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Studies on the Development of Low-cost and Stable Live Food Production Technologies for Tropical Aquaculture: A case Study of Rotifera (Family: Brachionidae)

熱帯域養殖での低コストで安定的な餌料生産技術の開発に関する研究：

ワムシ類（ツボワムシ科）を事例として

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in
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Studies on the Development of Low-cost and Stable Live Food Production Technologies for Tropical Aquaculture: A case Study of Rotifera (Family: Brachionidae)

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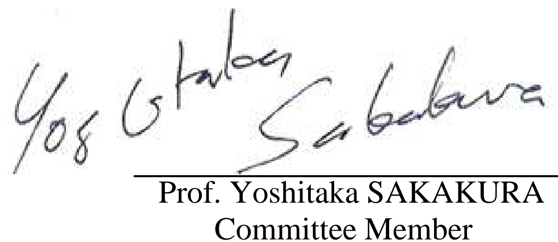
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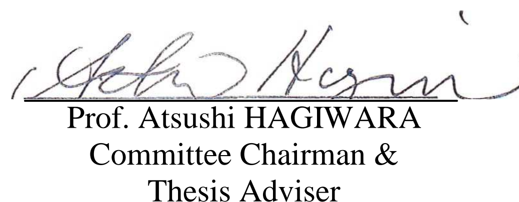
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CONTENTS

ABSTRACT: English……………………………………………………………..…..i

Japanese…………………………………………………………….....iv

CHAPTER I..1

GENERAL INTRODUCTION 1

CHAPTER II 12

BIOLOGICAL CHARACTERIZATION OF THE ROTIFER, *Brachionus angularis* FROM KENYA 12

CHAPTER III 30

CHICKEN MANURE EXTRACT (CME) ENHANCES REPRODUCTION OF THE ROTIFER, *Brachionus angularis*...30

CHAPTER IV 45

USING DRIED ALGAL DIETS FOR MASS CULTURE OF ROTIFERS, WITH gamma-aminobutyric acid (GABA) SUPPLIMENTATION 45

CHAPTER V 61

DEVELOPING FISHWASTE DIET (FWD) FOR PLANKTONIC LIVE FOOD CULTURE: PROTOCOL AND APPLICATIONS 61

V.1. FWD for culturing *Brachionus rotundiformis* (rotifera): a laboratory study ...61

V.2. FWD for culturing freshwater zooplankton in outdoor tank cultures................85

V.3. Dietary value of the FWD-fed rotifers for larviculture: a preliminary study ..100

CHAPTER VI..111

GENERAL DISCUSSION 111

REFERENCES 126

ACKNOWLEDGEMENTS 154

APPENDIX 156

ABSTRACT

Live food resources e.g. rotifers, copepods, cladocerans and *Artemia* are critical for successful fish larviculture. However, the hatchery production of these live foods depends on the availability of high density microalgae, which is unstable, laborious and costly to produce, thus impedes the development of larviculture, especially in most tropical countries. This study developed three low-cost and stable live food production protocols, with specific reference to brachionid rotifers, for mass culture initiatives and, applications in the larviculture sector.

Firstly, a study was conducted to investigate the population ecology of a common freshwater rotifer species in Kenya, using individual life table and small batch cultures, to advance the knowledge of rotifer fauna in Kenya (Chapter II). The rotifer cysts were sampled from pond sediments in Kenya and transported to Nagasaki University for further study. The rotifer was morphologically identified as *Brachionus angularis*, before conducting the ecological studies at 20, 25 and 30°C using fresh *Chlorella vulgaris* diet at 2.5×10^5, 2.5×10^6 and 2.5×10^7 cells ml^{-1}. The rotifer was most fecund (2.11±0.07 offspring female $^{-1}$ day $^{-1}$) and reproductive (8.43±0.24 offspring female $^{-1}$) at 25°C with 2.5×10^6 algal cells ml^{-1}. The highest intrinsic rate of natural increase (0.74±0.02 d^{-1}), specific population growth rate (0.49±0.01), longest life expectancy at hatching (12.41±0.28 d) and shortest generation time (2.87±0.03 d) also occurred at 25°C with 2.5×10^6 algal cells ml^{-1}. The duration of hatching to first spawning was shortest (2.86±0.21 h) at 30°C with 2.5×10^7 algal cells ml^{-1} and longest (8.83±0.39 h) at 20°C with 2.5×10^5 algal cells ml^{-1}. The highest population density (255.7±12.6 ind ml^{-1}) occurred at 25°C with 2.5×10^6 cells ml^{-1} on day 8. The Kenyan strain of *B. angularis* has small lorica size (length: 85.6±3.1 µm; width: 75.4±3.6 µm) and, reproduces optimally at 25°C with 2.5×10^6 algal cells ml^{-1}; thus suitable for feeding small-mouth freshwater fish larvae.

To enhance mass production of this rotifer species, for aquaculture, a chicken manure extract (CME) technique was developed, and its effects on the population growth, mixis induction and body size of the rotifer was determined (Chapter III). Four concentrations of CME (i.e. 0.5, 1.0, 2.0 and 3.0 ml l^{-1}) were added to different glass jars containing 20 ml of sterilized pond water, in which 30 clones of rotifers were cultured at 25°C, and daily fed with 2.5×10^6 cells ml^{-1} of *C. vulgaris* for 7 days without aeration or water exchange. The rotifer's specific population growth rate and population density increased significantly with 2.0 ml l^{-1} of CME, without altering the lorica size.

The mictic response decreased with increasing concentrations of CME. Therefore, 2.0 ml l^{-1} of CME is optimal for enhancing the mass culture of the rotifer *B. angularis*.

