Development of enrichment of *Brachionus plicatilis* species complex for initial feeding to marine fish larvae

海産魚類仔魚への初期給餌のための *Brachionus plicatilis* 複合種の強化方法の開発

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Abstract

Marine fish larvae are particularly vulnerable during their early stages of ontogeny due to strict larviculture requirements. Euryhaline rotifers *Brachionus*, especially those belonging to the *B. plicatilis* species complex are often used as the preferred initial-feed for most marine finfish species. Given current problems associated with differential growth and survival rates during hatchery culture, it is apparent that further research is required to investigate the use of enrichment media, the type of live feeds and the response by larvae during early larval ontogenetic development. This study used the L-type *B. plicatilis* and SS-type *B. rotundiformis* as the model rotifer species and the red sea bream *Pagrus major* and the Japanese flounder *Paralichthys olivaceus* as the model marine finfish species. The effects of different enrichment diets on population growth, fatty acid content and protein content of L and SS morphotypes of *B. plicatilis* sp. complex were investigated. Four common Japanese commercial rotifer enrichment formulations were tested: docosahexaenoic acid (DHA) enriched *Chlorella vulgaris*, frozen *Nannochloropsis oculata*, salmon roe emulsion oil, against a control diet of normal *C. vulgaris*. In both morphotypes, DHA-enriched *C. vulgaris* and frozen *N. oculata* had significantly higher population growth and fatty acid content compared to the negative impacts on population growth under salmon roe oil treatment. This is despite it having good levels of highly unsaturated fatty acids (HUFA). Variations in content of non-polar lipids and polar lipids between L and SS-type were observed. Differences were observed in soluble protein levels with higher levels detected in DHA-enriched *Chlorella* and control treatment. Using the two enrichment diets that had high levels of HUFA, DHA-enriched *C. vulgaris* and salmon roe oil, larviculture trials were conducted on *P. major* and *P. olivaceus* from 0-15 days after hatching (DAH) using L-type *B. plicatilis*. HUFA content was better in the combined salmon roe emulsion and DHA-enriched *C. vulgaris* treatment due to eicosapentanoic acid fortification from the roe emulsion. Despite this, the DHA-enriched *C. vulgaris* only treatment was found to

have higher population growth, egg bearing capacity, soluble protein and free amino acid content. Both *P. major* and *P. olivaceus* suffered high mortality under the combined diet. In contrast, better survival, generally higher gut content, high ingested soluble protein and trypsin activity fraction was observed in the DHA-enriched *C. vulgaris* only treatment. This could reflect its nutritional qualities as its high free amino acid and soluble protein composition are beneficial for early stage larvae due to ease of hydrolysis and absorption by the early digestive system. Using both L-type *B. plicatilis* and SS-type *B. rotundiformis* fortified on DHA-enriched *C. vulgaris*, larviculture trials were also conducted on *P. olivaceus* from 0-7 DAH to assess if there were any differences in protein hydrolysis and digestive trypsin activity at first feeding. At 5 DAH, hydrolysis was significantly higher in larvae fed SS-type whereas L-type treatment had not completely hydrolyzed the proteins after 3 hours at the same molecular weight (50 kDa). Larvae fed SS-type had significantly higher trypsin activity at 3-7 DAH. Contribution of live prey to trypsin fraction in larvae showed significantly higher fraction for SS-type at 5 DAH and 6 DAH. Exogenous trypsin contribution from live prey was relatively low when compared to the total trypsin activity in larvae. This study revealed that depending on dosage, morphotypes within *B. plicatilis* species complex do not respond well to oil-based enrichments despite their high HUFA content. This was also mirrored during larviculture of *P. major* and *P. olivaceus*. Furthermore, this study highlighted the adaptability of *P. olivaceus* to different rotifer sizes during its early developmental stages.

要旨

海産魚類仔魚は、生息条件が厳しいために、初期の個体発生時に特に脆弱である。海産ツボワムシ類 *Brachionus plicatilis* 複合種は、多くの海産魚類種苗生産で好適な初期餌料として使用される。種苗生産時の不安定な成長および生残を考慮すると、仔魚期早期での栄養強化剤の使用、初期餌料の種類、それらに対する仔魚の反応について、一層の検討が必要である。本研究では、*B. plicatilis* L 型と *B. rotundiformis* SS 型を使用し、マダイ *Pagrus major* とヒラメ *Paralichthys olivaceus* を海産魚種のモデルとして用いた。本研究では、L 型および SS 型ワムシの各種栄養強化剤使用時の個体数増加、脂肪酸含量およびタンパク質含量に及ぼす影響を調べた。栄養強化剤としてドコヘキサエン酸（DHA）強化淡水クロレラ *Chlorella vulgaris*、冷凍 *Nanochloropsis oculata*、サケすじこ乳化油を用い、対照として DHA などが未添加である淡水クロレラを使用した。両ワムシタイプでは、すじこ乳化油で強化すると高度不飽和脂肪酸（HUFA）含量が高レベルになったが、DHA 強化クロレラおよび冷凍 *N. oculata* による強化で、有意に高い個体群増殖を示し、脂肪酸含量も高くなった。また、両タイプとも非極性脂質および極性脂質の含有量の変動が観察された。さらに DHA 強化クロレラおよびクロレラ強化では、可溶性タンパク質が高いレベルで差異が観察された。高レベル HUFA 含量の DHA 強化クロレラとすじこ乳化油で強化した L 型ワムシを用いて、0～15 日齢の間に、マダイおよびヒラメへの給餌試験を実施した 。HUFA の含量は、すじこ乳化油と DHA 強化クロレラの混合強化がすじこ乳化油単独強化よりもエイコサペンタエン酸強化には、良好であった。しかし、DHA 強化クロレラ単独強化では、より高い個体群増殖、卵保有率、可溶性タンパク質および遊離アミノ酸含量となった。マダイとヒラメ仔魚の両方で、DHA 強化クロレラ単独強化の時には、高い腸内含有、

消化された可溶性タンパク質およびトリプシン活性画分が観察されたが、複合強化ではそれらに加え、高い死亡率となった。前者は、栄養的な質を反映している可能性があるが、発達初期の消化器系の加水分解能や吸収能が原因で、高遊離アミノ酸量および高可溶性タンパク質組成が初期の仔魚に効果があるためであると考えられる。DHA 強化クロレラに強化された L 型ワムシと SS 型ワムシの両方を使用して、0〜7 日齢のヒラメの仔魚飼育試験を行い、最初の摂餌時にタンパク質加水分解能およびトリプシン活性消化能に差異があるかどうかを評価した。5 DAH では、SS 型を給餌した仔魚では加水分解活性が有意に高く、L 型給餌では同じ分子量（50kDa）で 3 時間後にタンパク質を完全には加水分解しなかった。また、SS 型を給餌した仔魚は、3-7 日齢で有意に高いトリプシン活性を示した。 仔魚のトリプシン活性に対して、5 および 6 日齢で SS 型ワムシのトリプシン活性は有意に高い貢献度を示した。 しかし、ワムシからの外因的なトリプシンの寄与は、仔魚の総トリプシン活性と比較して比較的低かった。 本研究では、海産ツボワムシ類複合種の両型は、投与量に応じた高い HUFA 含量であったのにも関わらず、油脂ベースの強化剤にあまり良く反応しないことを明らかにした。 これはマダイおよびヒラメの仔魚期でも反映していた。 さらに、本研究は、初期の発達段階の間に、ヒラメ仔魚はより効果的なワムシタイプに適応出来ることを示した。

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Declaration

I declare that the work presented in this thesis is entirely my own with all exceptions being clearly indicated or/and properly cited in the context

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Viliame Pita Waqalevu

Dedication

I would like to dedicate this work to my wife Mrs Sainimere Veitata-Waqalevu, my parents, Mr. Penijamini Waqalevu and Mrs Vilimaina Waqalevu, my siblings and all those that have supported me throughout this journey. Vinaka vakalevu and ありがとうございました。

Papers related to Doctoral degree

1. Waqalevu, et al. (2018) Proteolytic digestive enzyme response in Japanese flounder *Paralichthys olivaceus* fed two types of rotifers *Brachionus plicatilis* sp. complex. Fisheries Science, 84 (6): 1037-1049.
2. Waqalevu, et al. (2019) Population growth, fatty acid composition and protein content of two types of *Brachionus plicatilis* sp. complex rotifers fed four common Japanese nutritional enrichment diets. Aquaculture Science, 67(2).
3. Waqalevu, et al. (2019) Effect of oil enrichment on *Brachionus plicatilis* rotifer and first feeding red sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*). Aquaculture 510: 73-83.

List of Acronyms and Abbreviations

ALA	Alpha-Linolenic Acid
ANOVA	Analysis of Variance
ArA	Arachidonic Acid
BSA	Bovine Serum Albumin
CBB	Coomasie Brilliant Blue
DAH	Days After Hatching
DHA	Docosahexaenoic Acid
DW	Dry Weight
EFA	Essential Fatty Acid
EPA	Eicosapentanoic Acid
ETA	Eicosatetraenoic Acid
FAA	Free Amino Acid
HUFA	Highly-Unsaturated Fatty Acids
L-Type	Large Type
LA	Linolenic Acid
NL	Non-polar Lipid
OD	Optical Density

PL	Polar Lipid
SD	Standard Deviation
SDA	Stearidonic Acid
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SNK	Student-Newman-Keuls
SS-Type	Super Small Type
TL	Total Length

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CHAPTER 1: General Introduction

1.0 Live feed

Marine fish larvae are particularly vulnerable during their early stages of ontogeny due to strict biotic and abiotic condition requirements for survival and development. Consideration of rearing conditions that meet the larval requirements for optimal presentation of feed and understanding of its nutritional quality is important. Hamre et al. (2013) highlighted in their review of advances in early larval nutrition that the current knowledge has been based mainly on laboratory studies following reductionist approaches under artificial conditions based on limited prey types and normally under relatively constant conditions. This focus discounts species-specific ontogeny, feeding physiology and nutritional requirements which makes extrapolation obtained from model species difficult. A number of previous studies have highlighted the important nutritional composition requirements of various fish species, with extensive recommendations on dosages, and composition of macronutrients (Sanchez-Vazquez et al., 1999; Rubio et al., 2003), protein (Hamre, 2005; Kvåle et al., 2009), amino acids (Cahu et al., 1999; Carvalho et al., 2004), fatty acids (Izquierdo et al., 1989; Villenueve et al., 2005; Dantagnan et al., 2010; Kotani et al., 2017), vitamins (Moren et al., 2004; Villenueve et al., 2005) and minerals (Hamre et al., 2008; Nguyen et al., 2008; Srivastava et al., 2012), either through direct or indirect measures (Hamre et al., 2013).

Nutritional value and acceptance of the feed at this ontogenetic development stage in larvae is an important consideration. Therefore due to the immaturity of the digestive system in early stage larvae, planktonic live feed are usually used as primary feed (Cahu & Zambino Infante, 1994). The use of live prey as feed for early stage marine fish is common practice globally (Watanabe et al. 1978; Dhert, 1996; Helland *et al*., 2010; Hamre et al., 2013; Hamre, 2016). There are a variety of live feeds that have been used in larviculture; these include brine

shrimp (*Artemia*), copepods, water fleas (Cladocerans e.g. *Daphnia* sp. and *Moina* sp.), anostracans, protozoans, bacteria, and euryhaline rotifers. The reliable production and availability of large quantities of live feed such as rotifer (*Brachionus plicatilis* and *B. rotundiformis*) and *Artemia* nauplii to meet the different stages of fry production has also contributed to the successful fry production of at least 60 marine finfish species and 18 species of crustaceans (Dhert, 1996). There are many favorable characteristics of live feed, some of which are: (i) their small in size thus best suited towards the small mouth gape of early stage larvae; (ii) high reproduction rate therefore suitable for mass culture; (iii) it's planktonic nature which allows for development of natural predation skills and; (v) the ability for artificial manipulation of its nutritional qualities through enrichment and bioencapsulation (Dabrowski, 1984; Dhert et al., 2001). However, they require a rather labor-intensive culture, which isn't always reliable and occasional culture crashes occur (Dhont et al., 2013). Nevertheless, recent considerable improvements in live feed technology and development have being developed (Dhont et al., 2013; Hamre et al., 2013). With these recent advancements in mind, there are still however many gaps and bottlenecks that still need to be addressed with several research avenues open into improving the quality of the live feed produced (Hamre et al., 2013).

1.1 *Brachionus plicatilis* species complex

Since the introduction of euryhaline rotifers in the field of aquaculture, there have been many developments in its mass culture (Fu et al. 1997; Dhert et al. 2001; Hagiwara et al., 2014; Hamre, 2016). The importance of rotifers, especially those belonging to the *Brachionus plicatilis* species complex, is often highlighted as they are often used as the preferred first-feed for most marine finfish (Tomoda et al. 2004; Park et al. 2006). The phylum Rotifera is one of several phyla with a high level of cryptic diversity (Mills et al., 2016) and is most likely due to its small size, the paucity of taxonomically relevant morphological features and the scarcity of

rotifer taxonomists (Wallace et al., 2006). Mills et al., (2016) reported that the *B. plicatlis* species complex could be divided into 15 species and that populations could include more varieties than previously suggested. Segers (1995) classified L-type as *B. plicatilis* and others as *B. rotundiformis*. Consideration of rotifer size is important in seedling production, as selectivity of prey is dependent on sizes of live feed (Tanaka et al., 2005; Akazawa et al., 2008; Wullur et al., 2009). However, a current major concern is that dependent on the conditions of culture and types of enrichment, several nutrients may be present in insufficient levels in rotifers (Hamre, 2008). Levels of nutrients, e.g. protein, amino acids and essential fatty acids, are also determined by the metabolism of the feed organism and therefore good knowledge of nutritional levels in enrichments and also larval nutritional requirements throughout ontogeny would contribute to diet optimization and improved feeding protocols (Helland *et al*., 2010; Hamre, 2016). Although it is now possible to produce rotifers at a large scale and of high nutritional value, their quality is often far from optimal which may be due to the low hygienic quality and overall culture condition which often results in low swimming activity, low reproduction rate and low ingestion rate (Dhert *et al.*, 2001; Hagiwara *et al.*, 2007).

1.2 Enrichment of *Brachionus plicatilis* species complex

B. plicatilis rotifers can be enriched with different kinds of media as long as the particle size ranges from 0.3 μm up to 21 μm in diameter (Vadstein et al., 1993; Hansen et al., 1997). Several species of microalgae have been used to enrich *B. plicatilis* rotifers, such as *Nannochloropsis* spp., *Chlorella* spp., *Tetraselmis* spp. and *Isochrysis galbana*, which are the most commonly used (Conceição et al., 2010). Some of these microalgal species are available commercially with many varying in nutrient composition. Each enrichment media has its pros and cons as they each fortify rotifers differently in terms of nutrition. For example, *Chlorella*

vulgaris is known to have high levels of the high-unsaturated fatty acids (HUFA), docohexaenoic acid (C22:6 *n*-3; DHA), however it is also known to be poor in eicosapentaenoic acid (C20:5 *n*-3; EPA) (Maruyama et al. 2006; Thépot et al. 2016) with *Nannochloropsis* spp. being the opposite (Hirayama *et al.* 1979; Lubzens et al. 1995; Kobayashi et al. 2008). Therefore to optimize the level of nutrients in the enrichment media some are composed of mixed micro-algal constituents for optimal HUFA content. Some of the available commercial rotifer enrichment media are Rotigrow Plus©, RotiGreen©, N-Rich©, *Isochrysis*/*Pavlova* Blend© (Reed Mariculture, United States of America) or powdered DHA-protein Selco© (INVE aquaculture Inc., USA). In Japan the most common commercial rotifer enrichments include, frozen *Nannochloropsis oculata*©, Fresh *Chlorella*-V12© and Super Fresh *Chlorella*-V12© (Chlorella Industry Co. Ltd, Japan) and salmon roe emulsion oil© (MarineTech Co., Ltd., Japan) to name a few. Since the nutritional requirements of larvae are species-specific, understanding the effects of these enrichments on rotifers and ultimately on larviculture performance are important considerations in the development of optimal live feed in first feeding marine fish.

1.3 Marine fish study species

The refinement of culture practices and technology of many commercially valuable species is still an area of focus in many countries where aquaculture production is an important sector. However bottlenecks in high and irregular mortality, deformities and nutritional deficiencies still occur, especially at the onset of exogenous feeding (Heming et al., 1982; Taylor and Freeberg, 1984; Rice et al., 1987; Gisbert et al., 2004). This highlights the importance of the nutritional qualities of feed in early ontogeny and feed acceptance in larvae. As such, we have decided to choose two model commercial fish species, the red sea bream

(*Pagrus major*) and the Japanese flounder (*Paralichthys olivaceus*), to highlight the effects of live feed enrichment at the important stage of first feeding.

1.2.1 Red sea bream (*Pagrus major*)

The red sea bream, *Pagrus major* (Fig 1.), is an important fish in the recreational fishing and aquaculture industry in Japan. *P. major* is a marine fish that is often found at 10-200 meter depths and distributed from northeastern part of the South China Sea extending northward to Japan (Tabata and Taniguchi, 2000).

Figure 1: Adult red sea bream (*Pagrus major*)
Source: www.fishbase.de

Since its introduction into the aquaculture industry, it has since been mass-produced for stock-enhancement purposes and for this reason its early life history has been studied quite well (Kohno et al., 1983; Fukuhara, 1985; Matsuoka, 1987). Moteki (2001) highlighted that in comparison with other marine fishes, *P. major* larvae have a relatively short transition period between endogenous and exogenous feeding, which is disadvantageous for survival. However, this is compensated by a high rate of feeding upon exhaustion of the yolk with a reduced time from initial feeding to 100 % feeding and a large larval size allowing it a smooth transition at first feeding. Despite this, Moteki (2002) also highlighted that the stage of first exogenous

feeding is a critical period of consideration and the high mortality rates associated with it should not be discounted.

1.2.2 Japanese flounder (*Paralichthys olivaceus*)

The Japanese flounder, *Paralichthys olivaceus* (Fig 2.), is also an important commercial species for coastal fisheries and for the aquaculture industry in Japan and Korea (Yamashita and Yamada, 1999).

Figure 2: Adult Japanese flounder (*Paralichthys olivaceus*)
Source: www.fishbase.de

P. olivaceus is a marine, demersal fish that is often found at 10-200 meter depths and distributed in the Western Pacific from the north of Japan extending southwards to the Korean peninsula (Amaoka, 1984). As such, the life history of this species has been studied extensively (Fukuhara, 1986; Ikewaki and Tanaka, 1993; Yamashita and Yamada, 1999; Howell and Yamashita, 2005). Like other flatfishes, *P. olivaceus* early ontogenetic development is one that includes significant behavioral, anatomical and physiological transformation (Fukuhara, 1986; Bolasina et al., 2006). Understanding how different enrichments modulate larval response in terms of survival, growth and digestive enzyme response are important considerations for the advancement of its larviculture.

1.4 Purpose of study

Given the problems associated with differential growth and survival rates during hatchery culture, it is apparent that further research is required to investigate the use of enrichment media, the type of live feeds and the response by larvae during early larval ontogenetic development. This research intends to build on and develop existing knowledge of *Brachionus plicatilis* species complex enrichment techniques and its suitability for use in first feeding marine fish larviculture. Using *P. major* and *P. olivaceus* as the model species, it is hoped that refinements and parallels on live feed technologies adapted in this study can contribute to the optimization of rotifer enrichment and its subsequent use in larviculture. Upon completion of this study, it is hoped that a better understanding of the nutritional requirements using live feed for the rearing of *P. major* and *P. olivaceus* larvae at first feeding can be established and that it can open up avenues of further research into other marine fish species. It is hoped that results obtained from this project will assist in developing and optimizing technologies for the enrichment of *B. plicatilis* species complex rotifers and add to the current scientific knowledge available on enrichment practices and its effects in larviculture at the important stage of first feeding.

1.5 Objectives

The current study specifically addressed the following:

1. To determine differences in population growth, fatty acid composition and protein content of L-type *B. plicatilis* and SS-type *B. rotundiformis* enriched with commonly used Japanese commercial enrichment.
2. To assess differences in fatty acid composition, protein content and free amino acid content in *B. plicatilis* species complex rotifers when combining enrichment media to enhance nutritional quality of enrichment.

3. To determine differences in larviculture performance (i.e. survival, growth, gut content, protein hydrolysis) when reared using *B. plicatilis* species complex rotifers enriched with different commercial enrichment media.
4. To determine differences in larviculture performance (i.e. survival, growth, gut content, protein hydrolysis) when reared using two different rotifer morphotypes of the *B. plicatilis* species complex.

CHAPTER 2: Population growth, fatty acid composition and protein content of two types of *Brachionus plicatilis* sp. complex rotifers fed four common Japanese nutritional enrichment diets

Abstract

This study investigated the effects of different enrichment diets on population growth, fatty acid content and protein content of SS and L types *Brachionus plicatilis* sp. complex. Four commercial rotifer enrichment formulations were tested: docosahexaenoic acid (DHA) enriched *Chlorella vulgaris*, frozen *Nannochloropsis oculata*, Salmon roe emulsion oil, against a control diet of normal *C. vulgaris*. DHA-enriched *C. vulgaris* and frozen *N. oculata* had significantly higher population growth, fatty acid content and soluble protein in both morphotypes. However, population growth under salmon roe oil treatment was affected negatively despite having high essential fatty acid content in both morphotypes. Variations in the contents of non-polar lipids and polar lipids between L and SS-type were observed; highlighting enrichment effects and possible dissimilar biochemical roles of these compounds in tissue metabolism in each morphotype. No significant differences were observed in crude protein levels however, significant differences were observed in soluble protein levels with significantly higher levels detected in DHA-enriched *Chlorella* and control treatment. SS-type had significantly higher soluble protein content except under the frozen *N. oculata* treatment. Culture method, enrichment dosage and consideration of optimal amounts of essential fatty acid in live feed is important as different rotifer morphotypes respond differently to different enrichment.

2.0 Introduction

The genera *Brachionus* include several morphotypes; culturists describe them according to lorica size L (large, 130-340 μm), and SS (super small, 90-110 μm) types and based on several classification criteria e.g. morphology, allozyme pattern, karyotypes (Hagiwara et al. 2001, 2014; Kotani et al. 2005). Morphologically, Fu et al. (1991) described L-type as larger and having obtuse-angled anterior spines compared to the smaller and rounded lorica with pointy anterior spines on the S-type. Furthermore, Segers (1995) also classified *B. plicatilis* as L-type and smaller varieties as *B. rotundiformis.* Kotani et al. (1997) demonstrated that a strong species boundary between L-type *B. plicatilis* and SS-type *B. rotundiformis* is present. Consideration of rotifer size is important in seedling production, as an important factor during first feeding larvae is prey selection according to prey size and mouth gape (Shirota, 1970). The moment of first exogenous feeding is an important period as factors such as starvation and subsequent mortality usually accompanies this period. Dependent on the conditions of culture and types of enrichment, several nutrients may be present in insufficient levels in rotifers (Hamre, 2008). Levels of nutrients, e.g. protein, amino acids and essential fatty acids, are also determined by the metabolism of the feed organism and therefore good knowledge of nutritional levels in enrichments and also larval nutritional requirements throughout ontogeny would contribute to diet optimization and improved feeding protocols (Hamre, 2016). Arachidonic acid (C20:4*n*-6; ArA), eicosapentaenoic acid (C20:5*n*-3; EPA) and docosahexaenoic acid (C22:6*n*-3; DHA) are three important highly unsaturated fatty acids (HUFA) that are vital for the development and survival of marine fish larvae during its early stages (Watanabe, 1983; Koven et al. 1992). Although it is now possible to produce rotifers at a large scale and of high nutritional value, their quality is often far from optimal which may be due to the low hygienic quality and overall culture condition which often results in low swimming activity, low reproduction rate and low ingestion rate (Dhert et al. 2001; Hagiwara

et al. 2007). These factors have to be considered during the enrichment process as it may induce stress in the rotifer mesocosm, which may lead to other stressors such as increase in free ammonia levels, change of bacterial flora and contamination by protozoan species (Hagiwara et al. 1998). To ensure high larval survival and growth at the onset of exogenous feeding during subsequent larviculture, maintenance of feed quality, feed culture conditions and nutritional values of rotifers is crucial (Hagiwara et al. 2007). In addition to understanding differences in fatty acid composition, the impacts of enrichment on essential fatty acids for larviculture (e.g. ArA, EPA and DHA) and how they differ between different morphotypes is also an important consideration.

Information on the effect of different enrichment on different morphotypes within the commercially valuable *B. plicatilis* sp. complex is limited therefore this study aims to provide a preliminary insight into the suitability of enrichment and its impacts on two morphotypes commonly used during seedling production. This study focused on evaluating nutritional enrichment effect on growth ratio, egg bearing, fatty acid and protein content of the Indonesian strain, formerly known as SS type, and Obama strain rotifers, formerly known as L type, using three common Japanese commercial enrichment diets; a control diet of *Chlorella vulgaris*-V12 against (i) DHA-enriched *Chlorella vulgaris*-V12, (ii) frozen *Nannochloropsis oculata* and (iii) Salmon roe emulsion oil.

2.1 Materials and methods

2.1.1 Experimental Design

L type rotifer Obama strain stock population was obtained from the National Research Institute of Aquaculture, Fisheries Research Agency, Nansei, Mie, Japan. SS type rotifers Indonesian strain stock population was obtained from Nagasaki University, Nagasaki Prefecture, Japan. Both morphotypes were cultured in the continuous system following

protocols outlined by Kotani *et al.* (2009). The continuous system was used as the primary rotifer cultivation and would serve as the supply stock for the enrichment cultures and provide baseline nutritional levels. Concentrated freshwater *C. vulgaris* (Chlorella Industry Co. Ltd, Tokyo, Japan) was used as the control feed for the culture protocols. Water temperature was adjusted and maintained at 25 ± 1 °C using heaters for both SS-type and L-type rotifer cultures. Seawater used in culture was pumped from Kagoshima Bay surrounding the Kamoike On-shore Laboratory, Education and Research Centre for Marine Resources and Environment at Kagoshima University, Kagoshima, Japan. The filtered seawater was then diluted with tap water and the salinity was adjusted to 20 ± 1 psu and was left standing under strong aeration for 24 hours before use. Confirmations of salinity were undertaken daily using a refractometer. Levels of dissolved oxygen (DO), DO saturation ratio, temperature and pH were measured on a daily basis. Measurements of DO, saturation ratio and temperature were done using a portable DO meter (Horiba D-75 pH/ORP/DO Meter, Horiba Ltd., Kumamoto, Japan) and pH was measured using a pH Meter (Horiba D-51 pH meter, Horiba Ltd., Kumamoto, Japan).

