA were determined by optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). The

samples with an absorption ratio ranged between 1.9 and 2.0 was used for cDNA synthesis (100 ng of RNA) following the iScript ™ cDNA Synthesis protocol (Bio-Rad Inc., Hercules, CA, USA).

The quantitative real time PCR was carried out to determine the expression level of related genes for digestive enzymes (trypsin 3 (*try3*), chymotrypsin 2 (*ctrb2*), pepsin (*pep*), pancreatic lipase (*pl*), amylase (*amy2α*)), energy metabolism (Cytochrome-C-Oxidase (*cox*), ATPase-pk (*atp*)), cholecystokinin (*cck*), growth (insulin-like growth factor 1 (*igf1*)). The primer sequences were listed in Table 5.1. Each sample was performed in triplicate in a Biorad CFX connect^{TM} Real time system (Bio-Rad Laboratories, Inc., Japan). PCR reaction consisted 1 µL of cDNA template, 0.4 µL of each primer, 10 µL of qPCR Mix, and 0.4 µL ROX reference dye (KOD SYBR® qPCR Mix, Toyobo Co. Ltd, Osaka, Japan) in a final 20 µL reaction. The thermal profile was 98 °C for 2 min; followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. The melting curve was generated from 60 °C to 95 °C with an increment of 0.3 °C and holding for 15 s. The expression levels of targeted genes were normalized to the expression of β-actin and elongation factor-1 (*ef1*) and measured using the standard curve with diluted cDNAs.

Table 5. 1 The sequence of primers for qRT-PCR

Gene	Primer forward	Primer reverse	Source
try3	TATGAGTGCACGCCCTACTC	GTTCTCACAGTCCCTCTCAGAC	Lee et al., 2015
ctrb2	ACTACACCGGCTTCCACTTC	GAACACCTTGCCAACCTTCATG	
pep	TGAGTCCATGACTAATGATGCT	CACCTGATTGGCCACAGAG	
pl	ATGGGAGAAGAAAATATCTTATTTTTGA	TACCGTCCAGCCATGTATCAC	
amy2α	CACTCTTCATGTGGAAGCTGGTTC	CCATAGTTCTCAATGTTGCCACTGC	Moguel-Hernández et al., 2016
cox	AAATCAACGACCCCCACCTC	CGGGGATTGCTCAAGAGTGT	Lu et al., 2018
atp	GTCAAAGAAGTCGGCTCGGA	GTCAGCTGCCTTACGGATGA	
β-actin	GGAATCCACGAGACCACCTACA	CTGCTTGCTGATCCACATCTGC	
igf1	GCCACACCCTCTCACTACTGCT	GCCTCTCTCTCCACACACAAAC	Zheng et al., 2012
cck	TGCTGCAAGCTTCAACGC CC	TTGATGACACCGACAGCCAC	Suzuki et al., 1999
ef1-α	TGCTGCAAGCTTCAACGCCC	TTGATGACACCGACAGCCAC	Srivastava et al., 2002

Noted: ***try3***: trypsinogen 3; ***ctrb2***: chymotrypsinogen 2; ***pep***: pepsinogen; ***pl***: pancreatic lipase; ***amy2α***: amylase; ***cox***: Cytochrome-C-Oxidase; ***atp***: ATPase-pk; ***cck***: cholecystokinin; ***igf1***: insulin-like growth factor 1; **β-actin**: beta actin; ***ef1***: elongation factor-1

5.2.7 Enzyme assay

Pooled samples of 5 larvae were homogenized in 100 µL of ice-cold buffer (20 mM Tris-HCl, 1 mM EDTA, 10 mM $CaCl_2$, pH 7.5) to extract crude enzyme (Bolasina et al., 2006) using a pellet pestle cordless motor (Sigma-Aldrich Inc., St. Louis, MO, USA). After homogenization, the pestle tip was rinsed with 400 µL of homogenization buffer, thereafter, the tubes containing 500 µL homogenate were centrifuged for 30 min at 1700 x g at 4 °C. The supernatant was transferred into new 1.5 mL tubes and was used as the crude enzyme extract in protein content and enzymatic assay analysis later (stored at -80 ºC). The extraction was conducted in triplicate for each sampling point. Bradford method (Bradford, 1976) was applied to determine the protein content of samples.

5.2.7.1 Trypsin

The activity of trypsin was measured using Z-L-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA, Sigma-Aldrich Inc., C9521) as a substrate (Bolasina et al., 2006). The reaction contained 500 µL of substrate (containing 50 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ- L Arg-MCA) and 50 µL of crude enzyme was incubated for 30 min at 30 °C in a water bath, followed by adding 100 µL of 30 % acetic acid for quenching. The fluorescence was read using a Hitachi F2000 spectrofluorophotometer (Tokyo, Japan) at 380 nm of excitation and 440 nm of emission. The activity of trypsin was expressed in unit (mU) in 30 min, as percentage increase of emission per protein (mU mg protein −1).

5.2.7.2 Chymotrypsin

Chymotrypsin activity was determined using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPFNA, Sigma-Aldrich Inc.) as a substrate (Murashita et al., 2018). Briefly, the reaction mixture was prepared by adding 50 µL of crude enzyme to 240 µL of 100 mM Tris buffer (pH 8.5, containing 20 mM $CaCl_2$) and 100 µL 2.4 mM SAPFNA, followed by incubation for 7 min

at 37 ºC. Production of *p*NA was measured at 405 nm using a Hitachi U5100 spectrophotometer. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in 1 min.

5.2.7.3 Pepsin

The activity of pepsin was assayed using 2 % haemoglobin in 0.06 N HCl as a substrate (Worthington, 1993; Natalia et al., 2004). A total of 100 µL of enzyme extract was mixed with 500 µL of substrate, then was incubated at 37 ºC for 10 minutes. The reaction was terminated by adding 1 mL of 5 % trichloroacetic acid and left for 5 min. Following the centrifugation for 5 min at 12000 x g, the absorbance was read at 280 nm using a Hitachi U5100 spectrophotometer. For blank reading, the enzyme extract was added after trichloroacetic acid.

5.2.7.4 Lipase

The activity of lipase was determined using *p*-nitrophenyl myristate (PNPM, Sigma-Aldrich Inc.) as a substrate (Albro et al., 1985). The reaction was prepared by adding 450 µL of 100 mM Tris–HCl buffer (pH 8), 100 µL of 3.5 mM PNPM (containing 0.5 % Triton X-100, Nakarai, Osaka, Japan) and 50 µL of enzyme extract, then incubated for 7 minutes at 37 ºC. The production of *p*-nitrophenol (*p*NP) was measured by Hitachi U5100 spectrophotometer at 405 nm.

5.2.7.5 Amylase

Amylase activity was evaluated using 1 % starch solution as a substrate (Murashita et al., 2018). A total of 50 µL of enzyme extract was mixed with 25 µL of 20mM sodium phosphate buffer (containing 6.0 mM NaCl, pH 6.9) and 25 µL of the substrate solution, followed by incubation at 37 ºC at 60 min. Subsequently, 50 µL dinitrosalicylic acid reagent (1 % dinitrosalicylic acid and 30% sodium potassium tartrate in 0.4 M NaOH) was added and incubated for 5 min in boiling water. Absorbance at 540 nm was read using a Hitachi U5100

spectrophotometer. The amount of maltose released from this assay was estimated from standard curve.

5.2.8 Diet nutrient composition analysis

The percentage of crude carbohydrate was undertaken following methods outlined by (Dubois et al., 1956) and crude protein was determined following the Lowry's method (Lowry et al., 1951). Total lipid and fatty acid composition of the live feed were also estimated according to methods described by Folch et al. (1953). Total lipid extractions were undertaken from 0.1 g dry weight (DW) of the live feed, which were homogenized with chloroform-methanol (2:1) mixture. After filtration of the mixture, 0.5 ml of C19:0 (fatty acid standard) and 1 mL of 5 % hydrogen chloride methanol was then added to the solution. It was then incubated at 80 ºC for 3 h. After lipid extraction, the samples were mixed with 1 mL of hexane and 5.5 mL distilled water, vortexed and centrifuged for 5 min at 2000 rpm. The fatty acid containing hexane layer was collected into a vile and subjected to gas chromatography (GC-17, Shimadzu Co. Ltd., Kyoto, Japan) analysis based on the C19:0 standard.

For free amino acid (FAA) analysis, the samples were processed followed the method described by Teshima et al. (1986). A total of 0.1 g and 0.1 mL internal standard (6 mg DL-norleucine/ml deionized water and 2.5 mL trichloroacetic acid) mixed with 0.9 mL cold deionized water. The mixture was homogenized and washed with 8 % TCA and centrifuged at 4 °C, 3000 rpm for 15 min. The supernatant was transferred to 15 mL tube and repeatedly washed with diethyl ether. The resultant solution was adjusted pH within 2.2 ± 0.05 using chloric acid or 4 N NaOH solution, then filtered through a syringe filter into 2 mL vial. The free amino acid analysis was performed using high-performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan).

5.2.9 Statistical analyses

Potential differences were tested by one-way ANOVA (SPSS 24.0, IBM, USA) applied Tukey's HSD test (Honestly Significant Difference). Statistical significance was accepted at α=0.05. Besides, the linear regression (α=0.05) was applied to evaluate the correlation between gene expression and enzyme activity of fish larvae.

5.3 Results

5.3.1 Nutritional compositions of enriched rotifer

Crude protein content of enriched rotifer ranged from 59.6 to 62.1 % and crude lipid ranged from 15.3 to 17.2 % but no significant differences in crude protein and lipid content were recorded among the treatments ($p > 0.05$). The salmon roe oil-enriched rotifer supplemented with 5 % and 10 % of SDBP (15.5 ± 1.6 % and 16.9 ± 1.5 %, respectively) showed a significantly higher content of carbohydrate compared to control and 1 % of SDBP (12.4 ± 1.3 % and 13.6 ± 0.7 %, respectively) ($p < 0.05$) (Table 5.2).

Table 5. 2 The nutritional compositions of enriched rotifer used for Japanese flounder larvae (Mean ± SD).

	Control	S1	S5	S10
Crude protein (%)	49.6 ± 2.7^{a}	58.8 ± 4.1^{ab}	61.3 ± 3.0^{b}	62.1 ± 4.1^{b}
Crude lipid (%)	17.2 ± 1.5	16.2 ± 1.2	15.3 ± 1.7	15.9 ± 2.1
Carbohydrate (%)	12.4 ± 1.3^{a}	13.6 ± 0.7^{a}	15.5 ± 1.6^{b}	16.9 ± 1.5^{b}

The salmon roe oil-enriched rotifer supplemented with SDBP contained higher proportions and content of docosahexaenoic acid (DHA), but reduced proportion of palmitoleic acid (C16:1 *n*-7) compared to the control treatment (Table 5.3). The highest DHA content (2.14 ± 0.14 mg/g DW) and Σ*n*-3HUFA (4.05 ± 0.10 mg/g DW) were observed in S10 treatment and was observed to be statistically higher to Control and S1 treatment (1.45 ± 0.1 and 1.7 ± 0.25 mg/g DW, 3.33 ± 0.12 and 3.47 ± 0.19 mg/g DW, respectively) ($p < 0.05$). DHA/EPA ratio was also significantly higher ($p < 0.05$) in S5 and S10 (from 1.13 to 1.24 mg/g DW) compared to Control (0.77 mg/g DW) and S1 (0.95 mg/g DW). In contrary, Σmonoene contents in Control treatment was significantly higher ($p < 0.05$) than treatments enriched with SDBP.

The FAA compositions were observed to have significant differences among treatments in 6 essential FAA species (cysteine, leucine, tyrosine, phenylalanine, histidine, tryptophan and arginine) and 1 non-essential (glutamic acid) (Table 5.4). In which, the increase in glutamic acid, cysteine, histidine, and tryptophan was observed in SDBP supplemented treatments with the highest contents observed in S10 treatment. On the other hand, the rotifers in the control treatment showed significantly higher ($p < 0.05$) contents of leucine, tyrosine, phenylalanine and arginine **(Table 5.4).**

Table 5. 3 Fatty acid compositions (Area %) and contents (mg/g dry weight) of enriched rotifers (Mean ± SD). Alphabetical letters indicate significant difference among treatments. (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

Abbreviations: ARA: Arachidonic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid

Fatty acid (%)	Control	S1	S5	S10
C14:0	8.02 ± 0.66	7.36 ± 0.38	7.09 ± 0.56	6.98 ± 0.76
C16:0	13.27 ± 0.48	13.65 ± 0.37	13.13 ± 0.76	13.59 ± 1.08
C16:1n-7	3.58 ± 0.26^{b}	0.95 ± 0.15^{a}	0.83 ± 0.03^{a}	0.72 ± 0.08^{a}
C16:2n-4	2.12 ± 0.17	2.99 ± 0.54	2.45 ± 0.07	2.23 ± 0.31
C16:3n-4	0.43 ± 0.06	1.68 ± 0.08	0.93 ± 0.42	1.34 ± 0.13
C18:0	5.75 ± 0.35	6.09 ± 0.35	5.42 ± 0.12	5.88 ± 0.76
C18:1n-9+n-7	7.65 ± 0.39	7.97 ± 0.56	7.23 ± 0.63	7.16 ± 0.66
C18:2n-6	10.46 ± 0.51	12.22 ± 0.79	11.04 ± 1.24	11.02 ± 1.81
C18:3n-3	3.34 ± 0.17	3.52 ± 0.16	3.69 ± 0.47	3.70 ± 0.61
C18:4n-3	0.57 ± 0.03	0.64 ± 0.10	0.59 ± 0.11	0.57 ± 0.13
C20:0	0.13 ± 0.01	0.18 ± 0.03	0.17 ± 0.04	0.16 ± 0.01
C20:1n-9	1.23 ± 0.04	1.23 ± 0.03	1.18 ± 0.17	1.20 ± 0.19
C20:2n-6	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
C20:3n-6	0.97 ±0.39	0.68 ± 0.08	0.63 ± 0.07	0.61 ± 0.10
ARA	0.30 ± 0.02	0.34 ± 0.02	0.36 ± 0.03	0.35 ± 0.07
C20:4n-3	1.13 ± 0.06	1.47 ± 0.30	1.14 ± 0.17	1.18 ± 0.21
EPA	6.28 ± 0.43	6.54 ± 0.83	5.56 ± 0.46	6.11 ± 0.37
C22:0	0.44 ± 0.06	0.49 ± 0.04	0.43 ± 0.06	0.42 ± 0.09
C22:1n-9	0	0	0	0
C22:5n-6	0	0	0	0
C22:5n-3	3.65 ± 0.27	4.82 ± 0.87	3.76 ± 0.45	3.82 ± 0.61
DHA	4.84 ± 0.14^{a}	6.24 ± 0.24^{b}	6.30 ± 0.14^{bc}	6.87 ± 0.39^{c}
SFA	27.62 ± 1.51	26.17 ± 0.27	26.24 ± 0.44	26.53 ± 0.61
MUFA	12.45 ± 0.67	9.21 ± 0.71	9.24 ± 0.77	9.08 ± 0.93
n-6	11.78 ± 0.35	12.06 ± 1.26	12.10 ± 1.35	12.03 ± 2.00
n-3	19.79 ± 1.04	21.24 ± 1.88	21.05 ± 1.32	22.26 ± 1.31
PUFA	34.13 ± 1.50	36.66 ± 3.59	36.53 ± 3.15	37.87 ± 4.49
Others	25.79 ± 3.67	27.95 ± 4.53	27.99 ± 4.34	26.53 ± 4.83
Contents (mg/g DW)				
ARA	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
EPA	1.88 ± 0.06	1.80 ± 0.34	1.73 ± 0.30	1.91 ± 0.04
DHA	1.45 ± 0.10^{a}	1.70 ± 0.15^{ab}	1.96 ± 0.03^{bc}	2.14 ± 0.14^{c}
Σn-3HUFA	3.33 ± 0.12^{a}	3.47 ± 0.19^{a}	3.77 ± 0.06^{ab}	4.05 ± 0.10^{b}
Σmonoene	3.73 ± 0.11^{b}	3.08 ± 0.07^{a}	2.88 ± 0.18^{a}	2.82 ± 0.09^{a}
Σn-3	5.93 ± 0.19	6.36 ± 0.74	6.56 ± 0.26	6.92 ± 0.17
Σn-6	3.54 ± 0.21	4.04 ± 0.07	3.76 ± 0.33	3.72 ± 0.46
DHA/EPA	0.77 ± 0.05^{a}	0.95 ± 0.05^{b}	1.13 ± 0.04^{c}	1.24 ± 0.01^{c}
ARA/EPA	0.047 ± 0.004	0.067 ± 0.003	0.066 ± 0.001	0.056 ± 0.007
n-3/n-6	1.67 ± 0.07	1.58 ± 0.21	1.74 ± 0.08	1.87 ± 0.11

Table 5. 4 Free Amino Acid (FAA) composition of enriched rotifers. Alphabetical letters indicate significant difference between treatments. Asterisks (*) indicate essential FAA species (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

FAA	Control	S1	S5	S10
Taurine*	0.43 ± 0.05	0.39 ± 0.05	0.44 ± 0.07	0.44 ± 0.01
Aspartic acid	4.18 ± 0.28	3.16 ± 0.76	3.37 ± 0.75	3.42 ± 0.61
Threonine*	1.16 ± 0.07	0.98 ± 0.23	1.06 ± 0.14	1.01 ± 0.02
Serine	1.26 ± 0.11	0.99 ± 0.22	1.09 ± 0.14	1.02 ± 0.07
Glutamic acid	7.75 ± 0.23^{a}	7.87 ± 0.20^{a}	8.78 ± 0.10^{b}	8.86 ± 0.24^{b}
Proline	0.75 ± 0.05	0.59 ± 0.13	0.70 ± 0.04	0.60 ± 0.03
Glycine	0.76 ± 0.06	0.69 ± 0.12	0.73 ± 0.03	0.74 ± 0.02
Alanine	3.02 ± 0.16	2.52 ± 0.44	2.70 ± 0.01	2.75 ± 0.13
Cysteine	0.27 ± 0.04^{a}	0.32 ± 0.02^{a}	0.33 ± 0.02^{a}	0.41 ± 0.02^{b}
Valine*	0.72 ± 0.10	0.59 ± 0.17	0.65 ± 0.12	0.67 ± 0.05
Methionine*	0.34 ± 0.01	0.36 ± 0.04	0.35 ± 0.05	0.41 ± 0.02
Isoleucine*	0.73 ± 0.08	0.54 ± 0.11	0.64 ± 0.08	0.60 ± 0.03
Leucine*	1.21 ± 0.07^{b}	0.87 ± 0.15^{a}	0.99 ± 0.09^{a}	0.98 ± 0.07^{a}
Tyrosine*	0.36 ± 0.33^{b}	0.39 ± 0.43^{b}	0.12 ± 0.03^{a}	0.10 ± 0.01^{a}
Phenylalanine*	0.81 ± 0.03^{b}	0.67 ± 0.20^{a}	0.65 ± 0.09^{a}	0.64 ± 0.05^{a}
Histidine*	0.77 ± 0.12^{a}	0.86 ± 0.23^{a}	0.83 ± 0.08^{a}	1.32 ± 0.05^{b}
Tryptophan*	0.25 ± 0.03^{a}	0.38 ± 0.09^{a}	0.53 ± 0.01^{b}	0.61 ± 0.03^{b}
Lysine*	1.88 ± 0.07	1.63 ± 0.11	1.74 ± 0.05	1.70 ±0.06
Arginine*	3.36 ± 0.35^{b}	1.55 ± 0.21^{a}	2.35 ± 0.58^{a}	1.91 ± 0.16^{a}

5.3.2 Survival rate and growth performance of fish larvae

The larval survival rates were not significantly different in the first 10 days. However, the survival rate in control treatment tended to decrease and started significant differences began to be observed from 15 DAH (Fig. 5.1). A dramatic drop in survival rate in all treatments was recorded at 20-25 DAH coinciding with the larval metamorphosis. From 25 DAH, the survival rate in control and S1 treatment were observed to be remarkably lower than S5 and S10. After 40 DAH, the highest survival rates of larvae were recorded in S10 (38.37 ± 1.5 %) and S5 (36.43 ± 2.8 %); and were significantly higher ($p < 0.05$) than control (24.5 ± 3.7 %) and S1 (25.43 ± 2.5 %). The final total length ranged from 11.3 to 11.7 mm, however, there were no observed significant differences ($p > 0.05$) in total length of *P. olivaceus* larvae among treatments (Fig. 5.2).

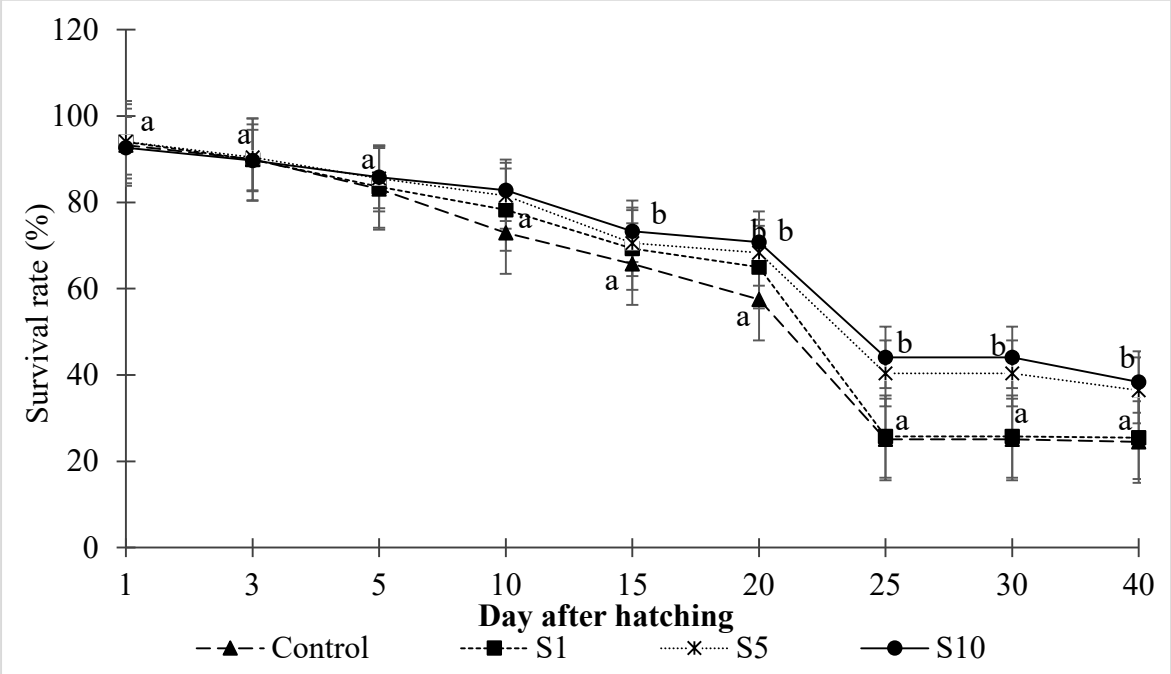


Figure 5. 1 Survival rates of *P. olivaceus* larvae among treatments. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of survival rate among treatments at particular stages. (ANOVA, Tukey's HDS test, a < b, $p < 0.05$).