To reduce overdependence on the freshly cultured microalgae for live food production, dried algae have often been used but cases of culture crash are common, thus requiring techniques to stabilize them. In chapter IV, dried *Nannochloropsis oculata* and *Chlorella vulgaris* were used to culture the euryhaline rotifer *Brachionus rotundiformis* (SS-type) with gamma-aminobutyric acid (GABA) supplementation. Firstly, the efficacy of GABA was tested during the lag phase of rotifer growth and every 2 days in small cultures (300 ml). Then, the rotifers were exposed to GABA for 24 and 48 h before up-scaling to 20 l cultures. GABA enhanced rotifer population density and egg/female ratio in both foods compared to the control. Pre-GABA incubation for 48 h caused higher rotifer population densities on days 5 and 6 (with both foods) and 8 and 10 (with *C. vulgaris*), than their respective controls.

In chapter V, a study was conducted to eliminate the expensive microalgae from aquaculture production chain. Here, a protocol was developed for making a low-cost fishwaste diet (FWD) that was used to culture the rotifers *B. rotundiformis* (SS-type), after several culture failures with *B. angularis*. About 0.5 g l^{-1} of fishwaste was wrapped in plankton net (200 μm) and placed in the rotifer culture medium. Then, 0.2 g l^{-1} of starch (wheat flour) was added into the culture as carbon source. Three diets i.e. FWD_1 (fishwastes only), FWD_2 (FWD_1 + starch) and control (*C. vulgaris* only) were each triplicated in 30 l polycarbonate tanks containing sea water (22 ppt), in which 20 rotifers ml^{-1} were stocked and cultured at 28±1°C without aeration for 18 days. The FWD, as food source, was used to determine the population growth, mixis rate and nutritive value of the euryhaline rotifer, *B. rotundiformis* (SS-type) (Chapter V.1). Half of the culture medium was replaced at every rotifer exponential growth phase and, fresh treatment added. The stability of the cultures was determined by the coefficient of variation (CV) of the mean specific growth rate (SGR). The total lipid content of the rotifers and the microbial flora were also analyzed. The FWD suppressed rotifer mixis rate but favoured parthenogenetic reproduction. FWD_2 produced significantly higher rotifer density than FWD_1 and control diet, where up to 1,188±69.7 rotifers ml^{-1} was obtained between 8-13 days with FWD_2. There was no significant effect of FWD on the CV (0.08-0.11) of the cultures. About 0.35 and 0.39 mg g^{-1} of DHA and EPA, respectively was obtained in the FWD-fed rotifers and, both were under detectable limit in the control-rotifers. The DHA/EPA ratios were 2.7, 0.9 and 0.0 for bioflocs, FWD-fed rotifers and control-

rotifers, respectively. The FWD may have contained probiotics and essential nutrients, which explain the high rotifer growth in the FWD cultures.

In the succeeding experiment, the FWD was blended with CME and tested for mass culture of freshwater rotifers, copepods and cladocerans in outdoor tanks in Kenya (Chapter V.2). Each treatment i.e. FWD_A (fishwastes + starch), FWD_B (FWD_A + CME) and control (CME only), were inoculated with 5, 2 and 0.4 ind ml^{-1} of rotifers, copepods and cladocerans, respectively in each tank containing 500 l of underground water, and cultured for 16 days. Half of the culture media was replaced at the end of the first exponential growth phase of the zooplankters. There was significantly higher population density and specific growth rate of zooplankters in FWD_B than in FWD_A and control tanks for all the zooplankton taxa. The most abundant genera were *Brachionus* sp., *Cyclops* sp. and *Daphnia* sp. for the rotifers, copepods and cladocerans, respectively. Up to 146.3±7.0, 12.1±2.7 and 7.7±1.7 ind ml^{-1} of rotifers, copepods and cladocerans, respectively were obtained on day 7 in FWD_B tanks. The FWD_B may have expanded the forage base for the zooplankton leading to higher growth rate, thus making it feasible for tropical aquaculture.

Subsequently, a preliminary larviculture experiment was conducted to determine the dietary value of the rotifer, *B. rotundiformis* (SS-type) fed with the FWD by testing its effects on the larval growth and development of the Japanese whiting, *Sillago japonica* (Chapter V.3). Here, fertilized eggs of *S. japonica* were transferred into polycarbonate tanks each containing 100 l of artificial sea water (33 ppt.) at 10 eggs l^{-1} with 50 ml min^{-1} of aeration. Two diets i.e. FWD-fed rotifers and control-rotifers (rotifers fed with super fresh Chlorella-V12 at 7.0×10^6 cells ml^{-1}) were tested. The fish were cultured at 25±1°C with 12-h diurnal photoperiod (900–2100) for 10 days. The diet was maintained at 10 rotifers ml^{-1} throughout in each tank. The fish were randomly sampled every 2 days for morphometric and gut content analysis. The composition of fatty acids was also analyzed but the total lipid content of the fish larvae were not analyzed due to insufficient sample quantity. There was a significantly higher total and standard length for fish larvae fed with FWD-rotifers than those given control diet. However, there were no significant differences in survival rate, viability, dry weight, number of ingested rotifers and, growth parameters e.g. head length, eye diameter, and body depth between the two diets. The FWD technique appears to be a cost-effective and stable live food production technique for mass production of fish seedling to enhance aquaculture development and capture fisheries.