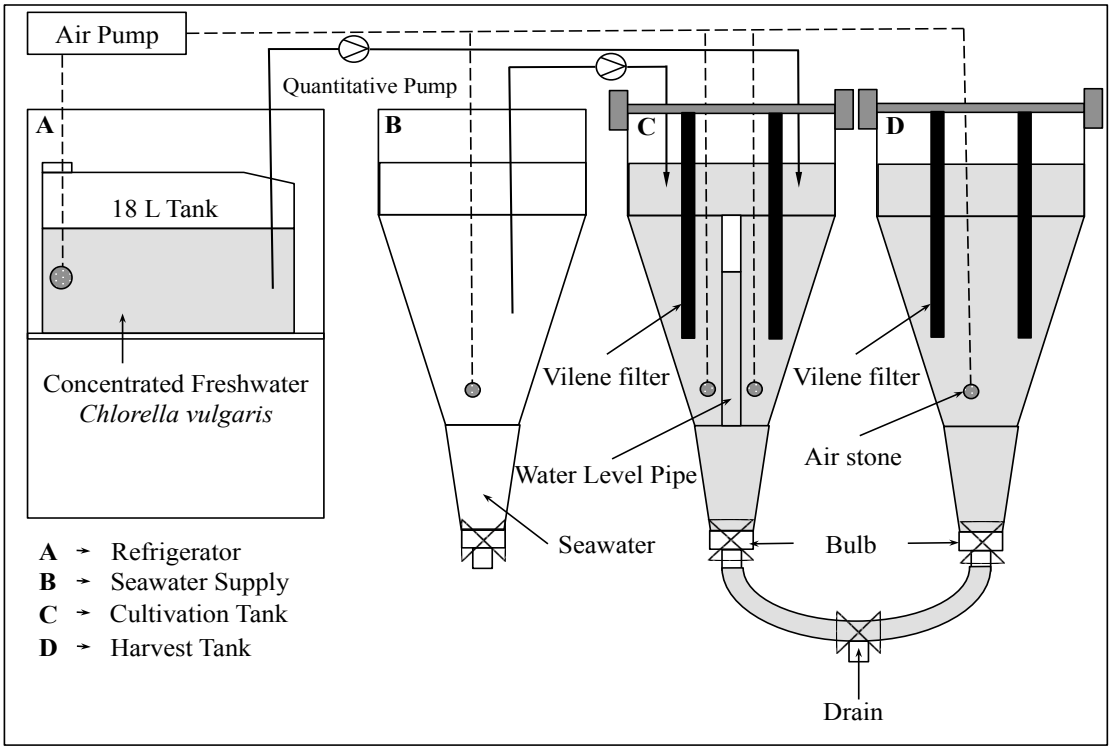
2.1.2 Continuous Culture

The system used was a slight modification of the system outlined by Kuwada (2000) and Kotani *et al.* (2009) (Fig. 3). The continuous culture system for SS-type rotifers consisted of three 55 l cylindro-conical tanks with (i) a tank supplying seawater, (ii) a tank for rotifer cultivation and (iii) a tank for harvesting the rotifers. In addition, the tanks were connected through the use of two quantitative pumps (Iwaki EWN-B11 Metering Pump, Iwaki Co. Ltd., Japan) were clear rubber hosing linked to a refrigerator that contained an 18 L gallon filled with a mixture of freshwater (15 L) and *C. vulgaris* (200 ml) at 20 psu. The pumps would supply the cultivation tank with seawater (33 psu) from the supply tank at a rate of (6 ml/min)

whilst the other would supply the culture with the mixture of freshwater and *C. vulgaris* (at 4 ml/min; 15 x 10^9 cells/ml).

The continuous culture system for L-type rotifers consisted of three 200 l cylindro-conical tanks with each tank serving similar purposes as outlined for the SS-type system. The two quantitative pumps would supply the cultivation tank with seawater (33 psu) from the supply tank at a rate of (60 ml/min) whilst the other would supply the culture with concentrated *C. vulgaris* in freshwater (at 40 ml/min; 15 x 10^9 cells/ml).

Figure 3: Schematic overview of the modified continuous culture system of Kuwada (2000) and Kotani et al. (2009)



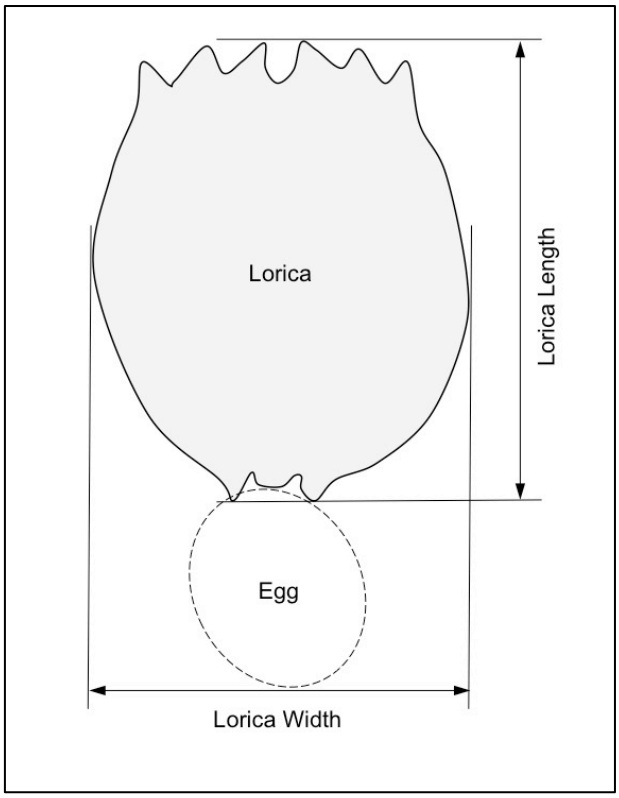
In both systems of continuous culture, aeration was provided by ceramic air-stones suspended at approximately 5 cm from the center of the tank bottom and 2 large sheets of Vilene mat filter (Japan Vilene Company Ltd., Saitama, Japan) were suspended vertically in the cultivation and harvest tanks to remove large particulate wastes. Dependent of density, approximately 20-30 l was harvested directly from the harvest tank during sampling as part of

the control treatment. A second collection of 20-30 l was harvested from the continuous culture, rinsed and placed into a 15 l tank filled with 60 percent seawater (20 psu) then split evenly into 3 consignments of 5 l batch cultures.

2.1.3 Measurement of rotifer lorica length and width

The Keyence VHX- S90F free-angle observation system with the Keyence VH-Z20R digital microscope (Keyence Corporation, Osaka, Japan) was used to measure the lorica length of 100 individuals from each rotifer type.

Figure 4: Measurement of rotifer lorica length and width



Rotifer samples were immobilized using Lugol's solution (I_3K) before measurement. Measurements of (i) lorica length (from lorica crown spines (base of corona) extending to the lower extremity of the lorica and (ii) lorica width (across laterally the widest points of the lorica) were undertaken as outlined by Fu *et al*. (1991) (Fig. 4).

2.1.4 Nutritional enrichment

The enrichment trials were performed in 5 l cultures. Rotifers harvested from the continuous culture were split into triplicates. The cultures were placed in a heated water bath (27 ± 1 °C) in order to maintain the culture water temperature in all cultures. Aeration was provided by ceramic air-stones suspended at approximately 5 cm from the center of the tank bottom. A small strip of Vilene mat filter (10 cm x 25 cm) was suspended vertically in the 5 l tanks to remove large particulate wastes, which was cleaned daily. Stock density was measured and the rotifer populations were harvested after 24 h of inoculation in the enrichment.

Three nutritional enrichment treatments were undertaken in the 5 l cultures and compared to the stock culture in continuous culture. The control treatment consisted of concentrated Fresh *Chlorella vulgaris* (Chlorella Industry Co. Ltd) that was being fed automatically in the continuous culture system. The trialed enrichment treatments were: (i) concentrated DHA-enriched *Chlorella vulgaris* (Chlorella Industry Co. Ltd) was fed at 46,000 cells /rotifer according to stock density at inoculation into the culture tanks. The volume of concentrated *Chlorella* to be fed was adjusted using the formula:

$$\text{Volume of } C.\ vulgaris \text{ (ml)} = \frac{(\text{stock density} \times 0.46 \times \text{culture volume})}{150}$$

(ii) frozen *N. oculata* (Chlorella Industry Co. Ltd) was fed to rotifers at 0.6 g/l of culture volume (3.63×10^{10} cells/g); (iii) following the manufacturers instruction, salmon roe emulsion oil (MarineTech Co., Ltd., Japan) was fed at 0.06 g/l of culture volume. All 5 l batch treatments were enriched twice (0800, 1700) and were harvested 24 h after inoculation of rotifers. After enrichment, the populations were rinsed with freshwater on a 63 μm mesh net. The mesh net was patted dry using paper towels and the samples filled and weighed in separate tubes and stored at -80 °C.

Population density was measured daily in each culture and the growth ratio from the previous day in the batch culture was calculated using:

$$\text{Growth ratio (\%)} = \left(\frac{D_p - D_{p-1}}{D_{p-1}}\right) \times 100 \qquad \text{(Kotani } et\ al.\text{, 2009)}$$

where D_p is the population density after enrichment and D_{p-1} is the population density at inoculation. Using an adaptation of the above equation, egg ratio was calculated using:

$$\text{Egg bearing ratio (\%)} = \left(\frac{R_2 - R_1}{R_1}\right) \times 100$$

Where R_1 is the average number of rotifers not bearing eggs; R_2 is the average number of rotifers bearing eggs (i.e. eggs and rotifer with 1,2 or 3 eggs are all counted). The formula is an adaptation of the similar formula used in growth ratio (Kotani *et al.*, 2010) and similar to that used by Wilde *et al*. (2010) for egg rate.

2.1.5 Fatty acid content

The extraction of lipids was undertaken following the methods outlined by Folch *et al*. (1957). Approximately 2 g (wet weight) of frozen samples are required for gas chromatography analyses and these were lyophilized using a freeze dryer (EYELA FDU-1200, Tokyo Rikaikai Co., Ltd, Japan). The samples were then suspended in chloroform and methanol solutions with varying concentration ratios (2:1, 1:1 v/v). These were then mixed thoroughly using a homogenizer (Homogenizer VH-10, VIOLAMO) before being filtered twice through circular filter paper (Whatman qualitative filter paper-Grade 1, Sigma Aldrich Co. LLC). Lipids were separated using Sep-pak silica cartridges with chloroform:methanol (98:2 v/v) and methanol to obtain neutral lipids and polar lipids. The extracted lipids were then transferred to test tube vials using 0.5 ml of C19:0 (fatty acid standard) before 1 ml of 5 % hydrogen chloride methanol solution was added. These were then incubated at 85 ± 1 °C for 3 hours. After

cooling, 1 ml of hexane and 5.5 ml of double distilled water were added to the test tube vials and vortexed using a mixer (FrontLab FLX-F60 mixer) then centrifuged for 10 mins at 3300 rpm for 10 mins using a centrifuge (LC 06, Tomy Seiko Co., Ltd, Japan). Fatty acid containing hexane layer was extracted and subject to gas chromatography analysis with the data detected analyzed based on the C19:0 standard.

2.1.6 Protein content

To determine the amount of soluble protein present we homogenized 0.01 g (dw) of harvested rotifer in 200 µl ice-cold phosphate buffer (pH 7.4) and the supernatant was extracted after centrifugation (at 4 °C, 1700 g for 10 min). We extracted 20 µl from the stock homogenate and any leftover supernatant was stored at -80 °C for later use. The extracted homogenate was then diluted 500 times due to the high amount of exhibited protein levels in the preliminary studies. The samples were then subject to the Bradford method of binding Coomasie Brilliant Blue G-250 (CBB) dye to proteins. Bovine serum albumin (BSA) was used as the standard and samples were analyzed using a Hitachi ratio beam spectrophotometer U-5100. Standard curves were then compared to sample data to ascertain protein content in each rotifer sample.

Crude protein levels were determined following the Kjeldahl method. We used 0.1 g (dw) of rotifer sample which we digested in 10 ml each of concentrated H_2SO_4 and 30 % H_2O_2 for 90 min at 550 °C. This was followed by distillation in 50 ml of 30-40 % NaOH solution using the KJELDAHL distilling apparatus (Kjeltec System Tecator 1002, Sweden). 150 ml of the solution was then distilled in H_3BO_3 solution, with methylene blue and methyl red indicators in ethanol that was titrated with 0.1 N H_2SO_4 until a neutral pH is reached. The percentage nitrogen (% N) and the percentage crude protein (% CP) were calculated using:

$$\% \text{ N} = \frac{(\text{S}-\text{B}) \times \text{ N} \times 1.4007}{\text{W}}$$

$$\% \text{ Protein} = \% \text{ N} \times \text{Protein factor}$$

Where, S is the amount (H_2SO_4 (ml)) back titration of sample, B is the amount (H_2SO_4 (ml)) back titration of blank, N is the normality of NaOH, W is the weight of the sample (g) and 1.4007 is the milliequivalent weight of nitrogen. A protein factor of 6.25 was used was in this study.

2.1.7 Statistical analysis

Statistical analysis was undertaken in SigmaPlot© version 11.0, from Systat Software, Inc., San Jose California USA. Non-parametric data were subject to Kruskal-Wallis ANOVA on ranks with any significant differences detected further subjected to a Student-Newman-Keuls (SNK) multi comparison post-hoc test. For comparison between the two morphotypes Mann-Whitney U-test was conducted. Parametric data were subject to a one-way analysis of variance (ANOVA) and any significant differences were further subjected to a Tukey HSD post-hoc test. For comparison between the two morphotypes unpaired t-tests were conducted.

2.2 Results

2.2.1 Rotifer lorica length measurements

L-type rotifers were observed to be within the size range of lorica length: 173.48 ± 19.24 μm (L-type) and 123.21 ± 17.89 μm for SS-type (mean ± SD). Lorica width was found to be 81.11 ± 6.74 μm (L-type) and 61.85 ± 9.42 μm (SS-type). It can be seen that there is some overlap in size due to the close proximity in the upper size limit of SS-type and lower size limit of L-type.

2.2.2 Population growth

DHA-enriched *Chlorella* treatment was observed to have significantly higher growth and egg ratio for both species ($p < 0.05$). L-type were observed to have significantly higher growth ratio while SS-type had higher egg ratio under this treatment ($p < 0.05$) highlighting the performance of this treatment in terms of population growth but also the differences in reproductive response by each species (Table 1).

Table 1: Growth Ratio of L and SS-Type rotifer after 24 h enrichment

Rotifer Species Population Growth	Rotifer morphotypes			
	L-Type		SS-Type	
	Growth ratio * (%)	Egg ratio* (%)	Growth ratio * (%)	Egg ratio (%)
Chlorella vulgaris (Control)	10.91 ± 8.23 ^{ab}	5.64 ± 1.14 ^{b}	(-)7.23 ± 5.51 ^{b}	3.76 ± 1.18
DHA-enriched *C. vulgaris*	55.24 ± 24.35 $^{b+}$	7.31 ± 5.86 ^{b}	25.34 ± 10.87 ^{b}	7.83 ± 2.01^{+}
Frozen *N. oculata*	20.44 ± 8.37 ^{b}	5.99 ± 0.27 ^{b}	14.87 ± 3.96 ^{b}	6.07 ± 1.91
Salmon egg emulsion oil	(-)41.07 ± 5.65 ^{a}	(-)14.53 ± 4.95 ^{a}	(-)63.88 ± 6.68 $^{a+}$	(-)9.33 ± 3.43

Mean ± SD. Asterisks indicate significant differences among treatments (One-Way ANOVA, *: $p < 0.05$). Crosses indicate significant differences between same treatments among rotifer type in that fatty acid (Unpaired T-test, +: $p < 0.05$). Alphabetical superscript indicates result of Tukey pairwise multiple comparison test among treatment ($p < 0.05$, b>a).

In contrast, the salmon roe emulsion treatment was observed to have significantly low growth ratio in L and SS-type and also significantly low egg ratio in L-type ($p < 0.05$). SS-type had significantly lower egg ratio of the two morphotypes ($p < 0.05$) further highlighting the possible differences in reproductive responses to each treatment (Table 1). Frozen *N. oculata* treatment also experienced increases in growth and egg ratio and there were no significant differences observed between the two morphotypes. Population growth in the control culture remained relatively constant with minimal extreme fluctuations throughout the enrichment period: 10.91 ± 8.23 % (L-type) and -7.23 ± 5.56 % (SS-type).

2.2.3 Fatty acid content

In the enrichment diets, significant differences were found in proportions of thirteen fatty acid species in the NL region (16:0, 16:2, 16:3, 18:0, 18;1*n*-9, 18:3*n*-3, 18:4*n*-3, 20:4*n*-6, 20:4*n*-3, 20:5*n*-3, 22:0, 22:5*n*-6, 22:6*n*-3) and eleven fatty acid species in the PL (16:1, 16:2, 16:3, 18:1*n*-9, 18:2*n*-6, 18:3*n*-3, 20:4*n*-6, 20:5*n*-3, 22:0, 22:5*n*-6; 22:6*n*-3 (Table 2). DHA (22:6*n*-3; $p < 0.01$) was not detected in the control and frozen *N. oculata* whereas significantly high proportions observed in both the DHA-enriched *C. vulgaris* (NL: 13.97 ± 0.40%; PL: 3.94 ± 0.55%) and salmon roe emulsion (NL: 15.22 ± 0.32%; PL: 26.14 ± 0.36%). Eicosapentaenoic acid (EPA, 20:5*n*-3; $p < 0.01$) was significantly high in the frozen *N. oculata* (NL: 42.27 ± 2.44%; PL: 34.12 ± 5.03%). Arachidonic acid (ArA, 20: 4*n*-6; $p < 0.05$) was found to be significantly high in both the NL and PL of the microalgal diets compared to the salmon roe emulsion diet. Salmon roe emulsion was found to have significantly higher ($p < 0.01$) fatty acid content (mg/g) of ArA, DHA and EPA compared to the microalgal based diets, with higher content observed in the PL than in the NL (Table 2). DHA-enriched *C. vulgaris* was observed to have the highest DHA/EPA ratio in both the NL and PL and also in ArA/EPA ratio (Table 2).

In L-type rotifers, significant differences were found in proportions of twelve fatty acid species in the NL region (14:0, 16:2, 18:0, 18;1*n*-9, 18:2*n*-6, 18:3*n*-3, 18:4*n*-3, 20:4*n*-6, 20:5*n*-3, 22:0, 22:5*n*-6, 22:6*n*-3) and eight fatty acid species in PL (16:3, 18:0, 18:2*n*-6, 18:3*n*-3, 20:4*n*-6, 20:4*n*-3, 20:5*n*-3, 22:5*n*-6) (Table 3, Table 4). Of the *n*–3 fatty acids in the NL, proportions of alpha-linolenic acid (ALA, 18:3*n*-3; $p < 0.05$), stearidonic acid (SDA, 18:4*n*-3; $p < 0.05$) and EPA, (20:5*n*-3; $p < 0.01$) were significantly higher in the frozen *N. oculata* treatment whilst docosahexaenoic acid (DHA, 22:6*n*-3; $p < 0.01$) was significantly higher in DHA-enriched *C. vulgaris* and salmon roe emulsion treatments. Proportion of *n*-6 fatty acids

such as linolenic acid (LA, 18:2*n*-6; $p < 0.05$) were significantly higher in control, docosapentaenoic acid (DPA, 22:5*n*-6; $p < 0.01$) in DHA-enriched *C. vulgaris* and EPA ($p < 0.05$) in frozen *N. oculata* treatment. DHA was not detected in the frozen *N. oculata* treatment. In the PL, the *n*–3 fatty acids ALA (18:3*n*-3; $p < 0.05$), eicosatetraenoic acid (ETA, 20:4*n*-3; $p < 0.05$) and EPA (20:5*n*-3; $p < 0.01$) were significantly higher in frozen *N. oculata* treatment whilst DHA (22:6*n*-3; $p < 0.01$) was significantly higher in DHA-enriched *C. vulgaris* and salmon roe emulsion treatment. Similar to NL, DHA was not detected in the frozen *N. oculata* treatment. Significant differences were also observed in *n*-6 fatty acid proportions in the PL in LA (18:2*n*-6; $p < 0.05$) of the control treatment, ArA (20:4*n*-6; $p < 0.05$) in frozen *N. oculata* and DPA (22:5*n*-6; $p < 0.01$) in DHA-enriched *C. vulgaris.*

In SS-type rotifers, significant differences were found in proportions of nine fatty acid species in the NL region (16:2, 18:0, 18:2*n*-6, 18:3*n*-3, 18:4*n*-3, 20:4*n*-3, 20:5*n*-3, 22:0, 22:6*n*-3) and five fatty acid species in PL (18:1*n*-9, 20:4*n*-3, 20:5*n*-3, 22:0, 22:6*n*-3) (Table 3, Table 4). Of the *n*–3 fatty acids in the NL, there were significantly higher proportions of ALA (18:3*n*-3; $p < 0.05$) observed in control treatment, ETA (20:4*n*-3; $p < 0.01$) and EPA (20:5*n*-3; $p < 0.01$) in frozen *N. oculata* treatment and SDA (18:4*n*-3; $p < 0.05$) and DHA (22:6*n*-3; $p < 0.01$) in salmon roe emulsion and DHA-enriched chlorella. ArA, ETA, EPA were not detected in the control whilst DHA was not detected in the frozen *N. oculata* treatment. LA (18:2*n*-6; $p < 0.05$) was the only *n*-6 fatty acid species that had significantly higher proportion in the NL of the *C. vulgaris* based control treatment and DHA-enriched *C. vulgaris*. In the PL region, *n*–3 fatty acids were significantly higher in proportion in ETA (20:4*n*-3; $p < 0.01$) in DHA-enriched *C. vulgaris*, EPA (20:5*n*-3; $p < 0.01$) in frozen *N. oculata* and DHA (22:6*n*-3; $p < 0.01$) in both DHA-enriched *C. vulgaris* and salmon roe emulsion treatment. DHA was not detected in the frozen *N. oculata* treatment whilst ETA, EPA and DHA were not detected in the control

treatment. There were no significant differences observed in *n*-6 fatty acid proportions in PL region of the SS-type morphotype.

Table 2: Fatty acid composition (area %) and contents (mg/g dry weight) of enrichment diets

Enrichment		Non-Polar Lipid				Polar Lipid			
		Chlorella vulgaris (Control)	DHA-enriched *C. vulgaris*	Frozen *N. oculata*	Salmon roe emulsion oil	*Chlorella vulgaris* (Control)	DHA-enriched *C. vulgaris*	Frozen *N. oculata*	Salmon roe emulsion oil
Fatty acid (%)									
14:0		3.46 ± 0.25	5.03 ± 0.72	4.33 ± 1.26	3.67 ± 0.11	2.82 ± 1.39	5.19 ± 2.12	5.42 ± 4.23	7.50 ± 0.05
16:0	*	17.22 ± 1.09 ^{c}	18.17 ± 2.60 ^{c}	12.96 ± 3.72 ^{b}	9.46 ± 0.05 ^{b}	26.15 ± 1.56	16.66 ± 3.22	9.14 ± 3.67	16.50 ± 0.18
16:1		2.48 ± 0.21	3.53 ± 0.30	5.69 ± 0.21	6.79 ± 0.05	* 3.21 ± 2.15 ^{b}	1.48 ± 2.23 ^{a}	4.70 ± 6.67 ^{c}	0.46 ± 0.01 ^{a}
16:2	*	2.15 ± 0.09 ^{c}	0.70 ± 0.16 ^{a}	1.82 ± 0.10 ^{b}	0.72 ± 0.02 ^{a}	* 0.98 ± 0.61 ^{c}	0.71 ± 0.30 ^{c}	8.86 ± 7.75 ^{b}	1.28 ± 0.02 ^{a}
16:3	*	2.04 ± 0.18 ^{c}	2.57 ± 0.03 ^{c}	1.56 ± 0.09 ^{b}	0.35 ± 0.01 ^{a}	* 0.52 ± 0.90 ^{a}	2.34 ± 0.37 ^{b}	3.18 ± 1.92 ^{b}	0.34 ± 0.01 ^{a}
18:0	**	8.78 ± 1.30 ^{c}	1.67 ± 1.14 ^{b}	8.75 ± 0.91 ^{c}	0.19 ± 0.00 ^{a}	4.94 ± 7.23	3.15 ± 0.03	9.11 ± 12.67	0.36 ± 0.01
18:1 n-9	*	4.45 ± 0.88 ^{b}	0.45 ± 0.52 ^{a}	3.52 ± 0.41 ^{b}	0.63 ± 0.98 ^{a}	** 1.01 ± 1.75 ^{a}	15.65 ± 0.01 ^{c}	7.33 ± 1.89 ^{b}	6.65 ± 0.23 ^{b}
18:2 n-6		12.77 ± 0.68	14.48 ± 1.95	10.52 ± 0.57	15.91 ± 0.12	* 18.77 ± 2.16 ^{b}	13.59 ± 1.33 ^{b}	6.48 ± 4.18 ^{a}	11.61 ± 0.08 ^{b}
18:3 n-3	**	4.65 ± 0.30 ^{c}	1.73 ± 1.51 ^{b}	0.94 ± 0.13 ^{a}	3.66 ± 0.21 ^{c}	** ND ^{a}	2.83 ± 0.59 ^{b}	3.57 ± 3.43 ^{b}	3.14 ± 0.06 ^{b}
18:4 n-3	*	2.02 ± 0.38 ^{c}	0.38 ± 0.05 ^{a}	1.06 ± 0.17 ^{b}	1.07 ± 0.19 ^{b}	3.83 ± 3.32	0.83 ± 0.08	2.68 ± 4.01	1.15 ± 0.01
20:4 n-6	**	0.55 ± 0.62 ^{a}	2.52 ± 0.19 ^{b}	2.06 ± 0.07 ^{b}	0.14 ± 0.01 ^{a}	* 3.82 ± 1.35 ^{b}	1.90 ± 0.55 ^{b}	6.51 ± 0.85 ^{c}	0.70 ± 0.44 ^{a}
20:4 n-3	**	1.97 ± 0.65 ^{b}	0.54 ± 0.07 ^{a}	4.14 ± 0.18 ^{c}	0.17 ± 0.00 ^{a}	2.62 ± 0.76	1.08 ± 0.75	2.88 ± 0.33	0.94 ± 0.72
20:5 n-3	**	13.63 ± 1.10 ^{c}	0.36 ± 0.02 ^{a}	42.27 ± 2.44 ^{d}	3.91 ± 0.06 ^{b}	** 0.28 ± 0.49 ^{a}	0.16 ± 0.64 ^{a}	34.14 ± 5.03 ^{c}	1.62 ± 0.10 ^{b}
22:0	**	ND ^{a}	0.80 ± 0.30 ^{b}	ND ^{a}	19.59 ± 1.54 ^{c}	** 0.29 ± 0.50 ^{b}	0.37 ± 0.15 ^{b}	ND ^{a}	5.55 ± 9.24 ^{c}
22:5 n-6	**	ND ^{a}	2.32 ± 0.71 ^{c}	ND ^{a}	0.74 ± 0.32 ^{b}	** ND ^{a}	3.27 ± 0.07 ^{c}	ND ^{a}	0.82 ± 0.70 ^{b}
22:6 n-3	**	ND ^{a}	13.97 ± 0.40 ^{b}	ND ^{a}	15.22 ± 0.32 ^{b}	** ND ^{a}	3.94 ± 0.55 ^{b}	ND ^{a}	26.14 ± 0.36 ^{c}
Contents (mg/g dw)									
ArA	*	0.09 ± 0.06 ^{a}	0.32 ± 0.07 ^{b}	0.22 ± 0.03 ^{b}	0.16 ± 0.02 ^{b}	0.25 ± 0.07	0.17 ± 0.04	0.20 ± 0.02	1.06 ± 0.70
EPA	**	1.44 ± 1.94 ^{a}	0.05 ± 0.02 ^{a}	2.44 ± 0.70 ^{b}	4.36 ± 0.27 ^{c}	* 0.03 ± 0.05 ^{a}	0.01 ± 0.06 ^{a}	1.06 ± 0.01 ^{b}	2.44 ± 0.23 ^{c}
DHA	**	ND ^{a}	1.77 ± 0.62 ^{b}	ND ^{a}	16.96 ± 1.18 ^{c}	** ND ^{a}	0.37 ± 0.05 ^{b}	ND ^{a}	39.37 ± 1.19 ^{c}
ΣSFA[1]	**	1.34 ± 0.43 ^{a}	2.62 ± 1.63 ^{a}	1.18 ± 0.42 ^{a}	36.61 ± 2.27 ^{b}	* 3.20 ± 1.12 ^{b}	1.98 ± 0.32 ^{b}	0.42 ± 0.88 ^{a}	35.94 ± 14.41 ^{c}
Σn-3	**	2.44 ± 1.11 ^{a}	2.10 ± 0.81 ^{a}	2.79 ± 0.81 ^{a}	26.75 ± 1.86 ^{b}	** 0.75 ± 0.51 ^{a}	1.10 ± 0.36 ^{b}	1.30 ± 0.48 ^{b}	49.68 ± 2.67 ^{c}
Σn-6	**	1.55 ± 0.25 ^{b}	2.43 ± 1.00 ^{b}	0.72 ± 0.21 ^{a}	18.71 ± 1.35 ^{c}	** 2.04 ± 0.27 ^{b}	1.87 ± 0.07 ^{b}	0.45 ± 0.56 ^{a}	57.91 ± 2.38 ^{c}
DHA/EPA	**	ND ^{a}	37.72 ± 30.23 ^{c}	ND ^{a}	3.89 ± 4.43 ^{b}	** ND ^{a}	27.20 ± 0.73 ^{c}	ND ^{a}	16.16 ± 5.15 ^{b}
ArA/EPA	*	0.04 ± 0.03 ^{b}	6.91 ± 7.10 ^{b}	0.05 ± 0.05 ^{b}	0.04 ± 0.07 ^{b}	** 8.64 1.48 ^{b}	12.85 ± 0.87 ^{b}	0.19 ± 0.22 ^{a}	0.43 ± 1.05 ^{a}
n-3/n-6	*	1.58 ± 0.55 ^{b}	0.86 ± 0.81 ^{a}	3.85 ± 2.91 ^{b}	1.43 ± 1.38 ^{a}	* 0.37 ± 0.88 ^{a}	0.59 ± 0.14 ^{a}	2.91 ± 0.87 ^{b}	0.86 ± 0.12 ^{a}

Values indicate mean ± SD in that treatment. . ΣSFA[1]: Total Saturated Fatty Acid (14:0, 16:0, 18:0, 22:0). Asterisks indicate significant differences among treatments in that fatty acid (One-Way ANOVA or Kruskal-Wallis test, *: $p < 0.05$; **: $p < 0.01$). Crosses indicate significant differences between same treatments among rotifer type in that fatty acid (Unpaired *T*-test or Mann-Whitney *U*-Test, +: $p < 0.05$). Alphabetical superscript indicates the result of a Tukey pairwise multiple comparison test among treatment ($p < 0.05$, $c > b > a$).