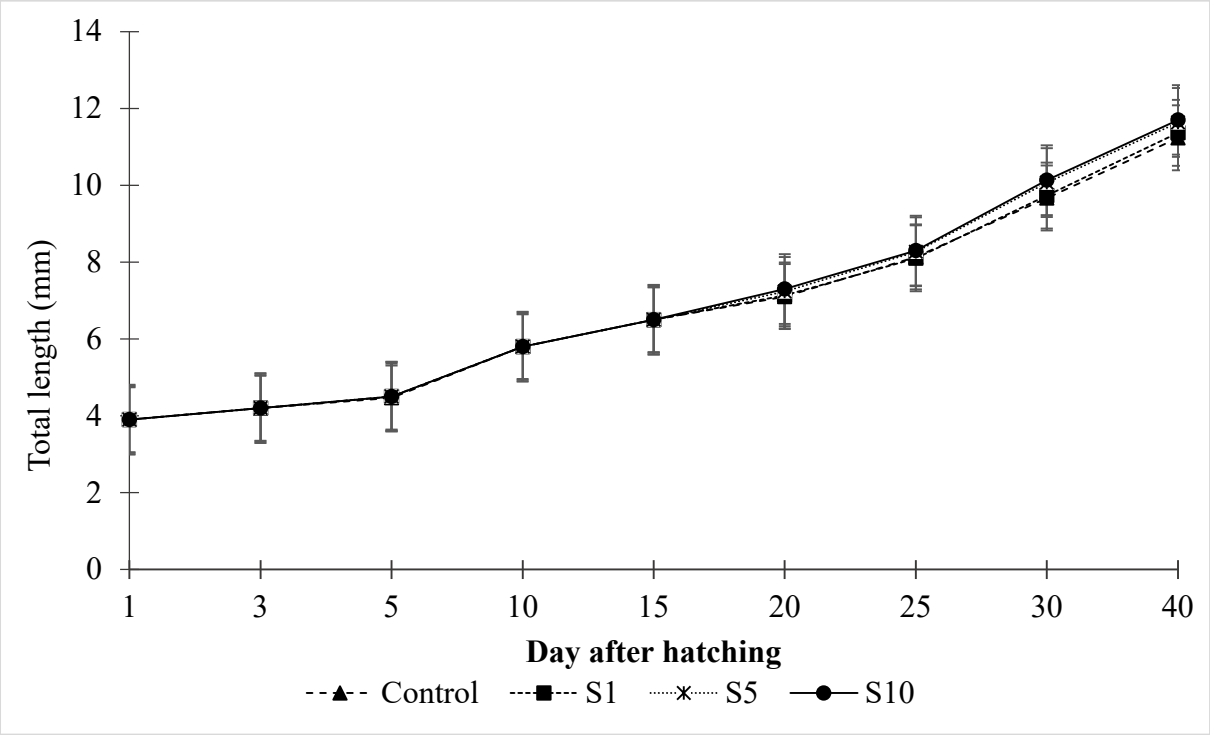


Figure 5. 2 Growth performance in total length of *P. olivaceus* larvae among treatments. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1.

5.3.3 Activity and expression of digestive enzymes

5.3.3.1 Trypsin

The trypsin activity and expression increased in all treatments within the first 10 days after hatching. Sudden increase in *try3* was observed in all treatments at 10 DAH except in the control treatment (Fig. 5.3A). However, the trypsin activity in SDBP supplemented treatments sharply increased and reached a peak at 15 DAH (Fig. 5.3B). In which, the S10 showed the highest activity of trypsin (0.43 ± 0.04 mU/mg protein), followed by S5 (0.34± 0.04 mU/mg protein), S1 (0.23 ± 0.03 mU/mg protein). The trypsin activity in the control treatment decreased and was significantly lower than others ($p < 0.05$). On the otherhand, *try3* expression in S5 and S10 treatment continued increasing and peaked at 20 DAH, followed by a dramatic drop at 25 DAH. Thereafter, both activity and expression of trypsin in all treatments were

gradually decreased until 35 DAH with a significant difference was noticed in S5, S10 compared to S1 and control ($p < 0.05$). At 40 DAH, a recovery of trypsin activity and expression was observed in SDBP supplemented treatments while the control treatment maintained significantly lower ($p < 0.05$) levels.

5.3.3.2 Chymotrypsin

The activity and expression of chymotrypsin were detectable from hatching and remained stable within the first 20 days with no significant differences observed among the treatments (Fig. 5.4). Chymotrypsin activity in S1 treatment did not remarkably change during the experimental time (ranged from 0.13 to 0.20 mU/mg protein) (Fig. 5.4B). On the contrary, the SDBP supplemented treatments at 5 % and 10 % showed a sharp increase in chymotrypsin expression and activity at 25 DAH and were significantly higher ($p < 0.05$) than S1 treatment. The highest expression level of *ctrb2* was observed in S10, followed by S5 and control treatment (Fig. 5.4A). The chymotrypsin activity in S10 and S5 ranged from 0.27 to 0.3 mU/mg protein whereby it was statistically higher ($p < 0.05$) compared to 0.18 mU/mg protein in S1. However, the chymotrypsin levels in S10 and S5 dramatically dropped at 30 DAH (0.16 ± 0.02 mU/mg protein and 0.18 ± 0.02 mU/mg protein) and significantly lower ($p < 0.05$) than chymotrypsin in the control (0.22 ± 0.01 mU/mg protein). At the end of the experiment, the chymotrypsin in all treatments showed a correlated trend in both expression and activity.

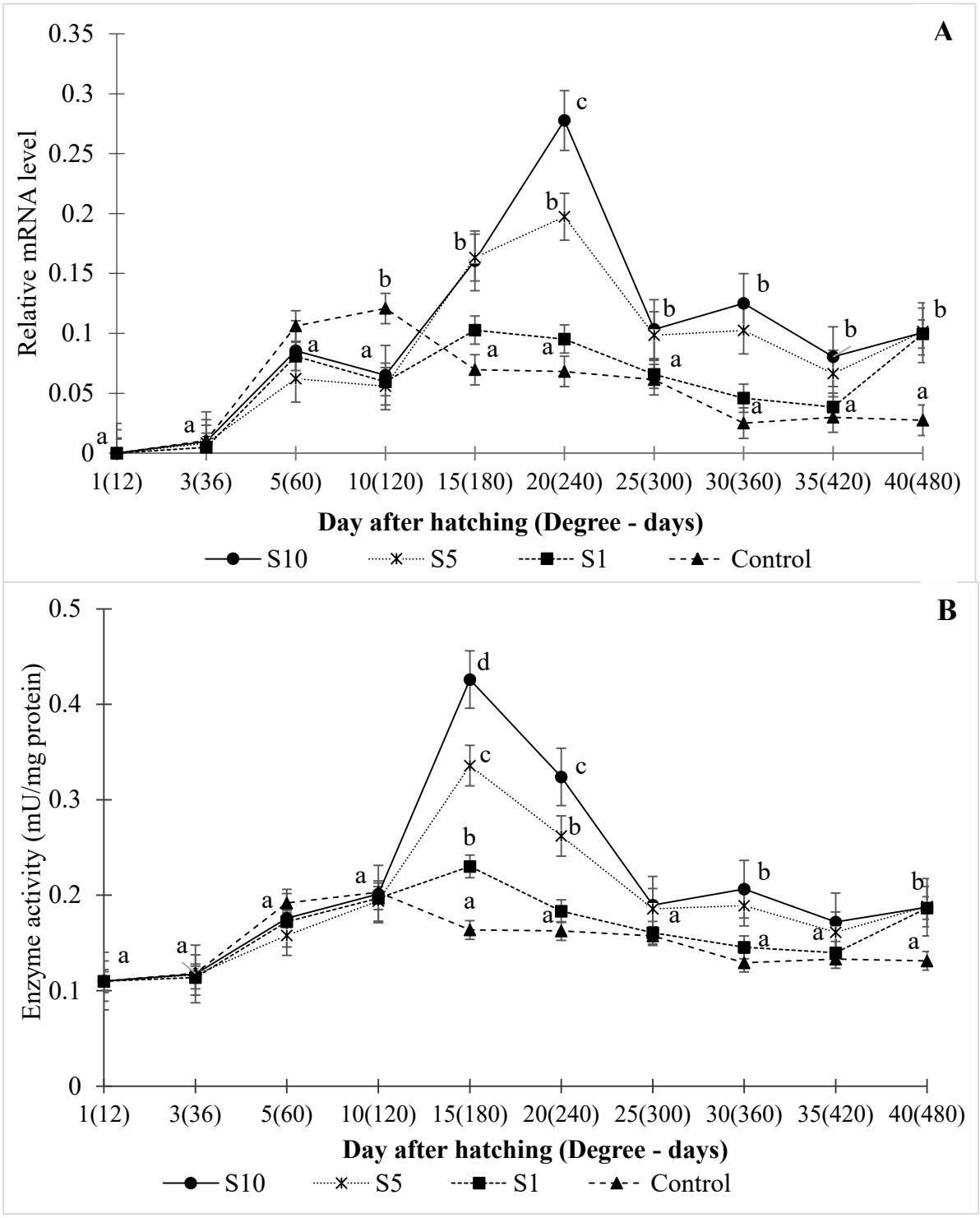


Figure 5. 3 *try* expression (A) and trypsin activity (B) during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of trypsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

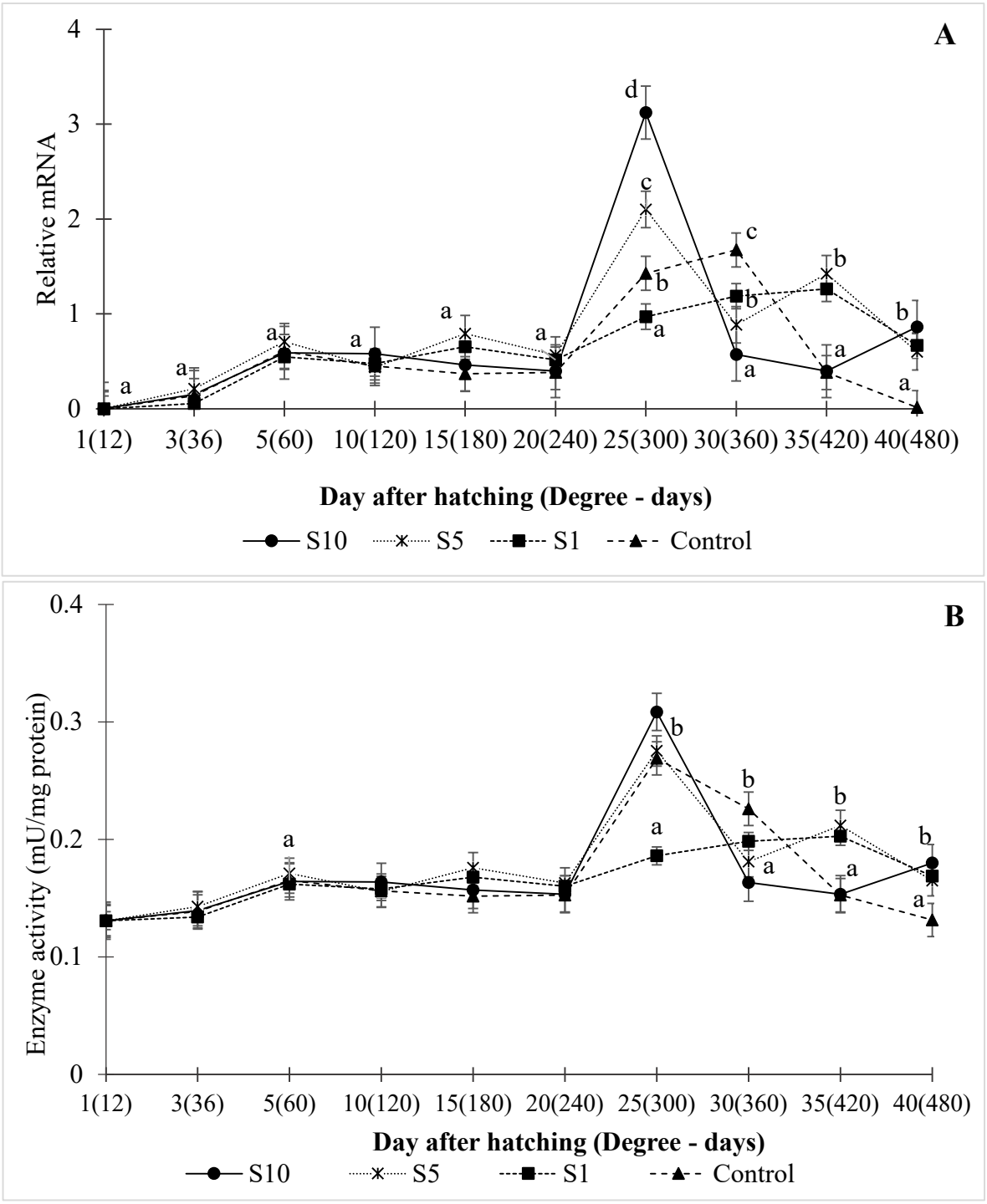


Figure 5. 4 *ctrb* expression (A) and chymotrypsin activity (B) during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of chymotrypsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c < d, $p < 0.05$).

5.3.3.3 Pepsin

Pepsin activity and expression were detectable from 25 DAH at very low levels and gradually increased until 35 DAH (Fig. 5.5). Thereafter, a sharp increase in pepsin activity and expression were observed. The highest levels in pepsin activity were recorded in S10 (61.9 ± 7.5 mU/mg protein), followed by S5 (44.1 ± 5.3 mU/mg protein) and were significantly higher ($p < 0.05$) than S1 (32.5 ± 3.8 mU/mg protein) and control (31.7 ± mU/mg protein) (Fig. 5.5B). The expression of *pep* was similarly increased and correlated to its activity at 40 DAH (Fig. 5.5A).

5.3.3.4 Lipase

The expression levels and activity of lipase were correlated during the experimental period (Fig. 5.6). No significant difference in both activity and expression of lipase was observed within the first 10 days among the treatments. From 10 DAH to 25 DAH, the treatment of S5 and S10 showed a sharp increase in lipase activity and expression. At 25 DAH, S10 treatment reached a peak in lipase activity and expression (45.3 ± 5.3 mU/mg protein and 0.72 ± 0.03), followed by S5 (25.7 ± 3.4 mU/mg protein and 0.46 ± 0.07,). Both S10 and S5 treatments were significantly ($p < 0.05$) higher than S1 and the control treatment (Fig 5.6A and B). However, the lipase level in all treatments dramatically dropped at 30 DAH until the end of the experiment where no statistical differences were observed.

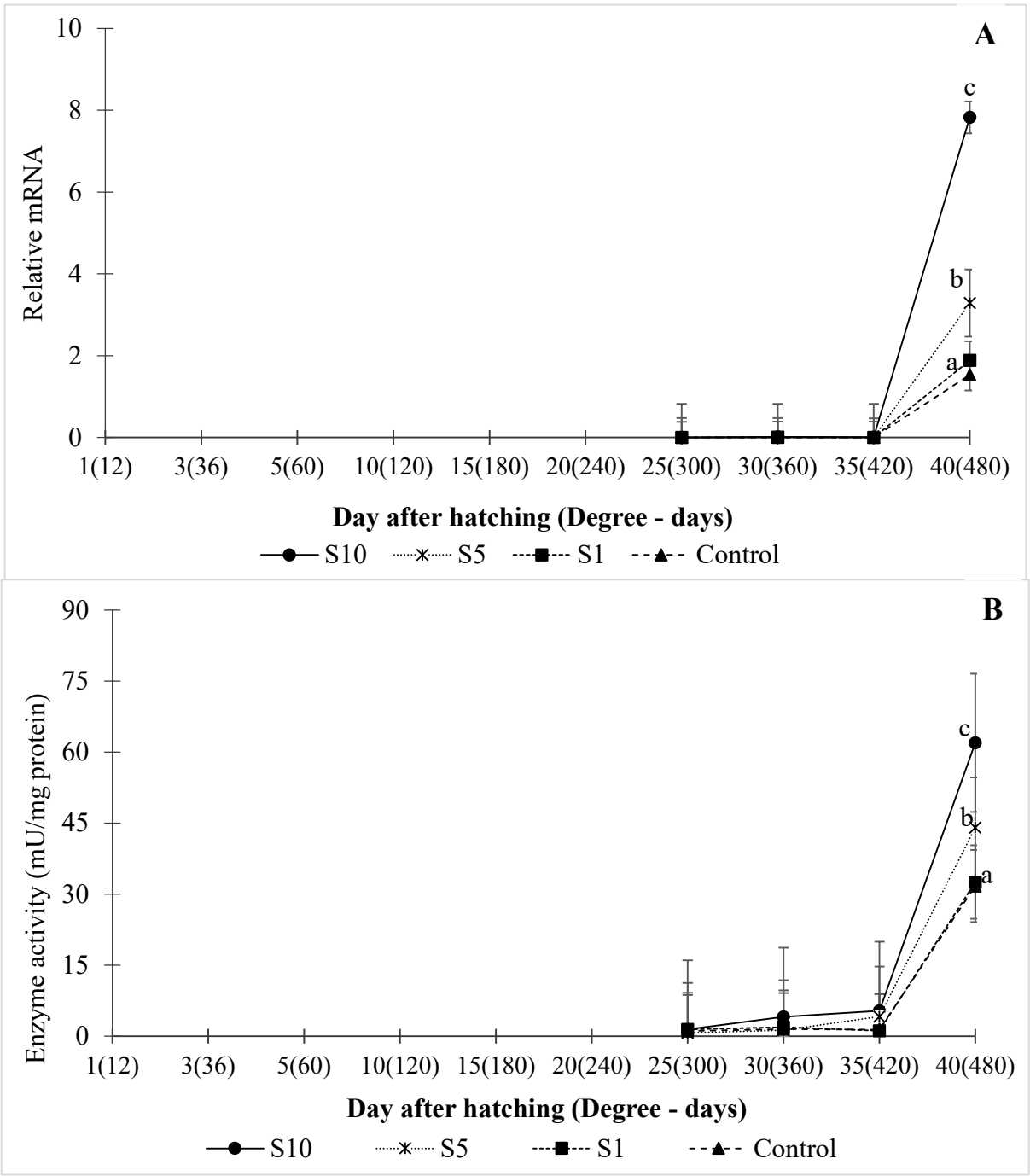


Figure 5. 5 *pep* expression (A) and pepsin activity (B) during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of pepsin activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

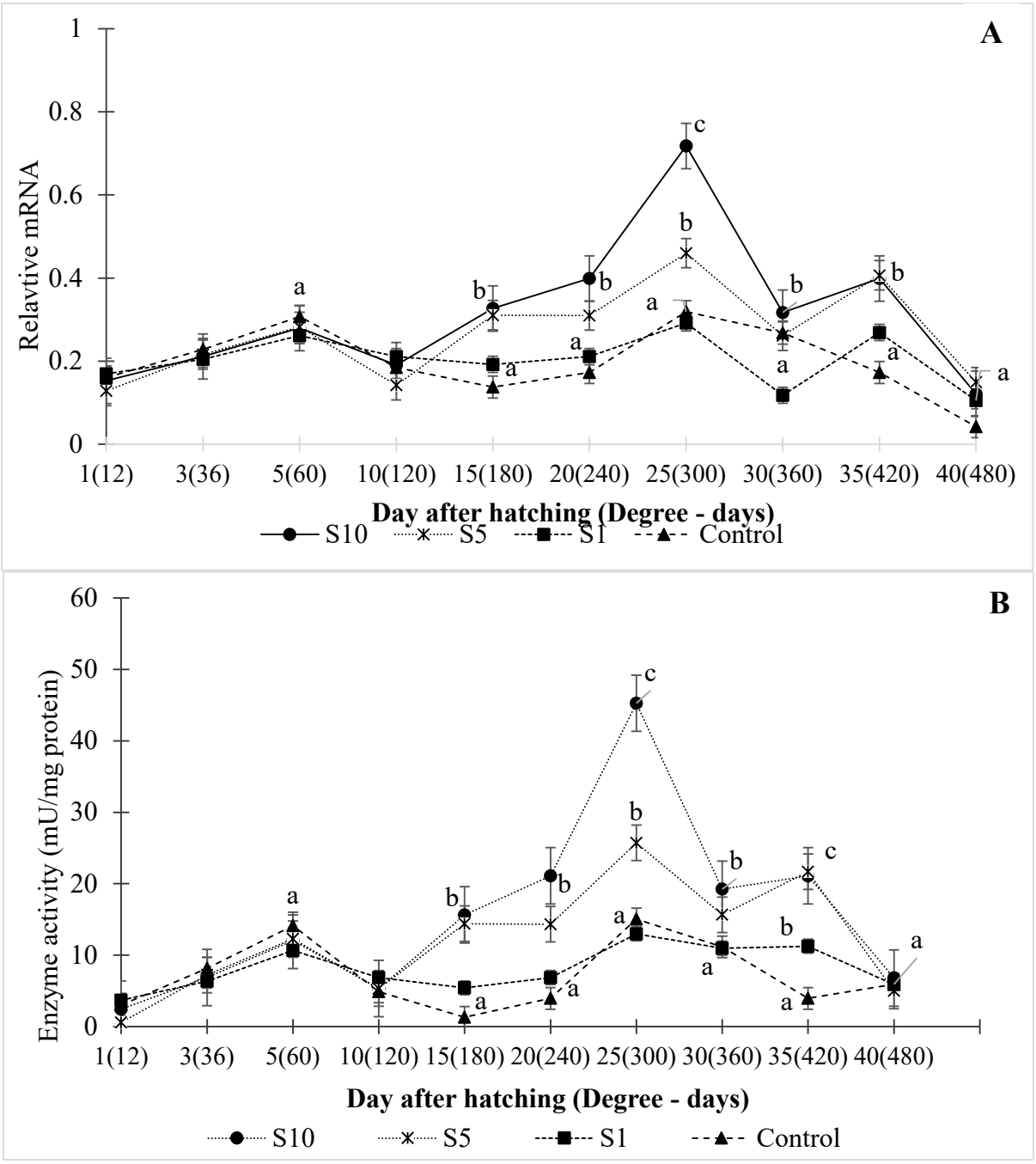


Figure 5. 6 *pl* expression (A) and lipase activity (B) during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of lipase activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

5.3.3.5 Amylase

The activity of amylase in all treatments was not significantly different within the first 10 days and ranged from 3.4 to 5.2 mU/mg protein (Fig. 5.7B). From 10 DAH, amylase activity sharply increased in the S5 and S10 treatment and remained at high levels (10.7 – 11.3 mU/mg protein) until 25 DAH. No statistical difference in amylase was observed between S5 and S10, but there were significantly higher than S1 and control treatments (6.1 – 6.8 mU/mg protein) ($p < 0.05$). After 25 DAH, the activity of amylase dropped and remained the same until 40 DAH in all treatments (3.4 -3.5 mU/mg protein). On the contrary, the expression of *amy2α* showed a very complicated fluctuation and was not correlated to the activity (Fig. 5.7A). Several peaks of *amy2α* expression were recorded at 5, 15, 25 and 35 DAH alternated with dramatic drops at 10, 20, 30 and 40 DAH. Generally, the *amy2α* expression in S5 and S10 were stronger than S1 and control, but all the treatments showed a very low ($p > 0.05$) expression at the 40 DAH.