要約

魚類の種苗生産で初期餌料の安定供給は必須である。しかし、餌料生物を量産するために使用される濃縮植物プランクトンは高価であり、ケニアなどの開発途上国での増養殖の発達の弊害となっている。本研究は、種苗生産の現場で主な初期餌料生物であるツボワムシ科プランクトン（以下、ワムシ）の安定・安価な培養技術の開発と応用を目的とした。まず、ケニアで採集された淡水産ワムシ *Brachionus angularis* の現地での応用を目指し、生物学・個体群生態学的パラメーターを利用して最適な培養条件を調べた（第 2 章）。続いで、これらを経済的に量産するため、低-コストである鶏糞が *B. angularis* の増殖に与える影響を調べた（第 3 章）。次に、高価な濃縮植物プランクトンの代替物を探索した。ワムシはバクテリアフィーダーでもあるため、バクテリアを発生する魚類廃棄物を利用出来るか検討した（第 4 章）。最後に、濃縮植物プランクトンより保存性が高く安価である乾燥植物プランクトンを利用してワムシの増殖能を最大限に発揮するため、gamma-aminobutyric acid（GABA）の有効性を検討した。（第 5 章）。

【ケニア株 *B. angularis* の最適培養環境】

ケニアで採集された淡水産ワムシ *B. angularis* の耐久卵を孵化させ、これらの生物学・個体群生態学的特長を調べた（第 2 章）。孵化した個体の大きさは背甲長 85.6±3.1 µm、背甲幅 75.4±3.6 µm であった。*B. angularis* の最適培養環境を調べるため、3 段階の水温（20、25、30°C）と餌密度（2.5×10^5、2.5×10^5、2.5×10^6 *Chlorella vulgaris* cells ml^{-1}）で個体別培養個体別・バッチ培養を行った。*B. angularis* は水温 25℃で *C. vulgaris* を 2.5×10^6 cells ml^{-1} の密度になるよう毎日給餌することで最大増殖能（個体群増殖率 0.49±0.01、最大密度 255.7±12.6 個体 ml^{-1}）を発揮することが分かり、口径が小さな魚類の初期餌料としての高い可能性を確認した。

【鶏糞が淡水産ワムシ *B. angularis* の増殖に与える影響】

安価な量産培養の方法として鶏糞を用い、淡水産ワムシ *B. angularis* のバッチ培養を行った。培養水の中、鶏糞濃度を 4 段階（0.5、1.0、2.0、3.0 ml l^{-1}）に調節し、最適環境下（水温 25℃、餌 *C. vulgaris* 2.5×10^6 cells ml^{-1} day^{-1}）で 7 日間換水せずに培養し *B. angularis* の生殖能力を比較した。これらの両性生殖は鶏糞の濃度が高くなるほど抑制された。一方、単性生殖は 2.0 ml l^{-1} の鶏糞濃度でサイズの変化なく活発になる事が分かった。

【乾燥植物プランクトンを用いたワムシの量産培養システムの構築及び応用】

乾燥植物プランクトンは濃縮物より、保管性に優れ、原価が安いという長所があるが、ワムシの増殖力は低下する。そこで、悪環境下でワムシの増殖力を高めると知られている GABA を用いワムシ量産培養の餌として乾燥植物プランクトン（*Nannochloropsis oculata* と *C. vulgaris*）の可能性を確認した。培養前 48 時間 GABA に暴露することで *B. rotundiformis* (S-type) の活発な増殖が誘導さ

れることが分かった。本研究を通じて構築された安定・安価な餌料生物の量産システムはアジアの若干国を含めた開発途上国の水産増・養殖の発達、及び持続的漁業のため必要となる資源管理（種苗生産）の効率化を加速する可能性がある。

【魚類廃棄物を用いた餌料生物の量産培養システムの構築及び応用】

現在、水産現場で汎用されている濃縮植物プランクトンの代替としてバクテリアの可能性を確認した。淡水産ワムシより培養が容易な汽水産ワムシ *Brachionus rotundiformis*（SS-type）を用い、設置した魚類廃棄物が *B. rotundiformis* の増殖と栄養価に与える影響を調べた。実験区は（１）魚類廃棄物（0.5 g l^{-1}）のみと、（２）魚類廃棄物と小麦粉（澱粉提供）を混合したの２つを設定し、植物プランクトン *C. vulgaris* のみの対照区と個体群増殖率を比較した。それぞれの餌環境下で 18 日間培養を行った結果、*B. rotundiformis* は混合物で植物プランクトンより高い密度（1188±67.7 個体 ml^{-1}）を安定的（CV 7.47±1.68%）に維持した。対照区のワムシは DHA と EPA を持たなかったが、魚廃棄物区は DHA 0.35 と EPA 0.39 mg g^{-1} を保有し、DHA/EPA 率は 0.9 だった。

さらに、魚類廃棄物を用いた量産培養方法の応用に向けて、餌料生物として高い可能性を持つ、淡水産ワムシ、カイアシ類、ミジンコについて実際にケニアで野外実験を実施した。それぞれの生物を 3 つの餌環境下：（１）魚類廃棄物と小麦粉の混合物、（２）魚類廃棄物に鶏糞添加、（３）鶏糞のみを提供（対照区）で井戸水 500 l を用いて 16 日間培養した。実験に用いられた全ての生物は魚類廃棄物に鶏糞を添加した実験区で高い増殖能を示した。

魚類廃棄物の利用可能性を確かめるため、ワムシの活発な増殖及び高い栄養価が確認された魚類廃棄物と小麦粉の混合物で培養した *B．rotundiformis*（SS-type）を用い、シロギス *Sillago japonica* の仔魚飼育実験を行った。濃縮クロレラで培養したワムシ給餌区（対照区）より、魚類廃棄物区で仔魚の活発な成長が見られた。

Chapter I

GENERAL INTRODUCTION

Aquaculture is the world's fastest growing food-producing sector, with an annual growth rate of 8.8% compared to 1.2% for capture fisheries and 2.8% for terrestrial meat production (FAO 2016). Today, aquaculture accounts for 44.1% (up to 74 million tonnes) of total annual world fish production (FAO 2016). In the recent past, the capture fishery has been relatively static (Figure 1-1) due to rampant ecological habitat degradation (Wilson et al. 2010), overexploitation (Smith et al., 2011) and effects of climate change (Brander, 2010). Consequently, the responsibility of increasing fish supply has shifted to aquaculture, which requires a fivefold increase by 2050 to match the expanding global fish demand (HLPE 2014). Regionally, the continental Asia is the giant in global aquaculture production while Africa and some tropical countries are yet to report significant statistics in the global aquaculture market (FAO 2016). Aquaculture productivity in Africa is frustrated by many challenges such as poor infrastructure, insufficient government budgets, unreliable supply and cost of pond inputs, limited expertise, biophysical limitations and diverse cultural and religious aspects (Brummet & Williams 2000; Ogello & Munguti 2016). Consequently, aquaculture in Africa mainly involves less productive small-scale production systems operated using locally available inputs and easily cultivable fishes (Prein & Ahmed 2000). With the projected human population at 2.4 billion in Africa by 2050 (UNDESA 2015), achieving sustainable nutritional security becomes an eminent challenge. Nonetheless, Africa's potential for aquaculture growth is prominent, with about 80% of its surface area being suitable for aquaculture (Brummet et al. 2008). So far, aquaculture's potential for quick production of fish has been widely reported, with vivid evidences of increased livelihood and economic growth for smallholder populations through production value chain linkages