Table 3: Fatty acid composition of non-polar lipids of L and SS type rotifers according to various enrichment treatments

Rotifer species		L type												SS type											
Enrichment Treatment		*Chlorella vulgaris* (Control)			DHA-enriched *C. vulgaris*			Frozen *N. oculata*			Salmon roe emulsion oil				*Chlorella vulgaris* (Control)			DHA-enriched *C. vulgaris*			Frozen *N. oculata*			Salmon roe emulsion oil		
Fatty acid (%)																									
14:0	*	6.39	±	1.91 ^{a}	4.37	±	2.92 ^{a}	7.43	±	0.72 $^{a+}$	14.06	±	4.86 $^{b+}$		3.64	±	1.08	4.08	±	1.87	2.60	±	1.53	3.62	±	0.76
16:0		21.22	±	5.28	14.79	±	1.73	21.72	±	1.93	14.01	±	1.56		16.99	±	0.07	16.51	±	3.02	16.81	±	8.92	14.73	±	1.16
16:1		2.13	±	0.15	2.81	±	0.23 $^{+}$	3.76	±	1.53	3.48	±	0.27		2.43	±	0.34 ^{a}	1.06	±	0.88 ^{a}	3.39	±	2.73 ^{b}	5.12	±	1.68 ^{b}
16:2	*	3.93	±	0.19 ^{b}	7.18	±	2.35 $^{ab+}$	1.40	±	0.29 ^{a}	1.64	±	0.34 ^{a}	*	5.71	±	1.77 ^{b}	2.02	±	1.30 ^{ab}	0.24	±	0.15 ^{a}	0.78	±	0.04 ^{a}
16:3		0.51	±	0.06	0.55	±	0.47	1.29	±	0.70	0.84	±	0.25		0.35	±	0.12	0.66	±	0.55	2.46	±	2.66	1.40	±	0.44
18:0	*	8.45	±	0.39 ^{b}	1.68	±	2.34 ^{a}	4.57	±	0.36 ^{ab}	4.73	±	1.88 $^{ab+}$	**	5.70	±	1.26 ^{b}	0.64	±	0.24 ^{a}	3.28	±	1.57 ^{ab}	0.70	±	0.18 ^{a}
18:1 n-9	*	2.30	±	0.25 ^{ab}	1.82	±	1.14 ^{a}	3.58	±	0.33 ^{ab}	5.23	±	0.95 ^{b}		3.58	±	0.77	1.13	±	0.10	3.25	±	2.08	3.75	±	0.49
18:2 n-6	*	22.37	±	1.25 ^{ab}	18.74	±	3.63 ^{ab}	12.19	±	2.30 ^{a}	20.24	±	5.05 ^{ab}	*	28.21	±	3.25 ^{b}	30.57	±	6.61 $^{b+}$	19.48	±	0.68 ^{a}	18.93	±	1.97 ^{a}
18:3 n-3	*	2.545	±	0.14 ^{a}	5.06	±	0.71 ^{ab}	4.86	±	1.07 ^{ab}	2.71	±	1.30 ^{a}	*	7.33	±	0.40 $^{b+}$	4.57	±	1.97 ^{ab}	4.10	±	0.77 ^{ab}	2.78	±	1.13 ^{a}
18:4 n-3	*	0.59	±	0.08 ^{a}	1.28	±	0.62 ^{b}	2.91	±	0.55 ^{c}	0.79	±	0.38 ^{a}	*	0.21	±	0.16 ^{a}	0.63	±	0.78 ^{a}	1.59	±	0.91 ^{b}	1.67	±	0.07 $^{b+}$
20:4 n-6	*	0.30	±	0.06 ^{a}	0.50	±	0.03 ^{b}	3.49	±	0.34 ^{ab}	2.21	±	0.33 ^{ab}		1.87	±	0.74 $^{a+}$	1.98	±	0.70 $^{a+}$	3.74	±	0.40 ^{b}	2.40	±	0.82 ^{b}
20:4 n-3		1.50	±	0.23 $^{+}$	0.71	±	0.31	0.76	±	0.17	0.88	±	0.37	**	ND		^{a}	0.61	±	0.26 ^{a}	1.62	±	0.46 ^{b}	0.78	±	0.12 ^{a}
20:5 n-3	**	0.31	±	0.53 ^{a}	0.81	±	0.46 ^{a}	8.74	±	3.15 ^{b}	1.50	±	0.39 ^{a}	**	ND		^{a}	0.50	±	0.36 ^{a}	6.11	±	1.74 ^{c}	1.39	±	0.18 ^{b}
22:0	**	0.26	±	0.05 $^{a+}$	5.03	±	0.56 ^{b}	1.36	±	0.48 ^{a}	1.61	±	0.43 ^{b}	**	ND		^{a}	4.35	±	1.10 ^{bc}	1.46	±	0.75 ^{b}	2.26	±	0.11 ^{b}
22:5 n-6	**	0.15	±	0.04 ^{a}	3.11	±	0.52 ^{c}	1.63	±	0.49 ^{ab}	1.76	±	0.95 ^{bc}		0.24	±	0.41 ^{a}	2.93	±	1.68 ^{b}	1.40	±	1.07 ^{a}	3.96	±	0.40 $^{b+}$
22:6 n-3	**	0.10	±	0.17 ^{a}	7.70	±	0.60 $^{b+}$	ND		^{a}	5.80	±	1.94 ^{a}	**	0.27	±	0.48 ^{a}	8.87	±	0.93 ^{b}	ND		^{a}	10.73	±	2.45 $^{b+}$
Contents (mg g^{-1} dw)																									
ArA	*	0.13	±	0.01 ^{a}	0.59	±	0.19 ^{ab}	3.97	±	2.80 $^{c+}$	1.00	±	0.05 $^{b+}$	*	0.04	±	0.02 ^{a}	1.28	±	0.12 $^{b+}$	0.47	±	0.18 ^{a}	0.33	±	0.09 ^{a}
EPA	*	0.14	±	0.07 ^{a}	1.39	±	0.96 ^{ab}	4.74	±	2.33 $^{c+}$	0.66	±	0.05 ^{bc}	**	ND		^{a}	0.39	±	0.09 ^{b}	1.22	±	0.08 $^{d+}$	0.77	±	0.08 ^{c}
DHA	**	0.04	±	0.02 ^{a}	4.56	±	1.53 $^{c+}$	ND		^{a}	3.07	±	0.12 ^{b}	**	0.01	±	0.02 ^{ab}	3.07	±	1.29 ^{b}	ND		^{a}	2.74	±	1.02 ^{b}
Σmonoene		4.12	±	1.48	6.59	±	1.23 $^{+}$	1.55	±	0.28	7.31	±	1.39		2.52	±	0.17	3.91	±	1.51	4.67	±	1.15 $^{+}$	4.63	±	2.00
Σn-3	*	0.23	±	0.08 ^{a}	7.59	±	2.37 ^{c}	4.88	±	2.30 $^{b+}$	3.75	±	0.17 ^{b}	**	0.21	±	0.19 ^{a}	5.66	±	1.22 ^{b}	1.22	±	0.08 ^{a}	3.72	±	0.58 ^{b}
Σn-6	*	0.24	±	0.19 ^{a}	2.74	±	0.64 ^{ab}	3.99	±	2.80 ^{b}	1.39	±	0.26 ^{a}	**	0.82	±	0.80 ^{a}	4.86	±	0.49 ^{c}	0.58	±	0.22 ^{a}	1.34	±	0.44 ^{b}
DHA/EPA	**	0.32	±	0.18 $^{a+}$	3.65	±	1.39 $^{b+}$	ND		^{a}	4.63	±	2.34 ^{b}	**	ND		^{a}	4.80	±	1.05 ^{b}	ND		^{a}	3.63	±	1.50 ^{ab}
ArA/EPA	*	2.52	±	0.60 ^{b}	0.49	±	0.21 ^{a}	1.06	±	0.97 ^{a}	1.52	±	0.95 ^{ab}	**	ND		^{a}	2.00	±	0.14 ^{b}	0.39	±	0.16 ^{a}	0.42	±	0.08 ^{ab}
n-3/n-6		0.97	±	0.44	2.86	±	0.58	1.72	±	1.10	2.69	±	0.67	*	0.26	±	0.04 ^{a}	1.16	±	0.30 ^{a}	2.12	±	0.33 ^{b}	3.07	±	1.38 ^{b}

Values indicate mean ± SD in that treatment. . ΣSFA[1]: Total Saturated Fatty Acid (14:0, 16:0, 18:0, 22:0). Asterisks indicate significant differences among treatments in that fatty acid (One-Way ANOVA or Kruskal-Wallis test, *: $p < 0.05$; **: $p < 0.01$). Crosses indicate significant differences between same treatments among rotifer type in that fatty acid (Unpaired *T*-test or Mann-Whitney *U*-Test, +: $p < 0.05$). Alphabetical superscript indicates the result of a Tukey pairwise multiple comparison test among treatment ($p < 0.05$, $c > b > a$).

Table 1: Fatty acid composition of polar lipids of L and SS type rotifers according to various enrichment treatments

Rotifer species		L type												SS type											
Enrichment Treatment		*Chlorella vulgaris* (Control)			DHA-enriched *C. vulgaris*			Frozen *N. oculata*			Salmon roe emulsion oil				*Chlorella vulgaris* (Control)			DHA-enriched *C. vulgaris*			Frozen *N. oculata*			Salmon roe emulsion oil		
Fatty acid (%)																									
14:0		3.49	±	0.51	9.74	±	2.35 $^{+}$	3.08	±	0.40	7.34	±	4.90 $^{+}$		4.17	±	2.98	2.98	±	0.37	1.49	±	0.44	1.95	±	0.83
16:0		16.69	±	1.06	18.64	±	1.61	15.70	±	2.41	14.47	±	4.92		16.08	±	5.07	15.41	±	2.11	16.59	±	1.80	17.86	±	1.50
16:1		1.73	±	0.34	0.68	±	0.16	3.04	±	1.68	2.55	±	1.26		3.41	±	0.16 ^{b}	2.25	±	0.44 $^{a+}$	3.98	±	0.51 ^{b}	3.30	±	0.10 ^{b}
16:2		1.53	±	0.27	1.41	±	0.27	2.30	±	0.26	0.50	±	0.33		1.10	±	0.59	1.32	±	0.99	1.39	±	0.35	1.29	±	0.52 $^{+}$
16:3	*	1.36	±	0.25 ^{a}	2.91	±	0.58 ^{a}	1.78	±	0.30 ^{a}	6.29	±	1.89 $^{b+}$		3.26	±	1.99 $^{+}$	1.80	±	0.50	1.18	±	0.27	1.22	±	0.37
18:0	*	6.65	±	0.46 $^{c+}$	3.48	±	0.47 $^{ab+}$	2.73	±	1.11 ^{a}	5.01	±	1.62 $^{bc+}$		2.37	±	0.97 ^{b}	1.73	±	0.75 ^{a}	1.47	±	0.41 ^{a}	4.52	±	1.54 ^{c}
18:1 n-9		5.67	±	0.79	3.41	±	0.22 $^{+}$	5.12	±	1.38	6.35	±	1.20	*	5.22	±	1.40 ^{b}	1.66	±	0.59 ^{a}	5.21	±	0.07 ^{b}	4.96	±	0.54 ^{b}
18:2 n-6		30.02	±	3.77 ^{c}	22.74	±	2.72 ^{ab}	17.04	±	0.54 ^{a}	21.76	±	5.43 ^{bc}		22.91	±	3.19 ^{b}	25.11	±	1.88 ^{b}	19.89	±	3.61 ^{a}	18.45	±	2.50 ^{ab}
18:3 n-3	**	3.18	±	0.15 ^{b}	1.16	±	0.12 ^{a}	3.52	±	0.99 ^{b}	3.31	±	0.77 ^{b}		3.02	±	0.75	4.36	±	0.73 $^{+}$	2.68	±	0.18	2.32	±	0.34
18:4 n-3		1.43	±	0.29	1.78	±	0.09	1.58	±	0.08	2.27	±	0.81 $^{+}$		2.99	±	1.11	4.64	±	0.40 $^{+}$	2.20	±	0.26	2.28	±	0.14
20:4 n-6		1.59	±	0.18	1.51	±	0.18	3.47	±	0.21	2.42	±	0.74		4.55	±	2.61 $^{+}$	2.50	±	0.16	3.50	±	0.22	3.71	±	1.34
20:4 n-3	*	2.24	±	0.12 $^{b+}$	1.32	±	0.29 ^{a}	2.02	±	0.58 ^{b}	0.75	±	0.24 $^{a+}$	**	ND		^{a}	3.11	±	0.60 $^{c+}$	2.52	±	0.43 ^{c}	0.15	±	0.04 ^{b}
20:5 n-3	**	0.46	±	0.22 $^{a+}$	0.36	±	0.02 ^{a}	6.26	±	0.85 ^{c}	2.46	±	0.82 $^{b+}$	**	ND		^{a}	0.26	±	0.21 ^{b}	5.11	±	1.45 ^{c}	0.53	±	0.22 ^{b}
22:0	**	ND		^{a}	4.11	±	0.43 $^{d+}$	0.44	±	0.16 ^{b}	2.25	±	0.72 ^{c}	**	ND		^{a}	1.22	±	0.78 ^{b}	1.64	±	0.46 ^{b}	2.87	±	0.05 ^{c}
22:5 n-6	**	ND		^{a}	3.40	±	0.89 ^{b}	2.57	±	0.11 ^{b}	3.13	±	0.94 ^{b}		2.47	±	0.81 $^{+}$	2.11	±	1.07	2.41	±	0.27	3.26	±	0.25
22:6 n-3	**	ND		^{a}	4.25	±	0.42 ^{b}	ND		^{a}	3.80	±	1.16 ^{b}	**	ND		^{a}	3.54	±	0.42 ^{b}	ND		^{a}	4.00	±	0.52 ^{b}
Contents (mg g^{-1} dw)																									
ArA	*	0.16	±	0.03 ^{a}	0.19	±	0.06 ^{a}	1.22	±	0.07 $^{b+}$	0.78	±	0.19 $^{b+}$	*	0.02	±	0.01 ^{a}	0.45	±	0.24 ^{b}	0.67	±	0.47 ^{b}	0.22	±	0.10 ^{a}
EPA	**	0.06	±	0.01 $^{a+}$	0.50	±	0.01 ^{ab}	3.62	±	1.75 ^{c}	0.97	±	0.06 $^{ab+}$	**	ND		^{a}	0.60	±	0.08 ^{c}	1.55	±	0.51 ^{c}	0.48	±	0.27 ^{b}
DHA	**	ND		^{a}	2.08	±	0.02 ^{c}	ND		^{a}	1.05	±	0.03 $^{b+}$	**	ND		^{a}	1.03	±	0.02 ^{b}	ND		^{a}	0.64	±	0.50 ^{b}
Σmonoene	*	4.45	±	0.93 $^{b+}$	11.60	±	4.97 $^{c+}$	1.50	±	1.05 ^{a}	5.78	±	2.54 ^{b}		3.97	±	1.50 ^{a}	4.21	±	1.56 ^{ab}	4.14	±	1.48 $^{ab+}$	6.24	±	1.73 ^{b}
Σn-3	*	0.08	±	0.01 $^{a+}$	2.38	±	0.03 $^{c+}$	0.71	±	0.08 ^{b}	2.11	±	1.07 ^{c}	*	0.14	±	0.04 $^{a+}$	1.66	±	0.07 ^{c}	0.93	±	0.09 ^{b}	1.18	±	0.53 ^{b}
Σn-6		1.53	±	0.04 $^{+}$	1.20	±	0.18	0.24	±	0.08	1.58	±	0.13 $^{+}$	*	0.19	±	0.05 ^{a}	1.68	±	0.25 ^{b}	0.12	±	0.02 ^{b}	0.52	±	0.43 ^{a}
DHA/EPA	*	ND		^{a}	4.19	±	0.16 $^{c+}$	ND		^{a}	1.08	±	0.09 ^{b}	**	ND		^{a}	1.73	±	0.25 ^{b}	ND		^{a}	1.34	±	1.49 ^{b}
ArA/EPA		0.63	±	0.07 $^{+}$	0.39	±	0.12	0.37	±	0.13	0.80	±	0.21	*	ND		^{a}	0.74	±	0.38 $^{b+}$	0.48	±	0.25 ^{b}	0.46	±	0.14 ^{b}
n-3/n-6	*	0.20	±	0.02 $^{a+}$	2.02	±	0.31 $^{c+}$	3.28	±	1.09 ^{c}	1.33	±	0.07 ^{b}	**	0.13	±	0.13 ^{a}	1.00	±	0.14 ^{b}	8.09	±	1.01 $^{c+}$	2.27	±	2.06 ^{a}

Values indicate mean ± SD in that treatment. . ΣSFA[1]: Total Saturated Fatty Acid (14:0, 16:0, 18:0, 22:0). Asterisks indicate significant differences among treatments in that fatty acid (One-Way ANOVA or Kruskal-Wallis test, *: $p < 0.05$; **: $p < 0.01$). Crosses indicate significant differences between same treatments among rotifer type in that fatty acid (Unpaired *T*-test or Mann-Whitney *U*-Test, +: $p < 0.05$). Alphabetical superscript indicates the result of a Tukey pairwise multiple comparison test among treatment ($p < 0.05$, $c > b > a$).

Of the essential fatty acids important for larviculture in the NL region of L-type rotifers; ArA (3.97 ± 2.80 mg/g) and EPA (4.74 ± 2.33 mg/g) were significantly higher in the frozen *N. oculata* treatment. Whilst DHA was significantly higher in DHA-enriched *C. vulgaris* (4.56 ± 1.53 mg/g) and salmon roe emulsion (3.07 ± 0.12 mg/g) treatments along with significantly higher DHA/EPA ratios 3.65 ± 1.39 mg/g (DHA-enriched *C. vulgaris*) and 4.63 ± 2.34 mg/g (salmon roe emulsion) (Table 3). Similar trends were observed in the PL region, however the content was slightly lower than observed in the NL, furthermore significantly high levels of total Saturated Fatty Acids (14:0, 16:0, 18:0, 22:0) was observed (Table 4). Of the essential fatty acids in the NL region of SS-type rotifers; ArA (1.28 ± 0.12 mg/g) was significantly higher in DHA-enriched *C. vulgaris*, whilst EPA (1.22 ± 0.08 mg/g) was high in frozen *N. oculata* and DHA in both DHA-enriched *C. vulgaris* (3.07 ± 1.29 mg/g) and salmon roe emulsion (2.74 ± 1.02 mg/g) treatment (Table 3). Significantly high DHA/EPA (4.80 ± 1.05 mg/g) and ArA/EPA (2.00 ± 0.14 mg/g) ratio was observed in DHA-enriched *C. vulgaris*. In the PL region of SS-type, ArA (0.67 ± 0.47 mg/g) and EPA (1.55 ± 0.51 mg/g) levels were significantly higher in the frozen *N. oculata* treatment whilst DHA (1.68 ± 0.25 mg/g) was highest in the DHA-enriched *C. vulgaris*. DHA/EPA (1.73 ± 0.25 mg/g) and ArA/EPA (0.74 ± 0.38 mg/g) ratio was significantly higher in the DHA-enriched *C. vulgaris* treatment.

2.2.4 Protein content

There were no significant differences observed between each enrichment treatments and between rotifer morphotype in crude protein (% dw) and crude lipid (% dw; % ww) (Table 5). Significant differences were observed in soluble protein levels of DHA-enriched *C. vulgaris* (184.1 ± 2.6 mg/g) and frozen *N. oculata* (144.7 ± 1.5 mg/g) whilst low soluble protein was observed in the salmon roe emulsion treatment (85.6 ± 4.4 mg/g). The fraction of soluble protein to crude protein was significantly high in DHA-enriched *Chlorella* and lowest in

salmon roe emulsion treatment. Similar trends were observed in SS-type, however the levels of soluble protein were significantly higher in some treatments (Table 5). DHA-enriched *C. vulgaris* (306.8 ± 1.3 mg/g) and the control treatment (268.3 ± 1.3 mg/g) were observed to have significantly high soluble protein compared to the low levels detected in the other two treatments. Furthermore, significantly high fraction of soluble protein to crude protein was observed in DHA-enriched *C. vulgaris* (46.0 ± 2.9 mg/g) and the control treatment (38.5 ± 2.9 mg/g). These results allude to possible effects of *Chlorella vulgaris* based enrichment on the levels of soluble levels in both morphotypes.

2.3 Discussion

Early rotifer enrichment practices were quantitative using media such as Baker's yeast (Imada et al. 1979; Watanabe et al. 1983) with aquaculturists in the field of larval rearing having little problem with these methods (Kitajima et al. 1979). However, recent studies have greatly improved the technology of rotifer enrichment (Dhert et al. 2001; Hagiwara et al. 2007; Kotani et al. 2009, 2010) and have highlighted the importance of essential fatty acids in larviculture (Park et al. 2006; Hamre et al. 2013, Hamre 2016; Kotani et al. 2017). Due to insufficient levels of essential fatty acids and *n*-3 HUFA, these studies highlight that enrichment is an important requirement in optimizing the nutritional characteristics of euryhaline rotifers for larviculture (Watanabe et al. 1978; Tomoda et al. 2004). Information on the effect of different enrichment on different morphotypes within the commercially valuable *B. plicatilis* sp. complex is limited, with only a few studies focusing on varying reproduction and stress tolerance between morphotypes and strains (Hagiwara et al. 1995; Hagiwara and Hino 1989; Araujo and Hagiwara 2005).

Table 5: Crude and soluble protein, and crude lipid contents of enriched L and SS-type rotifers

Rotifer Species		Culture Conditions							
		L-Type				SS-Type			
Enrichment Treatment		*C. vulgaris* (Control)	DHA-enriched *C. vulgaris*	Frozen *N. oculata*	Salmon roe emulsion oil	*C. vulgaris* (Control)	DHA-enriched *C. vulgaris*	Frozen *N. oculata*	Salmon roe emulsion oil
Crude protein (mg/g dw)		682.8 ± 16.7	694.5 ± 10.5	755.5 ± 7.8	677 ± 13.2	722.5 ± 6.1	696.3 ± 15.3	688.8 ± 73.7	628.9 ± 4.8
Soluble protein (mg/g dw)	*	105.3 ± 0.9^{a}	184.1 ± 2.6^{c}	144.7 ± 1.5^{ab}	85.6 ± 4.4^{a}	** 268.3 ± 1.3^{bc+}	306.8 ± 1.3^{c+}	127.1 ± 4.4^{a}	97.1 ± 1.9^{b}
Soluble protein (% of crude protein)	*	16.7 ± 1.4^{a}	27.3 ± 2.9^{b}	20.7 ± 2.5^{ab}	12.8 ± 6.7^{a}	* 38.5 ± 1.3^{b+}	46.0 ± 2.9^{c+}	19.9 ± 4.7^{a}	15.9 ± 3.7^{a}
Crude lipid (% dw)		17.3 ± 1.3	16.1 ± 3.2	16.0 ± 1.7	15.9 ± 3.0	14.3 ± 4.1	16.3 ± 2.2	16.2 ± 2.6	17.8 ± 2.3
Crude lipid (% ww)		2.4 ± 0.6	3.4 ± 0.7	2.6 ± 1.1	2.9 ± 0.7	3.4 ± 0.4	3.7 ± 1.5	3.5 ± 1.6	4.7 ± 1.6

Values indicate mean ± SD in that treatment. Asterisks indicate significant differences among treatments in that fatty acid (One-Way ANOVA, *: $p < 0.05$). Crosses indicate significant differences between same treatments among rotifer type in that fatty acid (Unpaired *T*-test, +: $p < 0.05$). Alphabetical superscript indicates result of Tukey pairwise multiple comparison test among treatment ($p < 0.05$, $c > b > a$).

In this study, DHA-enriched *C. vulgaris* is highlighted to be the best performing enrichment based on population growth, levels of fatty acids and levels of soluble protein observed. In both morphotypes, the DHA-enriched *C. vulgaris* treatment was observed to have significantly higher population growth (L-type: 55.24 ± 24.35%; SS-type: 25.34 ± 10.87%) and egg bearing (L-type: 7.31 ± 5.86%; SS-type: 7.83 ± 2.01%). L-type species displayed higher growth ratio compared to SS-type with DHA-enriched *C. vulgaris* treatment having significantly higher growth and egg ratio in both morphotypes (Table 1). Hirayama and Rumengan (1993) found that the fecundity patterns of S and L type rotifers were similar and that the difference in growth response between the two types stemmed from differences in net reproduction rates. Studies show that based on morphological features, reproduction and allozyme patterns, SS strains differed from L strains in mating behavior. As outlined in the methods of this study, this variability occurred despite rotifers being stocked equally (1300 ± 100 individuals) in each replicate and with enrichment conditions kept as uniform as possible (salinity: 20 ± 1 psu; temperature: 25 ± 1°C; dissolved oxygen: 7.5 ± 0.3 mg/*l*) to keep the effects of the culture environment minimal.

The DHA-enriched *C. vulgaris* diet was observed to have high DHA proportion in both the NL and PL (Table 2) and this was also reflected with high DHA observed in both morphotypes (Table 2, Table 3). This trend was also observed for ArA and EPA in the frozen *N. oculata* diet which had high proportions of ArA (NL: 2.06 ± 0.07%; PL: 6.51 ± 0.85%), elevated levels of EPA (NL: 42.27 ± 2.44%; PL: 34.14 ± 5.03%) and mirrored by high assimilation of ArA and EPA in the NL and PL region of both morphotypes (Table 2 and 3). This reciprocal relationship was also observed in fatty acid species in the diet that had high proportions such as 14:0, 16:0, 18:0 and 18:2 *n*-6, which was also observed to be high in both morphotypes. Therefore it can be said that the fatty acid composition of both morphotypes effectively reflect the fatty acid

composition of their enrichment diets as seen in this study, which is in agreement with what has previously been assumed to be true for invertebrate animals (Sakamoto et al. 1982; Frolov et al. 1991). Furthermore a study by Sechelles et al. (2009) highlighted that the levels of ArA, EPA and DHA in diets and those in *B. plicatilis* rotifers are linearly correlated.

DHA is known to have various positive effects on the development and survival of finfish larvae (Watanabe et al. 1978; Takeuchi et al. 1994) and ensure membrane absorbency, enzyme activation and production of prostaglandin among other functions (Sargent et al. 1989). Due to the fortification of DHA in DHA-enriched *C. vulgaris*, the results showed high DHA proportions and content in both morphotypes with the L-type having significantly higher DHA content in both NL and PL. In contrast, the EPA content was around fourfold less in both morphotypes in this treatment, this is perhaps due to the low detection of EPA in the diet (Table 2). Furthermore, previous studies have shown that *C. vulgaris* is a known poor source of EPA (Maruyama et al. 2006; Thépot et al. 2016). In both species, ArA/EPA ratio was high in DHA-enriched *C. vulgaris* with significantly high average content in the NL and PL of SS-type (Table 3, Table 4). Previous studies by Matsunari et al. (2013) and Thépot et al. (2016) highlighted that DHA-enriched *C. vulgaris* has low ArA which was not the case in this study. Kotani et al. (2013) reported that rotifers can naturally contain many fatty acids and there is a possibility that ArA can be naturally synthesized from linoleic acid (18:2*n*-6), which had significantly higher proportion, up to 20-30% in both morphotypes in this study (Table 3, Table 4). The *Chlorella*-based enrichments may have supplemented this natural process. Furthermore, Lubzens et al. (1985) supports this hypothesis in that, while ArA production is low, rotifers have the ability to *de novo* synthesize polyunsaturated fats, though in small amounts, through the process of elongation and desaturation of *n*-6 series as was reported by Robin (1995) and for *n*-3 series by Le Milinaire et al. (1983). The variability in the results of essential fatty acid proportions and content could also be due to differing growth states of the rotifer population. A

study by Tomoda et al. (2004) highlighted that rotifers change their nutritional value depending on their growth state, where in spite of enrichment the dietary value of rotifers was poor during or just prior to the stationary growth phase of batch culture. However, the effects of such phenomenon were avoided in this study as the stock was harvested during periods of exponential growth from the continuous culture, as is an essential feature of the chemostat continuous culture system (Kotani et al. 2009).