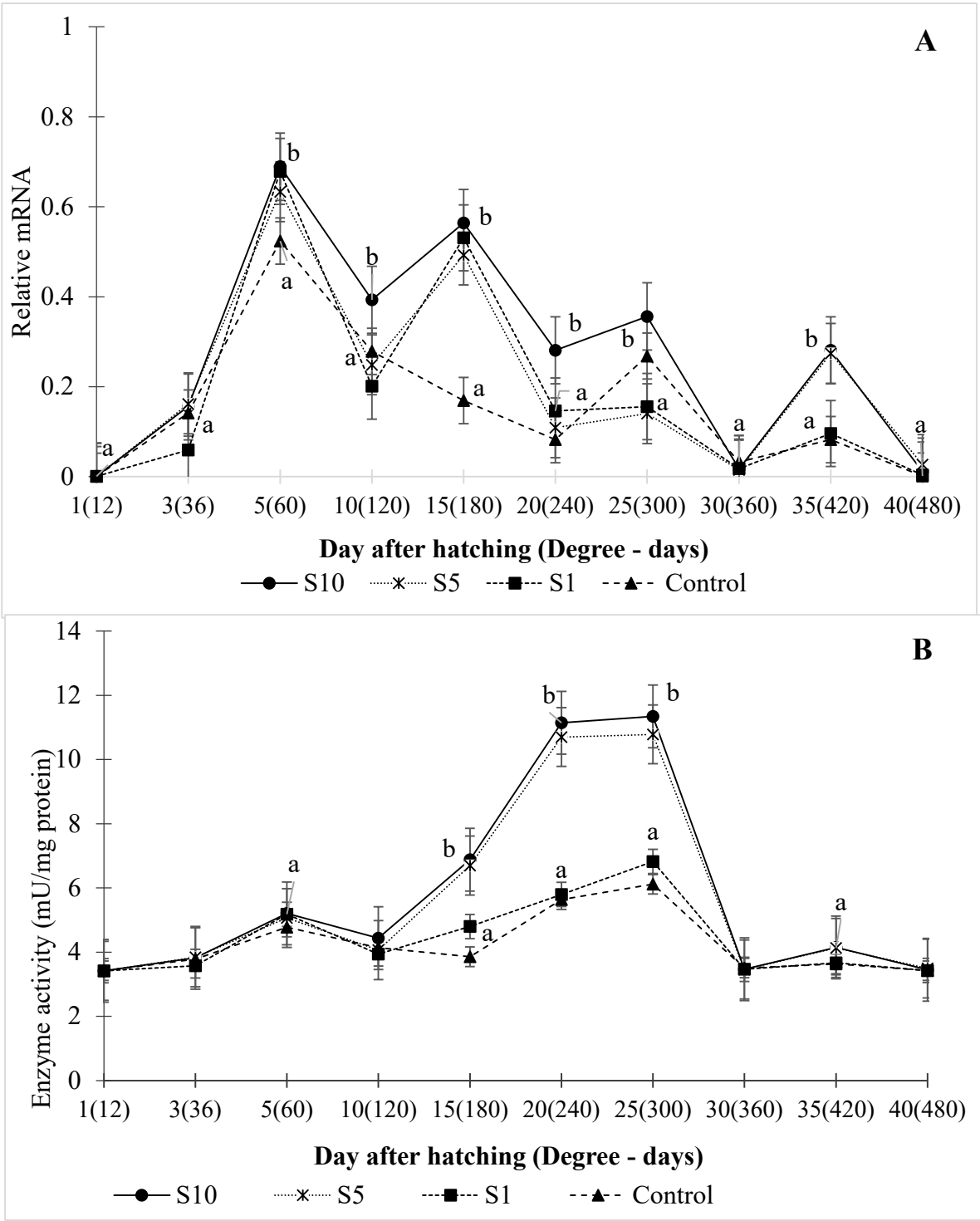


Figure 5. 7 *amy2α* expression (A) and amylase activity (B) during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of amylase activity/expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b, $p < 0.05$).

5.3.4 Expression of genes related to energy metabolism

atp expression levels gradually increased from hatching until 20 DAH with no significant differences observed among the treatments (Fig. 5.8). The *atp* expression in S10 was considerably increased and statistically higher than others at 25 DAH, and reached a peak at 30 DAH. S5 and S1 treatments also showed a significantly higher ($p < 0.05$) expression level compared to the control at 30 DAH. Significant differences in *atp* expression between control and SDBP supplemented treatments were observed until 35 DAH before reaching similar levels at the end of the experimental period (Fig. 5.8).

There was no dramatic variation in the expression of *cox* until 20 DAH. Thereafter, the expression of *cox* sharply increased in treatment S10 and was significantly higher ($p < 0.05$) than other treatments. However, this expression level dramatically dropped at 30 DAH. The control, S1 and S5 treatment also increased in *cox* expression levels and reached similar levels to S10 at 30 DAH. Towards the end of the experiment no significant differences in the *cox* expression levels among treatments were observed (Fig. 5.9).

5.3.5 Expression of cholecystokinin (*cck)*

cck expression was relatively stable until 20 DAH, followed by a sharp increase at 25 DAH (Fig. 5.10). However, S10 treatment showed a significantly higher ($p < 0.05$) level of *cck* expression compared to the other treatments. Thereafter, the *cck* level tended to decrease from 30 DAH, but S5 and S1 remained at a significantly higher ($p < 0.05$) level than S10 and Control until 35 DAH. At the end of the experiment, no significant difference in *cck* expression was observed (Fig. 5.10).

5.3.6 Expression of insulin-like growth factor 1 (*igf1*)

There were no significant changes in *igf1* expression observed until 20 DAH. A sharp increase in the expression of *igf1* was observed at 25 DAH (Fig. 5.11). In which, the S10

treatment showed the highest level of *igf1* expression and statistically higher ($p < 0.05$) than the other treatments. However, there was no significant difference ($p > 0.05$) between control, S1 and S5 treatments. The *igf1* expression level in S10 dropped at 30 DAH and reached similar levels as the other treatments. While the SDBP supplemented treatments *igf1* expression relatively remained stable until 40 DAH, the control treatment continued decreasing and became significantly lower ($p < 0.05$) than the other treatments.

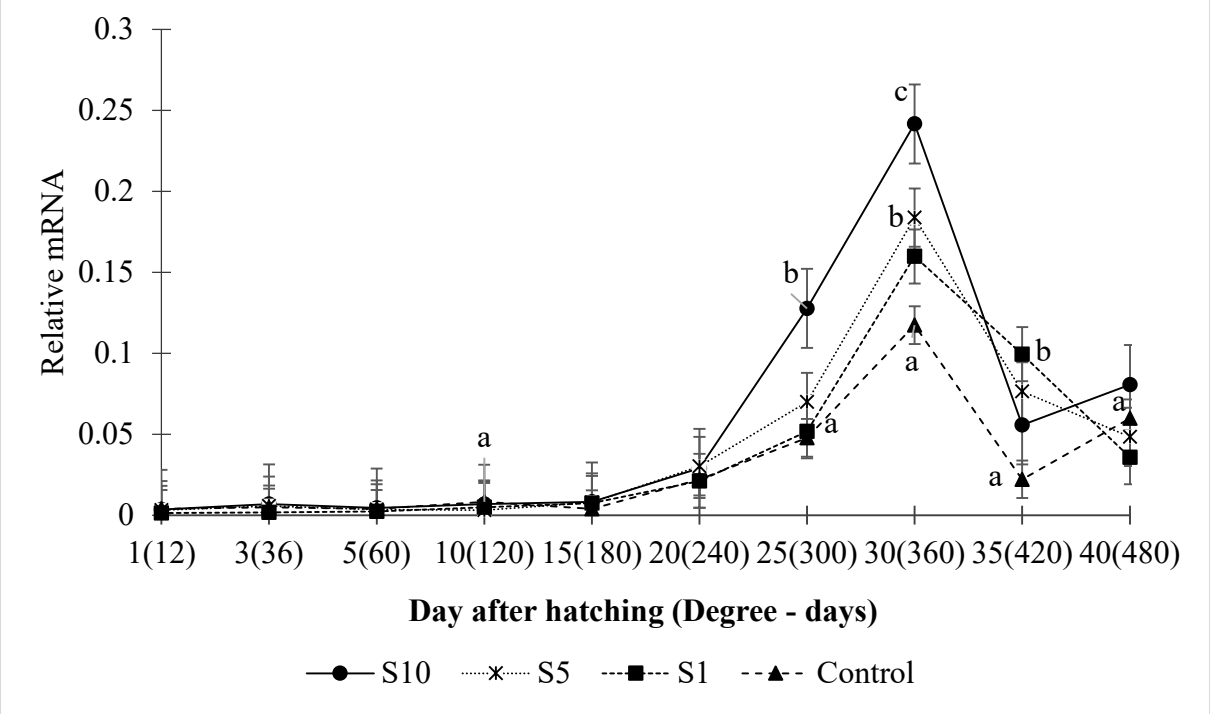


Figure 5. 8 *atp* expression during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of *atp* expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

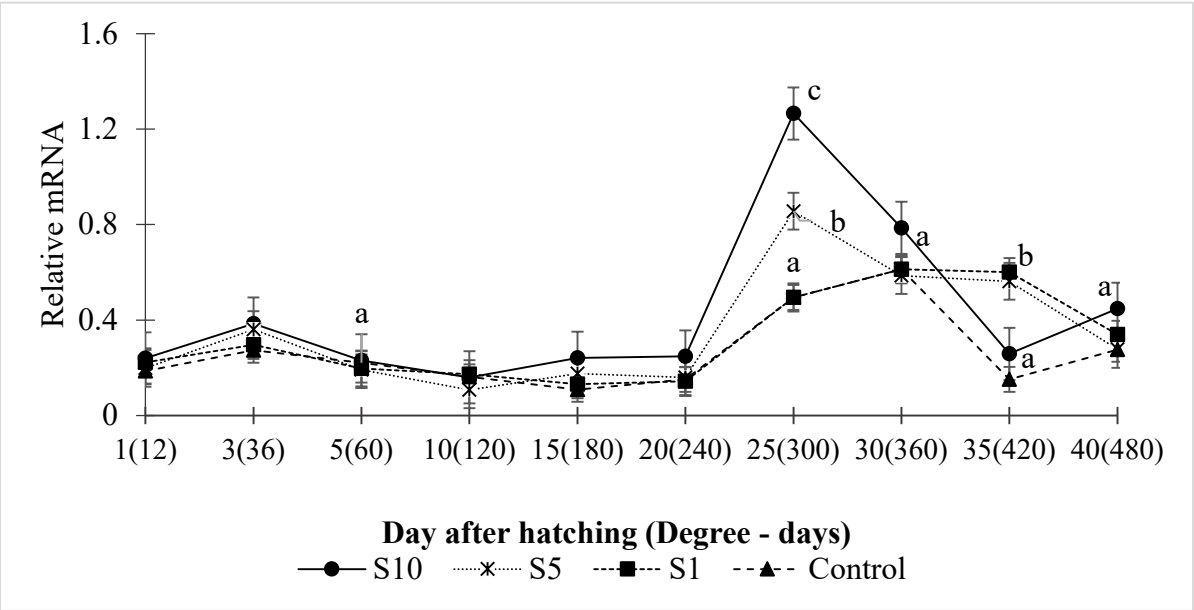


Figure 5. 9 *cox* expression during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of *cox* expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b < c, $p < 0.05$).

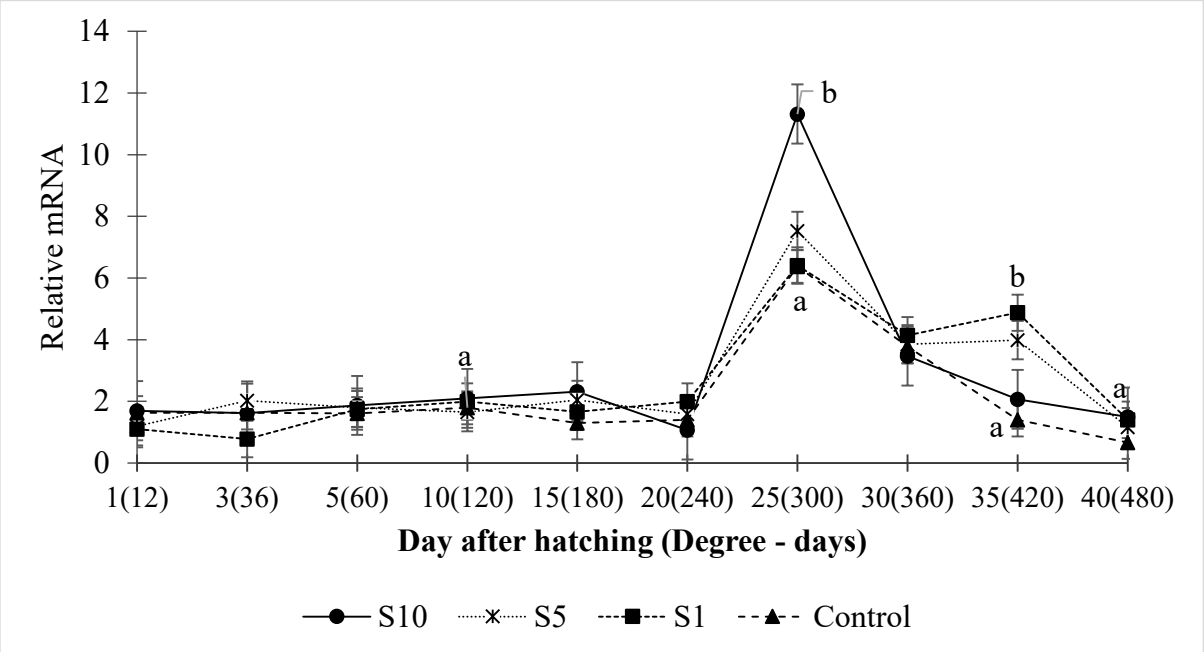


Figure 5. 10 *cck* expression during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of *cck* expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b, $p < 0.05$).

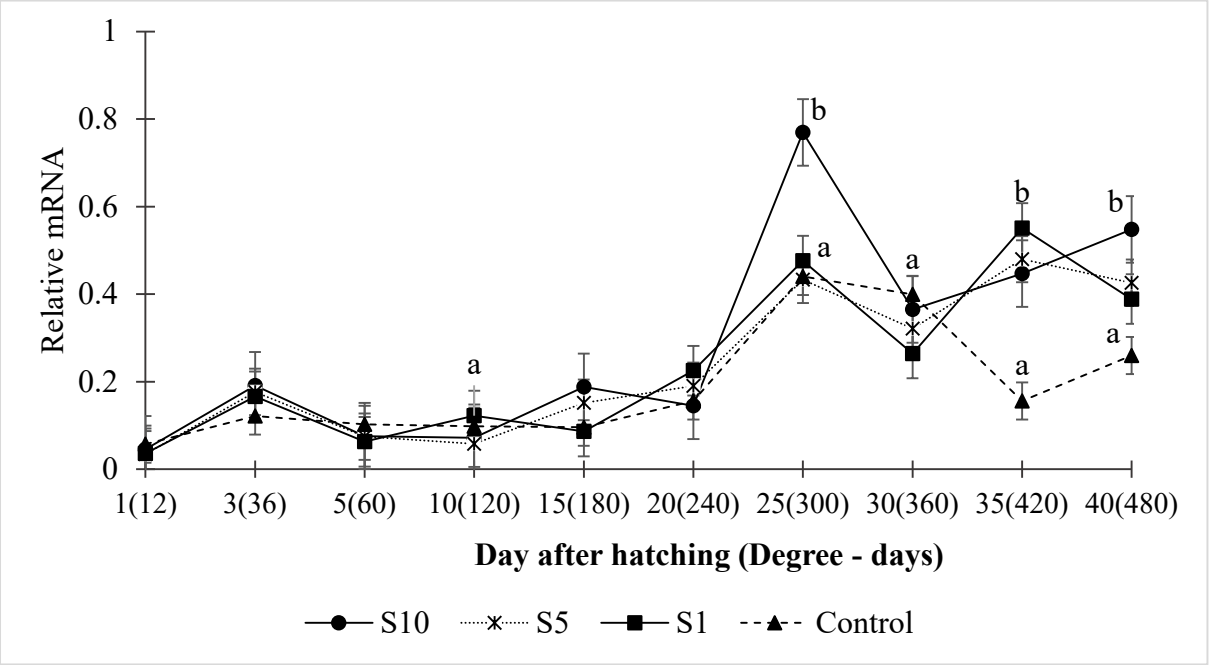


Figure 5. 11 *igf1* expression during larval development under different diets. The data was presented as mean ± SEM. Straight line and circle plot indicate S10. Dashed line and triangle plot indicate Control. Dashed line and asterisk plot indicate S5. Bold - dashed line and square plot indicate S1. Alphabetical letters indicate significant difference of *igf1* expression among treatments at particular stages (ANOVA, Tukey's HDS test, a < b, $p < 0.05$).

5.4 Discussion

A major bottleneck exists in larviculture, especially during the early stages of exogenous feeding (Dhert et al., 2001; Qin, 2013). Poor enzymatic activity and a non-functional stomach inhibit larvae from digesting formulated diets and thus the important focus placed on live feed as an important primary food source for early stage larvae (Conceição et al., 2008; Rønnestad et al., 2013). Despite the common use of rotifers and *Artemia*, nutritional deficiencies, particularly in terms of essential fatty acids still exists (Bell et al., 2003; Hamre et al., 2008; Saidi et al., 2018). As most marine fish larvae can not naturally synthesize highly unsaturated fatty acids (HUFA) (Støttrup and Josianne, 2004), it is often common practice to enrich live feed with media such as microalgae and oil-based emulsions (Dhert et al., 2001; Radhakrishnan et al., 2019; Waqalevu et al., 2019). Despite this, studies have also shown that even with

enrichment low survival rates and growth performance of larvae is still observed. This was highlighted in a study by Waqalevu et al. (2019) where rotifers enriched with oil emulsions from salmon roe resulted in high content of HUFA resulting in low larviculture performance. As such, there still modifications required for the improvement of rotifer enrichment practices in order to improve not only rotifer nutrient composition but also improve larviculture performance.

Previous studies have shown that the effectiveness of rotifer enrichment is reflected through its fatty acid composition (Kotani et al., 2013; Hamre, 2016). These studies have also highlighted that via different culture conditions and enrichment media, malnourishment due to the absence of essential nutrients can also occur. Results from the present study showed that rotifer enriched with salmon oil supplemented with of SDBP did not compromise lipid contents. The content of crude protein, crude carbohydrate and DHA in S5 and S10 were significantly improved compared to the control and S1 treatment (Table 5.2 and 5.3). The SDBP did not contain too high protein or DHA (Supplementary 5.1), therefore suggesting that the micro-community in the yeast of SDBP possibly plays a role in enhancing the nutritional value of the rotifer. According to Haydersah et al. (2012), Campbell et al. (2017) and Tamang et al. (2020), it's possible that lactic bacterium*, Saccharomyces* fungi assist in the degradation of amino acids and therefore enhance the digestibility of plant and seafood products. Several strains of useful bacteria and fungus have been isolated from the SDBP used in this study (Supplementary 5.2). Strains such as, *Lactobacillus* spp. and *Baciliius* spp. have been reported to be probiotic bacteria and can be applied as a nutrient source and a catalyst in enzymatic action to aid in digestive processes of aquatic organisms (Balca et al., 2006; Hai, 2015). Moreover, *Saccharomyces* yeast has been widely applied as a probiotic incorporated in existing aqua-commercial feeds (Akhter et al., 2015; Abass et al., 2018). Therefore, it is reasonable to hypothesize that the microbio-community in the SDBP can also contribute to the nutritional

enhancement of enriched rotifer mainly on the crude protein, fatty acid and free amino acid profiles. However, this study did not investigate the density and proportion of microbial community in the enriched media as well as the effect of probiotics and therefore further studies are recommended.

Fatty acid compositions in feed have been reported to have significant correlation to larval development, especially in pigmentation and eye migration in flatfish larvae (Hamre and Harboe, 2008). Of these fatty acid species, DHA has been highlighted as superior to EPA as an essential fatty acid (EFA) for marine fish larvae (Watanabe, 1993; Rainuzzo et al., 1997; Furuita et al., 1999). DHA plays an important role in the development of the nervous system, pigmentation, growth and survival of fish larvae (Furuita et al., 1999; Copeman et al., 2002). A lack of DHA is known to result in visual impairments in larvae that ultimately leads to a reduction in hunting efficiency and consequently reduction in growth and vitality (Watanabe, 1993; Rodríguez et al., 1997; Glencross, 2009; Glencross and Rutherford, 2011; Takeuchi, 2014).The relative ratio of DHA/EPA is also important due to the various physiological roles it is related to (Zuo et al., 2012; Choi et al., 2014; Jin et al., 2017; Magalhães et al., 2020). The DHA/EPA ratio is also known to promote higher survival rates, growth, nonspecific immunity immune related gene expression and disease resistance (Furuita et al., 1999; Zuo et al., 2012; Choi et al., 2014). In the present study, rotifer enriched with salmon roe oil supplemented with SDBP (S1, S5 and S10) showed a significant improvement of DHA levels, Σn-3HUFA and DHA/EPA ratio (Table 5.3). Among the SDBP supplemented treatments, DHA level was not significantly different, however, the ratio of DAH/EPA was statistically higher in S5 and S10 coinciding with high survival rates in these two treatments. It suggests that a DHA/EPA ratio from 1.13 to 1.24 should be considered for improved survival rates of *P. olivaceus* larvae. According to Bell et al. (1985), an increase of DHA/EPA ratio from 0.1 to 0.5 in the diets remarkably enhanced the survival rate of turbot (*Scophthalmus maximus*). However, several

studies have highlighted that the optimal ratios of DHA and EPA vary for different stages, species and living environments (Harel et al., 2001; Bell et al., 2003; Hamre and Harboe, 2008). A DHA/EPA ratio of ≥ 2 and EPA/ARA ratio of > 5:1 in diets were suggested for marine fish larvae (Izquierdo, 1996; Bell et al., 2003). The enriched rotifer used in this study contained high ratios of EPA/ARA, but DHA/EPA was lower than the suggested ratio (Fig. 5.2).

Free amino acids are beneficial for early stage larvae as early stage larvae limited differentiation in the early digestive system as FAA larvae have been known to utilize them better than whole proteins (Rønnestad et al., 2000, 2013). In the present study, enriched rotifers supplemented with SDBP showed a significant improvement of glutamic acid, cysteine, and tryptophan. On the contrary, the control diet contained significantly higher levels of leucine, tyrosine, phenylalanine and arginine (Table 5.4). The highest levels of taurine and glutamic acid were observed in the S5 and S10 treatment, though not significantly different (Table 5.4). Taurine and glutamine are considered as conditionally indispensable amino acids (Lacey and Wilmore, 1990; Kim et al., 2008; Li et al., 2009; Wu, 2009) due to their important role in promoting growth, feed utilization, anti-oxidation, stimulating immune status and intestinal development and structure of fish larvae (Cheng et al., 2011; Zhu et al., 2011). Recently, Han et al. (2014) reported interactive effects of dietary taurine and glutamine on the growth and oxidative status of *P. olivaceus*. Their study highlighted that fish fed higher levels of taurine and glutamine were observed to have higher growth and oxidation resistance (Han et al., 2014). Histidine, which is also an essential AA, was observed to be significantly higher in the S10 treatment compared to the other treatments (Table 4). Histidine has been reported to enhance growth performance and physiological status of *P. olivaceus* (Han et al., 2013). However, low growth of *P. olivaceus* was highlighted in diets with high leucine composition and low isoleucine level (Wang et al., 2017) which in this present study was observed in the control and S1 treatment (Table 4). It is evident from our results that the addition of SDBP to salmon roe

oil emulsion fortifies FAA, especially with essential FAA in rotifers. However, further investigation is needed to better understand the interactive effects of the use of SDBP and each FAA species and larviculture performance.