(Brummet & Williams 2000; Brummet et al. 2008; Ogello & Munguti 2016). Today, aquaculture in Africa is chiefly constrained by the non-availability of cheap and stable technologies for producing adequate larval fish feeds and seeds in hatcheries. Therefore, the urgent need is to evolve predictable and cost-effective technologies for larval fish feed and seed production for cultivable fish species, which indeed is the general focus of this study.

With the increasing human population, escalating demand for fish and declining arable land, extensive aquaculture is no longer tenable, hence the need to embrace semi-intensive or, at best, intensive aquaculture in Africa. However, intensive aquaculture requires close control with constant supply of high quality feeds at all fish growth stages. Fish feeds account for the highest operational costs in aquaculture with protein sources being the most expensive ingredients (Tacon & Metian 2008). For marine fishes, the feed disparity is more critical at larval stages because the onset of exogenous feeding is synchronized with a primitive digestive system and small mouth gap, which limits successful first feeding and, subsequent survival (Yufera & Darias 2007). At this point, timely supply of adequate, nutritious and easily ingestible and digestible feeds e.g. live foods is important for growth and survival of the fish larvae. High larval fish mortalities have been reported when foods of sub-optimal nutritional values are employed (Lavens & Sorgeloos 1996). Marine fish larviculture is further complicated by the strict requirement of essential fatty acids e.g. docosahexaenoic acid (DHA), which marine fishes cannot naturally synthesize from precursor molecules such as eicosapentaenoic acid (EPA) or linolenic acid, unlike freshwater fishes (Masuda et al. 1998). As a limiting factor of growth and survival for marine larval fishes, DHA must be provided in the diet (Sargent et al. 1999), but at an additional production cost. Although most freshwater fish larvae accept artificial diets, lower growth and survival rates are

reported when such diets are used alone (Lavens & Sorgeloos 1996). Larval rearing of some freshwater ornamental fishes with small mouth gaps is also affected by the limited availability of suitable live foods (Lim et al. 2003). Generally, survival of fishes through the larval stages is the most sensitive phase that impedes both aquaculture progress and recruitment in the wild.

The major live food resources available in nature include single and multi-cell microorganisms, e.g. bacteria, algae, rotifers, copepods, cladocerans, mysids, *Artemia* etc. The copepods and cladocerans are nutritious live foods but are difficult to culture and, are larger in size than the mouth gap of most larval fishes (Lavens & Sorgeloos 1996). *Artemia* nauplii contains essential nutrients, but the supply depends on the natural ecological conditions, making their availability unpredictable and expensive (Sorgeloos et al. 2001). Among the rotifers, the demand for *Brachionus* spp. as first choice diet for larval fishes is overwhelming (Fushimi 1989; Hagiwara et al. 2007) due to their small size (90-350 µm) (Akazawa et al. 2008), capacity for nutritional enrichment (Watanabe et al. 1983) and high reproductive rates (Hagiwara et al. 2001). Naturally, brachionid rotifers exhibit a dual life cycle strategy, where amictic reproduction through cyclical parthenogenesis (Figure 1-2) prevails during favorable conditions, but stressful stimulus induces mictic response to form cysts (Wallace et al. 2015) that hatch via photochemical reactions (Hagiwara 1996). Because of the favorable rotifer attributes, the demand for rotifers has increased, prompting more investigations into convenient culture techniques to ensure consistent supply in hatcheries (Hirayama & Hagiwara 1995; Hagiwara et al. 1997). Apparently, the cost of production is the main factor that determines rotifer availability. Whereas labor cost is critical in advanced countries such as Japan, USA and in Europe, cost of infrastructure and equipment is the main concern in the developing countries. In the USA, cultures that produce about

3,000 rotifers ml^{-1} in traditional algae-based batch and continuous systems cost approximately US$ 0.46 and 0.29 per million rotifers day^{-1}, respectively (Bentley et al. 2008). In Europe, the average cost of rotifer cultures in algae and yeast-based batch systems is about US$ 0.42 per million rotifers (Komis 1992). In Japan, high-density rotifer cultures (about 20,000–30,000 ind ml^{-1}) and recently, ultra-high-density rotifer cultures (about 160,000 ind ml^{-1}) using algal pastes cost about US$ 0.05 per million rotifers (Fu et al. 1997; Yoshimatsu & Hossain 2014).