Significant higher soluble protein levels detected in the DHA-enriched *Chlorella* and in control treatment for both morphotypes alludes to possible effects of *Chlorella vulgaris* based enrichment on soluble protein levels. *C. vulgaris* is one of the most exploited species due to its high protein content and favorable essential amino acid composition (Becker 2007; Bleakley and Hayes 2017). SS-type had significantly higher soluble protein content (306.8 ± 1.3 mg/g) with the soluble protein making up 46.0 ± 2.9% ratio of the crude protein. Only in the L-type rotifer was frozen *N. oculata* treatment observed to have high levels of soluble protein (184.1 ± 2.6 mg/g), second to DHA-enriched *C. vulgaris* (144.7 ± 1.5 mg/g). Srivastava et al. (2006) highlighted that variation in rotifer total soluble protein between species, stages, physiological status and variations according to culture conditions is to be expected. Previous studies have highlighted that live feed usually contain a large fraction of soluble intact proteins (Watanabe 1981; Govoni et al. 1986; Carvalho et al. 2003) and that soluble proteins are more available for larval digestion and absorption than insoluble proteins (Carvalho et al. 2004). Srivastava et al. (2006) stated that due to the drive for insoluble proteins to aggregate in insoluble inclusions, soluble proteins may be more available to intestinal proteases and also more available for uptake by pinocytosis. Soluble proteins have been suggested as having a higher importance in general larval dietary protein uptake (Watanabe 1981; Govoni et al. 1986) thus further highlighting the favorable effect of DHA-enriched *C. vulgaris* in both morphotypes observed in this study.

Compared to the low levels of EPA in the DHA-enriched *C. vulgaris*, salmon roe emulsion treatment was observed to contain EPA and near similar levels of DHA in the NL and PL of both morphotypes (Tables 2, Table 4). Thus this resulted in high DHA/EPA and ArA/EPA ratios. SS-type had higher proportion of DHA (NL: 10.73 ± 2.45; PL: 4.00 ± 0.52) compared to L-type (NL: 5.80 ± 1.94; PL: 3.80 ± 1.16) in both the NL and PL. Upon comparing the fatty acid constituents of the enrichment diet (Table 2) it can be said that the salmon roe emulsion treatment is an effective treatment due to its high lipid content and especially its fortification of essential fatty acids. However, both morphotypes were observed to experience significant drops in population growth (L-type: -41.07 ± 5.65%; SS-Type: -63.88 ± 6.68%) and egg ratio (L-type: -14.53 ± 4.95; SS-Type: -9.33 ± 3.43%) under the salmon roe emulsion treatment (Table 1). This is despite the relatively high HUFA composition in both NL and PL observed in both morphotypes under this treatment. These results suggest that this treatment was nutritionally effective but negatively affected population growth and reproductive capabilities. Salmon roe emulsion oil diet had much higher proportions ($p < 0.01$) of DHA (NL: 15.22 ± 0.32%; PL: 26.14 ± 0.36%) and DHA content (NL: 16.96 ± 1.18 mg/g; PL: 39.37 ± 1.19 mg/g) compared to DHA-enriched *C. vulgaris*, despite this both morphotypes had higher DHA content in the DHA-enriched *C. vulgaris*. These results would suggest that lipid rich diets do not necessarily mean increased uptake. The viscous nature of the oil-based salmon roe emulsion treatment may suggest that this is what is causing this effect. A study by Hagiwara et al. (1998) highlighted that high viscosity in culture water lowers ingestion rate, fecundity and population growth of *B. plicatilis* due to sub-lethal physiological stress. Larsen et al. (2008) showed that viscosity in culture water affects cilia action and reduces swimming velocity in *B. plicatilis* by 26%. Araujo et al. (2000, 2001) suggested that an increase of culture water viscosity decreased enzyme activity in *B. plicatilis* and affected reproductive responses in *B. rotundiformis*. Several other studies (Foscarini 1988; Dhert et al. 1990) have also shown that rotifers enriched in oil

emulsions often experience high mortality related to "sticking" at high densities, the production of lower quality rotifers with a too high lipid content and resultant reduction in water quality in fish culture tanks. As the environmental conditions were kept uniform, the dosage amount of oil enrichment media could prove to be the reason why there is a large discrepancy observed in population growth and reproduction between the salmon roe emulsion enrichment and the microalgae-based enrichment diets. The dosage of salmon roe emulsion oil used in this study was, 0.06 g/*l* of culture volume fed twice to both morphotypes over a 24 h period, as outlined by the manufacturer. Kotani et al. (2017) mentioned that generally, manufacturers of enrichment diets have determined the appropriate method and dosage for rotifers however, there is little information (Kotani et al. 2009, 2010) on the effects on rotifers of nutritional enrichment using methods not previously described by manufacturers. This is an area of research that requires important consideration, as dosage of enrichment will vary according to the scale of culture, environmental conditions and larval fish nutritional requirements.

In the frozen *N. oculata* treatment, there was high growth ratio and egg ratio in both morphotypes with no significant difference found between it and the higher DHA-enriched *Chlorella* treatment (Table 3). As expected, EPA proportion and content were significantly higher ($p < 0.01$) in the NL and PL of frozen *N. oculata*. *N. oculata* is known to support high rates of rotifer reproduction and contains significant amount of EPA (Hirayama *et al.* 1979; Lubzens et al. 1995; Kobayashi et al. 2008). ArA was also observed to have high proportion and contents in both NL and PL in both morphotypes with higher levels observed in L-type (Table 3, Table 4). During larviculture, Thépot et al. (2016) observed that larvae fed rotifers enriched with *N. oculata* diet had high ArA content and they hypothesized that this could be due to excess ArA supplied in the *N. oculata* diet. Furthermore, recent studies by Ferreira et al. (2008, 2009, 2018) highlighted the importance of nutritional quality of microalgae like *Nannochloropsis* sp. in influencing biochemical composition of rotifer cultures over the

quantity and concentration of enrichment with further complication rising from differences in metabolism of enrichment, as was observed in this study in the two different morphotypes.

Comparing the fatty acid composition between the lipid regions, it can be observed that it is generally higher in the NL than in the PL in both morphotypes. In the salmon roe emulsion treatment, DHA assimilation in the diet is around double the proportion in PL region compared to NL (Table 2). A study by Kotani (2017) highlighted that the main constituent of fish eggs or yolk are phospholipids and our results also coincide with this. However, despite high PL content in the diet, we observed that in both morphotypes, fatty acid content is higher in the NL region, which suggests that there is selective assimilation of fatty acids by both morphotypes. These variations in fatty acid content in the lipid regions, within species and between morphotypes, possibly arises from effects of enrichment and also dissimilar bio-chemical roles played by these compounds in tissue metabolism as suggested by Frolov et al. (1991). In tissue metabolism, the PL is associated with regulation and catalysis in the cellular membrane and their fatty acids are known to produce a strong effect on biological functions (Frolov et al. 1991). Therefore its composition is usually not highly affected by abiotic and biotic factors, as the composition of its fatty acids must remain fairly invariable for the efficient functioning of membranes. On the other hand, NL functions are related to cellular energy and its fatty acid composition is influenced by several factors acting simultaneously. These include the turnover of dietary lipids from PL as well as amino acid synthesis and as a result of impacts from these factors, a wide variability in its fatty acid composition is often observed (Frolov et al. 1991). There are limited studies that highlight the effect of lipid metabolism and population growth. However, a recent study by Lee et al. (2018) documented the phenomenon of aging extension and lipid metabolism modification in *B. koreanus*. Lee et al. (2018) highlighted that under reduced feeding conditions of *B. koreanus,* caloric restriction caused modulation of lipid metabolism, which led to a reduction of population growth and an extension of life span as a

trade-off. In the present study, we highlight that DHA-enriched *C. vulgaris* and frozen *N. oculata* had the highest population growth and egg bearing individuals and both these treatments had high HUFA assimilation in the NL than in the PL. We can assume that the assimilation and possible *de novo* synthesis of fatty acids in the lipid regions is an adaptive mechanism of the L and SS morphotypes. This is possibly achieved by active reorganization of dietary lipids in accordance with its metabolic requirements for its proper function and where necessary population growth (Le Milinaire et al. 1983; Lubzens et al. 1985; Robin 1995; Lee et al. 2018). Our results highlight that not only do differences occur within the lipid regions of each species but also between rotifer morphotype and its diet which are a result of the peculiarities of metabolism and functional roles of these fractions in each morphotype.

In conclusion, this study highlights that according to enrichment media and rotifer morphotype, response to the enrichment in fatty acid content, population growth and soluble protein content varies. Using the batch culture system, DHA-enriched *Chlorella* and frozen *N. oculata* was found to have good population growth, fatty acid content and soluble protein. In contrast, using the same culture method and culture conditions for the salmon roe emulsion oil treatment, both morphotypes were affected negatively in population growth and low soluble protein content, despite having high HUFA composition in both the diet and in the rotifer. Natural synthesis of certain fatty acid species is possible via elongation and desaturation of precursor fatty acid species as was observed in this study for archidonic acid (ArA, 20:4*n*-6) in normally ArA poor DHA-enriched *C. vulgaris*. Further studies are needed on culture methods and enrichment dosages and its impacts on different morphotypes due to the varying effects on fatty acid assimilation in the NL and PL and between rotifer morphotypes. The *Brachionus plicatilis* sp. complex, which includes a wide size range is an important first feed in the field of larviculture and therefore consideration of effects of enrichment on different morphotypes is

also as important as different fish species have different qualitative and quantitative requirements for survival, growth and development.

CHAPTER 3: Effect of oil enrichment on *Brachionus plicatilis* rotifer and first feeding red sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*)

Abstract

Euryhaline rotifer belonging to the genera *Brachionus* are often used as live feed for first feeding marine finfish larviculture. Two common rotifer enrichments, a single usage of docosahexaenoic acid (DHA)-enriched *Chlorella vulgaris* and a combination of DHA-enriched *C. vulgaris* and salmon roe emulsion oil were used to assess: (i) nutritional status of rotifers and, (ii) performance when rotifers were fed to *Pagrus major* and *Paralichthys olivaceus* larvae from 2-15 days after hatching (DAH). Both rotifer enrichment treatments were found to be nutritionally effective with high unsaturated fatty acid (HUFA) content, especially in the combined treatment with significantly higher eicosapentaenoic acid (EPA). Despite deficient levels of EPA, DHA-enriched *C. vulgaris* was found to be a suitable enrichment for rotifer and also had positive response in survival, growth and trypsin activity in both *P. major* and *P. olivaceus*. DHA-enriched *C. vulgaris* only treatment had higher rotifer population growth (13 % higher), egg bearing capacity (7 % higher), almost double the soluble protein content and generally higher free amino acid content. Oxidation of lipids in the combined diet due to its high-saturated fatty acid content could be a possible cause to the inferior performance of the combined treatment. Both *P. major* (20.1 ± 10.1 %) and *P. olivaceus* (12.3 ± 3.87 %) suffered low survival and lower gut content in the combined treatment especially during periods of significant growth in both species (7-11 DAH). Despite high HUFA content in the combined treatment, this study revealed that consideration of free amino acids and soluble protein content as parameters of nutritional indicators for first feeding *P. major* and *P. olivaceus* should not be discounted.

3.0 Introduction

Marine fish larvae are very vulnerable during first feeding and therefore have strict requirements for biotic and abiotic conditions (Hamre et al., 2013). Abnormal morphological development, alimentary tract deterioration, trunk musculature, reduction in food utilization efficiency and low feeding activity have been linked to nutrition deficiency and starvation immediately after reabsorption of the yolk (Heming et al., 1982; Taylor and Freeberg, 1984; Rice et al., 1987; Gisbert et al., 2004). Optimizing diet and its levels of essential nutrients is important for larval nutritional requirements during early ontogeny (Helland et al., 2010; Hamre, 2016). Live feed such as rotifers and *Artemia* are usually used as primary feed due to their small size, suitability for mass culture and ability for artificial manipulation of nutritional qualities (Yoshimura et al., 1997; Dhert et al., 2001; Kotani et al., 2009). Rotifers belonging to the genera *Brachionus* are usually chosen as a first-feed for many marine finfish species due to its favorable characteristics (Hagiwara et al., 2001; Tomoda et al., 2004; Park et al., 2006). Depending on conditions of culture and enrichment, essential nutrients can be in deficient levels in rotifers (Hamre et al., 2008). Levels of some nutrients, such as protein, amino acids, fatty acids (Srivastava et al., 2006; Hamre, 2016; Kotani et al., 2009, 2017) and phospholipid (Helland et al., 2010), are largely determined by the enrichment media and the metabolism of the feed organism (Hamre, 2016). There are many commercial rotifer media available that vary in nutrient compositions (Watanabe, 1993; Dhert et al., 2001; Koven et al., 2001; Hamre et al., 2008; Matsunari et al., 2005; Kotani et al., 2013). For example, common commercial rotifer enrichment media are DHA-enriched *Chlorella vulgaris*, frozen *Nannochloropsis oculata* (Chlorella Industry Co. Ltd., Japan) and taurine enrichment media (Aqua-Plus ET, Marubeni Nisshin Feed Co., Ltd., Japan). Others culturists also opt for products that have mixed constituents of microalgae for optimal high unsaturated fatty acids (HUFA) content such as Rotigrow Plus, RotiGreen, N-Rich, *Isochrysis*/*Pavlova* Blend (Reed Mariculture, United States

of America) or powdered DHA-protein Selco (INVE aquaculture Inc., USA). In this study we use the commercial diet (i) Super Fresh *Chlorella*-V12 (Chlorella Industry Co. Ltd, Japan) and (ii) salmon roe emulsion oil (MarineTech Co., Ltd., Japan). Super Fresh *Chlorella*-V12, also known as docosahexaenoic acid (DHA) enriched *C. vulgaris*, is a rich source of DHA (Hagiwara et al., 2014; Kim et al., 2014; Thépot et al., 2016; Kotani et al., 2017). DHA is one of the important *n*-3 HUFA that are vital for the development and survival of marine fish larvae (Watanabe, 1983; Izquierdo et al., 1989; Koven et al., 1992). Short term enrichment of rotifers using oil emulsions is also known to result in lipid-encapsulated rotifers, high in eicosapentaenoic acid (C20:5*n*-3; EPA) and DHA and generally creates a more stable composition which is important especially for fish larvae (Dhert et al., 2001; Støttrup and Attramadal, 1992).

The ability to digest feed and the subsequent growth are important indicators of acceptance of feed in larvae. Digestive enzyme activity is a reliable method that can be used as an indicator of digestive processes and nutritional condition of larvae (Ueberschär, 1988). The lack of a functional stomach in most early stage marine larvae causes the entire extra-cellular degradation of ingested prey to occur in the intestine with pancreas-derived enzymes (Bolasina et al., 2006). Trypsin is an important indicator digestive enzyme. It is an endoproteinase that is directly connected to protein metabolism and is known to be present in young fish larvae (Bolasina et al., 2006). Previous studies have highlighted it as a useful indicator of nutritional condition (Pedersen et al., 1987; Ueberschär, 1988, 1995; Bolasina et al., 2006).

Despite being commonly utilized enrichment mediums in larviculture, little is known of the effects of DHA-enriched *C. vulgaris* and emulsion oils and its impacts on larviculture performance for first feeding Japanese flounder (*Paralichthys olivaceus*) and red sea bream (*Pagrus major*). *P. olivaceus* and *P. major* are important commercial species for coastal

fisheries and for the aquacultural industry in Japan and Korea (Taniguchi and Sugama, 1990; Yamashita and Yamada, 1999; Howell and Yamashita, 2005; Gonzalez et al., 2015). The assessment of nutritional status under two commonly utilized rotifer enrichments could also contribute to determination of optimal feed and development of recommendations on rotifer enrichment most suited for *P. olivaceus* and *P. major* at the important stages of first exogenous feeding.

3.1 Materials and methods

3.1.1 Experimental Design

This experiment was conducted using 2 different enrichment treatments for L-type rotifers (i) DHA-enriched *Chlorella vulgaris* on its own and (ii) DHA-enriched *Chlolrella vulgaris* with addition of salmon roe 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.

3.1.2 Live feed

L type rotifer Obama strain stock population was obtained from the National Research Institute of Aquaculture, Fisheries Research Agency, Nansei, Mie, Japan. The rotifers were stock cultured in 150 L continuous culture systems at the Kamoike On-shore Laboratory, Education and Research Centre for Marine Resources and Environment at Kagoshima University following protocols outlined by Kotani *et al.* (2009). These cultures were automatically fed concentrated freshwater *Chlorella vulgaris* (Chlorella Industry Co. Ltd., Tokyo, Japan) and were cultured at a salinity of 20 ± 1 psu. Rotifers harvested from the stock continuous culture were then transferred to 5 L polycarbonate tanks where they were placed in

a heated water bath (27 ± 1 °C). Aeration was provided by ceramic air-stones suspended at approximately 5 cm from the center of the tank bottom at a rate of 7.3 ± 0.48 mg/l.

The rotifers were enriched with concentrated DHA-enriched *C. vulgaris* (Chlorella Industry Co. Ltd) and salmon roe emulsion oil (MarineTech Co., Ltd., Japan). DHA-enriched *C. vulgaris* was fed at 46,000 cells rotifer^{-1} according to stock density at inoculation. Volume of concentrated *Chlorella* to be fed was calculated using,

$$\text{Volume of Chlorella (ml)} = \left(\frac{\text{stock rotifer density (rotifers ml}^{-1}) \times 0.46 \text{ x culture volume (ml)}}{150}\right)$$

Salmon roe emulsion oil dosage followed the manufacturers instruction and was enriched at 0.06 g/l of culture volume in addition to DHA-enriched *Chlorella*. The 5 L enrichment cultures were enriched twice (08:00, 17:00) and were harvested 24 h after inoculation.

Population density was measured daily in each culture and the growth ratio from the previous day in the batch culture was calculated using:

$$\text{Growth ratio (\%)} = \left(\frac{D_p - D_{p-1}}{D_{p-1}}\right) \times 100 \qquad \text{(Kotani et al., 2009)}$$

where D_p is the population density after enrichment and D_{p-1} is the population density at inoculation. Using an adaptation of the above equation, egg ratio was calculated using:

$$\text{Egg bearing ratio (\%)} = \left(\frac{R_2 - R_1}{R_1}\right) \times 100$$

Where R_1 is the average number of rotifers not bearing eggs; R_2 is the average number of rotifers bearing eggs (i.e. eggs and rotifer with 1,2 or 3 eggs are all counted). The formula is an adaptation of a similar formula used in growth ratio (Kotani et al., 2010) and Wilde et al. (2010) for egg rate.

3.1.3 Protein content of enriched rotifer

Soluble protein content was determined using the homogenate of 0.01 g dry weight (dw) of harvested rotifer in 200 µl ice-cold phosphate buffer (pH 7.4) with the supernatant extracted after centrifugation (at 4 °C, 1700 g for 10 min). 20 µl from the stock homogenate was extracted and any leftover supernatant stored at -80 °C for later use. Extracted homogenate was diluted 500 times and subjected to the Bradford method of binding Coomasie Brilliant Blue G-250 (CBB) dye to proteins. Bovine serum albumin (BSA) was used as the standard and samples were analyzed using a Hitachi ratio beam spectrophotometer U-5100 (Hitachi High-Tech Science Corporation, Tokyo, Japan). Standard curves were compared to sample data to determine soluble protein content in each sample.

Crude protein levels were determined following the Kjeldahl method. We used 0.1 g (dw) of rotifer sample which we digested in 10 ml each of concentrated H_2SO_4 and 30 % H_2O_2 for 90 min at 550 °C. This was followed by distillation in 50 ml 30-40 % NaOH using the KJELDAHL distilling apparatus (Kjeltec System Tecator 1002, Sweden). 150 ml of the solution was then distilled in H_3BO_3 solution, with methylene blue and methyl red indicators in ethanol that was titrated with 0.1 NH_2SO_4 until a neutral pH is reached. The percentage nitrogen (% N) and the percentage crude protein (% CP) were calculated using:

$$\% \text{ N} = \frac{(\text{S}-\text{B}) \times \text{N} \times 1.4007}{\text{W}}$$

$$\% \text{ Protein} = \% \text{ N x Protein factor}$$

Where, S is the amount (H_2SO_4 (ml)) back titration of sample, B is the amount (H_2SO_4 (ml)) back titration of blank, N is the normality of NaOH, W is the weight of the sample (g) and 1.4007 is the milliequivalent weight of nitrogen. A protein factor of 6.25 was used was in this study. To obtain the soluble protein ratio against crude protein:

$$\text{Soluble protein (\% of crude protein)} = \left(\frac{\text{soluble protein (mg/g)}}{\text{crude protein (mg/g)}} \right) \times 100$$

3.1.4 Fatty acid content of enriched rotifer

The extraction of lipids was undertaken following methods outlined by Folch *et al*. (1957). Approximately 2 g dry weight (dw) of freeze-dried samples was used. Crude lipids were separated using into non-polar lipid (NL) and polar lipid (PL) fractions by column chromatography on Sep-Pak Silica Cartridges (Waters, S.A., U.S.A) (Juaneda and Rocquelin, 1985) with chloroform:methanol (98:2 v/v) to obtain NL and methanol for PL. Lipids were extracted using 0.5 ml of C19:0 (fatty acid standard) and 1 ml of 5 % hydrogen chloride methanol solution. Samples were incubated at 85 ± 1 °C for 3 h after which, 1 ml of hexane and 5.5 ml of double distilled water was added, vortexed and centrifuged for 10 mins at 3300 rpm. The fatty acid containing hexane layer was extracted and subject to gas chromatography (GC) analysis and the data analyzed based on the nonadecanoic acid (C19:0) internal standard. During the GC process, the samples were injected automatically by an autosampler into the gas chromatographer (GC-2010 Plus; Shimazu, Kyoto, Japan) that was equipped with a hydrogen flame ionizing type detector (260 °C). Helium was used as a carrier gas. Omegawax capillary gas ghromatography column (Sperco, 30 m × 0.32 mm × 0.25 μm) was used as the capillary column.

3.1.5 Free amino acid content of enriched rotifer

Free amino acids analyses of the rotifer enrichment diets were performed according to methods described by Teshima et al. (1986). 0.1 g (dw) sample was weighed and mixed with 0.9 ml pre-cold deionized water and 0.1 ml internal standard (6 mg DL-norleucine/ ml deionized water and 2.5 ml trichloroacetic acid (TCA, Nacalai Tesque, Kyoto Japan). The protein precipitates were then homogenized, and washed successively with 8 % TCA and

centrifuged at 4 °C, 3000 rpm or 15 minutes. The supernatant was then repeatedly washed with diethyl ether and vortexed to extract and remove lipids. The pH of the resultant solution was than adjusted to become 2.2 ± 0.05 via addition of perchloric acid or 4N NaOH solution. The resulting solution was then filtered through syringe filters and stored in 3 ml viles. The analysis of free amino acids were conducted with high-performance liquid chromatography (HLPC; Shimadzu Corp., Tokyo Japan) with an ion exchange resin column by post-column derivatization with *o*-phthaldiadehyde (OPA, Nacalai Tesque, Kyoto, Japan).

3.1.6 Source of eggs and larval rearing

Japanese flounder (*Paralichthys olivaceus*) eggs were obtained from natural spawning broodstock from the Hayato Aquaculture Farm, the Local Production Headquarters, MBC Kaihatsu Co. Ltd., in Kirishima, Kagoshima Prefecture, Japan. Red sea bream (*Pagrus major*) eggs were obtained from the Ogata Suisan Corporation, in Amakusa, Kumamoto 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. Due to seasonality and availability of eggs the two species were cultured separately, larviculture of *P. major* occurred in October (2017) and *P. olivaceus* occurred in November (2017). Larval rearing duration was until 15 days after hatching (DAH) and was conducted in clear 100 l polycarbonate tank with aeration system provided. Each treatment was replicated three times with 2000 eggs stocked in each tank. Frozen *Nannochloropsis oculata* (Chlorella Industry Co. Ltd, Tokyo, Japan) was directly introduced into all larval rearing tanks at 2 DAH as rotifer food along with the rotifers once the larval mouth groove is observed under the microscope. Frozen *N. oculata* was supplied at 0.03 g/l of culture volume (3.63×10^{10} cells g^{-1}). Rotifer densities in rearing tanks were adjusted twice daily at 8:00 am and 3:00 pm during feeding to ensure that a density of 10 rotifers ml^{-1} was maintained. To achieve this, rotifers

were counted using principles similar to that used by Kotani *et al.* (2017). A 1 ml glass pipette was used to sample rotifers from 5 random points in each rearing tank. Counts were then averaged and deducted from the required 10 rotifers ml^{-1} to obtain the number of rotifers needed.

$$\text{Volume of rotifer to feed (ml)} = \frac{\text{Amount of rotifers needed (ind./ml)} \times \text{fish tank volume (ml)}}{\text{Stock density of enriched rotifer (ind./ml)}}$$

According to the fish culture tank volume (ml) and the density of the enriched rotifer stock, the appropriate feeding volume from the enriched stock was calculated and concentration of rotifers adjusted to 10 rotifers ml^{-1} in each larval rearing tank.

3.1.7 Larval growth and gut content

A total of 480 larvae were collected (i.e. 10 larvae collected from each tank, over the course of the 8 sampling days (2, 3, 5, 7, 9, 11, 13, 15 DAH) for both treatments). Larval measurements were undertaken using the Keyence VHX- S90F free-angle observation system with the Keyence VH-Z20R digital microscope (Keyence Corporation, Osaka, Japan) to measure the total length (TL) of 160 individuals from both treatments. Constituents of larval guts were counted using the SMZ800 Nikon stereomicroscope.

3.1.8 Specific trypsin enzyme activity

The fluorometric assay of trypsin enzyme activity was conducted following methods similar to Bolasina *et al*. (2006) using Z-L-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA) as the substrate. A total of 480 larvae were collected (i.e. 10 larvae collected from three tanks per treatment) over the course of the 8 sampling days (2, 3, 5, 7, 9, 11, 13, 15 DAH). 10 frozen larvae and 0.01 g (dw) rotifer samples were homogenized with ice cold 200 µl of homogenization buffer (20 mM tris-HCl, 1mM EDTA, 10 mM $CaCl_2$, pH 7.5)

and the supernatant extracted after centrifugation (at 4 °C, 1700 g for 10 min). Any leftover supernatant was stored at -80 °C for later use in the assessment of soluble protein content of the homogenate. 20 µl of larval supernatant homogenate was added to 500 µl of the reaction buffer (50 mM tris-HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ-LArg-MCA). The mixtures were incubated into a water bath at 30 °C for 30 min and then 100 µl of 30 % acetic acid was added for quenching. Enzyme activities of rotifer were also measured using the homogenate of approximately 1500 ± 100 ind./ml from both enrichment treatments. Estimation of enzyme fraction via contribution of ingested prey was calculated following methods outlined by Bolasina *et al*. (2006) by multiplying the average number of prey consumed with the enzyme activity of the prey. Experimental blanks were prepared similarly without the addition of acetic acid prior to mixing the substrate. Fluorescence was measured using a spectrofluorophotometer and the difference in emissions at 440 nm (excitation 380 nm) was measured between the samples and the blanks. Activity was expressed in trypsin units (U) in 30 min, as increase of emission per protein (U µg/protein) and trypsin units as of emission per larvae (U/ind).