Digestive enzyme patterns in fish are age-dependent and can be modulated by feed manipulation (Cahu and Zambonino Infante, 2001). The enzymatic activity and related gene expression profile of *P. olivaceus* has been investigated widely using different diets (Bolasina et al., 2006; Lee et al., 2015; Khoa et al., 2020). Recently, Waqalevu et al. (2019) reported that *P. olivaceus* larvae fed rotifer enriched with salmon roe oil showed low food consumption, low trypsin activity and low survival but no effect on growth within the first 15 days. In this study, proteolytic enzyme activity and expression gradually increased and were not significantly different among treatments until 10 DAH (Fig. 5.3, 5.4). However, the larvae fed enriched rotifer supplemented with 5 % and 10 % SDBP affected the proteolytic enzyme activity and expression at 15 DAH, thereafter, pepsin was observed to significantly peak at 35 DAH (Fig. 5.5). Although the crude protein levels were different among treatments this did not result in different levels of trypsin and chymotrypsin enzyme within the first 10 days, which suggests that nutritional requirements were satisfied. From 10 DAH, the larvae underwent major changes in external morphology and digestive system structures (Khoa et al., 2020), hence larvae require high nutrient dietary to support these processes (Rønnestad et al., 2013). With higher nutritional compositions, S5 and S10 diet seem to be efficient for the larvae during the ontogenetic development. Importantly, *P. olivaceus* larvae need to conserve energy for the end of metamorphosis and eye migration, before the resumption of feeding (Gwak and Tanaka, 2002; Gwak et al., 2003; Geffen et al., 2007). During this period the larvae may be using energy stored earlier in the liver (Tanaka et al., 1989; Geffen et al., 2007). According to Geffen et al. (2007), if the *P. olivaceus* larvae is not able to feed properly and conserve energy during the early and mid-metamorphosing process, it would result in several effects by subsequent energy

needs of subordinate larvae during the non-feeding period. Based on the point-of-no-return suggested by Gwak et al. (1999), mass mortality might occur within 9-10 days and is correlated to settlement and metamorphic completion. The results of this study confirmed this theory as the higher survival and growth were archived in S5 and S10. The control and S1 diet were deficient nutritionally at the early and mid-metamorphosis stages leading to high mortality during metamorphosis.

Similarly, lipase activity and expression were genetically programmed and activated from hatching but no significant differences were recorded among treatments until 10 DAH (Fig. 5.6). The higher capacity in lipid digestion in S5 and S10 treatment compared to the control and S1 treatment demonstrated an efficient digestion and energy metabolism. Therefore, the fish larvae could reserve energy via the accumulation of lipids to prepare for metamorphosis as previously mentioned in studies by Gwak and Tanaka (2001) and Di Pane et al. (2019). Under food deprivation conditions during metamorphic process, liver glycogen and lipids are the first energy sources to be mobilized (Tanaka et al., 1989; Geffen et al., 2007; Zambonino-Infante et al., 2008). Low levels of proteolytic and lipolytic enzymes during the early stages of metamorphosis in the control and S1 might have caused a lack of energy storage coinciding with high mortality within 20-25 DAH in these two treatments (Fig. 5.1). Remarkably, the significant increase in proteolytic and lipolytic enzymes were observed from 15 - 20 DAH, coinciding with usual feeding activity. Within 20 - 25 DAH, the trypsin activity dramatically dropped and the cessation of feeding was observed. However, lipase activity was observed to still increase during metamorphosis. This could possibly be explained by the hydrolyzation of reserved energy such as triglyceride and phospholipids to provide energy for the metamorphic process (Gwak and Tanaka, 2002; Gwak and Tanaka, 2001; Gwak et al., 2003; Lazo et al., 2011). Previous studies have reported that *P. olivaceus* larvae save energy by actively synthesizing protein until their metamorphic climax, thereafter the protein synthesis rate

subsequently drops during the non-feeding period that normally occurs with settlement (Tanaka et al., 1996; Gwak and Tanaka, 2002). As such, energy reserved in lipid forms was most likely used to cope with the cessation of feeding until the completion of metamorphosis. After the eye migration and settlement, the digestion and energy metabolism returns to normal with effective contribution from pepsin enzymatic activity. Feeding cessation and reduction of pancreatic enzymes observed during metamorphosis highlights the importance of adapting feeding protocols based on the digestibility of feeds for larval fish according to its developmental stages to avoid mortality (Tanaka et al., 1996; Gwak et al., 1999; Kolkovski, 2001).

Amylase displayed complicated fluctuations in enzymatic expression and activity levels (Fig. 5.7). Amylase activity of *P. olivaceus* larvae is normally low at hatching and gradually increases until pre-metamorphosis (Tanaka et al., 1996; Li et al., 2013; Khoa et al., 2020), with some marine fish species reaching peak activity at this stage (Zambonino- Infante and Cahu, 2007). This was also observed in the current study with peaks in amylase activity between 20 and 25 DAH (Fig. 5.7). A strong peak of amylase activity could be due to continuation of feeding after the cessation during metamorphosis (Tanaka et al., 1996; Bolasina et al., 2006; Li et al., 2013). Cahu et al. (2004) suggested that carbohydrate (especially starch) concentration and nature of the feed (rotifer and *Artemia*) differed, this can result in amylase expression and activity variability. Fish age, diet composition, and diet quantity affect the molecular controlling mechanism of carbohydrase (Zambonino- Infante et al., 1996; Péres et al., 1998). Hence, amylase of fish can be modulated by adjusting diet composition (Zambonino- Infante et al., 1996) and the *amy* expression levels might be limited to very specific time points (Murashita et al., 2014). In the present study, the rotifer enriched with SDBP (S5 and S10) contained a significantly higher content of carbohydrate resulting in higher amylase activity in larval fishes compared to S1 and control treatments. Furthermore, the activity of amylase

enzyme dramatically dropped after settlement (30-35 DAH) which suggests that the juvenile turned to carnivorous feeding behavior.

The metamorphic processes in flatfish is critical and is very energy demanding (Geffen et al., 2007). The energy allocated for metamorphosis-related processes seems to be provided mainly from reserves accumulated during the pelagic phase (Parra and Yúfera, 2001; Richard et al., 2015). Expression of genes related to energy metabolism (*cox* and *atp*) in this study could help to understand the various energy allocations at different stages for normal growth and metamorphosis. The changes in energy metabolism related genes during larval development have been investigated by Khoa et al. (2021). Accordingly, *cox* expression level seemed to be sensitive to the feeding diets whereby *cox* transcription peaked when the larvae started the exogenous feeding and slightly increased when larvae fed *Artemia* while *atp* level remained low constantly for the first 20 DAH. This trend suggests that the *cox* gene is mainly involved in energy metabolism during the first 20 DAH of *P. olivaceus*, thereafter, the two genes play different roles during the metamorphosis stage. From 20–25 DAH, *atp* expression strongly increased which indicates an increase in the stored energy consumption while *cox* levels increased to keep energy status stable until the end of metamorphosis. A similar mechanism of *P. olivaceus* stocked in cold temperature conditions was reported by Lu et al. (2018). Using higher energy diets, especially in protein content, DHA and DHA/EPA ratio in S5 and S10 treatment could lead to improvement of lipid metabolism and antioxidant capacity of fishes (Jin et al., 2017; Luo et al., 2019). This is an important preparatory step for energy reservation for metamorphosis. A significantly higher level in expression of *cox* and *atp* gene during the metamorphic stage coinciding with higher survival in S5 and S10 indicated that the larvae were provided a satisfying energy source for these critical processes (Fig. 5.1, 5.8 and 5.9). After metamorphosis, larvae resumed feeding strongly again leading to peaks of enzyme activities,

subsequently the expression levels of *atp* and *cox* strongly recovered to provide energy for fish performance (Jesper et al., 1996; Gjevre and Næss, 1996; Dalziel et al., 2006).

A feeding cessation and delay in growth during metamorphosis of *P. olivaceus* larvae has been mentioned in previous studies (Gwak and Tanaka, 2002; Gwak et al., 2003; Bolasina et al., 2006; Khoa et al., 2020). In this study, these aspects were molecularly demonstrated through the expression of *cck* and *igf1* gene (Fig. 5.10 and 5.11). *cck* plays a catalytic role in the regulation of digestion of fish with a negative feedback mechanism to trypsin (Kurokawa et al., 2000; Tillner et al., 2013). A dramatic drop in trypsin activity coinciding with a peak of *cck* expression within 20-25 DAH (Fig. 5.3 and 5.10) were supportive of our hypothesis on the cessation of feeding of larvae. On the order hand, growth performance of larval fish was affected by several internal and external factors (Bertucci et al., 2019); besides the specific growth rate in body length and weight of fish; growth hormone and *igf1* could be applied as possible markers for evaluation of growth rate with advantage reduction in the need for lengthy and costly grow-out trials (Khoa et al., 2021). *igf1* has also been associated with fish metabolism, development, reproduction and osmoregulation in seawater (Reinecke et al., 2005; Beckman, 2011). Therefore, the fluctuation of *cck* and *igf1* expression levels in this study not only confirmed the feeding cessation and delay in growth but also highlighted the various levels in metabolism of *P. olivaceus* larvae among the different treatments.

Rotifer enriched with salmon roe emulsion oil supplemented with SDBP at 5 % and 10 % showed a significant enhancement in nutrient composition of crude protein, carbohydrate, DHA, HUFA and DHA/EPA ratio. The nutritionally effective enrichment of rotifer also reflected through adaptive feeding responses in *P. olivaceus* larvae. During larviculture, the larvae fed salmon roe emulsion oil only and the treatment supplemented with 1 % SDBP suffered high mortality due to a lack of energy required to survive metamorphosis. On the contrary, the larvae fed 5 % and 10 % SDBP showed effective responses in activity and

expression of digestive enzymes and energy metabolism resulting in significant improvement of survival rates. However, the effects of SDBP on FAA contents of rotifer as well as the growth of fish larvae require more consideration and optimization. This study suggests that SDBP has the potential as a live feed enrichment media for larval fish production. It is hoped that findings from this research will contribute to the development of appropriate rotifer diets to enhance growth and survival rates of larval fish whilst maintaining an eco-friendly and efficient approach towards finding effective uses for Shochu distillery by products in Japan.

Supplementary 5.1

Percentage composition of crude protein, crude lipid and carbohydrates of the Shochu distillery by product used as the rotifer enrichment (Mean ± SD).

	Shochu distillery by product
Crude protein (%)	47.1 ± 2.7
Crude lipid (%)	8.7 ± 2.5
Carbohydrate (%)	37.11 ± 2.3

Fatty acid proportion (%) and content (mg/g dry weight) of Shochu distillery by product used as the rotifer enrichment (Mean ± SD).

Fatty acid (%)	Shochu distillery by product
C14:0	2.59 ± 0.11
C16:0	29.52 ± 0.49
C16:1n-7	2.11 ± 0.26
C16:2n-4	0.53 ± 0.01
C16:3n-4	0.40 ± 0.04
C18:0	11.67 ± 0.23
C18:1n-9+n-7	6.69 ± 0.29
C18:2n-6	21.33 ± 0.52
C18:3n-3	2.90 ± 0.17
C18:4n-3	0.13 ± 0.02
C20:0	0.64 ± 0.01
C20:1n-9	1.40 ± 0.03
C20:2n-6	2.54 ± 0.01
C20:3n-6	0.06 ±0.01
ARA	0.05 ± 0.02
C20:4n-3	-
EPA	0.05 ± 0.02
C22:0	0.18 ± 0.05
DHA	-
SFA	44.60 ± 0.61
MUFA	10.19 ± 5.67
n-6	23.98 ± 0.54
n-3	3.09 ± 0.18
PUFA	27.99 ± 1.68
Others	17.21 ± 9.61
Contents (mg/g DW)	
ARA	0.05 ± 0.02
EPA	0.05 ± 0.03
DHA	-
Σn-3HUFA	-
Σmonoene	5.73 ± 1.11
Σn-3	10.55 ± 2.19
Σn-6	3.54 ± 0.21
DHA/EPA	-
ARA/EPA	1.02 ± 0.04
n-3/n-6	0.34 ± 0.05

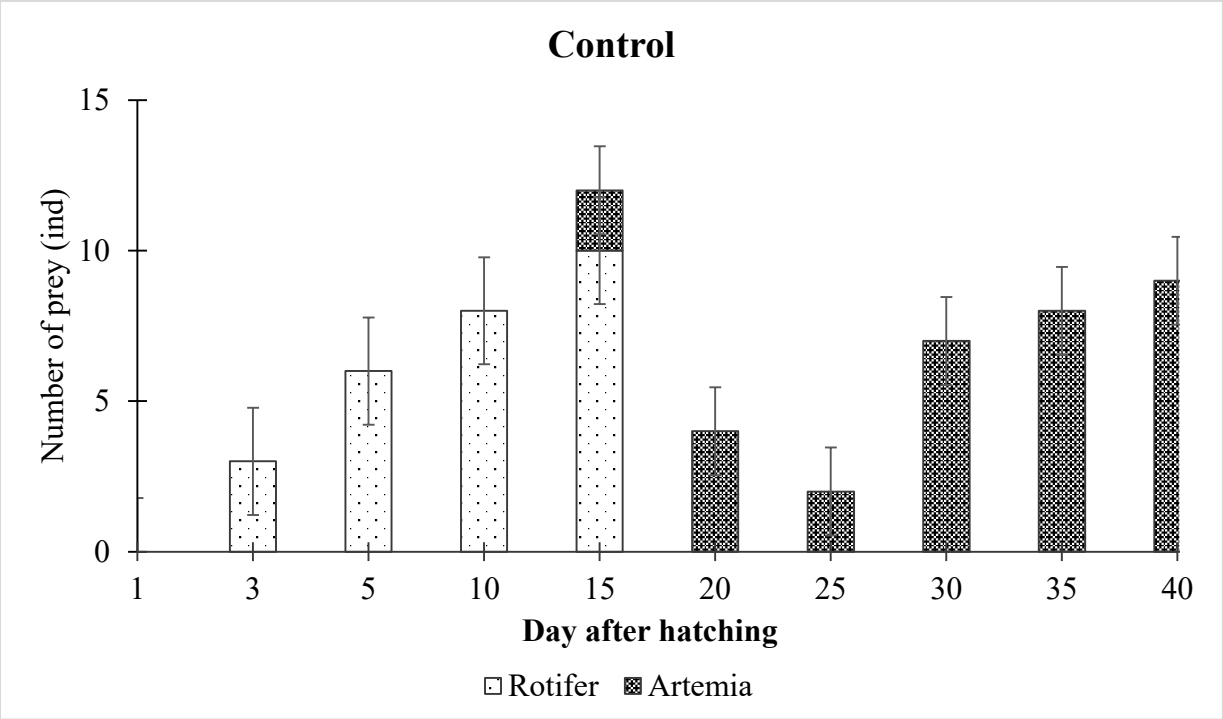
Supplementary 5.2

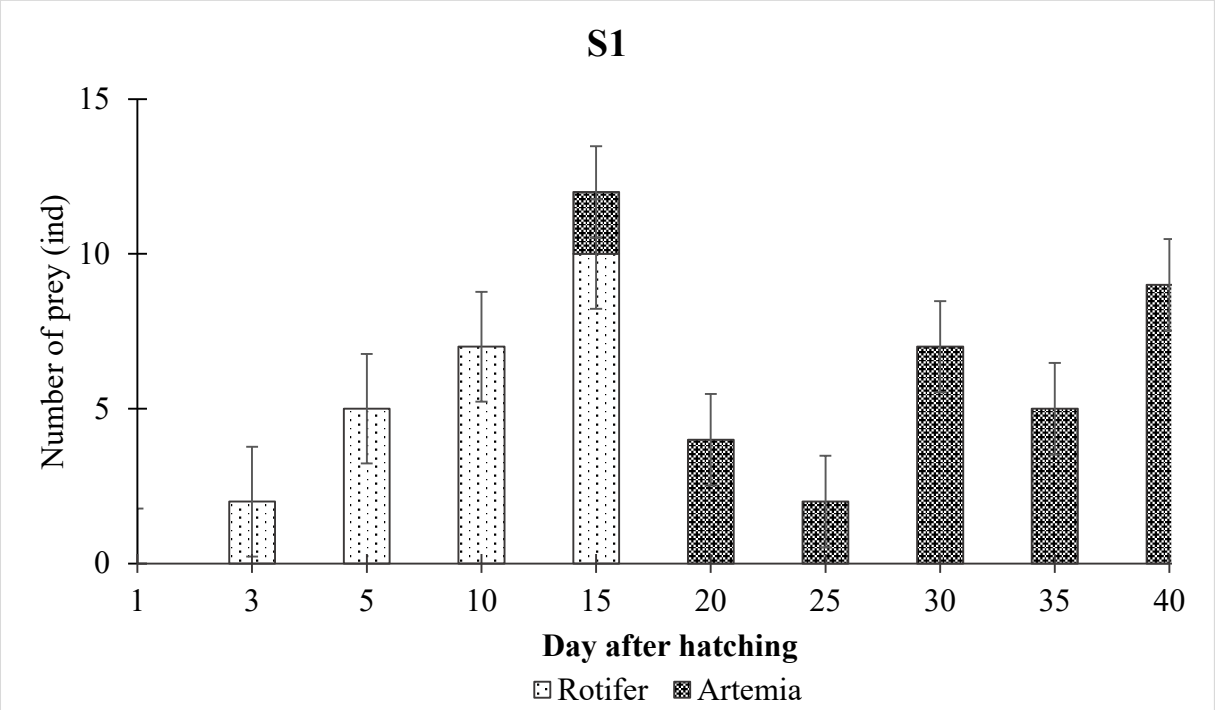
The strains of microbial-community in the Shochu distillery by product

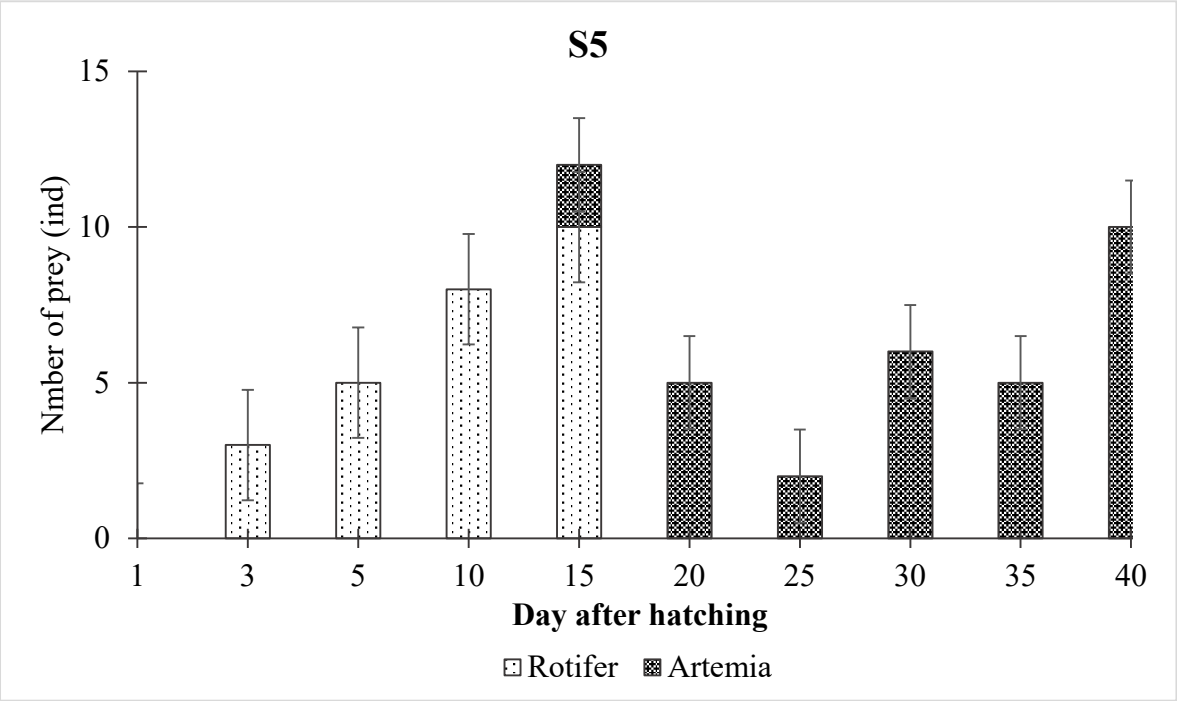
Strain Code	Scientific name
HR101327	*Lactobacillus delbruekii subsp. lactis*
HR101236	*Lactobacillus plantarum*
HR101475	*Lactobacillus debruekii subsp. bulgaricus*
HR101476	*Lactobacillus casei subsp. casei*
HR101182	*Lactobacillus brevis*
HR101174	*Lactobacillus acidophilus*
HR102263	*Enterococcus hirae*
HR102167	*Enterococcus faecalis*
HR103583	*Pediococcus acidilactici*
HR104164	*Streptococcus thermophilus*
HR201041	*Saccharomyces cerevisiae*
HR201010	*Saccharomyces cerevisiae*
HR301154	*Aspergillus oryzae*
HR106010	*Bacillus coagulans*
HR106013	*Bacillus subtilis*

Supplementary 5.3

Gut content of *P. olivaceus* larvae during the experimental periods.