The common method of cultivation of rotifers is based on an established artificial food chain that involves production of high density microalgal liquid or pastes (>135 g l^{-1} dry weight, DW) as rotifer diet (Maruyama et al. 1997). However, production of microalgal pastes requires laborious and expensive investments (Slade & Bauen 2013) with challenges in maintenance (Borowitzka 1997), harvesting (Barros et al. 2015), and storage (Camacho-Rodrigues et al. 2015). Other limitations of algal production include risks of contamination, seasonal variations of food value and limited shelf life of about 2-3 weeks (Laing 1991). For example, enriched *Chlorella vulgaris* paste (V12) costs about US$ 9.0 l^{-1} with about 20 billion cells ml^{-1} (Chlorella Industry Co. Ltd., Japan) and lasts for about 2-3 weeks under normal refrigeration. Production of *Tetraselmis suecica* in 200 l batch cultures cost about US$ 300 kg^{-1} DW (Helm et al. 1979), while indoor cultures of various phytoplankton species cost USD$ 200 kg^{-1} DW (De Pauw & Persoone 1988). Alternatively, other diets such as preserved algae (Lubzens et al. 1995), yeast-based diets (Hirayama & Funamoto 1983), micro-encapsulated and Selco (Inve-Co. Ltd, Thailand) products (Lavens & Sorgeloos 1996) have been recommended to reduce the need for the expensive on-site algal production. However these alternatives have specific limitations that affect rotifer production processes in different ways. For example, the micro-particulate feeds have problems of settling, clumping, nutrient leach

and low digestibility (Lavens & Sorgeloos 1996), while the yeast-based diets are nutritionally deficient due to imbalance of bacterial flora (Watanabe et al. 1983). Meanwhile, artificial diets e.g. Selco products (Inve-Co. Ltd, Thailand) are costly especially for African hatcheries and perhaps some tropical countries which are potential future leaders in marine fish larviculture. Indeed, the inconsistent supply of microalgae limits continuous production of sufficient live foods and, sometimes disrupts fish seedling production programs in the microalgae-based hatcheries (Lubzens et al. 1995). To date, availability of cost-effective and stable microalgae replacement diet for rotifers is not explicitly clear, thus merits specific research priority.

In Kenya, despite a wide network of aquatic resources for aquaculture growth, aquaculture sector is still at nascent stages of development with only 280 hectares of potential aquaculture sites being utilized, of which 95% is subsistence level (Ogello & Munguti 2016). Even after the government-sponsored national fish farming program called 'Economic Stimulus Package' (ESP) that started in 2008, only 7% (~12,000 tonnes) increase in the national aquaculture production was achieved by 2010 (Munguti et al. 2014). Nonetheless, the ESP program triggered an explosive public interest in fish farming, causing critical shortages of fish feeds and seeds, which are so far the most significant bottlenecks to aquaculture development in Kenya. Today, most fish farmers employ inert diets e.g. powdered chicken egg yolk, milk and fish meal (*Rastrineobola argentea*) for larval fish rearing. However, these diets quickly compromise water quality and are not easily ingested or digested by the larval fish, unlike the live foods. Despite existence of *Artemia franciscana* at the Kenyan coast, optimizing it for local aquaculture is yet to be achieved as only about 10 kg of poor quality cysts is harvested each season (Ogello 2013). As a result, most Kenyan hatcheries, especially those handling marine fishes, continue to report significantly high larval fish mortalities due

to lack of suitable starter diet (Munguti et al. 2014). Therefore, developing predictable and cost-effective live food production technologies appears to be the key for unlocking aquaculture potential in Kenya and perhaps other tropical countries.

Over the years, small-scale fish farmers have used animal manures to boost the natural pond productivity mostly in the developing countries. However, the optimal use of animal manure is sometimes poorly understood, leading to stunted fish productivity (Kang'ombe et al. 2006). Moreover, the direct use of some manure e.g. of pigs, is restricted by the diverse cultural and religious aspects (Dadzie 1992; Ogello et al. 2013). Today, Kenya is faced with critical shortage of fish as local tilapia costs about US$ 3.2 kg^{-1}, leading to cheap tilapia imports from China (costing about US$ 1.5 kg^{-1}) to bridge the fish production deficit. In order to make fish more accessible and affordable to the local people and, to spur considerable socio-economic benefits in the society, there is need to embrace localized innovative fish production technologies as critical priorities. These technologies should start from addressing larval fish feed and seed production disparities as critical concerns. Research focus should shift to cost-effective and adaptable live food culture technologies using cheap locally available materials that would otherwise compromise environmental quality, if poorly disposed.

Due to the high global demand for fish and fish products, about 64 million tons of fishwastes e.g. heads, viscera, bones, fins, scales etc. are generated annually from both formal and informal fish industries all over the world (Rai et al. 2010). These wastes compromise environmental quality if poorly disposed, but are excellent substrates for microbial growth, some of which have probiotic properties such as *Bacillus sp*. and lactic acid bacteria (LAB) (Balcazar et al. 2008). Being filter feeders, rotifers can ingest a variety of feed types, including algae, yeast, bacteria, or inert foods such as microcapsules, detritus as well as their own feaces (Hino et al. 1997). Fishwastes also

contain essential bio-molecules e.g. essential fatty acids, proteins and vitamins (Sahena et al. 2009) that can be recovered for reuse in aquaculture. This study hypothesized that the essential bio-molecules found in the wastes (e.g. of fishwastes and chicken manure) can be transferred to the cultured fish larvae through bacteria-rotifer-fish, and/or microparticles-rotifer-fish pathways. In this way, nutritious rotifers can be produced cheaply without microalgae or the need for expensive enrichment emulsions. Literature is replete with evidences that rotifers play important roles as top predators in microbial food web (Starkweather et al. 1979), and that they also utilize soluble bacterial excrete (Hagiwara et al. 1994). Indeed, some bacteria strains cause better growth and nutritional effects on the rotifers (Yasuda & Taga 1980; Yu et al. 1988, 1989, 1994). So far, the use of bacteria as food source for rotifers is limited to experimental scale and, is mainly added as supplement to microalgae or baker's yeast (Lubzens et al. 1989). This study explores the feasibility of promoting simultaneous growth of bacterial biomass and rotifers in the same culture facilities, where the rotifers feed directly on the bacteria as the sole diet.

This dissertation is organized into five chapters. Chapter II reports about the population ecology study and biological traits of a common Kenyan freshwater rotifer species to advance the knowledge of native rotifer fauna, which is rather scanty in Kenya. Available data primarily describes the abundance and distribution of the rotifers in aquatic ecosystems, without specification of their biological traits, suitability for local aquaculture initiatives and, their ecological stability during changing climatic conditions. Except for a preliminary account of the rotifer, *Brachionus africanus* spp. *nov*., and *Itura symmetrica* spp. *nov*., by Segers et al. (1994), there is practically no account of other rotifer species in Kenya. Therefore, studies on the biological traits of Kenyan rotifer strains are useful to advance the local aquaculture. The Kenyan rotifer strain was

morphologically identified as *Brachionus angularis* before investigating its life table demography and reproductive traits at varying temperatures and food densities.