3.1.9 Statistical analysis

Statistical analysis was undertaken in SigmaPlot© version 11.0, from Systat Software, Inc., San Jose California USA. Unpaired *t*-tests were conducted to assess mean differences between the two enrichment treatments and a one-way analysis of variance (ANOVA) was conducted within treatments to assess mean differences across the duration of the study. Any significant differences yielded from the ANOVA test were then subjected to a Tukey pairwise multiple comparison post-hoc test.

3.2 Results

3.2.1 Enrichment effect on L-type *B. plicatilis*

3.2.1.1 Population growth and egg bearing

DHA-enriched *Chlorella* treatment was observed to have significantly higher population growth ($p = 0.03$) and egg ratio ($p < 0.01$) compared to the combined enrichment of DHA-enriched *C. vulgaris* and salmon roe emulsion oil enriched rotifers after 24 hour enrichment (Table 6). Population of DHA-enriched *C. vulgaris* cultures increased 51.52 ± 18.82 %, whereas the combined enrichment increased by 38.29 ± 9.93 %. Despite no significant differences detected, egg bearing was lower in the combined enrichment (4.15 ± 18.82 %) compared to the DHA-enriched *C. vulgaris* cultures (11.49 ± 6.36 %).

Table 6: Population growth, protein content and crude lipid content of enriched L-type *Brachionus plicatilis* species complex

Rotifer Species		L-Type *Brachionus plicatilis*	
Enrichment Treatment		DHA-enriched *C. vulgaris*	DHA-enriched C. vulgaris + Salmon roe emulsion oil
After 24 h enrichment			
Population growth ratio (%)	*	51.52 ± 18.82	38.29 ± 9.93
Egg bearing ratio (%)		10.49 ± 4.36	4.15 ± 1.18
Crude protein (mg/g dw)		673.3 ± 16.7	689.6 ± 7.5
Soluble protein (mg/g dw)	*	189.4 ± 2.3	90.0 ± 4.7
Soluble protein (% of crude protein)	*	27.8 ± 1.3	13.1 ± 0.6
Crude lipid (% dw)		16.1 ± 3.2	15.9 ± 3.0
Crude lipid (% ww)		3.4 ± 0.7	2.9 ± 0.7

Values indicate mean ± SD in that treatment. Asterisks indicate significant differences between enrichment treatments (Unpaired T-test, *: $p < 0.05$).

3.2.1.2 Protein Content

There were no significant differences observed in crude protein between the 2 treatments (Table 6). Significant differences (p<0.05) were observed in soluble protein content with DHA-enriched *C. vulgaris* having higher soluble protein content (189.4 ± 2.3 mg/g dw) compared to the combined treatment (90.0 ± 4.7 mg/g dw). DHA-enriched *C. vulgaris* also had significantly higher (p<0.05) soluble protein/crude protein ratio (27.8 ± 1.3 %) compared to the combined diet, which had 13.1 ± 0.6 % (Table 1).

3.2.1.3 Fatty acid analysis

Generally, fatty acid proportion (%) and content (mg/g dw) was observed to be higher in the non-polar lipid (NL) than in the polar lipid (PL). It was also evident that DHA-enriched *C. vulgaris* on its own had very little EPA proportion and content in NL and PL. Salmon roe emulsion had significantly higher levels of EPA and boosted the levels of proportion and content in rotifer under this treatment. In the NL region, significantly higher fatty acid proportions were found in four fatty acid species (16:1, 16:3, 18:1*n*-9, 20:4*n*-6) in ArA/EPA (0.91 ± 0.05 mg/g dw) and n-3/n-6 (2.51 ± 0.97 mg/g dw) ratios in DHA-enriched *C. vulgaris* treatment (Table 7). In the combined treatment, significantly higher fatty acid proportions were observed in 18:0, 18:4 *n*-3 and 20:5 *n*-3 with significantly high content in ArA (0.37 ± 0.04 mg/g dw), EPA (0.72 ± 0.07 mg/g dw), DHA (1.81 ± 0.18 mg/g dw) and Σ*n-6* (0.47 ± 0.02 mg/g dw).

In PL region, significantly higher proportion was found only in one fatty acid species (16:2) in DHA-enriched *C. vulgaris* (Table 7). In the same treatment, significantly higher fatty acid content was observed in Σmonoene (1.81 ± 0.18 mg/g dw), Σ*n*-3 (1.81 ± 0.18 mg/g dw), Σ*n*-6 (1.81 ± 0.18 mg/g dw) and DHA/EPA ratio (1.81 ± 0.18 mg/g dw). In the combined

treatment, significantly higher fatty acid proportions were observed in three fatty acid species (16:1, 18:0, 20:5 *n* – 3) with significantly high content in EPA (0.17 ± 0.01 mg/g dw).

3.2.1.4 Free Amino Acid (FAA)

The FAA composition was observed to be generally higher in the DHA-enriched *C. vulgaris* enriched rotifers (Table 8) with 5 essential FAA species (arginine, histidine, isoleucene, lysine, methionine) and 1 non-essential (glutamic acid) found to be significantly higher ($p < 0.05$) in this treatment. The mixed DHA-enriched *C. vulgaris* and salmon roe emulsion treatment was observed to have significantly higher ($p<0.05$) FAA composition in 1 FAA species (tyrosine).

Table 7: Fatty acid composition (%) and content (mg/g dw) in non-polar and polar lipids of rotifers enriched with DHA-enriched C. vulgaris and the combined treatment.

Enrichment	Non-Polar Lipid		Polar Lipid	
	DHA-enriched *C. vulgaris*	DHA-enriched *C.vulgaris* + Salmon roe emulsion oil	DHA-enriched C. v*ulgaris*	DHA-enriched *C.vulgaris* + Salmon roe emulsion oil
Fatty acid (%)				
14:0	0.35 ± 0.30	0.33 ± 0.08	1.55 ± 0.39	1.40 ± 0.84
16:0	19.99 ± 2.48	25.56 ± 2.44	22.08 ± 7.34	23.87 ± 5.89
16:1	1.12 ± 0.68 *	1.69 ± 0.06	2.38 ± 1.23	6.31 ± 4.70 *
16:2	8.70 ± 2.63	7.35 ± 2.30	6.96 ± 0.35 *	4.31 ± 0.68
16:3	5.23 ± 1.45 *	1.47 ± 0.24	1.93 ± 0.51	1.40 ± 0.27
18:0	4.65 ± 2.25	8.13 ± 4.10 *	3.47 ± 0.13	5.37 ± 0.80 *
18:1 n-9	4.32 ± 2.36 *	2.11 ± 0.04	2.09 ± 1.65	1.41 ± 1.37
18:2 n-6	28.30 ± 6.73	26.54 ± 7.08	28.04 ± 3.58	22.78 ± 6.46
18:3 n-3	4.77 ± 2.73	4.98 ± 1.53	5.83 ± 1.44	5.26 ± 2.42
18:4 n-3	0.26 ± 0.07	1.21 ± 0.20 *	0.42 ± 0.08	0.51 ± 0.06
20:4 n-6	2.76 ± 0.63 *	1.95 ± 0.37	1.28 ± 1.51	1.20 ± 0.54
20:4 n-3	1.10 ± 0.58	1.81 ± 0.74	5.85 ± 0.66	4.93 ± 1.60
20:5 n-3	0.34 ± 0.13	2.50 ± 0.72 *	0.97 ± 0.22	1.95 ± 0.26 *
22:0	0.41 ± 0.08	0.33 ± 0.21	0.79 ± 0.04	0.63 ± 0.31
22:5 n-6	0.26 ± 0.10	0.32 ± 0.03	0.86 ± 1.22	0.91 ± 0.15
22:6 n-3	5.41 ± 3.70	7.04 ± 1.81	3.99 ± 1.12	5.15 ± 0.35
Contents (mg g^{-1} dw)				
ArA	0.18 ± 0.09	0.37 ± 0.04 *	0.20 ± 0.13	0.25 ± 0.01
EPA	0.20 ± 0.01	0.72 ± 0.07 *	0.09 ± 0.01	0.17 ± 0.01 *
DHA	0.43 ± 0.27	1.81 ± 0.18 *	0.37 ± 0.12	0.44 ± 0.06
Σmonoene	1.80 ± 0.64	1.23 ± 0.16	3.38 ± 1.13 *	0.24 ± 0.06
Σn-3	0.49 ± 0.27	0.31 ± 0.16	1.28 ± 0.22 *	0.12 ± 0.03
Σn-6	0.19 ± 0.09	0.47 ± 0.02 *	0.28 ± 0.13 *	0.03 ± 0.01
DHA/EPA	2.21 ± 0.40	2.50 ± 0.08	4.31 ± 1.43 *	2.58 ± 0.89
ArA/EPA	0.91 ± 0.05 *	0.52 ± 0.04	2.31 ± 1.71	1.48 ± 0.19
n-3/n-6	2.51 ± 0.97 *	0.66 ± 0.08	4.58 ± 1.12	3.74 ± 1.67

Values indicate mean ± SD in that treatment. Asterisks indicate significant differences between enrichment treatments (Unpaired T-test, *: $p < 0.05$).

Table 8: Free Amino Acid (mg/100 g) composition of rotifers enriched with DHA-enriched *C. vulgaris* and the combined diet

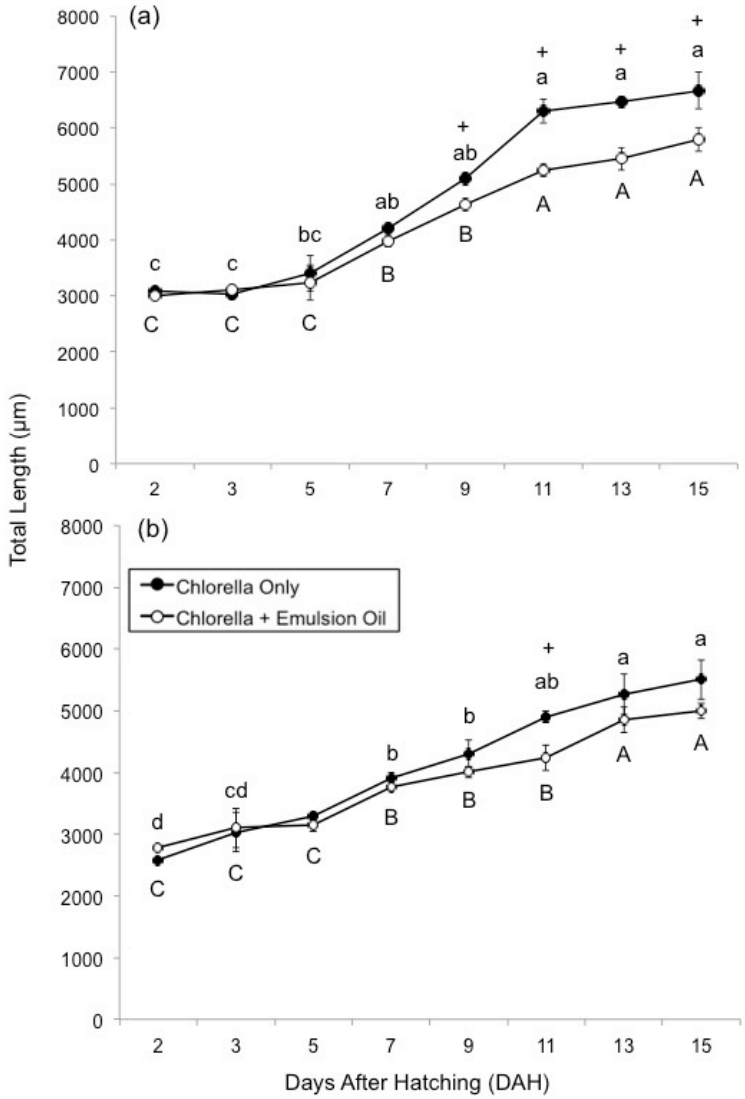
Enrichment	DHA-enriched *C. vulgaris* only	DHA-enriched *C. vulgaris* + Salmon Roe Emulsion Oil
FAA		
Arginine*	2.29 ± 0.28^{+}	1.99 ± 0.12
Histidine*	0.80 ± 0.16^{+}	0.58 ± 0.01
Isoleucine*	3.67 ± 3.82^{+}	0.65 ± 0.10
Leucine*	2.18 ± 1.71	0.82 ± 0.07
Lysine*	1.40 ± 0.24^{+}	1.25 ± 0.09
Methionine*	1.92 ± 0.32^{+}	0.82 ± 1.17
Phenylalanine*	2.10 ± 1.71	2.05 ± 1.34
Threonine*	3.06 ± 2.90	1.08 ± 0.07
Tryptophan*	0.40 ± 0.04	0.33 ± 0.06
Valine*	2.48 ± 1.76	0.93 ± 0.02
Tyrosine*	0.54 ± 0.52	0.93 ± 0.02^{+}
Taurine	0.43 ± 0.10	0.42 ± 0.03
Aspartic acid	0.62 ± 0.12	0.67 ± 0.03
Serine	1.93 ± 1.62	0.93 ± 0.04
Glutamic acid	4.49 ± 0.17^{+}	3.92 ± 0.25
Proline	0.78 ± 0.37	0.82 ± 0.04
Glycine	0.80 ± 0.24	0.85 ± 0.07
Alanine	2.18 ± 1.45	1.13 ± 0.09

Values indicate mean ± SD in that treatment. * Indicate essential Free Amino Acids; + indicate significant differences between enrichment treatments (Unpaired T-test: $p < 0.05$)

Overall, a high hatching rate was observed in both species (*P. major*: 96.0 ± 0.06 %; *P. olivaceus*: 97.5 ± 0.29 %). Considering the sampling rate of larvae of 80 larvae per tank over 8 sampling days (approximately 0.4 % of the total number of larvae initially stocked), the overall average survival in the DHA-enriched *C. vulgaris* treatment was significantly better ($p = 0.01$) in both species (*P. major*: 71.9 ± 3.5 %; *P. olivaceus*: 62.1 ± 5.69 %) compared to the combined diet of DHA-enriched *C. vulgaris* and salmon roe emulsion treatment which had low survival (*P. major*: 20.1 ± 10.1 %; *P. olivaceus*: 12.3 ± 3.87 %). DHA-enriched *C. vulgaris* treatment had significantly higher ($p < 0.05$) average total length at 9, 11, 13 and 15 DAH in

P. major (Fig. 5a) and at 11 DAH in *P. olivaceus* (Fig. 5b). There were no observed significant differences in total length in the first week of hatching in both species. .

Figure 5: Average total length (µm) in (a) *P. major* and (b) *P. olivaceus*

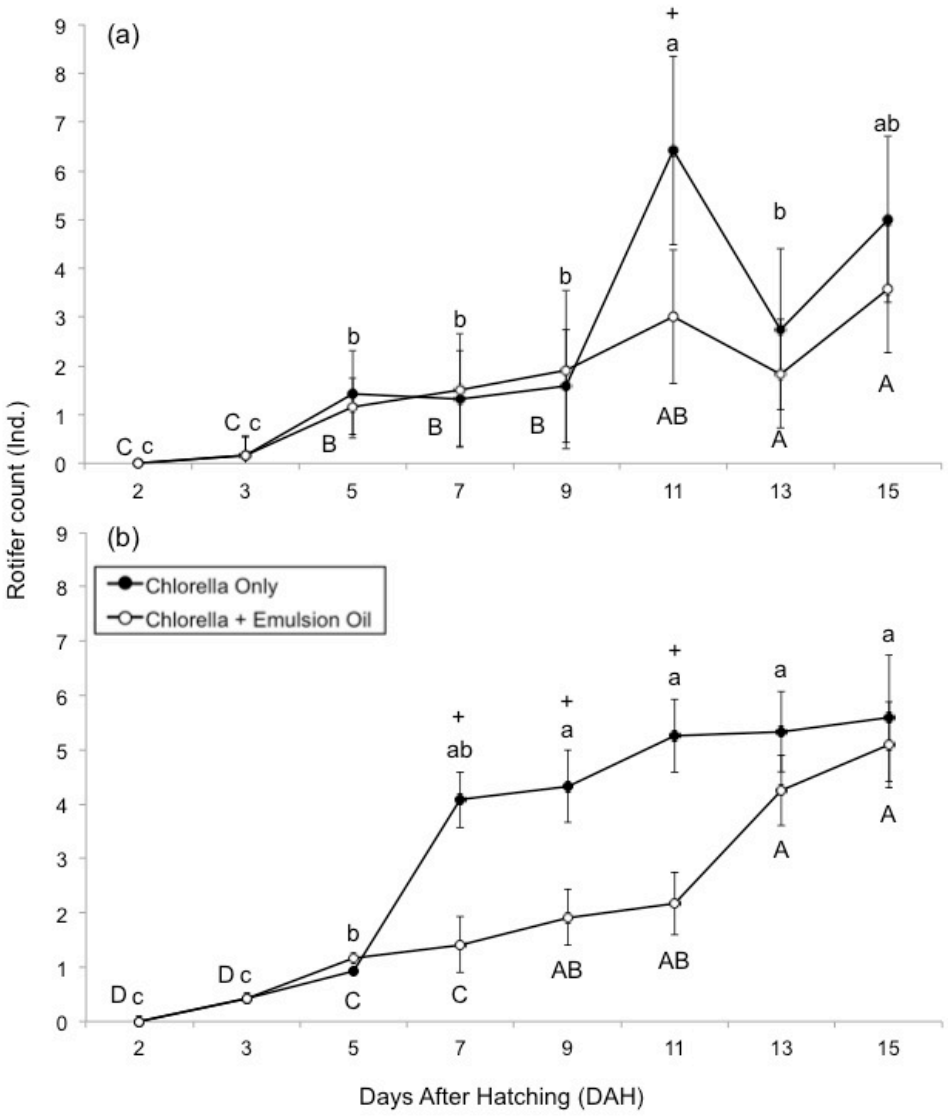


Values indicate mean ± SD in that treatment. Alphabetical script indicates the result of Tukey pairwise multiple comparison test among treatment (p<0.05, a>b>c>d); + Indicates significant difference between the two treatments (Unpaired t-test: p <0.05).

Significantly higher gut content (p < 0.05) in *P. major* fed DHA-enriched *C. vulgaris* were observed at 11 DAH (6.42 ± 1.94 ind.) (Fig. 6a). Similarly, significantly higher gut content (p < 0.05) were observed at 7 DAH (4.08 ± 1.16 ind.), 9 DAH (4.33 ± 1.92 ind.) and 11

DAH (5.25 ± 2.17 ind.) in *P. olivaceus* larvae fed DHA-enriched *C. vulgaris* (Fig. 6b). A general trend of increase in gut content is observed in both species especially between 9–15 DAH in *P. major* and 7–15 DAH in *P. olivaceus* an indication of the enhanced feeding capability.

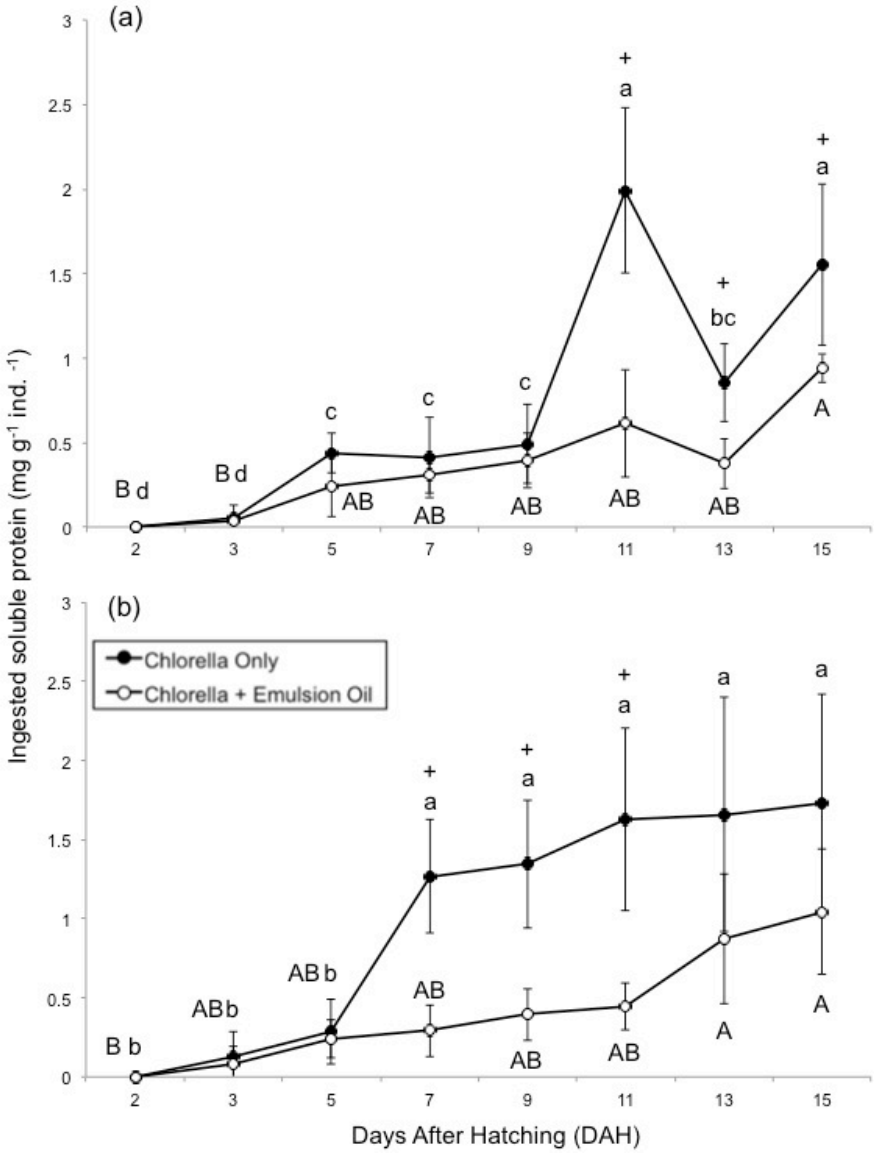
Figure 6: Gut content in (a) *P. major* and (b) *P. olivaceus*



Values indicate mean ± SD in that treatment. Alphabetical script indicates the result of Tukey pairwise multiple comparison test among treatment (p<0.05, a>b>c>d); + Indicates significant difference between the two treatments (Unpaired t-test: p <0.05).

Furthermore, significant differences ($p < 0.05$) were observed in the estimated soluble protein ingested by both fish species. Significantly higher estimated ingested soluble protein was observed at 11, 13 and 15 DAH in *P. major* (Fig. 7a) and at 7, 9, 11, 13 and 15 DAH in *P. olivaceus* (Fig. 7b).

Figure 7: Estimated ingested soluble protein in (a) *P. major* and (b) *P. olivaceus*



Values indicate mean ± SD in that treatment. Alphabetical script indicates the result of Tukey pairwise multiple comparison test among treatment ($p<0.05$, a>b>c>d); + Indicates significant difference between the two treatments (Unpaired *t*-test: $p <0.05$).

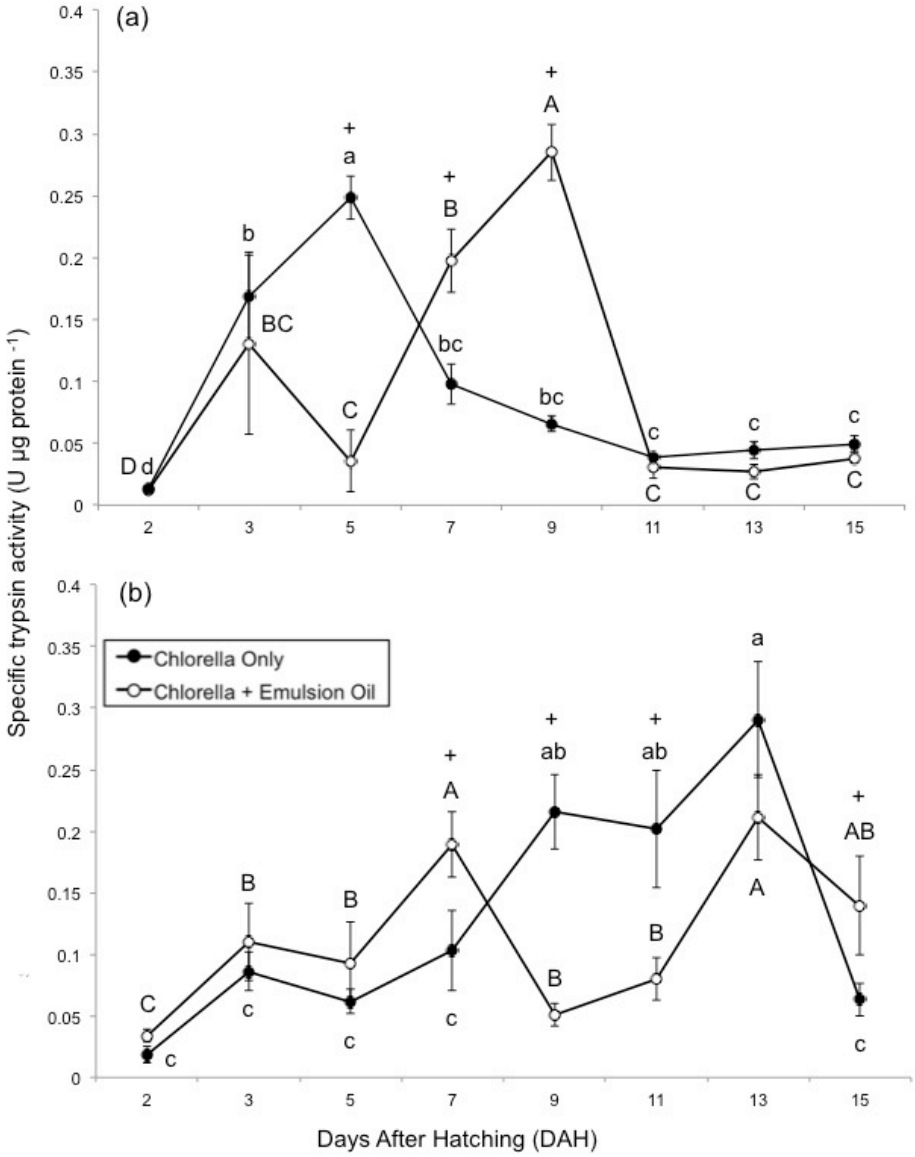
3.2.2.1 Trypsin response and enzyme fraction

The evolution of trypsin activity in *P. major* was marked by rapid increase peaking at 5 DAH (DHA-enriched *C.* vulgaris treatment) and 9 DAH (DHA-enriched *C. vulgaris* + salmon roe emulsion) followed by sharp decrease in enzyme activity profile until 15 DAH (Fig. 8a). Low levels of trypsin activity were detected before the onset of exogenous feeding at 2 DAH (DHA-enriched *C. vulgaris*: 0.01 ± 0.004 μg/protein; DHA-enriched *C. vulgaris* + salmon roe emulsion: 0.01 ± 0.01 μg/protein). Trypsin activity in both feed treatments increased dramatically at 3 DAH upon mouth opening.

Trypsin activity in DHA-enriched *C. vulgaris* fed larvae peaked earlier at 5 DAH (0.25 ± 0.02 μg/protein) and decreased from 7-15 DAH. On the other hand, trypsin activity in the combined enrichment treatment increased at 3 DAH (0.13 ± 0.07 μg/protein), dropped at 5 DAH (0.04 ± 0.02 μg protein^{-1}) before peaking at 9 DAH (0.28 ± 0.02 μg protein^{-1}) and reducing significantly at 11-15 DAH, similar to DHA-enriched *C. vulgaris* treatment. Comparing between treatments, significantly higher trypsin activity (p <0.05) was observed in DHA-enriched *C. vulgaris* at 5 DAH whereas it was significantly higher at 7 DAH and 9 DAH in the combined treatment.

The development of trypsin activity in *P. olivaceus* was marked by high fluctuations in enzyme activity profile (Fig. 8b). Low levels of trypsin activity were detected before the onset of exogenous feeding at 2 DAH (DHA-enriched *C.* vulgaris: 0.03 ± 0.01 μg protein^{-1}; DHA-enriched *C. vulgaris* + salmon roe emulsion: 0.02 ± 0.01 μg/protein). Three peaks of activity were detected in both feed treatments (Fig. 8b). In the DHA-enriched *C. vulgaris*, trypsin activity peaked at 3 DAH (0.09 ± 0.02 μg protein^{-1}), 9 DAH (0.2 ± 0.03 μg/protein) and 13 DAH (0.29 ± 0.05 μg/protein). In the combined enrichment treatment, trypsin activity peaked at 3 DAH (0.11 ± 0.03 μg/protein), 7 DAH (0.19 ± 0.03 μg/protein) and 13 DAH (0.21 ± 0.03 μg/protein).