S5
15
10
5
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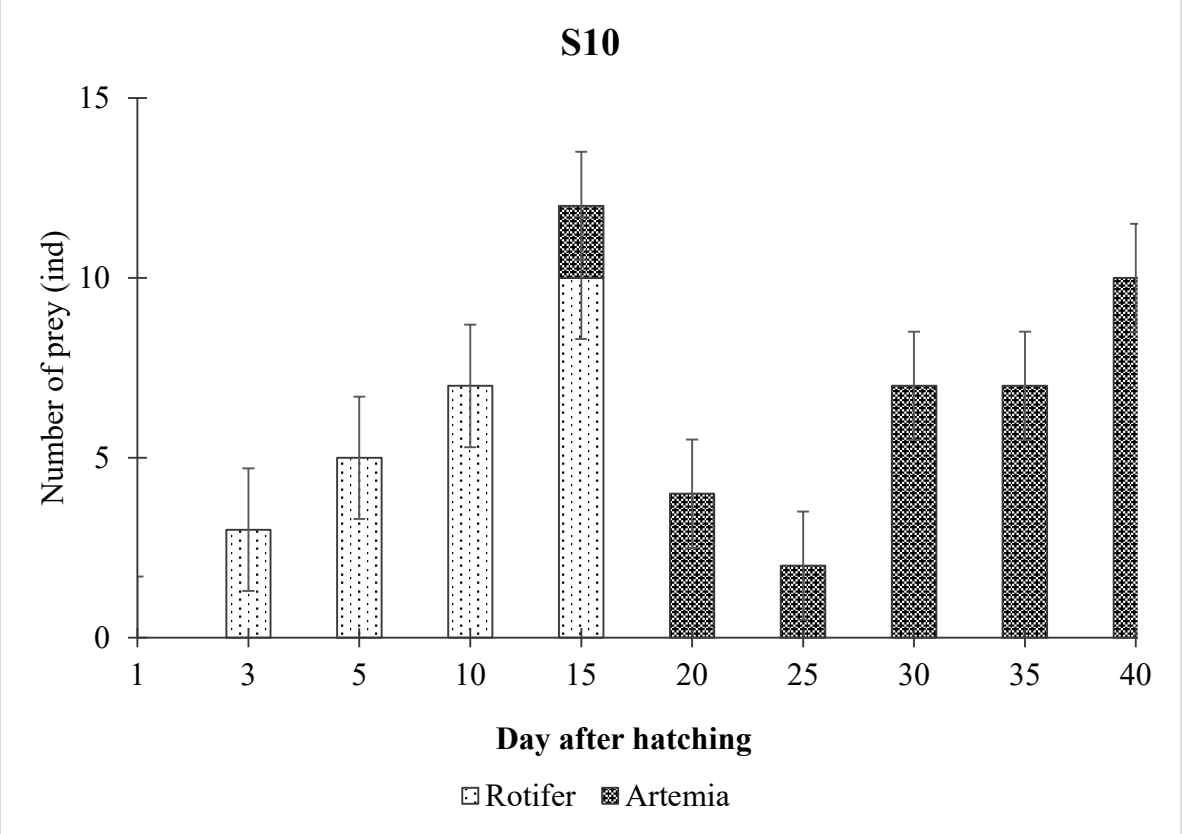
Nmber of prey (ind)
1
3
5
10
15
20
25
30
35
40
Day after hatching
Rotifer
Artemia


S10
15
10
5
0
Number of prey (ind)
1
3
5
10
15
20
25
30
35
40
Day after hatching
Rotifer
Artemia

CHAPTER 6: Changes in early digestive tract morphology, enzyme expression and activity of Kawakawa tuna (*Euthynnus affinis*)

Abstract

Kawakawa tuna (*Euthynnus affinis*) is a novel target species for aquaculture in Japan and currently there is very limited knowledge on its early ontogenetic development in captivity. The ontogeny of *E. affinis* larvae from hatching to 20 days after hatching (DAH) was illustrated in the present study through an integrated approach of histology, biochemistry and molecular approaches. Larvae were fed enriched L-type rotifer *Brachionus plicatilis* species complex from 2 - 10 DAH, followed by enriched *Artemia* nauplii. Target digestive enzymes; trypsin, pepsin, lipase and amylase were assessed in order to evaluate nutritional condition. The larvae began exogenous feeding from 2 DAH, coinciding with the opening of the oral capacity and anus. The yolk sac was rapidly consumed and completely resorbed at 3 DAH, while the oil globule disappeared by 5 DAH. Enzymatic activity and expression of the target enzymes were detectable at hatching, except pepsin. With the development of the gastric glands, pepsin activity was then detected at 10 DAH, which was followed by rapid increase in pepsin activity at 15 DAH. The larvae showed rapid growth, however, high mortality also occurred at 6 - 10 DAH which has been highlighted as a critical stage in the larviculture of *E. affinis*. From 15 – 20 DAH, the histological structures of the digestive tract were completely segmented with the digestive tract resembling that of its juvenile stages. The activity of trypsin, pepsin, and lipase tended to increase sharply, while amylase dramatically dropped, possibly indicating a change in dietary preference. Findings from the present study provides a preliminary insight into the early development of the digestive system and associated digestive enzymes of *E. affinis* larvae and establishes an initial step towards the rearing and feeding protocol optimization of this novel target species.

6.1 Introduction

Kawakawa tuna (*Euthynnus affinis*) belongs to epipelagic tuna Scombridae family and are widely distributed in the tropical and subtropical waters of the Indo–Pacific region (Williamson, 1970; Chiou and Lee, 2004). Recently, *E. affinis* has been considered as a novel target species for aquaculture due to its high market prices and fast growth (Yazawa et al., 2017; Manabe, 2019). In Japan, some small scale cage culture of Kawakawa have been conducted with wild-caught seeds (Yazawa et al., 2017). However, the reliable supply of *E. affinis* juvenile from local fishing boats was not achieved as the catch rate of juveniles were not high enough within Japans fishing waters. Therefore the need to develop artificial breeding approaches for stable mass seed production for the expansion of Kawakawa tuna farming arose (Yazawa et al., 2017 and 2019; Amezawa et al., 2018). Some primary results in artificial breeding of *E. affinis* have been archived and these have mostly been achieved via the administration of a gonadotropin-releasing hormone analog (GnRHa) in recirculating culture systems (Yazawa et al., 2015, 2017 and 2019; Amezawa et al., 2018). Since then, good progress in artificial breeding has been achieved, with high spawning and hatching index. As such, the development of larval rearing protocols subsequently required more attention as the demand for sufficient supply and high quality fingerlings for production grew. However, information on early larval development and rearing technology for this species is still very limited.

In the field of larval rearing protocol development, one of the biggest challenges is the optimization of diet to ensure maintenance of high quality fry, especially at early stages of larval ontogeny, such as first feeding (Hamre et al., 2013). Most fish larvae hatch with a very rudimentary digestive system, thus leading to digestive capacity limitations (Rønnestad et al., 2013; Yúfera et al., 2018; Uebersch et al., 2018). After several major changes in morphological structures of the digestive system, the larvae are then able to ingest, digest and assimilate food diversely (Conceição et al., 2008). During the stages when the digestive system undergoes

rapid structural changes, the ability of larvae to digest certain feed varies with feed type (Zambonino-Infante et al., 2008; Mahjoub et al., 2013). Fish larvae absorb nutrients from the yolk sac upon hatching, then rely heavily on live feed at early stages of exogenous feeding (Rønnestad et al., 2013). The development of gastric glands and the activation of the digestive enzyme pepsin, act as an indicator of the functionality of the larval stomach and also the ability of larvae to now effectively digest different types of feed such as inert/formulated feed (Zambonino-Infante and Cahu, 2001; Hamre et al., 2013). As such, a good knowledge of larval fish digestive physiology as well as early larval nutritional requirements will prove helpful for diet and feeding regime optimization and hence, hopefully improve production via increased survival and high quality fry.

Traditionally, studies on ontogenetic development of larval fish digestive systems were mainly illustrated through histological structures of the digestive tract, morphological changes of digestive organs and digestive enzyme activity on hydrolyzed nutrient components (Zambonino-Infante et al., 2008; Lazo et al., 2011; Yúfera et al., 2018). More recently, molecular approaches have been applied to access expression profiles of digestive enzyme precursors in fish, which could provive insight into both temporal and spatial expression patterns of genes involved in the development and functionality of digestive systems during early ontogeny (Moguel-Hernández et al., 2016 ; Yúfera et al., 2018; Khoa et al., 2019).

This study aimed to investigate the ontogenetic development of the digestive system of *E. affinis* larvae through the changes in its histological structures, enzymatic activity and its related gene expression of key digestive enzymes at its early stages (from hatching to 20 DAH) of larval development. It is hoped that the results from the present study and the comprehensive description of the changes in early ontogeny of *E. affinis* will contribute towards the development of rearing technologies for this economically important species.

6.2 Materials and methods

6.2.1 Kawakawa larval rearing

Fertilized eggs of *E. affinis* were obtained from Fisheries Research Center, Ehime Research Institute of Agriculture, Forestry and Aquaculture, Uwajima, Ehime, Japan and transferred to the Aquaculture Research Center, Kagoshima University, Japan. A total of 2000 eggs were stocked in 500 L composite tanks with continuous aeration and 35 psu of salinity. Temperature in the rearing tank was maintained at 18 ºC via titanium heaters (Aqua Heat, Sunlight Supply Inc., Japan).

From mouth opening to 10 DAH, the larvae were fed twice a day at 07:30 and 16:00 with the euryhaline L-type rotifer Amami strain, *Brachionus plicatilis* species complex. Rotifers were enriched with freshwater *Chlorella vulgaris* containing docosahexaenoic acid (C22:6 n-3, DHA; Super Fresh Chlorella V-12, Chlorella Industry Co. Ltd., Tokyo, Japan) and taurine (AquaPlus ET, Nisshin Marubeni Feed Co., Ltd., Tokyo, Japan). Rotifer enrichment culture and enrichment protocols followed those as described by Khoa et al. (2019). Rotifer density in the rearing tank was maintained at 10 ind./mL. From 10 - 20 DAH, water exchange in the rearing tank began at the onset of exogenous feeding (2 DAH) was set at 300 % per day. Water was drained through a drainage vinyl pipe screened through a polyethylene mesh (0.2 mm) (Khoa et al., 2019).

Artemia nauplii (Great Salt Lake Artemia, Ogden, UT, USA) enriched with salmon roe oil (MarineTech Co., Ltd., Japan) were introduced to the larvae. *Artemia* density in the rearing tank was maintained at 10 ind./mL from 10 DAH. *Artemia* enrichment consisted of cysts being incubated for 36 h at 25 ºC, before the nauplii were collected and then enriched with salmon oil at 0.06 g/L of culture volume. After 12 h of enrichment, the *Artemia* nauplii were harvested and carefully rinsed with freshwater before feeding. 3 mL of *C. vulgaris* was added to the fish rearing tanks to maintain green water conditions.

6.2.2 Diet nutrient composition analysis

The nutrient compositions of enriched rotifer and *Artemia* were analyzed. The percentage of crude carbohydrate was undertaken following methods outlined by Dubois et al. (1956) and crude protein was determined following the Lowry's method (Lowry et al., 1951) and crude carbohydrate following Dubois et al. (1956). Total lipid and fatty acid composition of the live feed were also estimated according to methods described by Folch et al. (1953). The total lipid extractions were undertaken from 0.1 g dry weight (DW) of the live feed which were homogenized with chloroform-methanol (2:1) mixture. After filtration of the mixture, 0.5 ml of C19:0 (fatty acid standard) and 1 mL of 5 % hydrogen chloride methanol was then added to the solution. It was then incubated at 80 ºC for 3 h. After lipid extraction, the samples were mixed with 1 mL of hexane and 5.5 mL distilled water, vortexed and centrifuged for 5 min at 2000 rpm. The fatty acid containing hexane layer was collected into a vile and subjected to gas chromatography (GC- 2010, Shimadzu Co. Ltd., Kyoto, Japan) analysis based on the C19:0 standard.

For free amino acid analysis, the samples were processed followed the method described by Teshima et al. (1986). A total of 0.1 g and 0.1 mL internal standard (6 mg DL-norleucine/ml deionized water and 2.5 mL trichloroacetic acid) mixed with 0.9 mL cold deionized water. The mixture was homogenized and washed with 8 % TCA and centrifuged at 4 °C, 3000 rpm for 15 min. The supernatant was transferred to 15 mL tube and repeatedly washed with diethyl ether. The resultant solution was adjusted pH within 2.2 ± 0.05 using chloric acid or 4 N NaOH solution, then filtered through a syringe filter into 2 mL vial. The free amino acid analysis was performed using high-performance liquid chromatography via a LC-20 CE Liquid Chromatograph pump, DGU-20A3 degasser, CBM-20A communications bus module, LC-20AB liquid chromatograph, SIL-20A autosampler, RF-20A fluorescence detector, CTO-20A column oven and a FCV-11AL valve unit (HPLC, Shimadzu Corp. Kyoto, Japan).

6.2.3 Fish sample collection

A total of 430 larvae were sampled for various assessments, each assessment varied in sampling method and period. Sampling was conducted 2 h after morning feeding. The sampled larvae were then rinsed with distilled water to remove any contaminants from the rearing water and processed for different analyses. For histological observation, 20 larvae were randomly collected at each sample point (1-6 DAH, 10 DAH, 12 DAH, 15 DAH and 20 DAH). The collected larvae were then mixed with Bouin's solution and stored at 4 °C. For gut content analysis samples 15 larvae were also collected every day from 1-6 DAH and then at 10, 12, 15, 20 DAH. For enzyme expression assays, a total of 150 larvae were sampled (5 larvae × triplicate × 10 sampling points), submerged in the stabilization reagent (*RNA*later®, QIAGEN GmbH, Germany) following the manufacturers protocol for quantitative real time PCR. Following the same sampling protocol, 150 larvae were sampled for enzyme activity assays, however, the larvae collected were stored semi-dry at –80 °C without any suspension mediums. The total length and wet body weight of 15 larvae per group (15 larvae per sampling, at 1, 2, 5, 10, 15, 20 DAH) were measured each time after removing water with filter paper, using a fish measuring board and an analytical balance, respectively.

6.2.4 Histological observation

The samples were submerged in Buoin's solution for 24 h, then washed and preserved in 70 % alcohol. After dehydration processes, the specimens were embedded in paraffin blocks and sectioned in serial sagittal sections (5 μm), using a rotary microtome (RM 2135, Leica, Nussloch, Germany). The hematoxylin–eosin (HE) stain was used for histological observations. Finally, the slide with sections was mounted permanently (Entellan, EMD Millipore, Billerica, MA, USA) and observed under Olympus light microscope (BX41, Olympus, Tokyo, Japan).

Table 6. 1 Primer sequences for qRT-PCR

Gene	Primer forward	Primer reverse	Source
β-actin	AGCTGCCTGACGGACAGGTCATCA	TCGTACTCCTGCTTGCTGATCCA	Yazawa et al. (2013)
try	TCAGGTGTCTCTGAACTCTG	ACCTGAACACGAGACTTG	Kondo et al., 2017
pep1	CTCTGATTGTTGGACCTCAGAGCAG	TGAGGCATTTGGCCAATGTTGTTAC	Murashita et al. (2014)
ctrb1	TCTGACCCGCTACAACGCTCCCAGT	TGCTGCCCCAGTGTCTCTTACACTC	
amy2α	CTACCAAGTCTGTTCCCATCAATAG	CCCACCAGTTAGAATGAGGCTGTCC	
pl1	TTCCAGGACACTCCTGTTTCTGTGC	ATCCCCAGACCAAGTTTGGAGTTGA	
bal1	CATGGATGGACACCTCTTTACTGGT	AAACCAGCCTGGCCCTTCTCTTTAG	

In which: trypsin (*try*), chymotrypsin (*ctrb1*), bile salt activated lipase (*bal1*), pancreatic lipase (*pl1*), pepsin (*pep1*) and amylase (*amy2α*)

6.2.5 RNA extraction and quantitative real time PCR (qRT-PCR) analysis

For total RNA isolation, pooled samples containing 5 larvae were homogenized in 1 mL of TRIzol™ reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) in triplicate following the manufacturer's instructions. The RNA quality was determined by optical density (OD) absorption ratio (OD 260 nm/OD 280 nm) using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). Besides, gel electrophoresis was also carried out on 1 % agarose gel to verify the RNA integrity. The absorption ratio used ranged between 1.9 and 2.0 for cDNA synthesis (100 ng of RNA) following the iScript ™ cDNA Synthesis protocol (Bio-Rad Inc., Hercules, CA, USA).

The expression profile of relative genes of digestive enzymes (trypsin (*try*), bile salt activated lipase (*bal1*), pancreatic lipase (*pl1*), pepsin (*pep1*) and amylase (*amy2α*) were investigated during the larval stages. Primer sequences were listed in Table 6.1. Each reaction for qRT-PCR contained 10 µL of qPCR Mix, and 0.4 µL ROX reference dye (KOD SYBR® qPCR Mix, Toyobo Co. Ltd, Osaka, Japan), 1 µL of cDNA template, 0.4 µL of each primer in a final volume of 20 µL. The qRT-PCR was performed in triplicate for each sample in a Biorad CFX connect™ Real time system (Bio-Rad Laboratories, Inc., Japan). The thermal profile was 98 °C for 2 min; followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s. The melting curve was generated from 60 °C to 95 °C with an increment of 0.3 °C and holding for 15 s. To confirm the successful amplification of targeted genes, the PCR products were purified by agarose gel electrophoresis and FastGene Gel/PCR extraction kit (Nippon Genetics Co., Ltd, Japan). The amplified PCR products were sequenced by Gene research center, Kagoshima University using BigDye terminator v3.1 Cycle Sequencing kit (Applied Biosytems, Foster city, CA, USA). The obtained sequence was used for genotyping based on BLAST similarity search tool in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) (Supplementary 6.1).

The expression levels of targeted genes were normalized to the expression of β-actin and measured using the standard curve with diluted cDNAs

6.2.5 Enzyme assay

To prepare crude enzymes, pool samples of 5 larvae were homogenized with 500 µL of ice cold buffer (20mM Tris-HCl, 1mM EDTA, 10mM $CaCl_2$, pH 7.5) (Bolasina et al., 2006) using pellet pestle cordless motor (Sigma-Aldrich Inc., St. Louis, MO, USA) in 1.5 mL tubes. After homogenization, the tubes were centrifuged for 30 mins at 1700 *g* at 4 °C. for 30 min at 1700*g* at 4 °C. The supernatant transferred into new 1.5 mL tubes and was used as the crude enzyme extract in protein content and enzymatic assay analysis later (stored at −80 °C). Soluble protein contents of crude enzyme samples were determined using the Bradford method (Bradford, 1976).

6.2.5.1 Trypsin

Trypsin activity was measured using Z-L-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA, Sigma-Aldrich Inc., C9521) as the substrate to performed the fluorometric assay according to Bolasina et al. (2006). A reaction mixture was prepared by adding 500 µL of substrate (containing 50 mM Tris–HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ-LArg-MCA) and 50 µL of crude enzyme, then incubated for 30 min at 30 °C in a water bath, followed by adding 100 µL of 30% acetic acid for quenching (excepting blanks). Fluorescence was measured at 380 nm (excitation) and 440 nm (emission**)** using a Hitachi F2000 spectrofluorophotometer (Tokyo, Japan). Trypsin activity was expressed in unit (mU) in 30 min, as percentage increase of emission per protein (mU mg protein^{-1}).

6.2.5.2 Chymotrypsin

Chymotrypsin activity was assayed using N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPFNA, Sigma-Aldrich Inc.) as a substrate (Murashita et al., 2018). Reaction was imitated

by adding 50 µL of crude enzyme, 240 µL of 100 mM Tris buffer (pH 8.5, containing 20mM CaCl2) and 100 µL 2.4 mM SAPFNA, then incubated for 7 min at 37 °C. *p*NA production was measured by using a Hitachi U5100 spectrophotometer at 405 nm. One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1 absorbance unit at 405 nm in 1 min.

6.2.5.3 Pepsin

The activity of pepsin was determined by using 2% of haemoglobin in 0.06 N HCl as substrate Worthington (1993). Briefly, the reaction mixture was prepared by adding 100 µL of enzyme extract to 500 µL of substrate, then was incubated at 37 °C (pH 2.5) for 10 min. A 1 mL of 5 % trichloroacetic acid was added to terminate the reaction (for blank reading, the crude enzyme was added after this step), then left for 5 min. The sample tubes were centrifuged at 12000 × g for 5 min, then read at 280 nm using a Hitachi U5100 spectrophotometer.

6.2.5.4 Lipase

Lipase activity was determined using *p*-nitrophenyl myristate (PNPM, Sigma-Aldrich Inc.) as a substrate (Albro et al., 1985). The mixture reaction consisted of 450 µL of 100 mM Tris–HCl buffer (pH 8), 100 µL of 3.5 mM PNPM (containing 0.5 % Triton X-100, Nakarai, Osaka, Japan) and 50 µL of enzyme extract, followed by incubation at 37 ºC for 7 minutes. The production of *p*-nitrophenol (*p*NP) was measured by Hitachi U5100 spectrophotometer at 405 nm.

6.2.5.5 Amylase

Amylase activity was assayed using 1% starch solution as a substrate (Murashita et al., 2018). The assay was prepared by adding 50 µL of enzyme extract, 25 µL of the substrate solution and 25 µL 20mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl). After incubating at 37 ºC for 60 min, 50 µL dinitrosalicylic acid reagent (1 % dinitrosalicylic acid

and 30 % sodium potassium tartrate in 0.4M NaOH) was added into the mixture, then continue incubating for 5 min in boiling water. A six-fold dilution of the reaction mixture was read at 540 nm using a Hitachi U5100 spectrophotometer. The level of maltose release was estimated basing on a referenced standard curve.

6.2.6 Gut content analysis

The larvae were examined to constituents of larvae gut under a digital microscope (VHX-S90F, Keyence Corporation, Osaka, Japan) connected to an ultra-small, high-performance zoom lens (VH-Z20R, Keyence Corporation).

6.2.7 Statistical analyses

Potential differences were tested by one-way ANOVA (SPSS 24.0, IBM, USA) applied Tukey's HSD test (Honestly Significant Difference). Statistical significance was accepted at α=0.05. Besides, the linear regression (α=0.05) was applied to evaluate the correlation between gene expression and enzyme activity of fish larvae.

6.3 Results

6.3.1 Growth performance of Kawakawa larvae.

The newly hatched larvae were recorded at 3.08 ± 0.56 mm of total length and with a large yolk sac and oil globule at the anterior of the abdominal cavity (Fig. 6.1B). From 2 DAH, the mouth opened and the larvae began exogenous feeding. The yolk sac was completely exhausted at 3 DAH, while the oil globule remained until 5 DAH. The larvae began to consume a lot of rotifer from 4 DAH and were able to feed on *Artemia* nauplii from 10 DAH (Fig. 6.2). At 20 DAH, the total length was 5.54 ± 0.92 mm and the body weight was 0.88 ± 0.24 g. Total length ($y = 3.0052e^{0.0335x}$ ($R^2 = 0.9677$)) and body weight ($y = 0.0436e^{0.1597x}$ ($R^2 = 0.9677$) of the larvae both increased exponentially as observed in Fig. 6.1A.

The survival rate of Kawakawa larvae dramatically dropped at 5 DAH with the most critical stage observed to be at 6 – 10 DAH. Moreover, there were also a high percentage of larval deformities observed in the larvae from 5 DAH and these deformities included skeletal deformities and bending of notochord observed just posterior to the gut (Fig. 6.3). At the end of the study (20 DAH), larval survival was less than 0.5 % of the initial stocking density.