To promote mass culture of the Kenyan rotifer, *B. angularis*, for local aquaculture, a chicken manure extract (CME) technique was developed (Chapter III). The CME is cheap, eliminates organic loads from raw chicken manure and retains a blend of various growth hormones (Hakk et al. 2005; Hagiwara et al. 2014), making it more effective and socially attractive for live food production. CME is also a source of useful microflora for rotifer culture (Elsaidy et al. 2015). However, CME technology requires addition of microalgae for effective rotifer production, hence the need for a microalgae-less technology. The microalgae-less technology can be a major leap towards profitable aquaculture, especially in countries without modern infrastructure for high density microalgal production.

To provide an alternative to the freshly cultured microalgae, dried algae, e.g. *Nannochloropsis oculata* and *C. vulgaris*, which are storable at room temperature for up to 2 years, have been used as rotifer diet (Lubzens et al. 1995), but with frequent culture instabilities. These instabilities can be cured through various techniques such as chemical supplements e.g. gamma-aminobutyric acid (GABA) that has been used to stabilize rotifer cultures using fresh algae (Gallardo et al. 1997). For this reason, a hypothesis whether GABA supplementation can stabilize rotifer cultures with dried algae was formulated and tested in Chapter IV. GABA has been used to enhance rotifer reproduction during suboptimal conditions (Gallardo et al. 1997, 1999, 2000a). GABA is cheap, biodegradable and has positive effects even on non-targeted aquatic invertebrates (Garcia-Lavandeira et al. 2005). Dried algae can be transported and conveniently stored at room temperature for long periods, thus providing an opportunity to minimize the direct dependence on the freshly cultured algae. Despite the usability of

dried algae, they still have problems related to nutrient leach and low digestibility. Also, importation costs may be affected by fluctuating exchange rates. For this reason, studies are needed to discover live food production techniques without microalgae. In chapter V, fishwastes (heads) were used to develop a protocol for making a fishwaste diet (FWD) as a low-cost and stable diet for culturing the planktonic live foods. Fish heads are mostly inedible but contain appreciable amounts of essential nutrients (Khoddani et al. 2009). At the beginning of the study, wastes of *Cyprinus carpio* Linnaeus, 1758 were used to develop a FWD to culture the freshwater rotifer *B. angularis*. However, the cultures repeatedly crashed after 3-5 days. Instead, heads of the Chub mackerel *Scomber japonicus* Houttuyn, 1782 were used to develop FWD to culture the euryhaline rotifer, *Brachionus rotundiformis* (S-type) Perth strain, as a representative of the tropical rotifers (Chapter V.1). This study explored the feasibility of converting fishwastes into microbial biomass by promoting the simultaneous growth of the high density bacteria (at optimal carbon/ nitrogen ratio) and rotifers in the same culture facility, where rotifers directly ingest bacteria. So far, the FWD preparation protocol has been considered for patenting under the registration Number P00201609066 in Indonesia. In order to test the viability of the FWD technology for local aquaculture, fish heads of the cyprinid *Barbus altinalis* Boulenger 1900 and CME were blended at optimal carbon/ nitrogen ratio to develop FWD for mass culture of freshwater rotifers, copepods and cladocerans in outdoor tanks in Kenya (Chapter V.2). In chapter V.3, a preliminary experiment was conducted to determine the dietary value of the FWD-fed rotifers for larviculture. Here, the effect of the FWD-fed rotifers on the larval growth and development of Japanese whiting, *Sillago japonica* Temminck & Schlegel 1843 was determined. The general objective of this study was to establish economically attractive, stable and eco-friendly live food production technologies relevant for improving aquaculture sector in tropical countries without modern infrastructure for live food production.