Figure 8: Trypsin activity detected in (a) *P. major* and (b) *P. olivaceus*

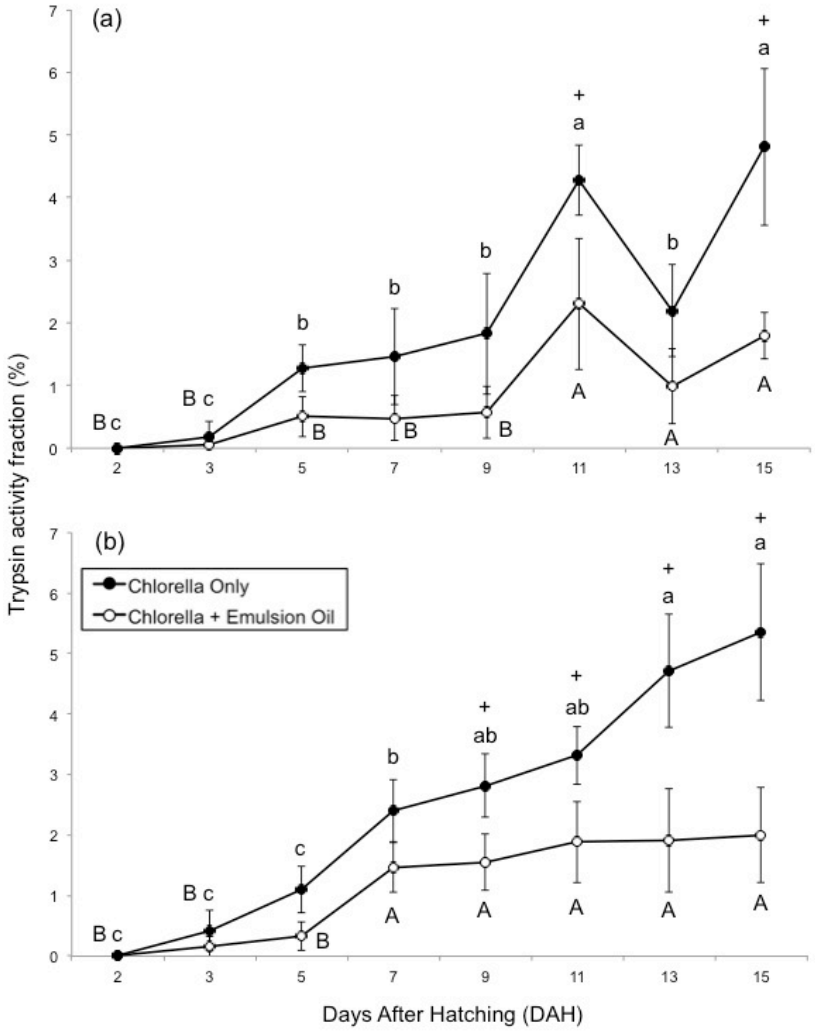


Specific enzyme activity of trypsin (U µg/protein) of (a) *Pagrus major* and (b) *Paralichthys olivaceus*. (mean ± SD); alphabetical script indicates the result of Tukey pairwise multiple comparison test among treatment ($p<0.05$, a>b>c>d); + Indicates significant difference between the two treatments (Unpaired *t*-test: $p<0.05$).

Both treatments experienced significant drop in tryptic activity at 15 DAH. Comparing between treatments, significantly higher trypsin activity ($p<0.05$) was observed in DHA-enriched *C. vulgaris* at 9, 11 and 13 DAH whereas it was significantly higher at 7 DAH and 15 DAH in salmon roe emulsion fed treatment.

The average fraction of trypsin activity potentially attributable to ingested prey was observed to be higher in DHA-enriched *C. vulgaris* in both species of fish. In *P. major*, trypsin fraction was significantly higher (p <0.05) at 11 DAH (4.28 ± 0.56 %) and 15 DAH (4.81 ± 1.25) (Fig. 9a) whereas in *P. olivaceus* it was significantly higher (p <0.05) from 9 DAH (2.81 ± 0.52 %), 11 DAH (3.31 ± 0.47 %), 13 DAH (4.71 ± 0.93 %) and 15 DAH (5.35 ± 1.13 %) (Fig. 9b). It is observed that the average fraction of trypsin activity potentially attributable to ingested prey is relatively small (less than 6 %) in both fish species and both feed treatments.

Figure 9: Average fraction of trypsin activity attributable to ingested prey



Values indicate mean ± SD in that treatment. Alphabetical script indicates the result of Tukey pairwise multiple comparison test among treatment (p<0.05, a>b>c>d); + Indicates significant difference between the two treatments (Unpaired t-test: p <0.05).

3.3 Discussion

Rotifers from the genus *Brachionus* are often used as the first feed for most marine fish larvae due to its many favorable characteristics, especially the ability to artificially manipulate its nutritional qualities (Hagiwara et al., 2001; Kobayashi et al., 2008; Kotani et al., 2009). Studies have highlighted that fatty acid composition of rotifers is an effective reflection of its enrichment (Sakamoto et al. 1982; Frolov et al. 1991; Kotani et al., 2013), however, depending on the conditions of culture and types of enrichment, several nutrients may be present at potentially deficient levels (Hamre, 2016). In this study, the effect of DHA reinforcement provided via the addition of DHA-enriched *C. vulgaris* was reflected in high DHA composition in both treatments (Table 7). The combined feed was found to have higher HUFA content, especially in the essential fatty acid (EFA) EPA (20:5*n*-3) and DHA (22:6*n*-6). The absence of EPA in *C. vulgaris* has been previously documented (Maruyama et al. 2006; Thépot et al. 2016) and therefore addition of oil enrichment in the combined treatment fortified EPA in the rotifer. ArA (20:4*n*-6), which is also an EFA for larviculture, was found to be higher in DHA-enriched *C. vulgaris*, with significantly higher ($p < 0.05$) proportions in the NL region (2.76 ± 0.63 %). Despite this, ArA content (mg/g dw) was significantly higher in the NL in the combined diet (0.37 ± 0.63 mg/g dw). ArA levels are important for stress tolerance, pigmentation, growth, and survival and is vital in the formation of eicosanoids in early stage marine fish larvae (Park *et al*., 2006).

Watanabe (1993) suggested that DHA was superior to EPA as an EFA for marine finfish larvae. However a deficiency in both these two EFA's will result in reduced growth as a result of poor dietary value (Tomoda et al., 2004). A study by Watanabe at al. (1989) on *P. major* larvae fed rotifers enriched with EPA but deficient in DHA (0 %) and rotifers enriched with sufficient DHA amounts observed lower growth but similar survival rates. Similar results

were also observed in a study by Izquierdo et al. (1997) where there was an improvement of growth, survival and activity of *P. major* larvae fed rotifers including 0.4% of total *n*-3 HUFA of whole body dry basis. Kotani et al. (2013) highlighted that a level of 18 % DHA has a negative impact on *P. major* larvae and that levels in rotifers should be controlled at around 6 or 13 %. In the present study, both treatments had levels close to 6 % in both the NL and the PL (Table 2) thus suggesting the suitability of DHA levels in both feed treatments. Though it was slightly lower in DHA-enriched *C. vulgaris* treatment, there were no significant differences detected in DHA levels between the two treatments. Improved levels of ArA, is known to improve culture performance and Kotani et al. (2013) alluded to that direct addition of ArA into rotifer enrichment can improve *P. major* larviculture performance. In this study, ArA levels were significantly higher in the NL of the DHA-enriched *C. vulgaris* treatment however there were no significant differences observed in the PL. Further studies are required to assess the importance of ArA to the larviculture performance of *P. major* and the associated responses when fed with rotifers enriched under this regiment.

Kim et al. (2002) highlighted that dietary *n*-3 HUFA is essential for normal growth, as the dietary lipid sources have significant effects on growth performance, liver cell property and blood chemistry in *P. olivaceus*. Izquierdo et al. (1992) highlighted that the *n*-3 HUFA requirement was approximately 3.0-3.5 % in *P. olivaceus*. Furthermore, Furuita et al. (1999) reported that the DHA requirement was around 1.6 % and 1.0% for EPA, which is also supported in studies conducted by Estévez et al. (1997) and Kim and Lee (2004) at 0.8-1.0 %. In the present study DHA levels were around 6 % whereas EPA levels (1-2 %) were found to be significantly higher in the combined treatment (Table 7) due to the non-existence of EPA in the *Chlorella* only treatment. The EFA levels observed in the current study were quite high compared to prerequisite levels that have been highlighted in previous studies and which Kim and Lee (2004) stated that any excess of this amount could result in impaired growth. However,

any impairment in growth between the two treatments was not observed as there were no significant differences in TL measurements (Figure 5). ArA and *n*-6 fatty acids have generally received less attention despite it being known to promote better growth (Cowey et al., 1976). ArA is known to be required in small, specific and levels have yet to be quantified for flatfish nutrition (Estévez et al., 1997). In the current study, ArA levels were observed to be between 1-3 % in both treatments with significantly higher levels observed in the NL of the DHA-enriched *C. vulgaris* treatment. Estévez et al. (1997) conclude their study on the effects of ArA on the pigmentation in Japanese flounder by stating that correct dosage of ArA should be studied more in detail as an imbalance or an overdose in ArA may cause undesirable pathophysiological diseases as has already been observed in cultured salmon.

Generally, fatty acid compositions varied between the NL and PL in both treatments and this is possibly due to selective assimilation (Frolov et al. 1991) and perhaps conversion (Le Milinaire et al. 1983; Lubzens et al. 1985; Robin, 1995) of HUFA in accordance with rotifer metabolic requirements as reported by Kotani et al. (2013). A lack of dietary EFA's is known to result in hindered physiological development and altered behavior (Rainuzzo et al., 1997; Sargent et al., 1997). Upon hatching, the yolk of many wild marine fish contain a DHA/EPA ratio of about 2.0 (Parrish et al., 1994; Sargent et al., 1997) and previous studies have suggested that a DHA/EPA ratio of around 2 is optimal, though it is species specific (Lazo et al., 2000; Wu et al., 2001; Bell and Sargent, 2003; Kotani et al., 2013). In this study both enrichment treatments had DHA/EPA ratios higher than 2 in both NL and PL with ArA/EPA ratio's higher than 1 in the PL (Table 7) thus highlighting the suitability of both treatments for larviculture, especially the combined treatment as a suitable enrichment regiment due to its high EFA composition. However, it was also observed that the combined treatment had slightly lower rotifer population growth (13 % lower) and egg bearing (7 % lower) compared to the DHA-enriched *C. vulgaris* only treatment (Table 6).

A high composition of free amino acids (FAA) are also 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., 1999; Kvåle et al., 2007). FAA content was observed to be generally higher in the DHA-enriched *C. vulgaris* only treatment, with significant differences found in 5 essential FAA species, compared to the combined treatment (Table 8). Essential free amino acids are classified as those that cannot be naturally synthesized to an adequate level in fish (Mambrini and Guillaume, 1999) and thus enrichment is an important consideration for fortifying FAA acids in live feed. Amino acids have several important functions; these include protein synthesis and growth, osmoregulation, endocrine/metabolic response, cell signaling, antioxidative defense and ammonia removal (Bouckenooghe et al., 2006). Our results highlight that despite the high HUFA content of the combined feed, it had lower FAA content compared to the DHA-enriched *C. vulgaris* only treatment.

Variability in soluble protein content (twice as high in DHA-enriched *C. vulgaris* (Table 7) and varying trypsin activity between treatments supports the idea that there is a link between the amount of soluble protein present in the feed and the digestive response in early stage larvae. It has been suggested that soluble proteins are more available for larval digestion and absorption compared to insoluble proteins (Carvalho et al., 2004). Variance in enzyme activity between treatment as observed in this study may be due to a lower capacity for enzyme activation as a result of the rotifer enrichment (Dabrowski 1984), under the assumption that the absorption capacity of the larvae does not change. A study by Tonheim et al. (2005) using pre-hydrolyzed proteins to feed Atlantic halibut larvae supports this hypothesis, their results revealed that the limiting factor was low proteolytic activity and not absorption capacity. Tryptic activity in *P. major* peaked at 5 DAH in DHA-enriched *C. vulgaris* treatment whereas it peaked later in the combined treatment at 9 DAH in (Fig. 8a). The opposite trend was observed in *P. olivaceus*, where the trypsin activity in the combined diet peaked earlier at 7

DAH and at 13 DAH in the DHA-*C. vulgaris* treatment (Fig. 8b). According to these results, *P. major* larvae were able to digest the live feed earlier than *P. olivaceus* in both treatments, perhaps due to differing intestinal development rates and/or ability to digest the proteins in the feed among the two fish species. Srivastava (2002) highlighted that the ability for cleavage of protein by pancreatic extract in early larval stomachs depends on protein species available in the feed which favor rapid cleaving (e.g. albumin thyroglobulin, and lactate dehydrogenase). The ability of larvae to digest different dietary proteins at different larval developmental stages highlights that there is a possibility that some protein species are not digested due to the lack of a functional stomach as proven by Srivastava (2002). Cahu et al. (2004) also highlighted that early larval pancreatic enzyme secretion is linked to dietary protein amount and that different levels of dietary proteins moderated enzymatic expression, which in turn affected chlolecystokinin (CCK) content, the peptide hormone responsible for stimulating the digestion of fat and protein. CCK producing cells have been detected in early stage larvae such as 1 DAH Japanese flounder (Kurokawa et al., 2000), 5 DAH seabass (Garcia-Hernandez et al., 1994) and 11 DAH turbot larvae (Reinecke et al., 1997).

Trypsin activity was observed in both larval species before mouth opening (2 DAH) and was observed to increase significantly at 3 DAH (Fig. 8). This sudden influx in activity at 3 DAH could be related to the availability of prey in the gut of the larvae at the first moment of feeding providing a substrate for enzymatic activation (Cara et al., 2003; Melianawati et al., 2015). However in *P. major*, despite an increasing gut content count (Fig. 6a) and estimated ingested soluble protein (Fig. 7a) at 5 DAH, a significant drop in trypsin activity was observed in the combined treatment compared to the DHA-enriched *C. vulgaris* only treatment. This effect is perhaps due to the larvae consuming the feed but not digesting it as reflected in the tryptic activity suggesting differential acceptance of the feed at this developmental stage (Fig. 4a). Tryptic activity was observed to peak at 5 DAH (DHA-enriched *C. vulgaris* treatment) and

9 DAH (combined treatment) before reducing significantly until 15 DAH. This marked decline in activity could be due to the activation of other proteolytic enzymes present in early stage larvae such as chymotrypsin and aminopeptidase (Kurokawa and Suzuki, 1996, 1998). Previous studies have suggested that in fish larvae, presence of trypsin could activate chymotrypsin (Sunde, 2001; Rungruangsak-Torrissen et al., 2006). Pepsin would have been activated later as it requires acidic environments to be activated (Cara et al., 2003; Rønnestad et al., 2013). Trypsin activity increased gradually until 13 DAH in *P. olivaceus* before experiencing a significant drop in activity at 15 DAH (Fig. 8b). Similar results were observed in studies conducted by Bolasina et al. (2006) with significant decrease of trypsin activity between 15–23 DAH of *P. olivaceus* larvae. They attributed the low activity to metamorphosis; *P. olivaceus* like most other flatfish undergo drastic morphological changes during this period and it is commonly associated with minimal feeding and low trypsin activity (Bolasina et al., 2006).

Considering the effect of exogenous trypsin activity from live feed under the assumption that they are in the gut, the estimated trypsin activity fraction was found to be less than 7 % of the total trypsin activity in both larval species (Fig. 9). The influence of exogenous enzymes was most likely limited to an autolytic process in the prey and which is perhaps neutralized by the alkaline contents of the larval gut or that they may also play a possible role in enzyme activation (Dabrowski 1984; Diaz et al., 1997).

DHA-enriched *C. vulgaris* treatment had significantly higher average total length at 9, 11, 13 and 15 DAH in *P. major* (Fig. 5a) and at 11 DAH in *P. olivaceus* (Fig. 5b). Using the significant differences observed in Fig. 5, it can be observed that there are three distinct growth patterns observed in both species and in both treatments. From 2–5 DAH there is minimal growth, followed by a growth spurt from 7–11 DAH and peaking at 13–15 DAH (Fig. 5).

These results also highlighted that phases of exponential growth (7–11 DAH) were critical periods of growth and feed utilization as reflected in gut content. The combined diet had low survival (*P. major*: 20.1 ± 10.1 %; *P. olivaceus*: 12.3 ± 3.87 %) with majority of the mortality observed at 9 DAH, which would suggest water quality control and adjustment of dosage during this period. In addition, the possibility of lower survival and slower development could also be related to oxidation of lipids leading to the deterioration of dietary lipid quality. As our results highlight, the combined diet has a high composition of HUFA, which places it at a higher risk of oxidation due to the increased number of oxidation prone double bonds existent in such lipids rich in unsaturated fatty acids (Rainuzzo et al., 1994). Dhert et al. (2001) highlighted in his review that rotifers enriched with oil emulsions often produce lower quality rotifers with too high lipid content, short retention time of nutrients and transfer of oil to larval rearing tanks reducing water quality and problems associated with viability of larvae. Therefore dosage of such enrichments needs to be regulated according to rotifer culture conditions and high water exchange in the larval rearing tanks during this period of high growth. Generally manufacturers of enrichment diets have determined the appropriate methods and dosages for rotifers. However, there is little information on the effects on rotifers of nutritional enrichment using methods not previously described by manufacturers (Kotani et al., 2017). Therefore adjustments to dosage should be done to suit each condition. Further studies are required on dosage optimization of oil-based enrichments and its impacts on nutritional status of rotifers and consequential effects in first feeding larvae.

In conclusion, both rotifer enrichment treatments were found to be nutritionally effective. However, HUFA content was better in the combined treatment due to EPA fortification whereas it was in deficient levels in the DHA-enriched *C. vulgaris* only treatment. Despite this, the DHA-enriched *C. vulgaris* only treatment was found to have higher population growth, egg bearing capacity, soluble protein and free amino acid content. To avoid the

observed negative effects of oil contamination in larval rearing water, a suggested alternative would be to reinforce rotifers with EPA using a combined microalgal enrichment of *Nannochloropsis oculata* rather than an oil-based enrichment. During larviculture, both *P. major* and *P. olivaceus* suffered high mortality under the combined diet. In contrast, generally higher survival, gut content, ingested soluble protein and trypsin activity fraction was observed in the DHA-enriched *C. vulgaris* only treatment. Periods of significant growth in both *P. major* and *P. olivaceus* was observed between 7 DAH and 11 DAH which was also reflected in the gut content. This could possibly allude to important ontogenetic stages of growth in both larval species. Furthermore, we would also like to highlight that despite high HUFA content, consideration of free amino acids and soluble protein content as parameters of nutritional indicators for first feeding *P. major* and *P. olivaceus* should not be discounted.

CHAPTER 4: Proteolytic digestive enzyme response in Japanese flounder larvae *Paralichthys olivaceus* fed two types of rotifers *Brachionus plicatilis* species complex

Abstract

DHA-enriched *Chlorella vulgaris* has been highlighted in the results of the two previous chapters to be the best enrichment media used in this study due to its nutritional qualities and larval response during initial feeding. Therefore the general objective of this chapter is to assess whether there are any differences in larval responses when fed two morphotypes of the *Brachionus plicatilis* species complex, the SS-type *B. rotundiformis* and L-type *B. plicatilis* enriched with DHA-enriched *Chlorella vulgaris*. To determine the nutritional capacity of digestion in larvae, differences in protein hydrolysis and digestive trypsin activity in first feeding Japanese flounder was assessed. There were no significant differences in hydrolysis activity at 2, 3 and 7 days after hatching (DAH). At 5 DAH, hydrolysis activity was significantly higher in larvae fed SS-type ($p < 0.05$) at 50 kDa in 1.5 and 3 hours incubation whereas L-type treatment had not completely hydrolyzed the proteins after 3 hours at the same molecular weight. This variation could be linked to the differing digestive capabilities throughout early ontogeny of fish larvae to digest different protein species available via the live feed. Larvae fed SS-type had significantly higher ($p < 0.05$) trypsin activity at 3, 5, 6, 7 DAH. Contribution of live prey to trypsin fraction in larvae showed significantly higher ($p < 0.05$) fraction for SS-type at 5 DAH (2.18 ± 0.44 %) and 6 DAH (2.04 ± 0.29 %) and the effect of exogenous trypsin from live prey was relatively low when compared to the total trypsin activity in larvae. This study discusses the differences in ability to digest proteins in Japanese flounder when fed different rotifer morphotypes and highlights the adaptability of this species to alternative rotifer morphotypes during its early developmental stages.

4.0 Introduction

Rotifers from the genera *Brachionus* are often the preferred first-feed during marine finfish larviculture due to its many favorable characteristics (Hagiwara *et al*., 2001; Tomoda *et al*., 2004; Park *et al*., 2006; Kotani *et al*., 2009). The *Brachionus* genus includes several morphotypes and there are a variety of classification methods via morphology, allozyme pattern, and karyotypes (Hagiwara *et al*., 2007). Mills *et al*., (2016) reported that the *B. plicatlis* species complex could be divided into 15 species and that populations could include more varieties than previously suggested. Segers (1995) classified L-type as *B. plicatilis* and others as *B. rotundiformis.* Furthermore, a study by Kotani *et al*. (1997) further demonstrated that there is a strong species boundary observed between L-type *B. plicatilis* and SS-type *B. rotundiformis*. Morphologically, Fu *et al.* (1991) described the L-type as comparatively larger with obtuse-angled anterior spines whereas the smaller S-type had a more round lorica with pointed anterior spines. Based on lorica size, culturists classify L-type as *B. plicatilis* (large; 130-340 μm) and the SS-type as *B. rotundiformis* (super small; 90-110 μm) (Hagiwara *et al*., 2001, 2014; Kotani *et al*., 2005). Consideration of rotifer size is important in seedling production, as selectivity of prey is dependent on sizes of live feed (Tanaka *et al*., 2005; Akazawa *et al*., 2008; Wullur *et al*., 2009). The moment of first exogenous feeding is an important period to be considered if starvation and subsequent mortality is to be avoided. Periods of food deprivation after yolk reabsorption is known to result in behavioral and morphological development abnormality, deterioration of alimentary tract and trunk musculature coupled with reduction in decreased food utilization efficiency and feeding activity (Heming *et al*., 1982; Taylor and Freeberg, 1984; Rice *et al*., 1987; Gisbert *et al*., 2004). At the moment of first feeding (~3 days after hatching (DAH)), the primitive digestive canal of flounder larvae normally comprises of three sections, the esophagus, intestine and rectum (Kurokawa & Suzuki, 1996). Studies by Kurokawa & Suzuki (1996, 1998) indicate that

there is active secretion of trypsinogen and aminopeptidase from the pancreas and intestine at the moment of first feeding and that the digestion of nutrients at this stage is largely dependent on pancreatic enzymes. Live feeds are usually used as primary feed due to its small size, high reproduction rate, planktonic nature, suitability for mass culture, and ability for artificial manipulation of its nutritional qualities (Yoshimura *et al.*, 1997; Dhert *et al*., 2001; Kotani *et al*., 2009). The ability for the larval body cells to absorb nutrients from live feed can be measured using digestive proteolytic enzymatic activity as a biological indicator of ability to digest proteins (Ueberschar, 1988).

The Japanese flounder *Paralichthys olivaceus* is an important commercial species for coastal fisheries and for the aquaculture industry in Japan and Korea (Ikewaki and Tanaka, 1993; Yamashita and Yamada, 1999; Howell and Yamashita, 2005). *P. olivaceus* larval development is one that includes significant behavioral, anatomical and physiological transformation (Fukuhara, 1986). According to Bolasina *et al.* (2006), due to such dramatic metamorphosis, flatfish experience greater effect on digestive enzymatic activities during this change. Hence, the purpose of this study is to determine if there are any significant variations in protein hydrolysis and digestive trypsin activity during the first feeding period (0-7 DAH) of Japanese flounder when fed two different morphotypes of rotifer, the L-type and SS-type. The assessment of nutritional status under two commonly utilized live feeds could contribute to the determination of optimal feed types and development of recommendations on live feeds which have positive effects on digestive enzyme activity of Japanese Flounder at the important stages of first exogenous feeding.

4.1 Materials and methods

4.1.1 Source of eggs and larval rearing

Japanese flounder eggs were obtained from natural spawning broodstock from the Hayato

Aquaculture Farm, the Local Production Headquarters, MBC Kaihatsu Co. Ltd., in Kirishima, 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. As the focus of this study was to observe enzymatic activity at first feeding, the larval rearing duration was until 7 days after hatching (DAH). Larval rearing was conducted in clear 30 L polycarbonate tank with aeration system provided and each tank was stocked with 600 eggs. *Nannochloropsis oculata* (Chlorella Industry Co. Ltd, Tokyo, Japan) was directly introduced into all larval rearing tanks at 2 DAH as a stabilizer along with the rotifers once the larval mouth is observed under the microscope. Frozen *N. oculata* was supplied at 0.03g L^{-1} of culture volume (3.63 x 10^{10} cells g^{-1}). Rotifer density in the rearing tanks was adjusted twice daily during feeding to ensure that a density of 10 rotifers mL^{-1} was maintained.

4.1.2 Live feed

L type rotifer Obama strain stock population was obtained from the National Research Institute of Aquaculture, Fisheries Research Agency, Nansei, Mie, Japan. SS type rotifer Indonesian strain stock population were obtained from Nagasaki University, Nagasaki Prefecture, Japan. Both rotifer species were stock cultured in continuous culture systems following protocols outlined by Kotani *et al.* (2009). These cultures were automatically fed concentrated freshwater *Chlorella vulgaris* (Chlorella Industry Co. Ltd., Tokyo, Japan) and were cultured at a salinity of 20 ± 1 psu. Rotifers harvested from the stock continuous culture were then transferred to 5 L polycarbonate tanks where they were placed in a heated water bath (27 ± 1 °C). Aeration was provided by ceramic air-stones suspended at approximately 5 cm from the center of the tank bottom. Both species were enriched with concentrated DHA-enriched *C. vulgaris* (Chlorella Industry Co. Ltd). The 5 L enrichment cultures were enriched

twice (0800, 1700) and were harvested 24 hours after inoculation of rotifers. The DHA-enriched *C. vulgaris* was fed at 46,000 cells rotifer^{-1} according to stock density at inoculation into the culture tanks. The volume of concentrated *Chlorella* to be fed was adjusted using the formula:

$$\text{Volume of Chlorella (ml)} = \left(\frac{\text{stock density x 0.46 x culture volume}}{150}\right)$$

Finally, taurine enrichment diet (Aqua-Plus ET, Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan) was also added at 0.06 g L^{-1} of culture volume as per manufacturer instruction, however, it was only added once, 15 hours prior to feeding of larvae, during the second enrichment. Rotifer culture water quality (salinity, dissolved oxygen, temperature, pH) and stock density was measured daily for the duration of the experiment for ascertaining the appropriate harvest volume.

4.1.3 Study design

This experiment was conducted using 2 treatments ((i) L-type and (ii) SS-type) with each treatment having 6 replications and all culture conditions maintained uniformly.

4.1.4 Gut content and rotifer size

A total of 100 larvae were sampled from each treatment at 2, 3, 5, 7 DAH and dissected. Constituents of its guts counted using a SMZ800 Nikon stereomicroscope. The two-rotifer types were observed using the Keyence VHX- S90F free-angle observation system with the Keyence VH-Z20R digital microscope (Keyence Corporation, Osaka, Japan) to measure the lorica length and width of 100 individuals from each rotifer type. This was undertaken so that a better understanding of dimensions of L-type and SS-type could be gained and as such, also if there were any significant differences in enzymatic activity. Rotifer samples were immobilized

using Lugol's solution (I_3K). Measurements of lorica length (i.e.) from lorica crown spines (base of corona) extending to the lower extremity of the lorica and lorica width (cross laterally the widest points of the lorica) were undertaken following Fu *et* al. (1991).