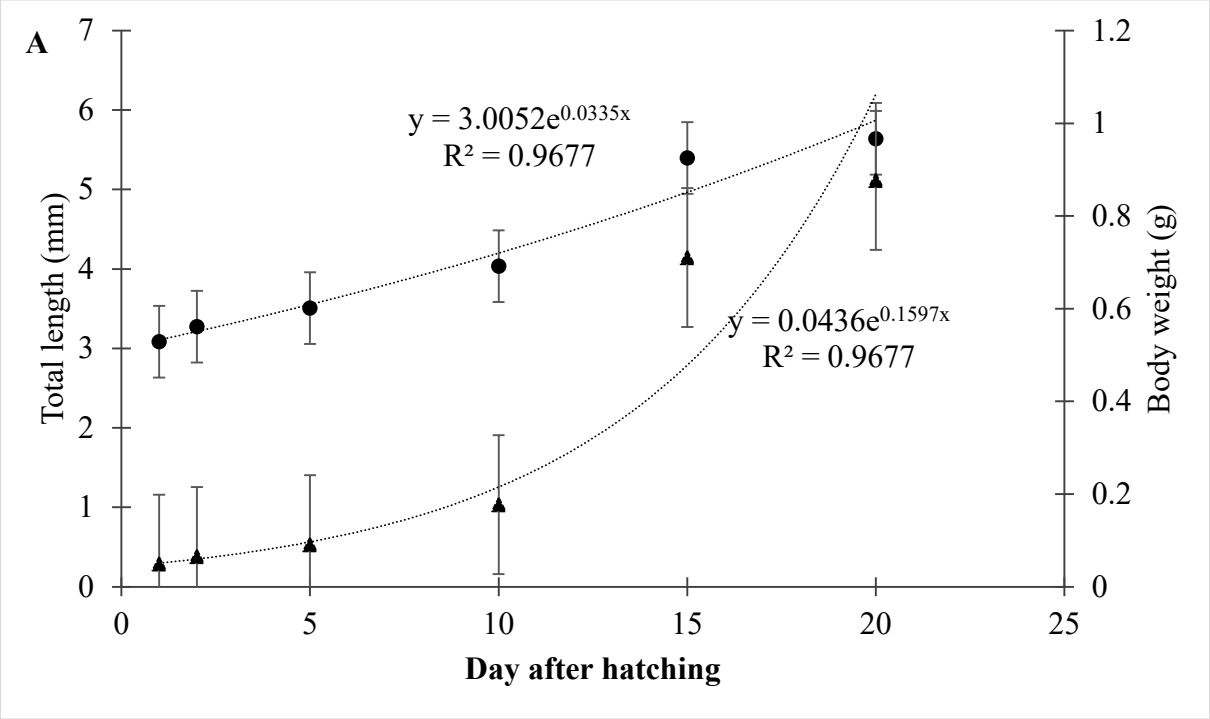
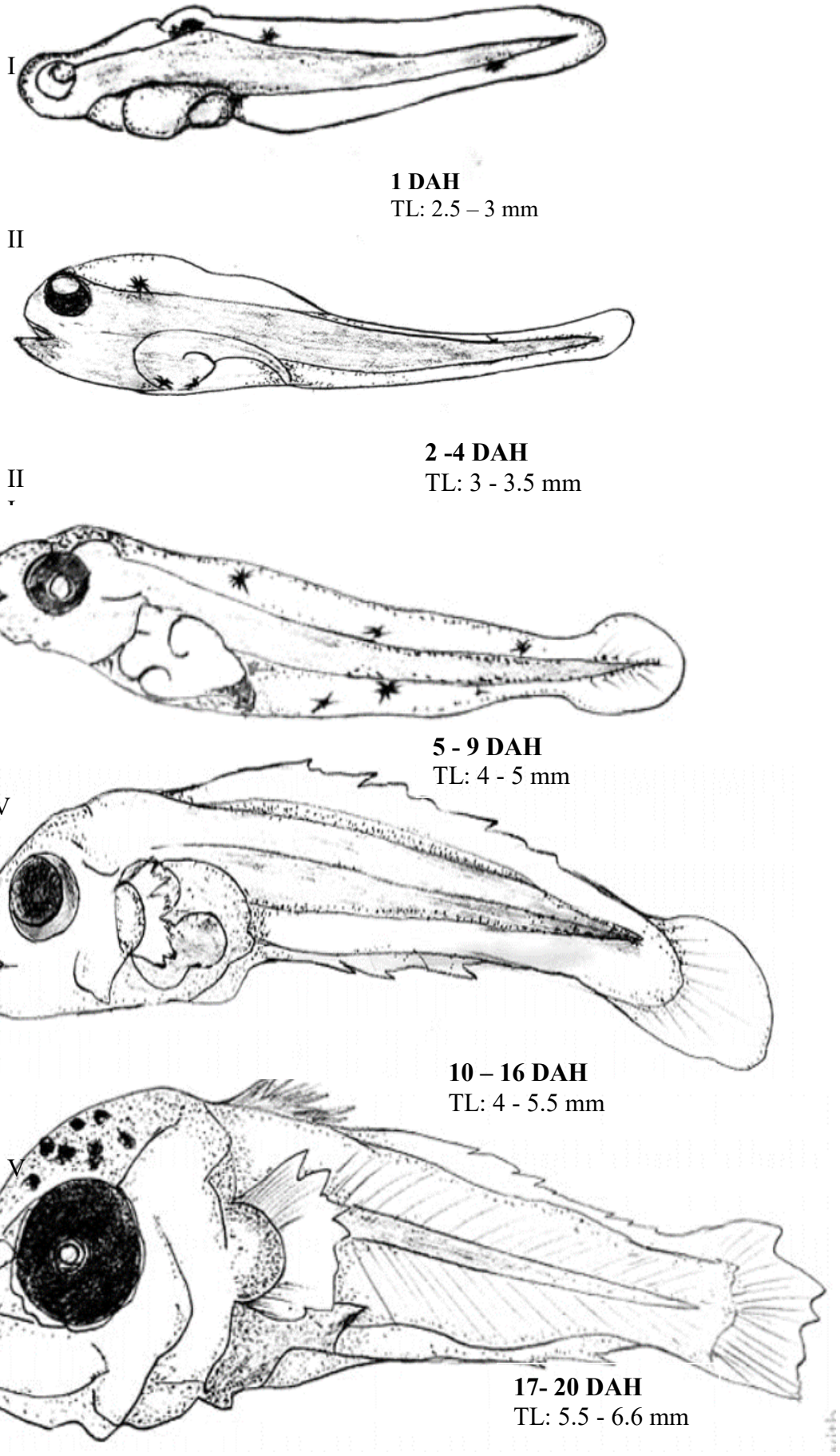


Figure 6. 1 Relationship of total length (mm), body weight (g), age post hatching (day) (A) and major morphological changes (B) from 1 to 20 DAH in Kawakawa larvae

B


I
1 DAH
TL: 2.5 – 3 mm
II
2 -4 DAH
TL: 3 - 3.5 mm
II
5 - 9 DAH
TL: 4 - 5 mm
IV
10 – 16 DAH
TL: 4 - 5.5 mm
V
17- 20 DAH
TL: 5.5 - 6.6 mm

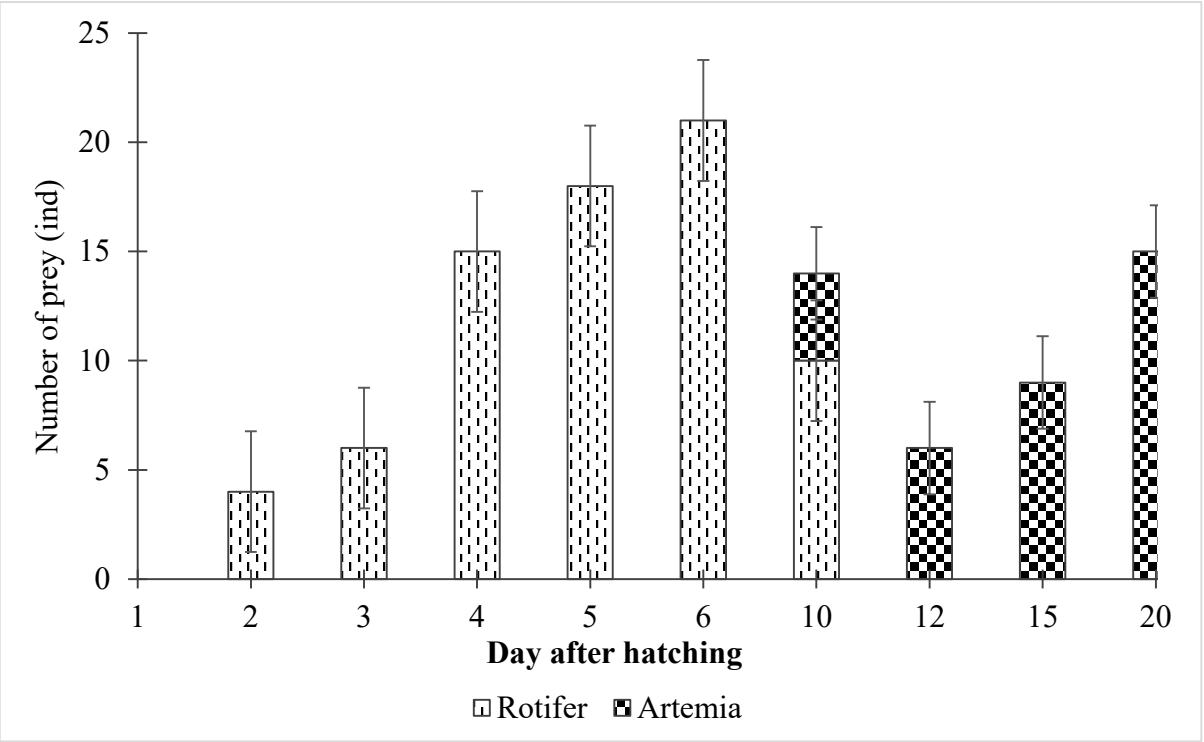


Figure 6. 2 Gut contents of Kawakawa larvae

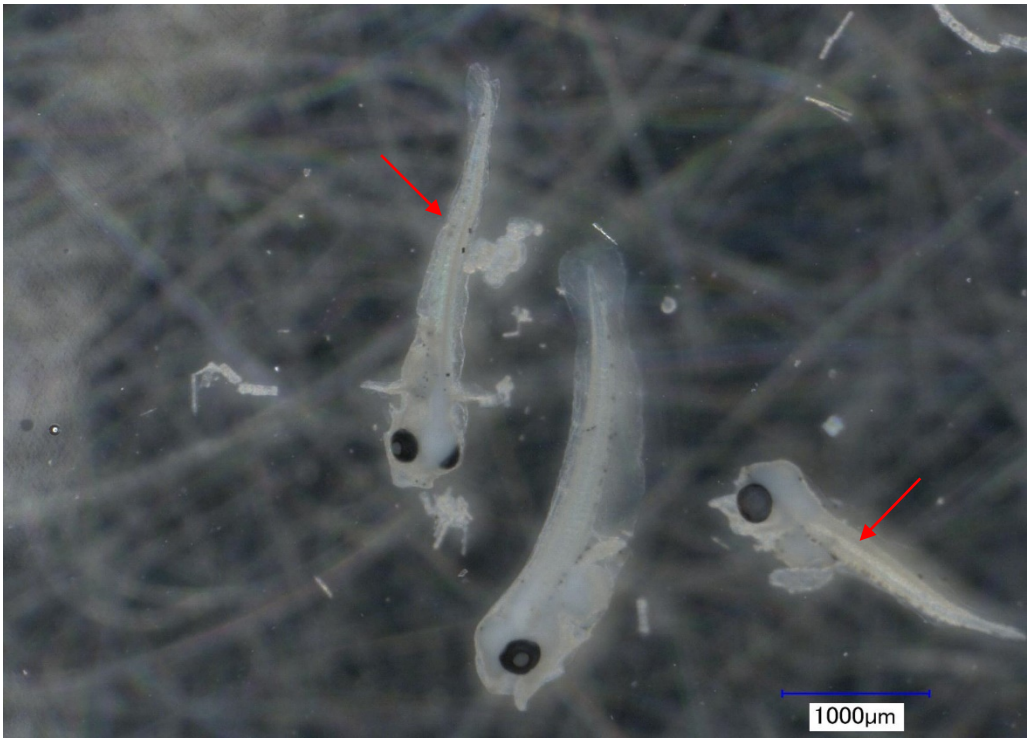


Figure 6. 3 Skeletal deformities during the ontogenetic development of Kawakawa larvae

6.3.2 Diet compositions

The enriched rotifer contained 61.75 ± 6.77 % of crude protein, 16.18 ± 3.24 % of crude lipid and 14.58 ± 1.83 % of carbohydrate (Table 6.2). *Artemia* nauplii enriched with salmon roe oil, presented a lower protein content (51.06 ± 2.41 %) as compared to rotifer (61.75 ± 6.77 %). There were no significant differences in lipid composition (*Artemia* nauplii: 14.7 ± 3.61; *B. plicatilis*: 16.18 ± 3.24 %) and carbohydrate (*Artemia* nauplii: 15.6 ± 1.85 %; *B. plicatilis*: 14.58 ± 1.83 %).

Of the essential fatty acids, rotifer diet showed higher contents in docosahexaenoic acid (DHA, 22:6n-3: 4.38 ± 0.40 mg/g DW), Σn-3HUFA (8.24 ± 0.66 mg/g) and DHA/EPA proportion (1.134 ± 0.029), but lower eicosapentaenoic acid (EPA, 20:5n-3: 3.86 ± 0.26 mg/g DW) compared to *Artemia* diet (EPA: 4.33 ± 0.06 mg/g; DHA: 0.69 ± 0.02 %; Σn-3HUFA: 5.03 ± 0.08 mg/g DW; DHA/EPA: 0.159 ± 0.007). However, *Artemia* contained higher Σmonoene (43.27 ± 1.13 mg/g DW) and Σn-3 (53.84 ± 1.18 mg/g DW) than rotifer (Table 3). Low levels of arachidonic acid (ARA, 20:4n-6) was observed in both composition and content for rotifers (0.71 ± 0.03 %; 0.59 ± 0.05mg/g DW) and *Artemia* (0.59 ±0.03 %; 0.88 ± 0.03 mg/g DW) (Table 6.3).

The enriched *Artemia* consisted high levels of taurine (14.82 ± 1.40 mg/g), alanine (3.24 ± 0.55 mg/g), arginine (2.45 ± 0.57 mg/g), proline (2.22 ± 0.31 mg/g) and glutamic acid (2.19 ± 0.41 mg/g), while the enriched rotifers had high contents of glutamic acid (9.57 ± 0.40 mg/g), aspartic acid (4.22 ± 0.26 mg/g), alanine (3.49 ± 0.09 mg/g) and taurine (2.64 ± 0.04 mg/g) (Table 6.4).

Table 6. 2 Diet compositions

Nutrient composition	Diet (DW)	
	Enriched rotifer	**Enriched *Artemia***
Crude protein (%)	61.75 ± 6.77	51.06 ± 2.41
Crude lipid (%)	16.18 ± 3.24	14.7 ± 3.61
Crude carbohydrate (%)	14.58 ± 1.83	15.6 ± 1.85

Table 6. 3 Total fatty acid profiles in enriched rotifer and enriched Artemia (Mean ± SD).

Fatty acid (%)	Enriched rotifer	Enriched *Artemia*
14:0	4.31± 0.21	1.12 ± 0.02
16:0	13.22 ± 1.25	9.54 ± 0.08
16:1 n-7	3.63 ± 0.08	3.71 ± 0.01
16:2 n-4	4.50 ± 0.05	1.39 ± 0.01
16:3 n-4	1.01 ± 0.04	0.76 ± 0.01
18:0	3.91 ± 0.76	4.71 ± 0.01
18:1 n-9+n-7	5.23 ± 0.23	24.57 ± 0.09
18:2 n-6	15.69 ± 0.76	6.75 ± 0.05
18:3 n-3	6.14 ± 0.55	26.90 ± 0.08
18:4 n-3	0.22 ± 0.04	5.04 ± 0.05
20:0	0.11 ± 0.03	0.45 ± 0.04
20:1n-9	0.87 ± 0.09	0.68 ± 0.02
20:2 n-6	0.07 ± 0.06	0.05 ± 0.01
20:3 n-6	0.33 ±0.38	0.09 ± 0.01
ARA	0.71 ± 0.03	0.59 ±0.03
20:4 n-3	0.71 ± 0.06	0.65 ± 0.01
EPA	4.68 ± 0.30	2.90 ± 0.03
22:0	0.33 ± 0.01	0.36 ± 0.01
22:1 n-9	-	-
22:5 n-6	0.06 ± 0.01	-
22:5 n-3	3.07 ± 0.11	0.10 ± 0.01
DHA	5.30 ± 0.20	0.46 ± 0.03
SFA	21.88 ± 1.80	16.18 ± 0.01
MUFA	9.73 ± 0.41	28.96 ± 0.09
n-6	16.86 ± 0.81	7.49 ± 0.01
n-3	20.12 ± 0.16	36.05 ± 0.05
PUFA	42.48 ± 0.98	45.68 ± 0.05
Others	25.61 ± 3.19	9.17 ± 0.04
Contents (mg/g DW)		
ARA	0.59 ± 0.05	0.88 ± 0.03
EPA	3.86 ± 0.26	4.33 ± 0.06
DHA	4.38 ± 0.40	0.69 ± 0.02
Σn-3HUFA	8.24 ± 0.66	5.03 ± 0.08
MUFA	8.04 ± 0.70	43.27 ± 1.13
n-3	16.66 ± 2.02	53.84 ± 1.18
n-6	13.90 ± 0.78	11.18 ± 0.25
DHA/EPA	1.134 ± 0.029	0.159 ± 0.007
ARA/EPA	0.152 ± 0.003	0.203 ± 0.008
n-3/n-6	1.196 ± 0.079	4.816 ± 0.002

Abbreviations: ARA: Arachidonic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid

Table 6. 4 Free Amino Acid (FAA) contents (mg/g DW) of enriched rotifers and enriched Artemia (Mean ± SD).

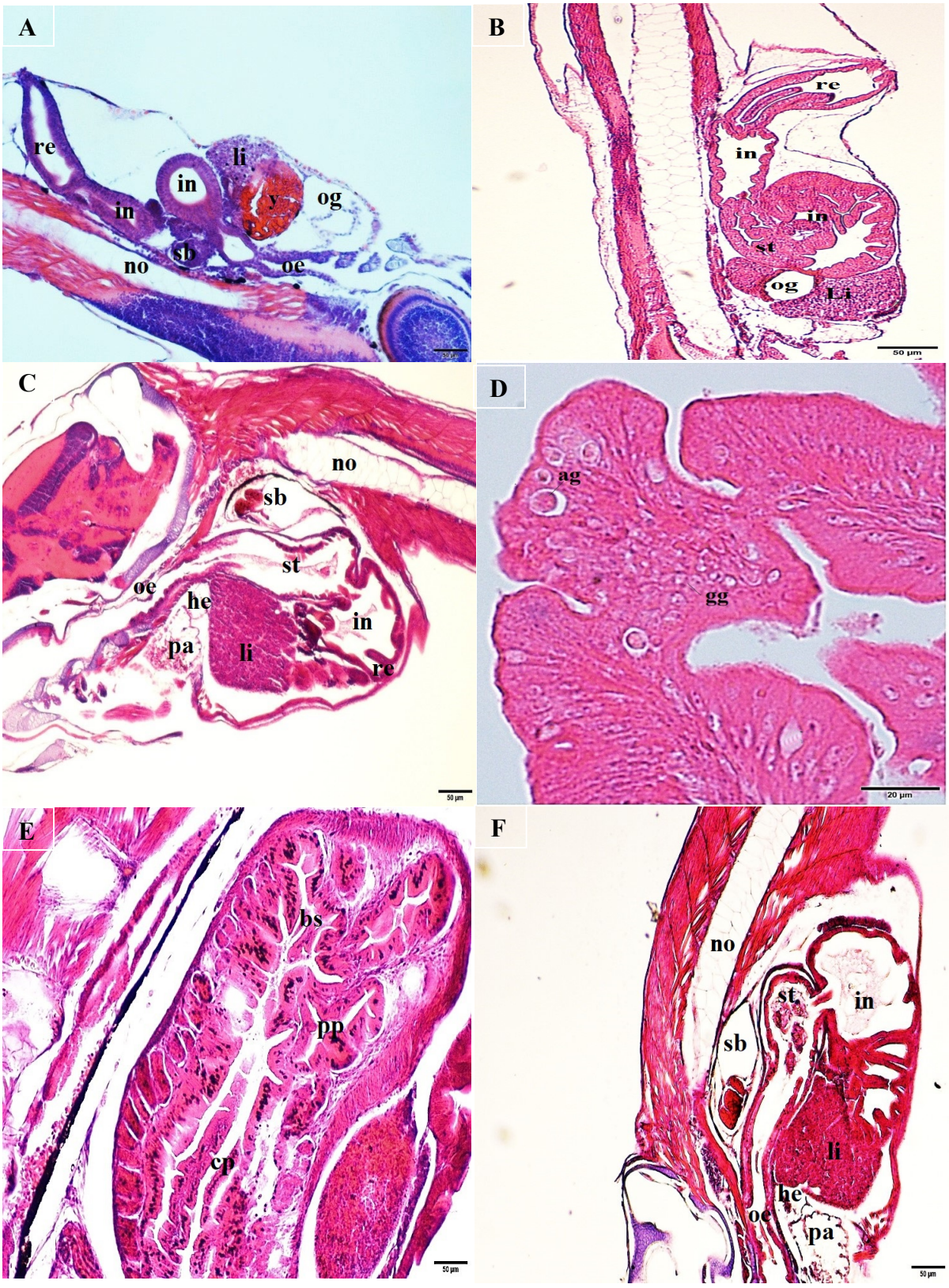
FAA	Enriched rotifer	Enriched *Artemia*
Taurine	2.64 ± 0.04	14.82 ± 1.40
Aspartic acid	4.22 ± 0.26	0.98 ± 0.08
Threonine	1.07 ± 0.06	1.04 ± 0.20
Serine	0.92 ± 0.07	1.50 ± 0.29
Glutamic acid	9.57 ± 0.40	2.19 ± 0.41
Proline	0.94 ± 0.07	2.22 ± 0.31
Glycine	1.25 ± 0.08	0.44 ± 0.08
Alanine	3.49 ± 0.09	3.24 ± 0.55
Cysteine	0.28 ± 0.03	0.13 ± 0.01
Valine	0.65 ± 0.04	0.74 ± 0.16
Methionine	0.38 ± 0.01	0.22 ± 0.09
Isoleucine	0.67 ± 0.04	0.47 ± 0.12
Leucine	1.03 ± 0.08	0.64 ± 0.18
Tyrosine	0.87± 0.02	1.67 ± 0.24
Phenylalanine	0.83 ± 0.09	0.56 ± 0.14
Histidine	1.33 ± 0.41	1.35 ± 0.31
Tryptophan	0.38 ± 0.15	0.62 ± 0.24
Lysine	1.78 ± 0.08	1.04 ± 0.21
Arginine	1.78 ± 0.07	2.45 0.57

6.3.3 Histological observation of digestive tract

At hatching, the larval digestive tract was mainly characterized by the yolk sac and oil globule. The oral capacity and anus opened at 2 DAH; whereby buccopharyngeal cavity, oesophagus and intestine were histologically observed. The undifferentiated digestive tract displayed as a straight tube extending posteriorly from the remaining yolk sac and oil globule (Fig. 6.4A). The yolk sac was completely depleted at 3 DAH, while the oil globule disappeared at 5 DAH (Fig. 6.4B). From 5 DAH, the intestine loop with an intestinal valve separated the anterior intestine and posterior intestine. A sphincter was also formed at 5 DAH to differentiate middle and posterior intestinal portions and the incipient stomach and swim bladder were also visible. From 5 - 10 DAH, the digestive tract elongated and expanded, in which the incipient stomach was observed with a surrounding of connective tissue and muscular circular layer. Intestinal brush border and epithelial fold increased (Fig. 6.4C). Gastric glands were first detected at 10 DAH, which is also evidence that the stomach starts functioning at this period, furthermore, this also coincided with an accumulation of lipids in the hepatocytes (Fig. 6.4D). From 15 DAH, the stomach clearly differentiated into 3 portions consisting of cardiac portion, pyloric portion, and blind sac (Fig. 6.4E), with the increase in size and density of the gastric glands. The intestinal epithelium was intensively vacuolated by lipid absorption. The stomach continued to enlarge and with a dense area of goblet cells observed in the intestinal epidermis at 20 DAH (Fig. 6.4F).

Figure 6. 4 Histological micro-sections of the digestive system in Kawakawa larvae during development. (A) 2 -3 DAH larvae, mouth and anus opened with a straight digestive tract, yolk sac and oil globule (og) remains. (B) 5 DAH, intestine loop with an intestinal valve separated the anterior intestine and posterior intestine, a sphincter was formed to differentiate middle and posterior intestinal portions. (C) 10 DAH, with the appearance of anterior intestine, middle intestine, liver, pancreas, posterior intestine, swim bladder, stomach. (D) 10 DAH appeared gastric lands. (E) 15 DAH, stomach segmentation. (F) 20 DAH with high density of gastric glands, goblet cells and brush borders in intestine. Abbreviations: y: yolk sac; bc: buccopharyngeal cavity; oe: oesophagus; he: heart; sb: swim bladder; li: liver; pa: pancreas; og: oil globule; st: stomach; gg: gastric gland; ag: acidophilic granules; no: notochord; pp:

pyloric portion; cp: cardiac portion; bs: blind sac. Scale = 50 µm. Staining: Haematoxylin-Eosin.