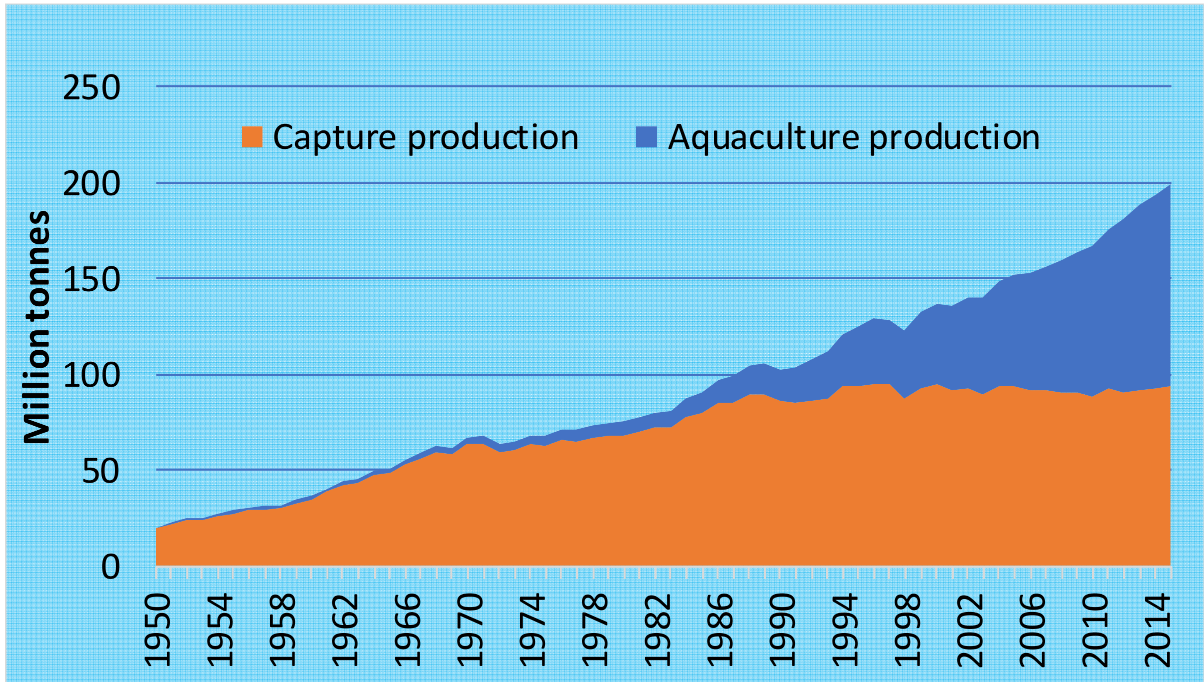

Figure 1-1: The status of world capture fisheries and aquaculture production since 1950 to 2015 (Adapted from FAO 2016)

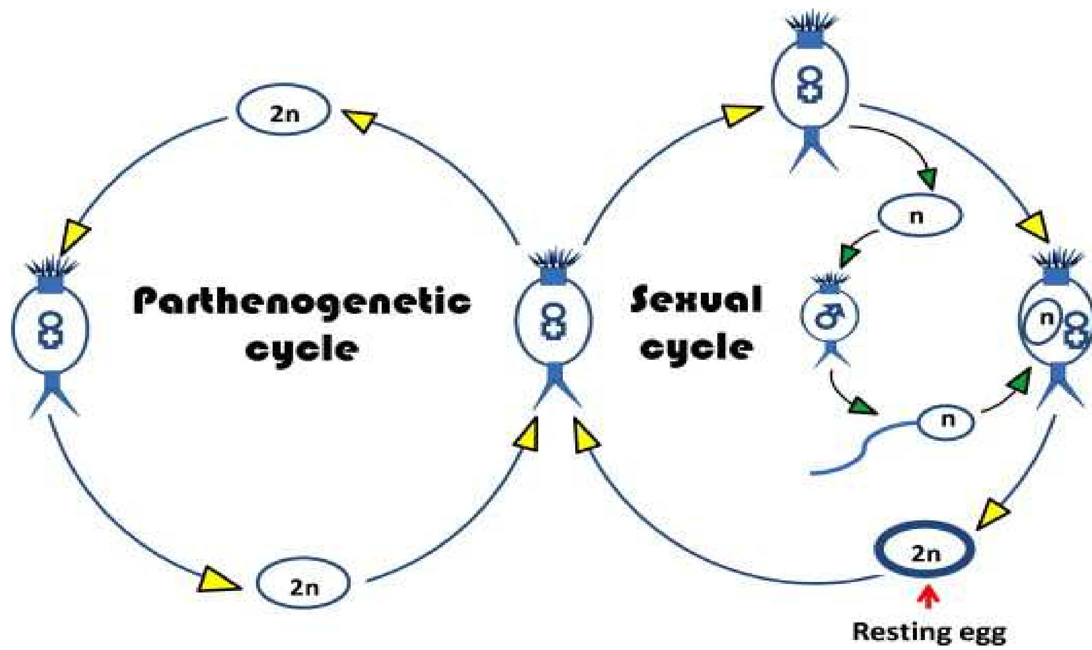

Figure 1-2: The dual life-cycle strategy of the monogonont rotifers where parthenogenetic and sexual reproduction cycles alternate depending on the prevailing ecological stimulus (Dahms et al. 2011)

Chapter II

BIOLOGICAL CHARACTERIZATION OF THE ROTIFER, *Brachionus angularis* FROM KENYA

Life table demography and population growth of the rotifer *Brachionus angularis* from Kenya: the influence of temperature and food density

Ogello et al. 2016: African Journal of Aquatic Science

Introduction

Life table is an informative tool commonly used to understand the demographic characteristics of zooplankton communities in their environments (Sarma & Nandini 2001; Xi et al. 2005, Ma et al. 2010). Life table demography provides information such as age-specific survivorship, fecundity, average lifespan, generation time, population growth rate and intrinsic rate of natural increase (Walz 1987; Sarma & Nandini 2001). Indeed, such information is critical to the understanding of the rotifer biological behaviors under the dynamic environmental conditions not only in their natural habitats but also in controlled culture facilities (Edmondson 1964, 1965).

The relationship between rotifer reproduction and ambient environmental factors is well documented (Edmondson 1965; Walz 1987; Pejler 1995; Sarma & Nandini 2001; Ma et al. 2010). Ecologically, salinity and temperature (Snell 1986), food quality and quantity (Sarma & Nandini 2001) are among the most important factors that

influence the growth (Yufera 2001), lifespan (King & Miracle 1980) and reproduction (Lubzens et al. 1985) of rotifers. For example, an increase in food density enhances rotifer egg production but reduces their lifespan (King & Miracle 1980). In natural populations, egg production rates of rotifers depend on both the present and the previous status of food supply (Dumont et al. 1995). However, if the environmental temperature varies, then the reproductive rate at any given food amount may also vary, perhaps due to the interaction of food and temperature (Edmondson 1964; Martinez et al. 1998). Indeed, temperature affects many parameters such as dissolved oxygen, algal growth, reproduction etc., which may individually or in combination affect the rotifer life histories (Edmondson 1965).

Studies have shown that increasing temperature accelerates the rate of egg hatching, reduces the life span and age at first reproduction of rotifers (Walz 1987; Stelzer 1998). Similarly, geographical location and other intrinsic factors may influence rotifer growth and reproductive responses (Sarma & Nandini 2001). Ma et al. (2010) reported significant effects of the interactions of temperature, food concentration and geographic location on the life expectancy at hatching, generation time, net reproductive rate and intrinsic rate of population increase of the freshwater rotifer *Brachionus calyciflorus*. Indeed, different life history parameters of the rotifer strains in their geographical sites suggest rotifer ecological adaptations to the local niches (Xi & Hu 2008).