Enzyme activities of rotifer were also measured using the homogenate of approximately 1500 ± 100 individual rotifers from L-type and SS-type in microcentrifuge tubes. To estimate the fraction of enzyme activity that was contributed by ingested prey, we followed the method of Bolasina *et al*. (2006) by multiplying the average number of prey consumed with the enzyme activity of the prey.

4.1.5 Estimation of protein digestion

The capability for the hydrolysis of proteins was estimated using Sodium Dodecyl Sulfate-Polyacrylamide (SDS-PAGE) gel electrophoresis as outlined by (Melianawati *et al*., 2015) with slight modification. Larvae were sampled at 2, 3, 5, 7 DAH from each treatment. SS and L type rotifers were also sampled after 24-hour enrichment period. Larval and rotifer samples were rinsed in distilled water, pipetted into 1.5 mL sampling tubes and stored at -80 °C immediately after sampling. Rotifer samples were lyophilized for 24 hours and samples were stored at -80 °C. Whole body of the larvae and 0.01 g of rotifer samples were extracted in Phosphate Buffer Saline (pH 7.4) homogenized using a 1.5 ml microtube and pestel biomasher and centrifuged at 5000 g for 5 minutes at 4 °C. The supernatants were collected and incubated in the substrate for 0 minutes, 15 minutes, 1.5 hours and 3 hours. Protein standards (Precision plus protein ^{TM} dual color standard, Biorad #161-0374) were used both as the marker and the substrate. After incubation, sample buffer was added in a ratio of 1:1 to the sample volume and then heated in a block heater at 95 °C for 5 minutes. Electrophoresis was conducted using 10 % acrylamide running gel (0.375 M of Tris-HCl, pH 8.8). Gels were placed into the electrode buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3). After electrophoresis, the gels

were fixed in (Methanol: Acetic: Distilled water, 4:1:5, v/v/v) for 10 minutes before it was stained in Coomasie Brilliant Blue solution. Each sample was replicated 3 times under the same conditions.

Densitometric analysis of detected bands was carried out using Biorad ChemiDoc gel imaging system and ImageJ 1.50i (Mac OS X version of NIH Image, http://imagej.nih.gov.ij). In this study we focused on two bands throughout the incubation period, 50 kDa and 37 kDa as these were the two bands of the molecular marker with clear degradation patterns. Relative banding density was compared for each sample incubation time (15 minutes, 1.5 hours, and 3 hours) against the control of 0 minutes incubation.

4.1.6 Specific trypsin enzyme activity

The fluorometric assay of trypsin enzyme activity was conducted following methods similar to Bolasina *et al*. (2006) using Z-L-arginine-7-amido-4-methylcoumarin hydrochloride (CBZ-LArg-MCA) as the substrate. The frozen larvae were homogenized in a biomasher with ice cold 200 µl of homogenization buffer (20 mM tris-HCl, 1mM EDTA, 10 mM $CaCl_2$, pH 7.5) and the supernatant extracted after centrifugation (at 4 °C, 1700 g for 10 minutes). Any leftover supernatant was stored at -80 °C for later use in the assessment of soluble protein content of the homogenate. 20 µl of larval supernatant homogenate was added to 500 µl of the reaction buffer (50 mM tris-HCl (pH 8.0), 10 mM $CaCl_2$, 0.2 mM CBZ-LArg-MCA). The mixtures were incubated into a water bath at 30 °C for 30 minutes and then 100 µl of 30 % acetic acid was added for quenching. Experimental blanks were prepared similarly without the addition of acetic acid prior to mixing the substrate. Fluorescence was measured using a spectrofluorophotometer and the difference in emissions at 440 nm (excitation 380 nm) was measured between the samples and the blanks. This was done similarly to rotifer samples and we ran the assay on 0.01 g DW (dry weight) of rotifer samples. Activity was expressed in

trypsin units (U), as increase of emission per protein (U μg protein^{-1}) and trypsin units as of emission per larvae (U ind^{-1}).

4.1.7 Soluble protein content assay

To determine the amount of protein present in the homogenates of both the larvae and rotifer samples, we extracted 20 μl from the same stock homogenate that was used for the trypsin assay. The Bradford method of binding Coomasie Brilliant Blue G-250 dye to proteins as outlined by Bradford (1976) was followed. Bovine serum albumin (BSA) was used as the standard and samples were analyzed using a Hitachi ratio beam spectrophotometer U-5100. Standard curves were then compared to sample data to ascertain protein content in each rotifer sample.

4.1.8 Statistical analysis

Statistical analysis was undertaken in SigmaPlot© version 11.0, from Systat Software, Inc., San Jose California USA. Unpaired t-tests were conducted to assess mean differences between the two feed types.

4.2 Results

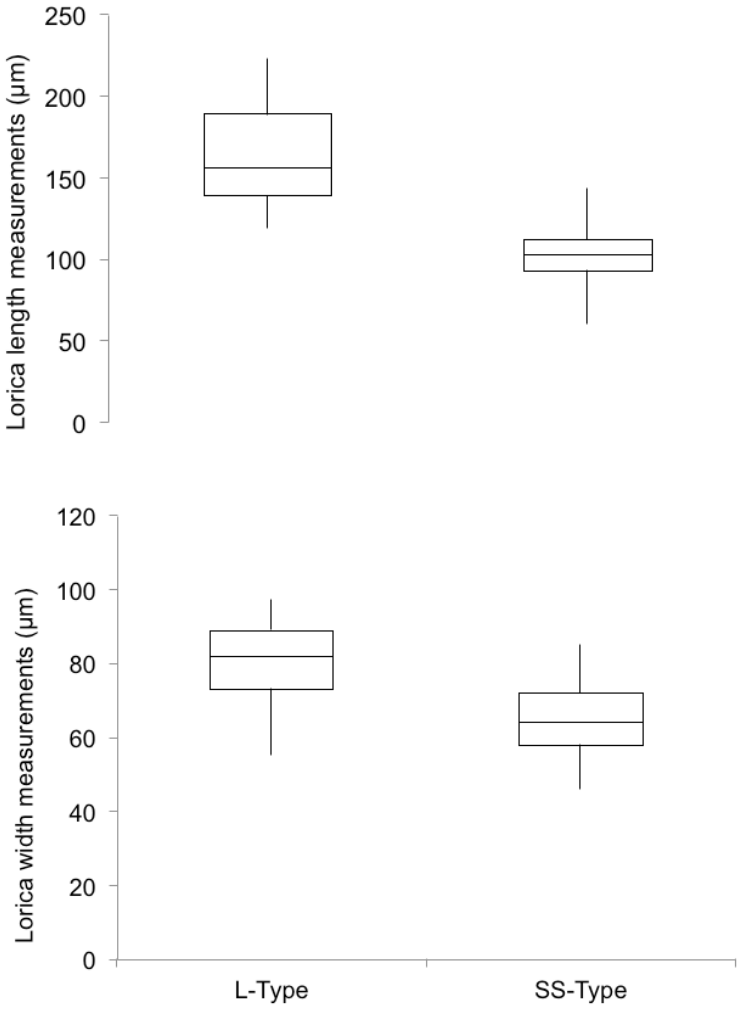
4.2.1 Physical dimensions of L and SS-type rotifer

100 rotifer individuals of each species were measured and it was found the dimensions of L-type rotifer was, lorica length: 161.37 ± 26.11 μm; lorica width: 80.9 ± 9.85 μm. SS-type rotifer was found to be, lorica length: 102.73 ± 15.75 μm; lorica width 64.37 ± 8.88 μm. Despite having almost similar lorica widths, the lorica lengths between the two species varied greatly (Fig. 10). Furthermore, it can also be seen that at certain life stages of both morphotypes, there may be some overlap in size (upper size limit of SS-type and lower size limit of L-type).

4.2.2 Protein content, hydrolysis and trypsin activity of L and SS-type rotifer

SS-type were found to have significantly higher soluble protein content (200.96 ± 1.03 mg g^{-1} DW) compared to L-type (80.04 ± 0.89 mg g^{-1} DW), t(2) = 14.89, p = 0.005. There were also significant differences in protein hydrolysis of both morphotypes (Fig. 11a). SS-type (t(4) = 3.98, p = 0.02) detected (low OD: 0.27 ± 0.04) and showed capability to hydrolyze at 50 kDa molecular weight of protein after 3 hours incubation, however at the same molecular weight, L-type had stronger banding and high OD (0.82 ± 0.02) (Fig. 11b). There were no significant differences observed in the banding between the two species at 37 kDa at all incubation times (Fig. 11c).

Figure 10: L-type *B. plicatilis* and *B. rotundiformis* lorica length and lorica width measurements (mean ± SD).



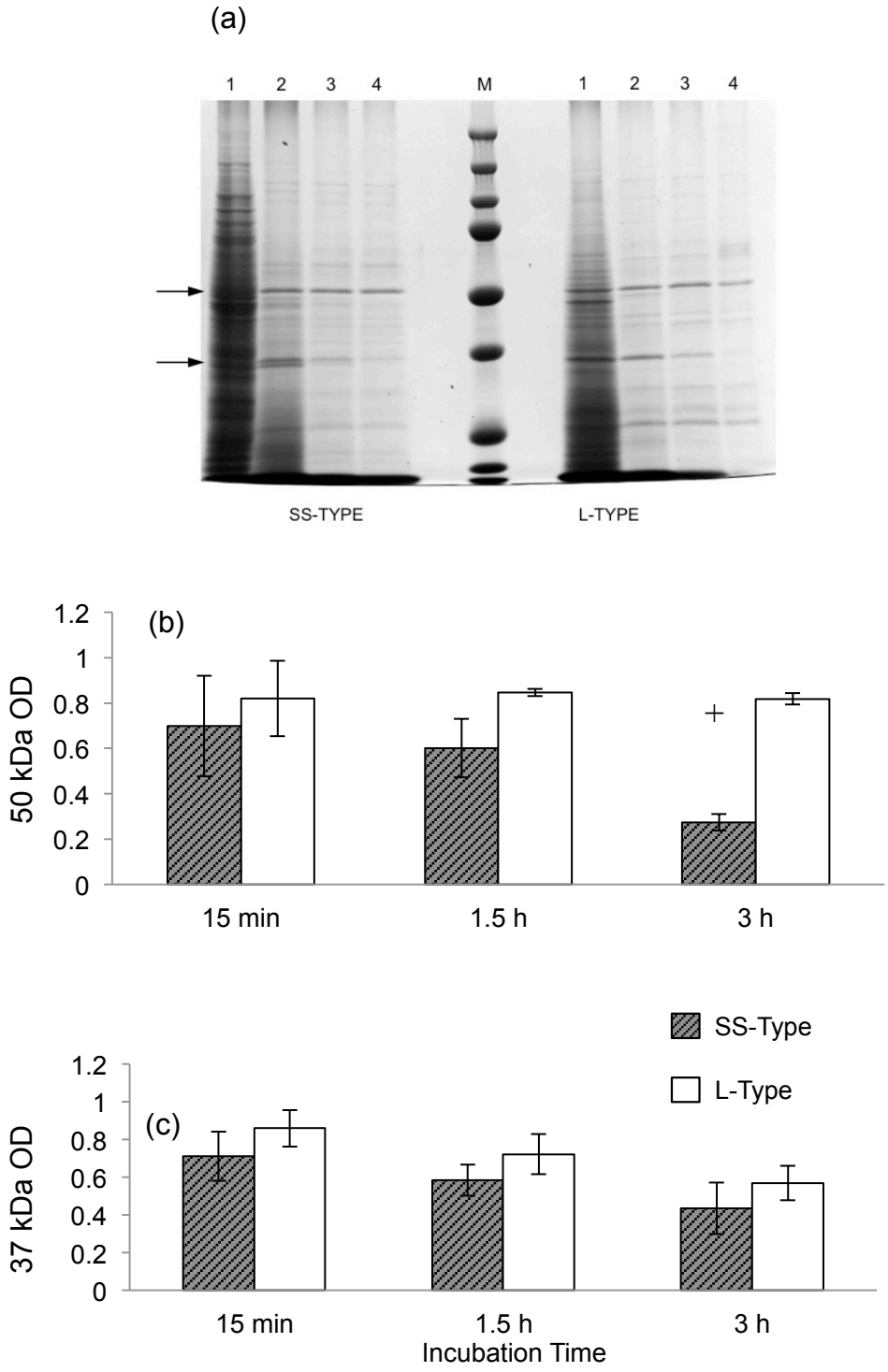
SS-type had significantly higher ($t(2) = 62.13$, $p = 0.0003$) trypsin activity (0.048 ± 0.0001 U ind^{-1}) compared to L-type (0.022 ± 0.0007 U ind^{-1}) (Fig. 4).

4.2.3 Survival, growth and gut content of Japanese flounder (*P. olivaceus*)

Overall, high hatching rate in both treatments was observed (SS-type: 96.81 ± 3.96 %; L-type: 96.53 ± 4.30 %, however, notwithstanding the daily sampling rate of 30 larvae per tank per day (approximately 35 percent of the total number of larvae initially stocked), the average survival rate in both treatments were quite low (SS-type: 36.92 ± 10.89 %; L-type: 34.75 ± 5.42 %) with no significant differences observed.

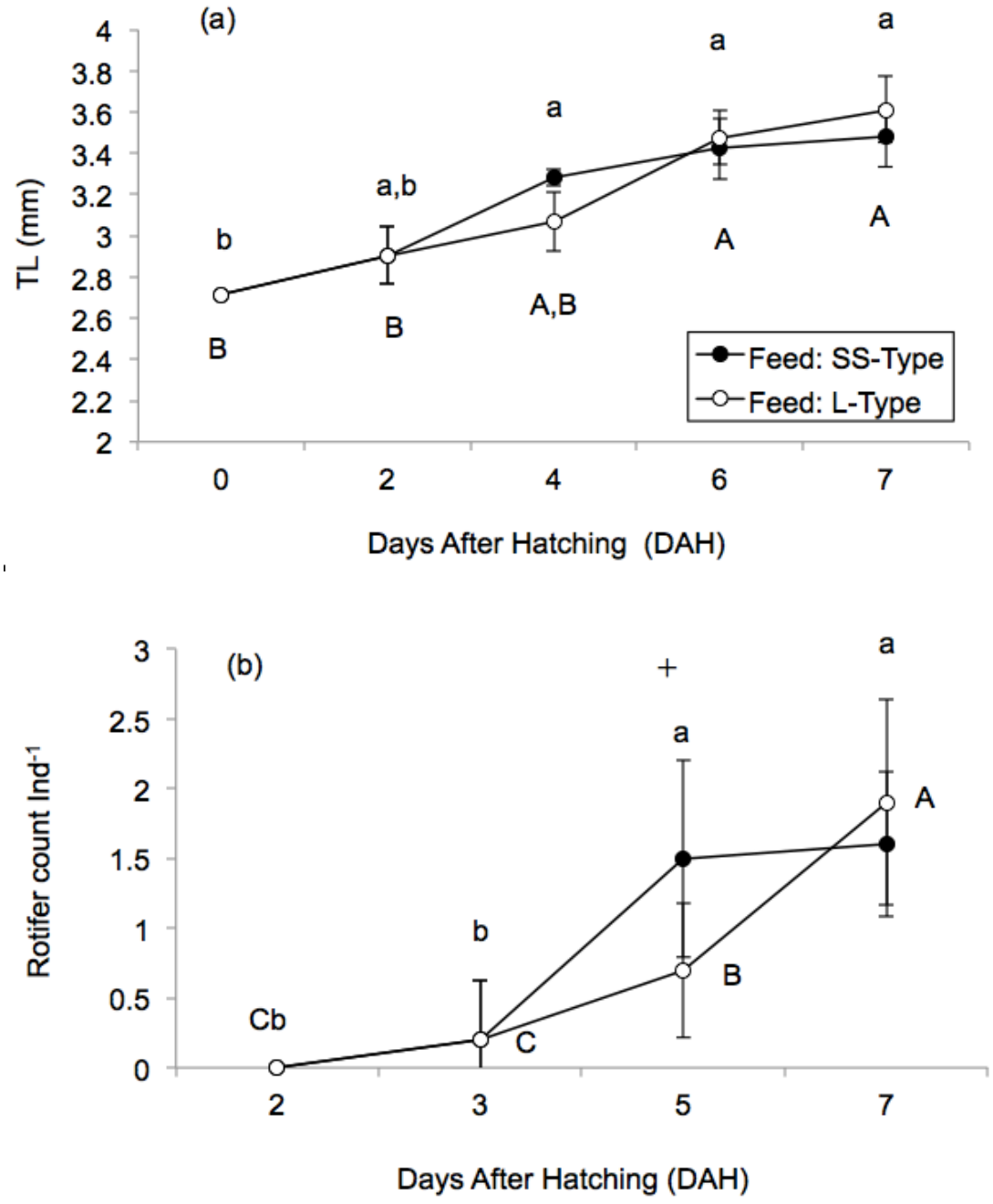
Larval growth (0-7 DAH) presented no significant differences between both treatments (Fig. 12a). There were also no significant statistical differences between the two treatments in the gut content of the larvae at 3 and 7 DAH. However, at 5 DAH SS-type fed had significantly higher ($p = 0.01$) gut content (1.5 rot $larvae^{-1}$) compared to L-type fed (0.7 rot $larvae^{-1}$) (Fig. 12b).

Figure 11: L-type and SS-type (a) gel (M: Marker; 1: 0 minutes incubation; 2: 15 minutes incubation; 3: 1.5 hours incubation; 4: 3 hours incubation); (b) Relative banding intensity at 50 kDa (mean ± SD); (c) Relative banding intensity at 37 kDa.



Mean ± SD. + Indicates significant difference between treatments (Unpaired t-test: p <0.05)

Figure 12: *P. olivaceus*, larval ontogeny during the study; (a) total length (TL), (b) gut content.

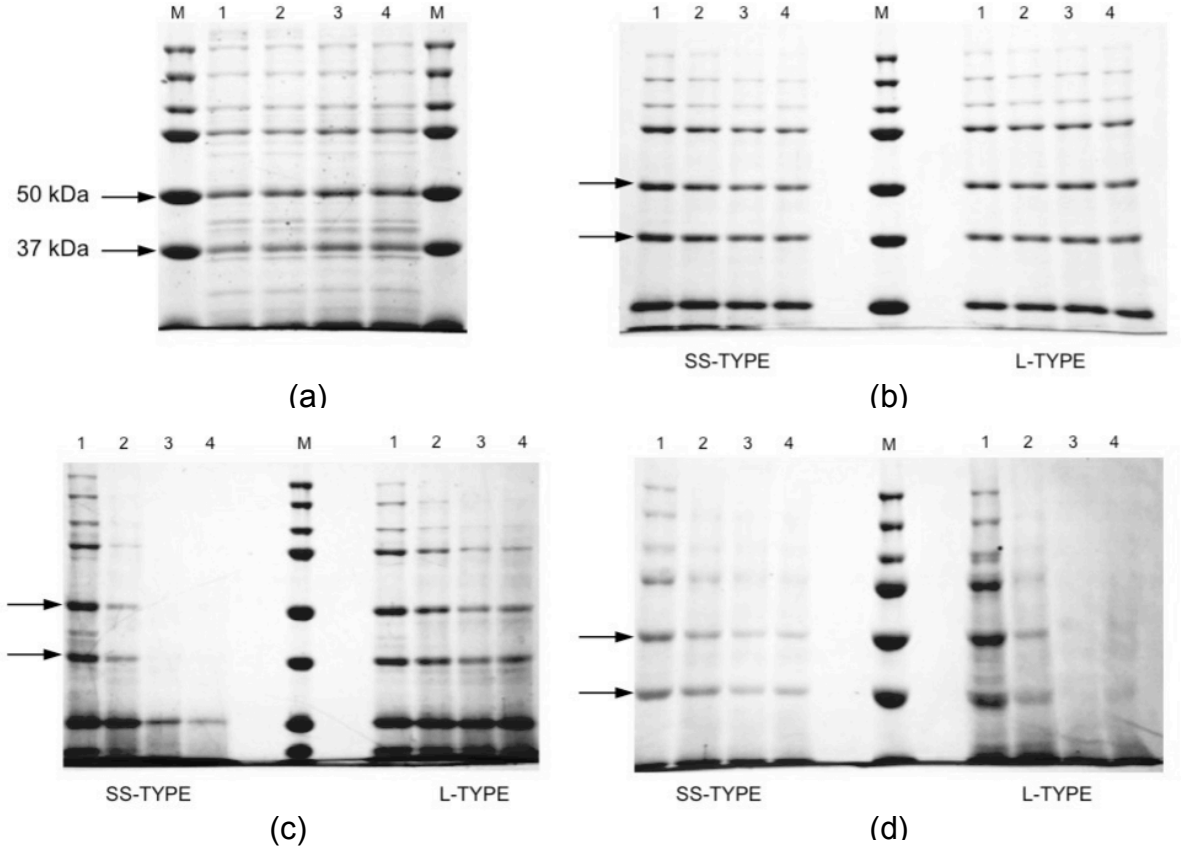


Value indicate Mean ± SD. Alphabets indicates significant difference within treatments (Tukey HSD test, $p<0.05$; SS-type: a>b>c>d; L-type: A>B>C>D)

4.2.4 Protein hydrolysis of Japanese flounder (*P. olivaceus*)

Larvae showed little capability of protein hydrolysis in both treatments at 2 DAH, at 50 kDa and 37 kDa. Relative to the control, there were no significant differences observed in the OD between the incubation times at both bands for 2 DAH and 3 DAH larvae (Fig. 13a; Fig. 13b.

Figure 13: Capability of protein hydrolysis of larvae at (a) 2 DAH; (b) 3 DAH; (c) 5 DAH; (d) 7 DAH. M: Marker; 1: 0 minutes incubation; 2: 15 minutes incubation; 3: 1.5 hours incubation; 4: 3 hours incubation



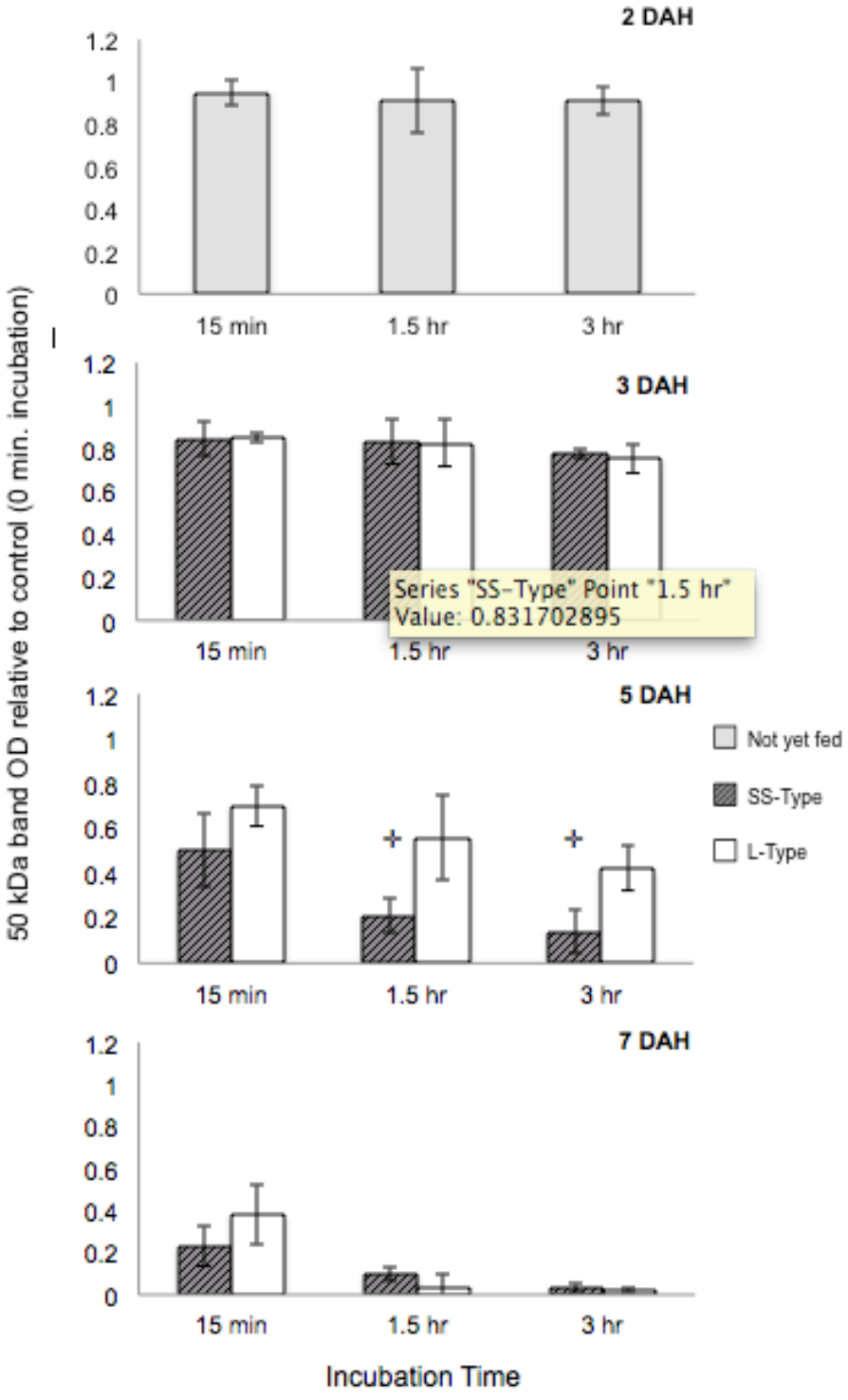
At 5 DAH (Fig. 13c; Fig. 14), hydrolysis activity was observed to be higher in SS-type fed larvae, significant differences ($t(4) = 2.29$, $p = 0.04$) in average band OD were observed at 50 kDa at 1.5 hours incubation, SS-type (0.21 ± 0.18), L-type (0.56 ± 0.19) and at 3 hours incubation ($t(4) = 2.28$, $p = 0.02$), SS-type (0.14 ± 0.09), L-type (0.42 ± 0.09). At 37 kDa (Fig. 15), significant differences ($t(4) = 3.78$, $p = 0.02$) were also observed after 1.5 hours

incubation, SS-type (0.45 ± 0.08), L-type (0.77 ± 0.12) treatments and after 3 hours incubation ($t(4) = 5.34$, $p = 0.01$), SS-type (0.31 ± 0.13), L-type (0.79 ± 0.09). There were no significant differences observed at 7 DAH, despite higher enzymatic activity detected in the OD of banding in L-type fed larvae at 50 kDa (Fig. 13d; Fig. 14).