6.3.4 Expression and activity of digestive enzymes

6.3.4.1 Trypsin

Trypsin activity (1.77 ± 0.5 mU/mg protein) and expression (0.0056 ± 0.0012) were detectable within the first 3 days after hatching, despite being at very low levels (Fig. 6.5A). At 4 DAH, both activity and expression of trypsin sharply increased and peaked at 6 DAH (33.05 ± 4.7 mU/ mg protein and 24.60 ± 1.25). However, these levels dramatically dropped at 10 DAH and remained low until 20 DAH. Within the first 20 days of rearing, the trypsin activity showed a significant correlation to *try* expression (R^2 = 0.6004, p = 0.008) (Fig. 6.5B).

6.3.4.2 Pepsin

The first detection of pepsin activity and expression was at 10 DAH (9.59 ± 1.8 mU/mg protein; 0.34 ± 0.06,), our results showed a progressively increasing trend with fish age (Fig. 6.6A). The pepsin activity expression level sharply peaked from 15 DAH and reached the highest level at 20 DAH (38.31 ± 7.54 mU/mg protein; 224.57 ± 32.20). The correlation of activity and expression was observed to be strongly significant (R^2 = 0.9741, p = 0.013) (Fig. 6.6B).

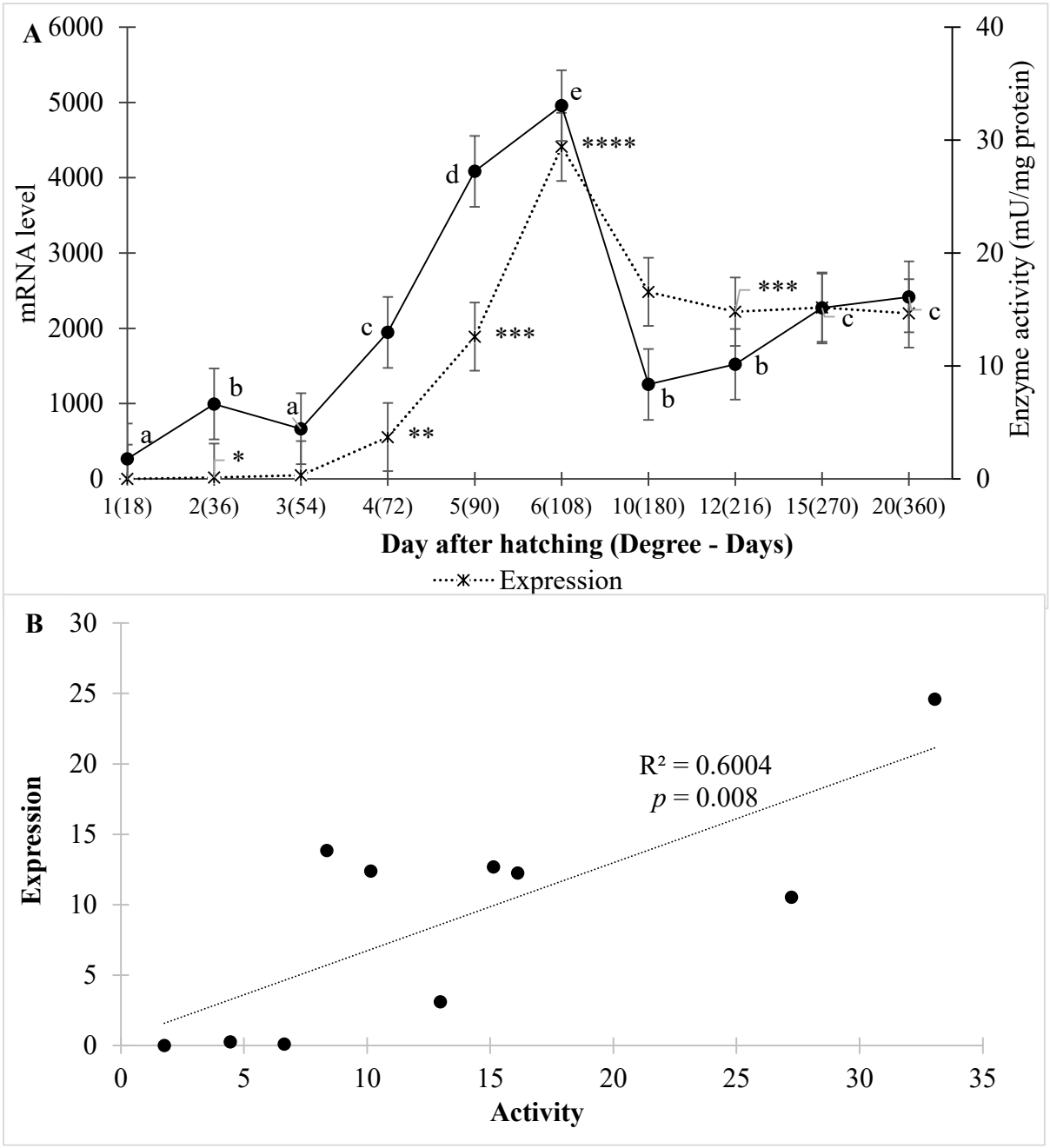


Figure 6. 5 Trypsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate trypsin activity. Dashed line and asterisk plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *try* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of trypsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d < e, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

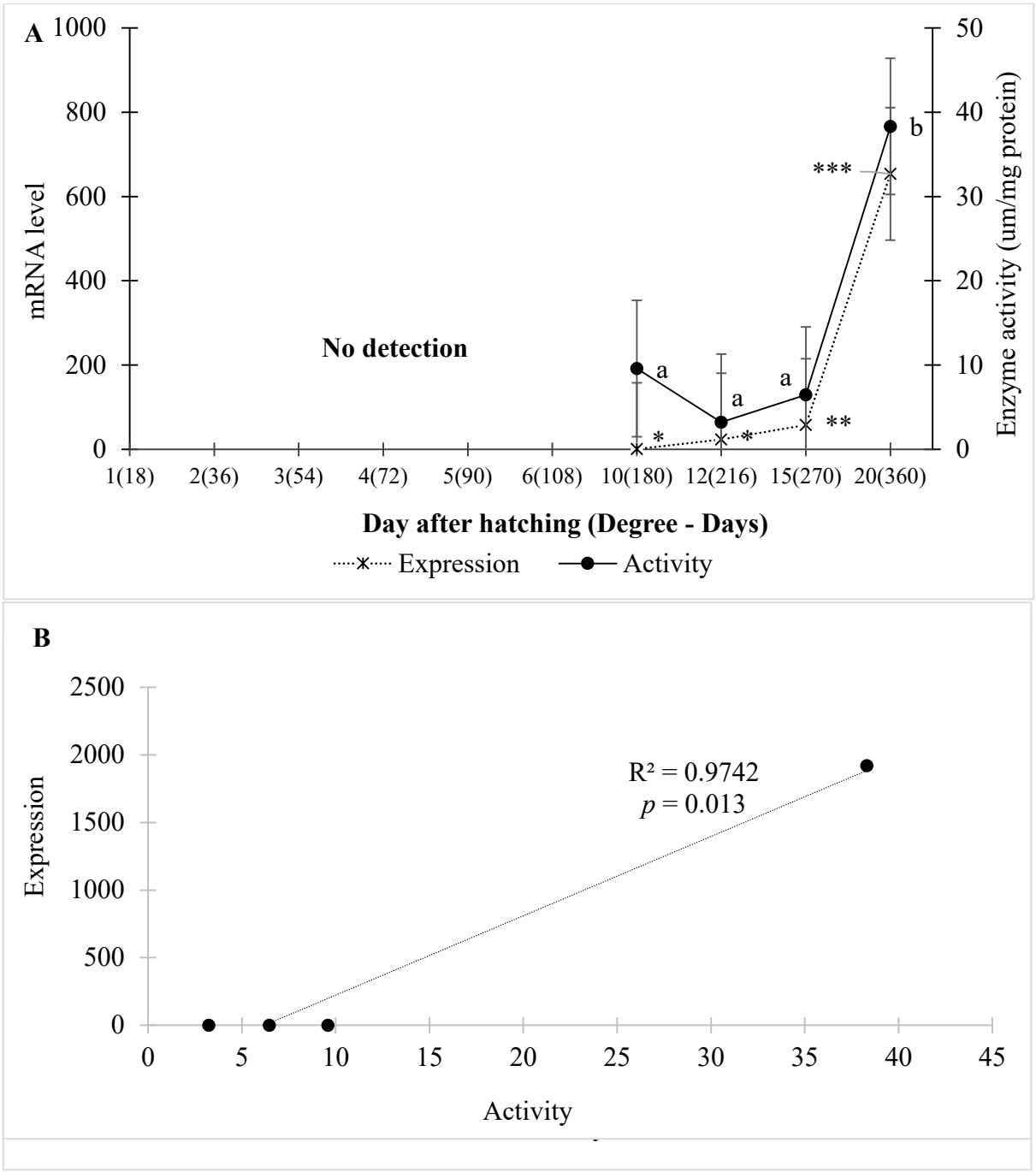
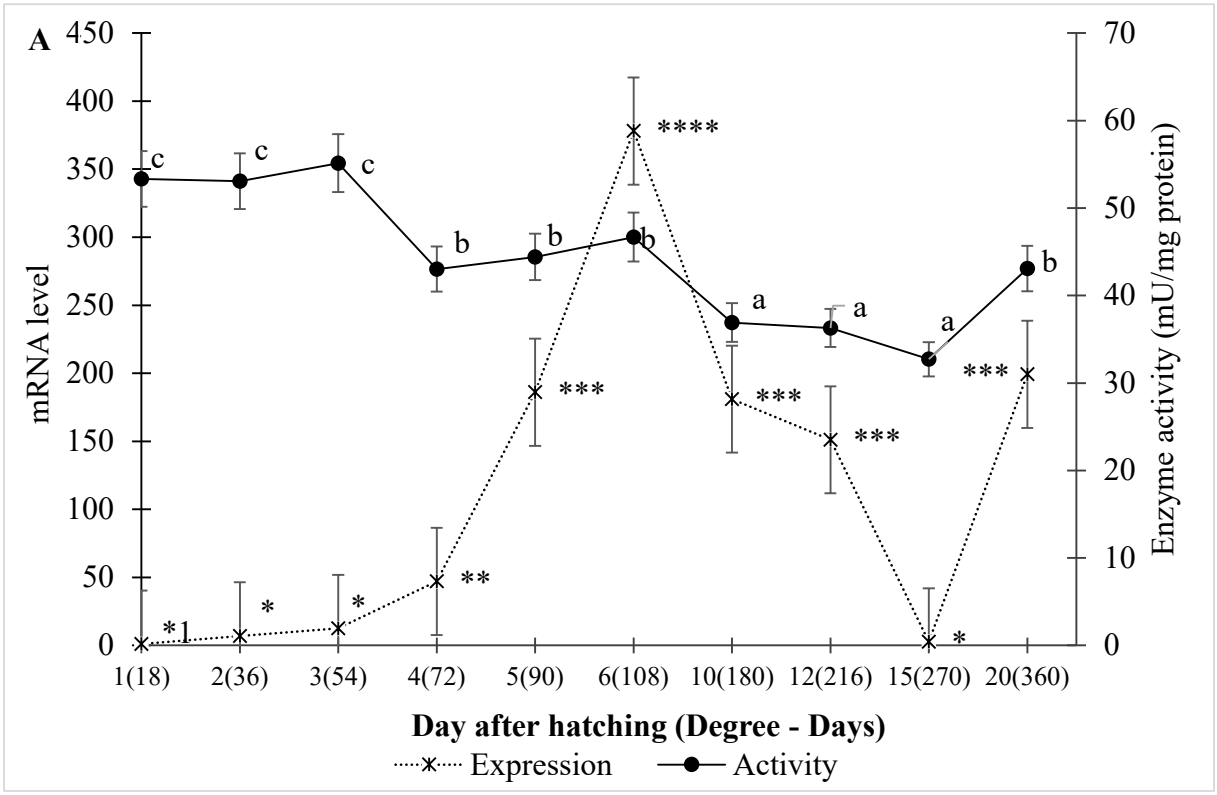
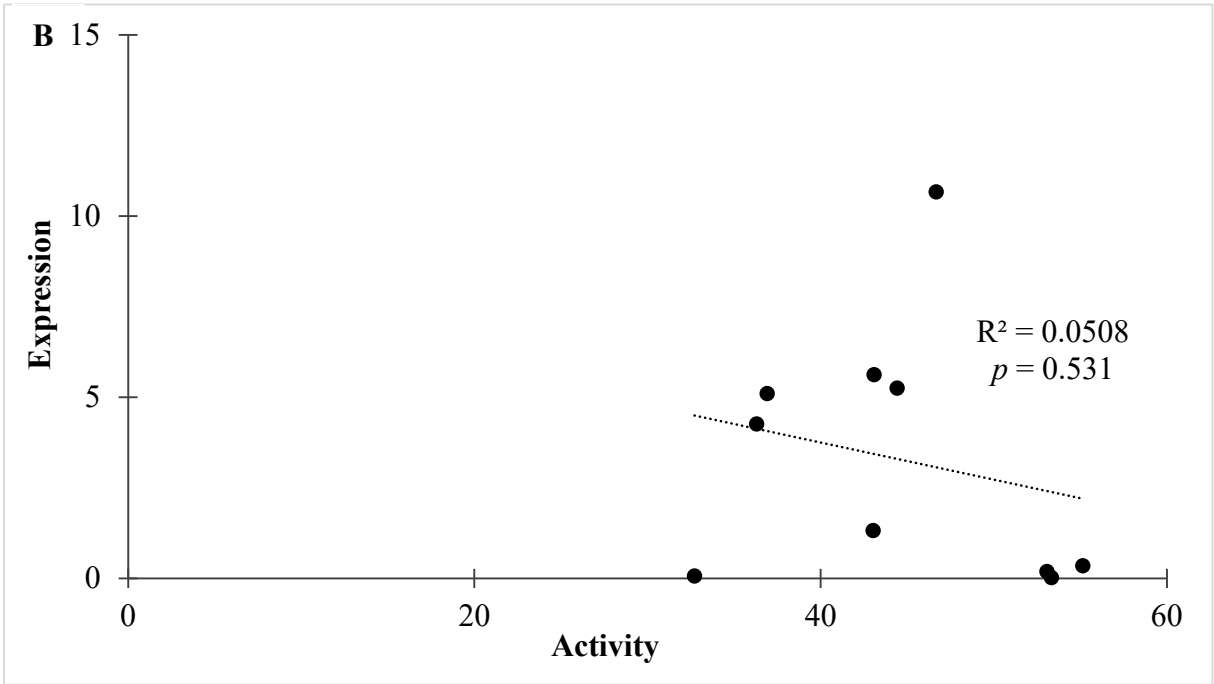


Figure 6. 6 Pepsin expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate pepsin activity. Dashed line and asterisk plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *pep1* at 10 DAH were used as reference samples. Alphabetical letters indicate significant difference of pepsin activity among developmental stages (ANOVA, Tukey's HDS test, a < b, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).


A
mRNA level
Enzyme activity (mU/mg protein)
Day after hatching (Degree - Days)
1(18)
2(36)
3(54)
4(72)
5(90)
6(108)
10(180)
12(216)
15(270)
20(360)
Expression
Activity


B
Expression
Activity
R² = 0.0508
p = 0.531

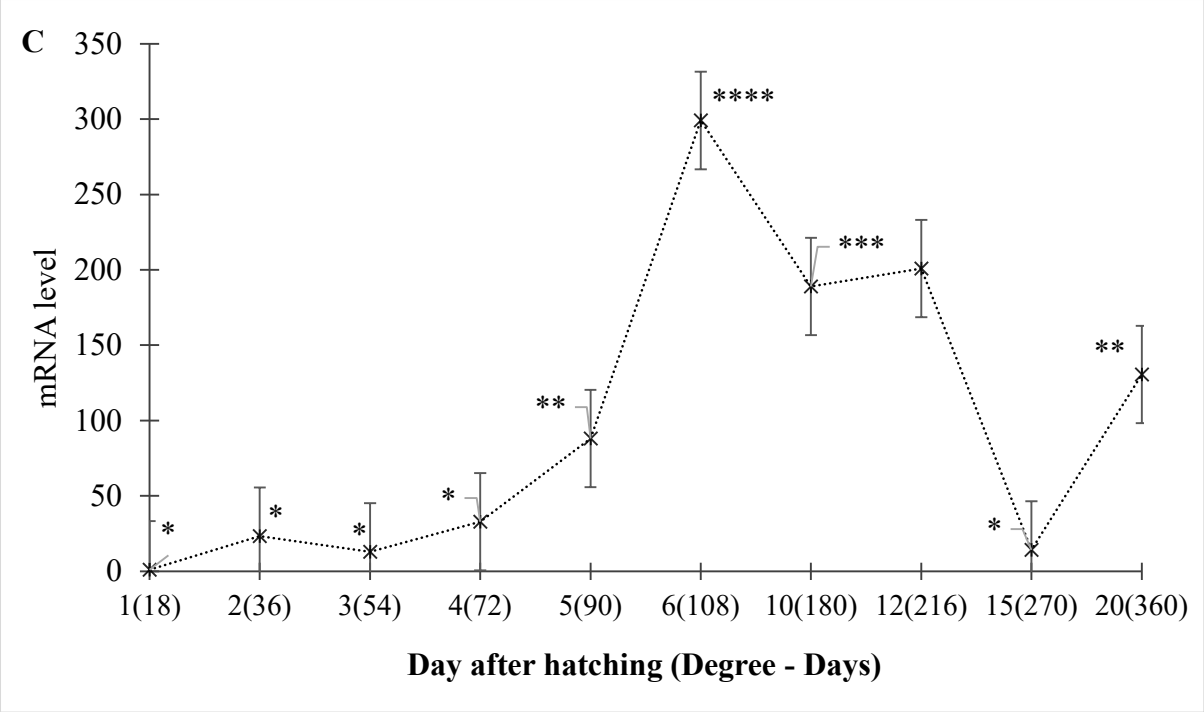


Figure 6. 7 Lipase expression and activity during larval development (A), (C) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate lipase activity. Dashed line and asterisk plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *pl1* and *bal1* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of lipase activity among developmental stages (ANOVA, Tukey's HDS test, $a < b < c$, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

6.3.4.3 Lipase

The activity of lipase was detectable at hatching (53.32 ± 5.28 mU/mg protein) and tended to decrease until 15 DAH (32.71 ± 4.30 mU/mg protein) (Fig. 6.7A). Compared to the proteolytic enzymes, lipase activity did not drastically fluctuate, whereas its associated *pl1* expression displayed high fluctuations (Fig. 6.8A). *pl1* expression progressively increased from hatching (0.028 ± 0.002) and peaked at 6 DAH (10.76 ± 2.25), this was followed by a dramatic drop from 6 DAH until 15 DAH (0.075 ± 0.002). The expression of *bal1* showed a similar pattern to *pl1* (Fig. 6.8C). Lipase activity and *pl1* expression recovered at 20 DAH (43.08 ± 5.31 mU/mg protein and 5.63 ± 0.83, respectively). Lipase activity was observed to not be significantly correlated its *pl1* expression (R^2 = 0.0508, p = 0.531) (Fig. 6.7B).

6.3.4.4 Amylase

Like the other enzymes analyzed, amylase activity and expression was also detected at hatching. Amylase activity showed a generally decreasing trend, however there were slight fluctuations between 1 DAH and 4 DAH. Activity slightly increased at 2 DAH (138.87 ± 10.92 mU/mg protein) and gradually decreased until 20 DAH (43.01 ± 5.75 mU/mg protein) where the lowest activity was detected (Fig. 6.8A). In contrast, the level of *amy2α* expression sharply increased from hatching (0.00062 ± 0.0001) and peaked at 4 - 5 DAH (1.04 – 1.63 of mRNA level). The *amy2α* expression then dramatically dropped between 5 DAH and 10 DAH (1.037 – 0.039) and remained at low level until 20 DAH (0.39 – 0.63 of mRNA level). No significant correlation was observed between activity and expression of amylase (R^2 = 0.0109, p = 0.774) (Fig. 6.8B).

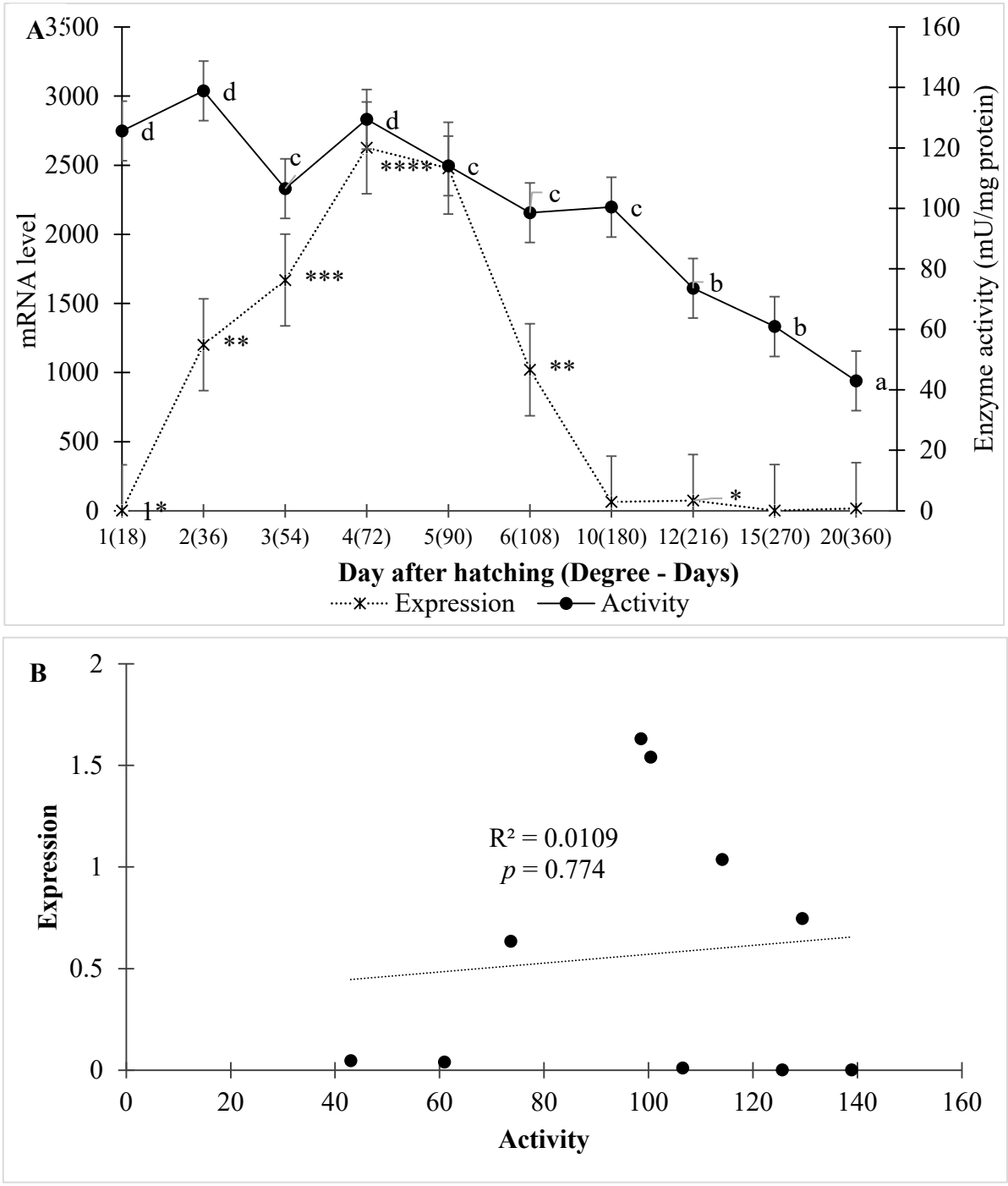


Figure 6. 8 Amylase expression and activity during larval development (A) and their correlation (B). The data was presented as mean ± SEM. Straight line and circle plot indicate amylase activity. Dashed line and asterisk plot indicate gene expression. Delineated are regression line and R^2- values. Expression of *amy2α* at 1 DAH were used as reference samples. Alphabetical letters indicate significant difference of amylase activity among developmental stages (ANOVA, Tukey's HDS test, a < b < c < d, $p < 0.05$). The number of asterisks indicates the level of statistical significance of gene expression (ANOVA, Tukey's HDS test, $p < 0.05$). The degree - days in the X axis was calculated from daily average temperature (degree Celsius) x DAH (day after hatching).

6.4 Discussion

The ontogenetic development of digestive system in marine fish larvae have been intensively investigated and thus an abundance of information on digestive physiology of larval fishes for diet and rearing optimization is available (Zambonino-Infante and Cahu, 2001; Zambonino-Infante et al., 2008; Lazo et al., 2011; Yúfera et al., 2018). These studies have highlighted that different fish species display various phenomenon in enzymatic activity due to the developmental performance of organs and tissues throughout ontogeny (Rønnestad et al., 2013; Moguel-Hernández et al., 2016). Moreover, these aspects are also influenced by rearing conditions, diet compositions and origin of species (Cahu et al., 2004; Rønnestad et al., 2013). As such, more species-specific investigations on digestive functions during ontogeny are required to fully understand development responses.

Previous studies have reported that tuna larvae mainly feed on copepods during its first week in the wild (Catalán et al., 2011; Yúfera et al., 2014). Therefore, many tuna rearing protocols have been developed based on a copepod diet, *Artemia* or fish larvae (Yúfera et al., 2014), with rotifers only provided at first feeding. On the other hand, other studies obtained good growth of tuna larvae by feeding enriched rotifer from first feeding until 10 DAH (Miyashita et al., 2002; Kaji et al., 2002), or 18 DAH (Murashita et al., 2014) then followed by *Artemia* or larval fish. A study by Sawada et al. (2005) highlighted that a feeding strategy of using enriched rotifer for the first 10 days post hatch and then following this with *Artemia* nauplii/yolk sac larvae was an acceptable regiment for tuna larvae within the first 20 DAH. In this study, *E. affinis* larvae showed rapid growth and reached 5.54 ± 0.92 mm of total length and 0.88 ± 0.24 g of body weight after 20 DAH. The larvae highly consumed live feed (12-17 rotifer and *Artemia* nauplii per larvae) from 4 DAH, whereby the yolk sac was completely reabsorbed (Fig. 2). According to studies by Støttrup and McEvoy (2004) and Mahjoub et al. (2013), enriched rotifers and *Artemia* nauplii are used in larviculture due to their

appropriateness in size and ability for manipulation of its nutrient values to suit the larvae's physiological and nutrient needs. The enriched diets used in this study showed high nutrient contents (Table 6.2, 6.3 and 6.4), and could provide adequate nutrition for the fish larvae. According to Betancor et al. (2017), tuna species require high DHA level and DHA/EPA ratio diets and a high concentration of n-3 long chain polyunsaturated fatty acid in neural tissues would be crucial for effective prey capture from the time of first feeding (Mourente, 2003). Rotifers in this study had high DHA content (4.38 ± 0.40 mg/g; Table 6.3) as compared to the enriched *Artemia* (0.69 ± 0.02). This is essential as rotifers were the first feed introduced to the *E. affinis* larvae as a deficiency in this would have resulted in reduced growth performance due to poor dietary values (Tomoda et al., 2005). Several studies have highlighted the importance of DHA at first feeding, with some studies even suggesting the superiority of DHA over EPA (Watanabe, 1993). A number of studies reported that the feeding behavior and food consumption of larval fish could be improved by increasing the DHA or n-3 HUFA dietary (Bell et al., 1995; Koven et al., 2016). Dietary DHA plays important roles in osmoregulation, bile salt conjugation, membrane stabilization, modulation of neurotransmitters, antioxidation and development of visual, neural and muscular systems at early stages; and therefore, was recorded to enhance the growth and survival rate of Atlantic bluefin tuna (*Thunnus thynnus*) (Koven et al., 2018). EPA was observed to be higher in the enriched *Artemia* (4.33 ± 0.06 mg/g; Table 6.3), which could be attributed to the lack of EPA in the rotifer enrichment (*Chlorella vulgaris*, Maruyama et al., 2006) and its fortification in the *Artemia* via the addition of salmon oil (Waqalevu et al., 2019). A high composition of free amino acids (FAA) is known to be beneficial for early stage larvae as they utilize them better than whole proteins due to the limited differentiation in the early digestive system (Rønnestad et al., 2013). Moreover, amino acids play an important role as building blocks for body protein as well as a precursor for other physiological function molecules including purines, hormones and neurotransmitters (Hamre

et al., 2013). Fish have certain essential free amino acids that are critical in larval development and are directly absorbed from the digestive tract without prior digestion (Conceiçao et al., 1997; Rønnestad et al., 2013). In this study, essential free amino acids such as isoleucine (0.67 ± 0.04 mg/g), leucine (1.03 ± 0.08 mg/g), lysine (1.78 ± 0.08 mg/g), methionine (0.38 ± 0.01 mg/g), phenylalanine (0.83 ± 0.09 mg/g) and threonine (1.07 ± 0.06 mg/g), was higher in rotifers, highlighting its nutritional and size appropriateness as a first feed. On the other hand, essential free amino acids such as arginine (2.45 ± 0.57 mg/g), histidine (1.35 ± 0.31 mg/g), tryptophan (0.62 ± 0.24 mg/g), valine (0.74 ± 0.16 mg/g) and tyrosine (1.67 ± 0.24 mg/g) were observed to be higher in *Artemia*. The effect of enrichment on Taurine levels was observed to be significantly higher in *Artemia* (14.82 ± 1.40 mg/g). Taurine is known to be a critical nutrient in fish feed as a myriad underlying mechanism in its functions have been attributed to larval physiological development such as membrane stabilization, osmoregulation, anti-oxidation and immunomodulation (Salze and Davis, 2015). As such, the appropriate dietary requirements of *E. affinis* needs special focus, however this area of study still requires further investigation for the optimization of feed.

E. affinis larvae were recorded with high mortality from 10 DAH with less than 1 % of the initial stocked population surviving until 20 DAH. Similar issues were also reported on attempts at larviculture of Pacific bluefin tuna, *Thunnus orientalis* (Takashi et al., 2006) and bluefin tuna (*T. thynnus*) (Mazurais et al., 2015). Remarks on the nutritional deficiencies (De La Gandara et al., 2008), skeletal deformities (Mazurais et al., 2015), cannibalism and swimming behavior leading to trauma caused by collision (Miyashita et al., 2007) were some reasons highlighted for the significantly high mortality rates. As seen in the nutritional quality of our feed this did not seem to be the issue in the present study. However, other studies on different marine fish species have suggested that the high mortality of larvae during early ontogenetic stages may not be necessarily due to physiological or digestive deficiencies

(Micale et al., 2006; Conceição et al., 2008; Solovyev et al., 2016) but rather a reflection of physiological performance due to substantial changes in morphology and physiology. Generally, survival rates of tuna larvae are known to be extremely low (1 – 3% before juvenile metamorphosis) with the critical stage occurring within the first 10 days after hatching (Sawada et al., 2005; Masuma et al., 2011; Tsuda et al., 2012; Benetti et al., 2016). Therefore, besides the diet, other factors such as *E. affinis* brood-stock management, post-flexion larvae physiological development and rearing condition also need further consideration. Moreover, from 5-15 DAH, *E. affinis* achieved a rapid growth, especially at 15 DAH (Fig. 6.1). Satoh et al. (2013) highlighted that a large size was found to positively affect the survival rate of tuna larvae at early larval stage. It suggested that good larval growth could result in good survival rate due to the selective-size mortality and growth rate-selective mortality occurred from a very early larval stage (Litvak et al., 1992; Tanaka et al., 2006; Takasuka et al., 2004; Meekan et al., 2006; Satoh et al., 2013).

E. affinis larvae hatched with a yolk sac and oil globule (Fig. 6.1B), and began exogenous feeding at 2 DAH coining with the opening of mouth and anus. The digestive enzymes expression and activity were detected (except pepsin) though at low levels. It indicated that the digestive enzymes were genetically programmed and activated from hatching and at first feeding to support the digestive process, therefore, highlighting that *E. affinis* larvae were able to hydrolyze and digest protein, lipid and carbohydrate at its early developmental stages. Previous studies have similarly reported this on Pacific bluefin tuna (*T. orientalis*) (Murashita et al., 2014) and Atlantic bluefin tuna (*T. thynnus*) (Mazurais et al., 2015). However, due to the lack of stomach functions, protein digestion was mainly achieved via trypsin and chymotrypsin in alkaline conditions (Rønnestad et al., 2013; Uebersch et al., 2018). Trypsin has been highlighted as a particular important enzyme for protein digestion during the early stages of many marine fish species (Rojas-Garcia and Rønnestad, 2002; Rønnestad et al., 2013; Khoa et

al., 2019). For *E. affinis* larvae, trypsin also presented as a potential factor to evaluate the nutritional status of larvae during the early stages (Fig. 6.5A and B). From 10 DAH, pepsin was activated coinciding with the formation of the gastric glands and pyloric caeca (Fig. 6.4D and Fig. 6.6), indicating the improvement of digestion via association of acid digestion. A similar pattern in gastric glands and pepsin detection was reported in *T. thynnus* and *T. orientalis* (Kaji et al., 1996; Miyashita et al., 1998; Yúfera et al., 2014; Murashita et al., 2014) at 10 - 11 DAH and observed much later in *T. albacares* at 14 -16 DAH (Kaji et al., 1996). Subsequently, the weaning practice of switching from live feed to formulated feed can be initiated at this larval stage, as it is within the digestive capacity of the larvae to digest complex proteins and could prove beneficial through accelerated larval growth and reduction of production costs. However, Yúfera et al. (2014) also suggested that the stomach seemed to begin functioning perfectly once the gastric glands proliferated and pepsin expression and activity progressed rapidly, which was around 17 – 20 DAH in this study. Therefore, it is recommended that co-feeding protocols should be considered during weaning periods to enhance the digestive efficiency of formulated diets and most importantly to avoid malnutrition (Khoa et al., 2020).

Previous studies have highlighted that the bile salt activated lipase and pancreatic lipase are mainly constituted of lipolytic enzymes in fish (Zambonino Infante and Cahu, 2007; Kortner et al., 2011). In which, the bile salt lipase contributed mostly to the catalyzation of a wide range of neutral lipids whereas pancreatic lipase catalyzed the hydrolysis of *phospholipids* at the *sn-2 position* (Olsvik et al., 2010; Kortner et al., 2011). The results from this study showed a complex fluctuation in expression of *pl1* and *bal1*, but the lipase activity remained relatively stable throughout the experimental period. Similarly, Murashita et al. (2014) reported the specific lipase activity of *T. orientalis* tuna larvae was detectable at hatching but remained low and stable within the first 20 DAH, thereafter, lipase activity increased concomitant with the

fastest larval growth. The transcription of *pl* and *bal* were detectable before the onset of exogenous feeding in many larval fish species such as Atlantic halibut (*Hippoglossus hippoglossus*) (Murray et al., 2006), European sea bass (*Dicentrarchus labrax*) (Darias et al., 2008), *Pagrus major* (Khoa et al., 2019). However, no significant change in *pl* and *bal* transcription was observed within the first 20 DAH. Besides the ontogenetic development, Kortner et al. (2011) also suggested that the feeding regime might effect on the *bal*/*pl* expression profiles. In this study, the larvae were fed enriched rotifer with high EPA and DHA composition (4.68 ± 0.30 % and 5.30 ± 0.20 %) until 10 DAH followed by enriched *Artemia* (2.90 ± 0.03 % and 0.46 ± 0.03 %; Table 3), therefore, the expression of *pl1* and *bal1* might fluctuated due to the change in dietary of EPA and DHA (Kortner et al., 2011). Lipase expression and activity recovered from 20 DAH concomitant with lipid accumulation in the intestinal epithelium (Fig. 6.4 and 6.7) to support fast growth during its metamorphosis into the juvenile stage. The correlation between expression and activity of some genes was not significant. This is probably due to the fact that the isoforms of genes analyzed in this study, thus did not illustrate the full function genes (Murashita et al., 2014, Khoa et al., 2019). Therefore, highlighting that perhaps more analysis is needed on the different gene isoforms.

Similar to the other tested digestive enzymes, amylase activity was detected from hatching. The observed trend was that it tended to decrease gradually throughout ontogeny. Similar trends were observed in amylase activity in bluefin tuna (Miyashita et al., 1998; Mazurais et al., 2015) and *T. orientalis* tuna (Murashita et al., 2014). However, the expression of *amy2α* was not correlated to the enzymatic activity, whereby, *amy2α* strongly expressed from the first exogenous feeding (2 DAH) to 5 DAH (Fig. 9). Previous studies have shown that the *amy2α* expression was also related to the fish age (Zambonino Infante et al., 1996; Péres et al., 1998; Khoa et al., 2020). For *E. affinis*, further investigations are still needed to confirm this hypothesis as other variables such as diet compositions and food quantity also affect *amy2α*

expression (Zambonino Infante et al., 1996; Péres et al., 1998; Khoa et al., 2020). The amylase activity declined and remained very low activity as the larvae turned to carnivorous feeding behavior (Murashita et al., 2014). Currently, the function of amylase at early stages during fish larvae ontogeny still remains unclear. However, it is hypothetically known that the larvae are required to digest efficiently potential sources of carbohydrate in their diet in order to optimally utilize the energy obtained from the food needed to support rapid growth in the critical stages (Rønnestad et al., 2013). As such, we hypothesize that the carbohydrates and lipids present in the diet used in this study, provided the primary important energy sources to assist the metabolism and growth without having the need to consume amino acids required for protein deposition and directing it towards cell construction (Darias et al., 2006)

The present study sheds new information on the enzymatic functions of trypsin, chymotrypsin, lipase, amylase and pepsin whilst observing physiological developments in the digestive system of *E. affinis* larvae. *E. affinis* larvae grow rapidly upon the resumption of exogenous feeding at 2 DAH however this is also associated with high mortality at critical stages (6 – 10 DAH), which is an area that needs further investigation. Important periods such as the development of the gastric glands coincided with detection of pepsin expression and activity, which also indicate the nutritional capabilities of *E. affinis* larvae at this stage. Findings from this study provide an insight into the understanding of morphological-functional changes of *E. affinis* tuna during early ontogeny, which could help in the optimization of rearing and feeding protocols for this species.

Supplementary 6.1

The PCR product size and similarity amplified from the primers using in this study.

Primer	Product size (bp)	Similarity (%)	Species/ Accession number
β - actin	161	95.0%	*Scomberomorus niphonius* beta-actin mRNA, complete cds (KT009015.1)
try	68	93.75%	*Thunnus orientalis* mRNA for trypsinogen 1, complete cds (AB678422.1)
pep1	86	94.29	*Thunnus orientalis* PGA mRNA for pepsinogen 1, complete cds (AB440200.1)
pl	79	98.41%	*Thunnus orientalis* pl mRNA for pancreatic lipase, complete cds (AB859991.1)
bal1	85	96.39%	*Thunnus orientalis* bal1 mRNA for bile salt-activated lipase 1, complete cds (AB859992.1)
amy2α	104	94.06	*Thunnus orientalis* mRNA for amylase-2, complete cds (AB678420.1)

CHAPTER 7: General discussion

The ontogeny of larval fishes has been histologically and biochemically assessed in many fish species (Kolkovski, 2001; Hamre et al., 2013; Rønnestad et al., 2013; Mata-Sotres et al., 2016; Yúfera, 2018). However, there is limited information available on functional enzymatic digestion in relation to histological-morphological changes, hydrolyzing capacities, feeding activity and the digestive function of marine fish larvae (Kolkovski, 2001; Hamre et al., 2013; Mata-Sotres et al., 2016; Yúfera, 2018). Moreover, previous studies do not elucidate on real digestive capacities and functional activities during larval stage developments. Recently, molecular approaches have been applied to complement traditional methods in expressing the profiles of digestive enzyme precursors in fish (Murashita et al., 2014; Mata-Sotres et al., 2016; Moguel-Hernández et al., 2016) and thus providing insight into both temporal and spatial expression patterns of genes involved in the development and functionality of digestive systems during early ontogeny. The comparison between molecular expression and actual activity of the different enzymes could provide new insights on the feeding regulation mechanisms of larval fishes (Rønnestad et al., 2013). Essentially, the connection between feeding behavior and digestive response at genomic and biochemical level is an important step towards the optimization of feeding and rearing protocols aimed to enhance nutrient utilization and developmental performance at early larval stages (Yúfera et al., 2018).

In the present study, we have investigated the early ontogenetic development of red seabream (*P. major*), Japanese flounder (*P. olivaceus*) and Kawakawa tuna (*E. affinis*) using an integrative approach. Accordingly, the changes of key digestive enzymes of larval fishes were evaluated in relations to the histological morphology changes, feeding status, enzymatic activity and its related gene expression from hatching until juvenile stage, therefore we could provide more insight on the ontogenetic development of these fish species. Remarkably, some

critical stages were observed during the early ontogeny of *P. major* (15 to 20 DAH and 25–30 DAH) and *P. olivaceus* (20-30 DAH) related to the deficiency of proteolytic and lipolytic enzyme activity and also energy conservation (Khoa et al., 2019; Khoa et al., in press). Important observations such as the cessation of feeding during metamorphosis in *P. olivaceus* larvae was also noticed in this study. As such, our findings highlight the importance of adapting feeding protocols based on the digestibility of feeds for larval fish according to its developmental stages to avoid mortality (Tanaka et al., 1996; Gwak et al., 1999; Kolkovski, 2001). Furthermore, this study also sheds new information on the enzymatic functions of trypsin, lipase, amylase and pepsin whilst observing physiological developments in the digestive system of *E. affinis* larvae. *E. affinis* larvae showed rapid growth upon the resumption of exogenous feeding at 2 DAH followed by detection of gastric glands, pepsin activity at 10 DAH. However, this is also highlighted high mortality at critical stages (6 – 10 DAH), however the reasons remain unclear. Several suggestions from previous studies were discussed such as the physiological or digestive deficiencies (Micale et al., 2006; Conceição et al., 2008; Solovyev et al., 2016), larval size (Satoh et al., 2013), brood-stock management, post-flexion larvae physiological development and rearing condition (Sawada et al., 2005; Masuma et al., 2011; Tsuda et al., 2012; Benetti et al., 2016). The results of this study could not indicate the reason of mass mortality of *E. affinis* larvae, and therefore we recommend further studies as it is an important commercial species in Japan.

The use of live feed has proven to have major bottlenecks for larviculture of many marine finfish and shellfish species (Coutteau et al., 1997; Bengtson, 2004) as it is generally and operationally expensive, unreliable, associated space requirements and laboriously intensive (Holt et al., 2011; Srichanun et al., 2013). Furthermore, it has been known that the risk of harmful pathogen infection is linked with the use of live feed (Lubzens and Zmora, 2004; Srichanun et al., 2013). Therefore, the development of micro-diets that are economical,

palatable and provide essential nutrients for larval development is an important consideration. Weaning between live feed and formulated diets in fish larvae is considered as a critical phase during fish development since it is often accompanied by increased mortality and reduced growth (Parma and Bonaldo, 2013). Among many biological processes that characterize the metamorphosis, the appearance of the gastric glands is one of the most important factors indicating the potential ability to digest complex protein into amino acids (Tanaka, 1971; Sarasquete et al., 1995; Parma and Bonaldo, 2013). The functionality of the stomach, which follows the appearance of the gastric glands, is a histological criterion to differentiate larvae from juveniles. A functional stomach is able to break down proteins into amino acids by the action of pepsin in an acid environment improving the digestive efficiency. Subsequently, the weaning practice of switching from live feed to formulated feed can be initiated at this larval stage, as it is within the digestive capacity of the larvae to digest complex proteins and could prove beneficial through accelerated larval growth and reduction of production costs. However, Yúfera et al. (2014) also suggested that the stomach seemed to begin functioning perfectly once the gastric glands proliferated and pepsin expression and activity progressed rapidly, which was around 17 – 20 DAH in *E. affinis* and *P. major* larvae, 20 - 25 DAH in *P. olivaceus* larvae in this study. Therefore, it is recommended that co-feeding protocols with appropriate transition time should be considered during weaning periods to enhance the digestive efficiency of formulated diets and most importantly to avoid malnutrition (Khoa et al., 2020). The advantages of co-feeding of live feed and artificial feed has been reported on many fish species (Kolkovski, 2001; Richard et al., 2015; Mata-Sotres et al., 2016). Feeding prey organisms for a short time to early stage larvae and gradually adding artificial feed is known to increase digestive activity and increase the larvae's ability to digest the dry diets (Kolkovski et al., 1997; Kolkovski, 2001) as live prey include certain neuro-hormonal factors which may stimulate digestive enzyme secretions in fish larvae (Chan and Hale, 1992; Ruyet and Mugnier, 1993;

Hamre et al., 2013). Diets containing high energy levels, supplied as neutral lipid and phospholipid mixtures could promote larval growth (Cahu and Zambonino- Infante, 2001). The use of improved diets would sustain the production of constant high quality fingerlings in the hatchery (Cahu and Zambonino- Infante, 2001; Kolkovski, 2013; Takeuchi, 2014).

A novel application of Shochu distillery by product (SDBP) showed a promising live feed enrichment media for larval fish production. Our results highlight that it could help to enhance rotifer nutritional compositions and growth, survival rates of larval fish whilst maintaining an eco-friendly and efficient approach towards finding effective uses for SDBP in Japan. However, there still remains unclear roles of the density and proportion of microbial community in the enriched media as well as the effect of probiotics on larval fish performance. Additionally, it requires more optimizations on the live feed enrichment to adjust the proportion of EFA and FAA in order to enhance the larval growth. More trials on different larval fish species are necessary to evaluate their effective feeding responses and larval biometrics.

This study provide insight into the improved understanding of digestive functions in developing fish larvae (*P. major, P. olivaceus and E. affinis*), however, there are still many questions to solve and gaps to fill in the digestion processes of larval fish. Digestion and nutrient absorption are regularly processes that follow a daily cycle and are affected by biotic (food availability) and abiotic (temperature, illumination cycle) factors. This current study focused on a single sampling per day schedule and therefore could not illustrate fully the variation in feeding behavior of fish larvae within a one-day period (Mata-Sotres et al., 2016). To fully understand the changes in daily production of enzymes in relation to the feeding status and regulated factors requires more investigation. Moreover, the connection between molecular expression and actual enzymatic activity is not well understood as the peaks of molecular expression were sometimes detected prior to or after the corresponding activity, as was observed in this study. Therefore, highlighting that perhaps more analysis is needed on the

different isoforms and the delay in precursor gene translations (Srichanun et al., 2013, Lee et al., 2015; Moguel-Hernández et al., 2016). Furthermore, it is also necessary to enhance the understanding of the effects of water physical-chemical conditions and diet quality on the regulatory mechanisms and final enzymatic capacity.

It is hoped that the finding of this study will be able to contribute positively towards the development of appropriate feeding strategies and rearing protocols. Early larval ontogenetic development and feed development are critical for the enhancement growth and survival rates and the improvement of hatchery practices for marine fish larvae. It highly hoped that parallels can be drawn from our results for other commercially important marine fish species, especially in today's rapidly expanding aquaculture industry.

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