4.2.5 Trypsin activity and enzyme fraction in Japanese flounder (*P. olivaceus*)

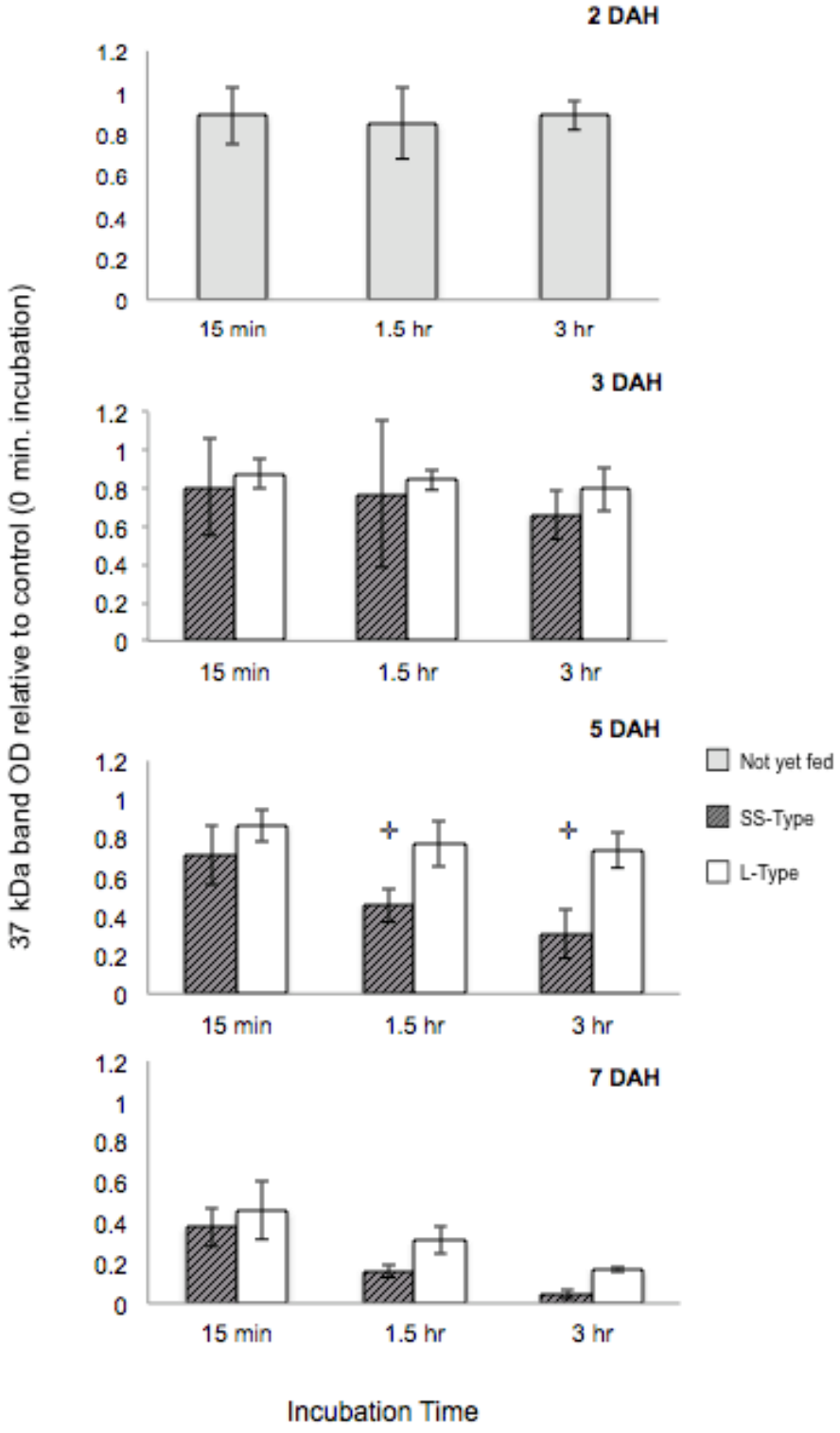
The evolution of trypsin observed was marked by fluctuations in the enzyme activity profile and significantly higher levels of activity detected in SS-type fed larvae (Fig. 9a). Low levels of trypsin were detected at 2 DAH (0.02 ± 0.01 U μg protein^{-1}) even before the onset of exogenous feeding. Significantly higher ($p < 0.05$) trypsin activity was detected at 3 DAH (0.08 ± 0.02 U μg protein^{-1}) coinciding with the moment of first feeding, 5 DAH (0.08 ± 0.02 U μg protein^{-1}), 6 DAH (0.1 ± 0.004 U μg protein^{-1}) and 7 DAH (0.06 ± 0.004 U μg protein^{-1}) in SS-type fed larvae compared to L-type treatment. The average fraction of trypsin activity potentially attributable to ingested prey was significantly higher ($p < 0.05$) in SS-type fed larvae at 5 DAH (2.18 ± 0.44 %) and 6 DAH (2.04 ± 0.29 %). Whereas L-type fed larvae had significantly higher trypsin activity fraction at 7 DAH (3.15 ± 0.33 %; Fig. 10).

Figure 14: Densitometric analysis of optical density (OD) of the three incubation times relative to control treatment (0 minutes incubation) at 50 kDa.



Mean ± SD. + Indicates significant difference between (Unpaired t-test: $p < 0.05$)

Figure 15: Densitometric analysis of optical density (OD) of the three incubation times relative to control treatment (0 minutes incubation) at 37 kDa.

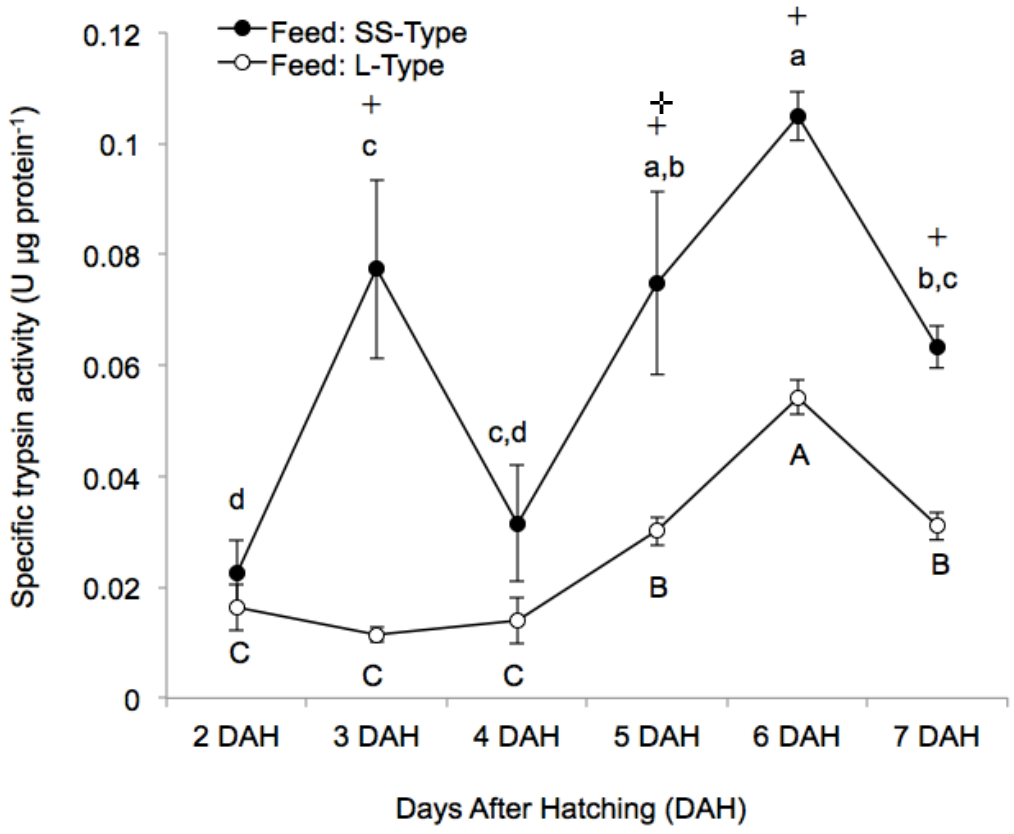


Mean ± SD. + Indicates significant difference between treatments (Unpaired t-test: $p < 0.05$)

4.3 Discussion

The development of digestive enzymes in larvae determines its ability for effective digestion of dietary lipids at first feeding (Gawlicka *et al.*, 2000). Several studies have shown that digestive enzymes are known to be present in low levels in larval fish even at very early stages (Zambino Infante & Cahu, 1994; Cara *et al*., 2003; Bolasina *et al*., 2006). This was also observed in this study where at 2 DAH, larvae exhibited tryptic activity (0.02 ± 0.01 U µg protein^{-1}), which indicates the possibility of enzyme synthesis even before diet stimulation.

Figure 16: Specific activity of trypsin of *P. olivaceus* fed L and SS-Type rotifers at 3-7 DAH.

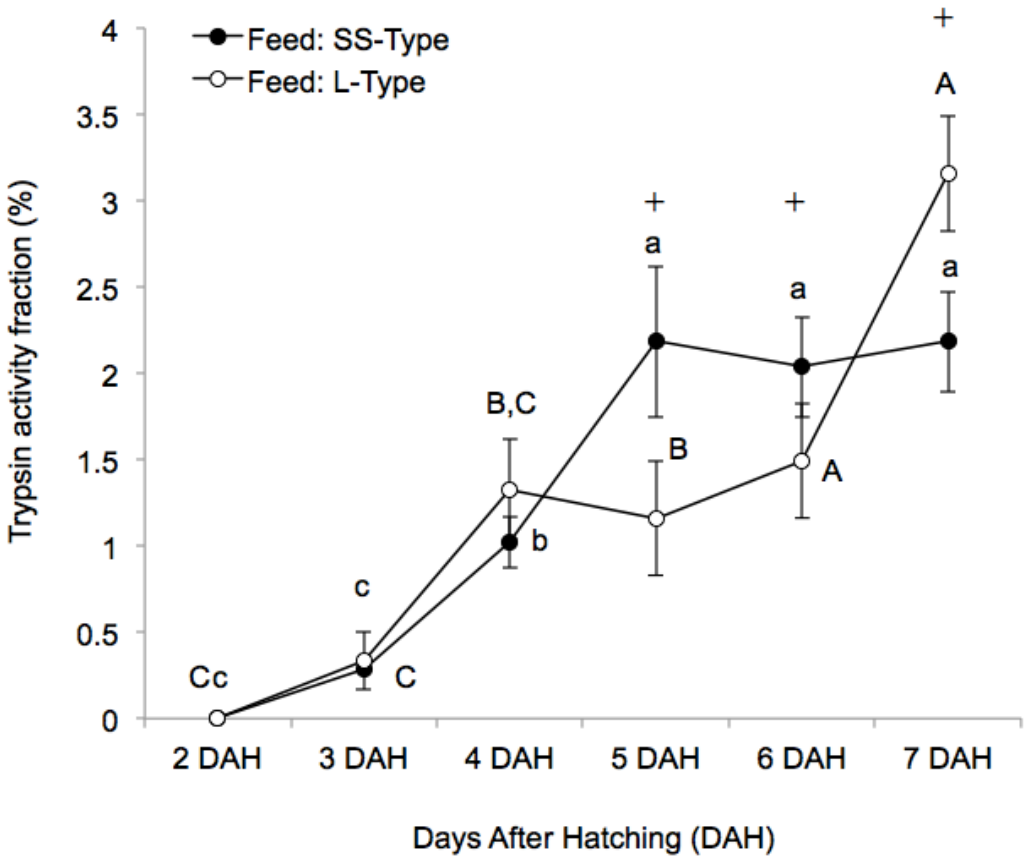


Specific activity of trypsin (mean ± SD) of *Paralichthys olivaceus* during first feeding on L-type *Brachionus plicatilis* and SS-type *B. rotundiformis* (2 to 7 DAH) Alphabets indicate significance within treatments (Tukey HSD test, p<0.05; SS-type: a>b>c>d; L-type:A>B>C>D)

Upon introduction of rotifers at 3 DAH, trypsin activity in the SS-type fed treatment increased dramatically (0.08 ± 0.02 U µg protein^{-1}) whereas L-type rotifer (0.01 ± 0.001 U µg

protein^{-1}) decreased slightly (Fig. 9). The protein hydrolysis assay conducted on larvae at 2 DAH and 3 DAH did not detect any significant changes in proteolytic activity at all incubation times and this is probably due to the gel electrophoresis not being able to detect the trace amounts of enzymatic activity (Fig. 6a, Fig. 6b).

Figure 17: Average fraction of trypsin activity attributable to ingested prey.



Average fraction of trypsin activity attributable to ingested prey (mean ± SD). + Indicates significant difference between treatments (unpaired *t*-test: $p < 0.05$). Alphabets indicate significance within treatments (Tukey HSD test, $p<0.05$; SS-type: a>b>c>d; L-type:A>B>C>D)

This low level of enzymatic activity is attributable to the larvae possessing a premature digestive tract and feeding apparatus at 2 and 3 DAH (Fukuhara, 1986; Moteki *et* al., 2002; Bolasina *et al.*, 2006) and consequentially correlating to the low availability of substrates for digestive enzymes (Dabrowski, 1984; Cara *et al*., 2003).

According to Fukuhara (1986) complete yolk reabsorption in Japanese flounder larvae occurs at 4 DAH and the oil globule resorbed at 5 DAH, complete reliance on exogenous feeding also begins around the same time. At 5 DAH, significantly higher numbers of rotifer were observed in the gut content of SS-type fed larvae compared to L-type fed larvae (Fig. 5b). Furthermore, this result also correlated with significantly higher proteolytic activity detected at 5 DAH in the SS-type treatment in the gel electrophoresis (Fig. 6c), trypsin activity (Fig. 9) and trypsin activity fraction (Fig. 10). The higher incidence of larvae with the smaller SS-type in its gut could also be linked with preference related to the relative mouth gape/prey size ratio as mentioned in previous studies (Busch, 1996; Russo *et al*., 2009). And as such, leads to a higher availability of substrate for enzyme activation (Cara *et al*., 2003; Melianawati *et al.*, 2015). Prey size is a major criterion in determining food selection, preference, capture and ingestion and its effects on early enzyme activity. Previous studies have found that the mouth gape and oesophagus diameter are defining factors in food preference during early larval development as was found in previous studies conducted on Japanese flounder and other marine finfish larvae (Takahashi, 1985; Ikewaki and Tanaka, 1993; Moteki *et al*., 2002; Rønnestad *et al*., 2013).

At 7 DAH, there was no significant difference in protein hydrolysis, however it was observed that L-type fed larvae had higher hydrolysis than SS-type fed (Fig. 6 (d)), thus perhaps indicating a switch in preference for the larger sized prey. General proteolytic activity favored the L-type treatment at 7 DAH, however specific trypsin activity was shown to be higher in SS-type (0.06 ± 0.003 U μg protein^{-1}). Furthermore, despite the electrophoresis results showing an increased general proteolytic enzyme activity between 5 – 7 DAH (Fig. 6), specific trypsin activity reduced markedly in both treatments (Fig. 9). A possible explanation for this variability is that it is most likely a result of the activation of other proteolytic enzymes present in early stage larvae such as chymotrypsin and aminopeptidase (Kurokawa & Suzuki (1996, 1998). Studies by Rungruangsak-Torrissen *et al*. (2006) and Sunde (2001) suggest that in fish

larvae, presence of trypsin could activate chymotrypsin. Zambonino Infante and Cahu (2001) highlighted that trypsin was detected earlier in sea bass and sole and that the activation of chymotrypsin is later in the culture period therefore making it strongly linked to age. Pepsin is normally absent in the digestive tract of early stage larvae and is only detected in the functional stomach, as it requires acidic environments to be activated (Cara *et al*., 2003; Rønnestad *et al*., 2013). The general trend of specific trypsin activity is expected to show succession of increases and decreases (Rønnestad *et al*., 2013) as was observed in this study. The decreases in specific activity is largely due to the increase of body protein as specific activity is the ratio activity per mg protein and does not reflect a lowering in digestive capacity (Ma *et al.*, 2005). In Japanese flounder, it is expected that digestive enzyme activity pattern will continue to increase significantly until the onset of metamorphosis (Bolasina *et al.,* 2006).

Considering the contribution of L-type and SS-type rotifers to trypsin fraction, SS-type had significantly higher ($p < 0.05$) trypsin activity fraction at 5 DAH (2.18 ± 0.44 %)) and 6 DAH (2.04 ± 0.29 %), and significantly higher in L-type at 7 DAH (3.15 ± 0.33 %)(Fig. 10). However as is observed in previous studies (Kurokawa *et al.,* 1998; Lazo *et* al., 2000; Bolasina *et al.*, 2006), the effects of exogenous trypsin from live prey (under the assumption that they are present in the gut) is relatively small (less than 4 % in both treatments in this study) when compared to the total trypsin activity in larvae. Diaz *et al*. (1997) highlighted that the contributions of exogenous enzymes was probably limited to an autolytic process in the prey, which is perhaps neutralized by the alkaline contents of the larval gut or they may also possibly play a role in enzyme activation (Dabrowski 1984). Furthermore, the patterns of protein hydrolysis in larvae occur more rapidly than that in the live feed highlighting the faster rate of protein degradation in the larvae. The two morphotypes, did not have any significant differences in protein hydrolysis however, SS-type not only had significantly higher soluble protein content (20.96 ± 1.03 mg g^{-1} DW) but also higher specific trypsin activity (0.048 ±

0.0001 U ind^{-1}). The observed variability in specific trypsin activity between treatments could also possibly stem from such differences in stimulation response to dietary protein quality between the two live feed types. A study by Cahu *et al.* (2004) showed that early larval pancreatic enzyme secretion is linked to dietary protein amount and quality. They further highlight that different levels of proteins in the diets moderated trypsin expression, and in turn this affected chlolecystokinin (CCK) content, the peptide hormone responsible for stimulating the digestion of fat and protein. CCK producing cells have been detected from 1 DAH in Japanese flounder (Kurokawa *et al.*, 2000). Furthermore, observed differences in trypsin activity between treatments at 5 DAH could also be linked to the ability of larvae to digest different protein species at this life stage. A study by Srivastava (2002) on Japanese flounder concluded that there was a possibility that some of protein species are not digested in early larvae that lack functional stomach and that some proteins showed resistance to pancreatic enzymes of which are not suitable as components of larval diets. Following on from findings by Srivastava (2002), it is possible that levels of protein species and enzymes that have the ability to be rapidly cleaved by pancreatic extract (e.g. thyroglobulin, albumin and lactate dehydrogenase) are higher in SS-type rotifer. Alternatively, it is also possible that in L-type there are higher hydrolysis resistant protein species and enzymes (e.g. ferritin and catalase), however further studies are needed to clarify these assumptions.

In conclusion, there were no significant differences in growth, survival and protein content in larvae fed with L-type and SS-type rotifers during first feeding. As the purpose of this study was to assess the variations in protein hydrolysis and digestive trypsin activity at first feeding, the effects of long-term survival and growth of such a feeding regiment requires further study. There were clear differences in the proteolytic enzyme response, specifically trypsin, observed between to the two treatments. Based on these results, SS-type is a suitable alternative live feed at 2–7 DAH in first feeding Japanese flounder before the introduction of

the commonly used L-type rotifer. It is suitable in size, favored more by the larvae from 3-6 DAH, exhibited higher general proteolytic enzyme activity and higher trypsin activity alluding to its digestibility and suitability for the nutritional status of first feeding Japanese flounder. Furthermore, these results highlight the adaptability of the Japanese flounder to the use of alternative rotifer morphotypes during its early developmental stages.

CHAPTER 5: General Discussion

Morphological abnormalities, alimentary tract deterioration, trunk musculature, reduction in food utilization efficiency and low feeding activity have been linked to nutrition deficiency and starvation immediately after reabsorption of the larval yolk (Heming et al., 1982; Taylor and Freeberg, 1984; Rice et al., 1987; Gisbert et al., 2004). Nutritional requirements of fish larvae are quantitatively and qualitatively complex as they differ not only among species but also at different ontogenetic stages of development due to morphological and physiological changes. The importance of rotifers, especially those belonging to the *Brachionus plicatilis* species complex, is often highlighted as they are often used as the preferred first-feed during marine finfish larviculture (Watanabe et al. 1978; Tomoda et al. 2004; Park et al. 2006). This is due to favorable characteristics such as its small size (70-340 μm), high reproduction rate, planktonic nature, suitability for mass culture and ability for artificial manipulation of its nutritional qualities (Yoshimura et al. 1997; Dhert et al. 2001; Kotani et al. 2009). However, dependent on the conditions of culture and types of enrichment, several nutrients may be present in insufficient levels in rotifers (Hamre, 2008). Furthermore, the digestibility and subsequent growth are important indicators of acceptance of feed in larvae that also need to be considered to highlight how effective the enrichment that was used on the live feed. The lack of a functional stomach in most early stage marine larvae causes the entire extra-cellular degradation of ingested prey to occur in the intestine with pancreas-derived enzymes (Bolasina et al., 2006). Digestive enzyme activity is a reliable method that can be used as an indicator of digestive processes and nutritional condition of larvae (Ueberschär, 1988). The larval ability for utilization of enzymes derived from prey is known to aid in digestion or in the activation of zymogens in the gut and thus improving digestion and growth (Dabrowski, 1979; Lauf & Hoffer, 1984; Kolkovski *et al*., 1993). The lack of a functional stomach in most early stage marine larvae causes the entire extra-cellular degradation of ingested prey to occur in the

intestine via pancreas-derived enzymes (Bolasina et al., 2006). The mechanism through which these exogenous enzymes are activated is still not clearly understood, however their presence in the gut of the larvae can be measured in its homogenate (Lazo *et al*., 2000). Early enzymatic activity using enzymes such as amylase, neutral lipase, chymotrypsin, trypsin and pepsin has also been reported in other marine species such as the gilthead sea bream, (Moyano *et al*., 1996), white sea bream (Cara *et al*., 2003) and Senegalese sole (Martinez *et al*., 1999). Trypsin is an important indicator digestive enzyme. It is an endoproteinase that is directly connected to protein metabolism and is known to be present in young fish larvae and previous studies have highlighted it as a useful indicator of nutritional condition (Pedersen et al., 1987; Ueberschär, 1988, 1995; Bolasina et al., 2006). Therefore, the investigations outlined in this thesis are the first in exploring the effect of commonly utilized enrichment on different morphotypes within the commercially valuable *B. plicatilis* sp. complex before expanding on its effects on the growth of two model marine fish species.

In this study, the enrichment effect on two morphotypes of the common live feed, *Brachionus plicatilis* species complex, and its suitability for use in first feeding marine fish larviculture was determined. The *B. plicatilis* species complex was chosen as it has extensively studied since its introduction in the 1960's (Segers, 1995; Lavens and Sorgeloos, 1996; Kotani et al., 1997; Fu et al. 1997; Dhert et al. 2001; Dhont et al., 2013; Hagiwara et al., 2014; Hamre, 2016; Mills et al., 2016). The red sea bream (*P. major*) and the Japanese flounder (*P. olivaceus*) were used as the model species based on several factors. Firstly, they are both important recreational, commercial and aquaculture species in Japan and therefore their culture protocols have been standardized and as such the availability of literature on the culture, growth and development of both species is plentiful. Furthermore, since their introduction into the aquaculture industry, it has since been mass-produced for stock-enhancement purposes and for this reason their early life history has been studied quite well (Kohno et al., 1983; Fukuhara,

1986; Fukuhara, 1985; Matsuoka, 1987; Ikewaki and Tanaka, 1993; Yamashita and Yamada, 1999; Howell and Yamashita, 2005).

The first part of this study explored the suitability of enrichment and its impacts on two morphotypes, the L-type *B. plicatilis* and the SS-type *B. rotundiformis*. It was found that the response in fatty acid content, population growth and soluble protein content to enrichment varied between the two morphotypes. L-type species generally displayed higher growth ratio compared to SS-type under DHA-enriched *C. vulgaris,* frozen *N. oculata* and salmon roe oil treatments. Under salmon roe oil emulsion treatment though, both morphotypes experienced negative population growth and low soluble protein content, despite having high HUFA composition. The viscous nature of the oil-based salmon roe emulsion treatment may suggest that this is what is causing this effect as previously suggested (Foscarini, 1988; Dhert et al. 1990; Hagiwara et al., 1998; De Araujo et al., 2000, 2001; Larsen et al., 2008). Therefore dosage of oil-based enrichment media and maintenance of good stocking density are important considerations in sustaining high quality *B. plicatilis* species complex populations in batch cultures. As generally, manufacturers of enrichment diets have determined the appropriate method and dosage however, there is little information on the effects on rotifers of nutritional enrichment using methods not previously described by manufacturers (Kotani et al., 2017). Despite DHA-enriched *Chlorella vulgaris* being poor in EPA (Maruyama et al. 2006; Thépot et al. 2016) and frozen *Nannochloropsis oculata* being poor in DHA (Hirayama *et al.* 1979; Lubzens et al. 1995; Kobayashi et al. 2008) both were found to have positive responses in population growth in both morphotypes. However, normally ArA poor DHA-enriched *C. vulgaris* (Matsunari et al. 2013; Thépot et al. 2016) was found to have high a composition in this study and thus alluding to the possible natural synthesis of certain fatty acid species is possible via elongation and desaturation of precursor fatty acid species (Le Milinaire et al., 1983; Robin, 1995).

Combining DHA-enriched *C. vulgaris* and salmon oil emulsion to optimize the higher HUFA content in *B. plicatilis* showed that both rotifer enrichment treatments were found to be nutritionally effective. However, HUFA content was better in the combined treatment due to EPA fortification from the salmon roe emulsion. Despite this, the DHA-enriched *C. vulgaris* only treatment was found to have higher population growth, egg bearing capacity, soluble protein and free amino acid content. During larviculture, both *P. major* and *P. olivaceus* suffered high mortality under the combined diet. In contrast, generally higher gut content, ingested soluble protein and trypsin activity fraction were observed in the DHA-enriched *C. vulgaris* only treatment. This may well be a reflection of its nutritional qualities as highlighted above as a high composition of free amino acids (FAA) and soluble proteins is beneficial for early stage larvae due to ease of hydrolysis and absorption by the early digestive system (Rønnestad et al., 1999; Carvalho et al., 2004; Kvåle et al., 2007). Important periods of growth in both fish species was observed between 7 DAH and 11 DAH which and alludes to important ontogenetic stages of growth in both larval species. These stages of increased growth are to be highlighted as critical phases during the first feeding period as feeding incidence is increased, thus optimization of feeding during this period is important. Furthermore, it is recommended that to avoid the observed effects of increased viscosity and oil contamination in larval rearing water, a suggested alternative would be to reinforce rotifers with EPA using a combined microalgal enrichment of *Nannochloropsis oculata* rather than an oil-based enrichment.

Performance of DHA-enriched *C. vulgaris* has been highlighted in this study, therefore evaluating it's performance during larviculture between L-type *B. plicatilis* and SS-type *B. rotundiformis* showed no significant differences in growth, survival and protein content in in first feeding *P. olivaceus*. However, there were clear differences in the proteolytic enzyme response, specifically trypsin, observed between the two morphotypes. In the larviculture of *P. olivaceus*, the L-type *B. plicatilis* is often utilized, however in this study, SS-type proved to be

a suitable alternative live feed at 2–7 DAH. The affinity towards SS-type *B. rotundiformis* could be linked to its size and the physiological limitations of the early stage larvae and therefore size grading of prey is an important consideration (Takahashi, 1985; Ikewaki and Tanaka, 1993; Busch, 1996; Moteki *et al*., 2002; Russo *et al*., 2009; Rønnestad *et al*., 2013). These results highlight the adaptability of the Japanese flounder to the use of alternative rotifer morphotypes during its early developmental stages. Furthermore, the importance of the consideration of nutritional value of the feed such as amounts of soluble protein content was found to be having a positive impact on larviculture performance as previously mentioned in this study and by others (Rønnestad et al., 1999; Carvalho et al., 2004; Kvåle et al., 2007).

In conclusion, this is the first study to evaluate nutritional differences in the commonly used L-type *B. plicatilis* and the SS-type *B. rotundiformis* morphotypes of the *Brachionus plicatilis* species complex when enriched with common Japanese commercial feed and the larval responses at first feeding. This study suggests that population growth, fatty acid content and soluble protein in response to the different enrichment treatments differed between the two morphotypes. However, further studies are needed on culture methods and enrichment dosages and its impacts on different morphotypes due to the varying effects on fatty acid assimilation in the NL and PL and also significant variances between rotifer morphotypes and strains. Additionally, studies on culture technologies such as culture methods and systems used and adjusted dosages of enrichment affect nutritional status of *B. plicatilis* species complex rotifers and if it can be standardized to maximize production and optimal quality of live feed. The use of oil based enrichment, despite being high in HUFA content was found to have detrimental effect on the rotifer population sustainability and also in larviculture performance and therefore adjustment of dosage according to culture methods is important. The larviculture of important marine finfish larvae at first feeding such as *P. major* and *P. olivaceus* is an important area of research. This study highlighted that as important HUFA content are, consideration of free

amino acids and soluble protein content as parameters of nutritional indicators for first feeding *P. major* and *P. olivaceus* should not be discounted. Furthermore, our results highlight the adaptability of the Japanese flounder to the use of alternative rotifer morphotypes and preference for small morphotypes during its early developmental stages. Many parallels can be drawn from this study for the culture of rotifers suitable for larviculture, however there is still a lot that we do not know about the behavior of marine fish larvae in relation to feed intake at first feeding. Therefore studies on effects of rotifer enrichment on feeding intensity and rhythm, how long feed remains in the gut and the amount of nutrients that is taken up by the larval body compared to wastes are future prospective research topics that can provide a good platform for studies on feed requirements in marine fish larvae at initial exogenous feeding. Important considerations for future studies include the measure of relevant biological larval response to different enrichment and live feed, such as pigmentation, skeletal and neural system development as these differ amongst fish species and culture conditions. It is hoped that the results obtained from this study could provide avenues for future research into optimization of *Brachionus plicatilis* species complex and its impacts on commercially important marine fish species, which could also in turn offer new approaches for improvement of live feed technology in hatchery practices and aquaculture management.

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