Despite the numerous studies performed on rotifer species across the world (e.g. Dumont & De Ridder 1987; Sharma 2000; Ma et al. 2010; Ogata et al. 2011), there is a dearth of information regarding the identity and reproductive characteristics of the African freshwater rotifers. Most studies in Africa have focused primarily on the general abundance and diversity of rotifers in the ecosystems (De-Ridder 1987; Murray

2011; Sutherland et al. 2013) without specification of the individual life table demographics under the changing environmental stressors; hence their ecological stability and/or suitability for aquaculture is largely unknown. In Kenya, except for a preliminary account of the rotifer, *Brachionus africanus* sp. *nov*., and *Itura symmetrica* sp. *nov*., by Segers et al. (1994), there is no account of other rotifer species. Therefore, the objectives of this study were: 1) to identify a common Kenyan rotifer strain and 2), to investigate its reproductive and growth characteristics at various temperatures and food densities using individual life table and small-scale batch culture approaches.

Materials and methods

Rotifer and algal supply

The rotifer's resting eggs were collected from the sediments of freshwater fish ponds of Kenya Marine and Fisheries Research Institute (KMFRI), Kenya, located at 00°42'S; 034°47'E and transported to the Laboratory of Aquaculture Biology, Nagasaki University, Japan for further study. The eggs were hatched in a Petridish (diameter 45 mm) under constant illumination (115.5 µmols $^{-1}$ m^{-2}) and, the rotifers were acclimatized for one month at 25±1°C with daily feeding at ad libitum amount of *C. vulgaris*. The culture medium (pond water) was GF/C filtered (Whatman) and autoclave sterilized at 121°C for 15 min. The liquid *C. vulgaris* paste was regularly supplied by a Chlorella Company in Fukuoka, Japan and stored at 4°C for further use.

Rotifer identification

From the hatched rotifers, one amictic female was isolated based on observable features (Shiel 1995), and cultured for one month with daily feeding at ad libitum amount of *C. vulgaris* at 25±1°C to produce clones. From the clones, 20 individuals with visible and identifiable features were randomly isolated and subjected to further morphological analysis according to Shiel (1995). The rotifers were fixed with 10% formalin before analyzing the morphological characteristics under a compound microscope (Axioskop, Zeiss microscope, Germany) at ×40 magnification. The photo was taken and the size (lorica length and width) was measured using ocular micrometer.

Life table demography experiment

The life table demography of the rotifers was investigated at three temperatures i.e. 20, 25 and 30°C, and food densities i.e. 2.5×10^{5}, 2.5×10^{6} and 2.5×10^{7} ml^{-1} of *C. vulgaris*. The ranges of conditions represent the dynamic ecological status of the rotifers' natural habitat. To initiate individual culture of the rotifers, an amictic female from the stock culture was isolated and cultured at 25±1°C with daily feeding of *C. vulgaris* at ad libitum amount to establish clonal population. From this culture, about 250 amictic eggs were collected at logarithmic growth phase from the bottom of the culture container, and incubated in a Petridish (diameter 45 mm) under the same conditions as the stock cultures. Hatchlings (F_1) (<6 h) were employed in the study. Individual F_1 was introduced into each well of a 24-well polystyrene microplate (Iwaki, Japan) containing 1 ml of each food suspension at 2.5×10^{5}, 2.5×10^{6} and 2.5×10^{7}cells ml^{-1}. The rotifer cultures at each food concentration were incubated at 20, 25 and 30°C under complete darkness in 24 replicates. The rotifers were observed every 24 h under stereo

microscope at ×25 magnifications to assess survival of parental females and the neonate number. The numbers of the parental females alive and neonates were recorded before the parental females were transferred into a new well of the microplates containing fresh culture medium with appropriate food concentration. Dead individuals, if any, were enumerated and removed. This process was continued until the last parental female died. Based on the data collected, the life table parameters e.g. age-specific survivorship and fecundity, life expectancy at hatching (e_o), duration of first egg spawning (D_j), net reproductive rates (R_o), generation time (T), and intrinsic rate of natural population increase (r) were estimated using Lotka (1913) formulae as follows:

Net reproductive rate (R_o) $= \sum_{0}^{\infty} l_x m_x$

Generation time $(T) = \dfrac{\sum l_x m_x x}{R_o}$

Intrinsic rate of population increase (r): $\sum l_\chi m_\chi e^{-r_\chi} = 1$, Where x = time interval, lx = the probability of surviving to age x, m_x = the number of female offspring per female of age x born during the interval.

Population growth experiment

About 20 rotifers were selected and cultured for 1 week using fresh *C. vulgaris* at ad libitum amount. From this population, rotifers were selected and batch-cultured in 50 ml fresh culture medium at an initial density of 5 ind ml^{-1} in 300 ml glass jars under complete darkness without water exchange and aeration. Same food concentrations and temperature levels were tested in 3 replicates. The respective amounts of *C. chlorella* suspension were added to each jar daily. The population density of rotifers was defined by counting live rotifers in 1 ml from each replicate jar daily using a counting plate with

10% lugol fixation. The experiments were terminated after 14 days. The specific population growth rate (*r*) was calculated during the exponential growth phase using the formula $r = [\ln N_t - \ln N_o] / t$, where, N_o = initial population density, N_t = population density after the time (t), and t = 8 days.

Statistical analyses

Data were analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test of homogeneity of variances was used to test for the normality of the data. Two-way repeated ANOVA was performed to test the effects of temperature and food density on the life table variables and population density. The Log-Rank Test for groups was performed to explore the differences in age-specific survivorship among the treatments. The Tukey's HSD Post Hoc Test was performed to locate any significant differences at $p<0.05$.

Results

Rotifer identification

Morphologically, the Kenyan rotifer strain has two median occipital spines embedded on a pot-shaped lorica, while sub-median spines are either reduced or lacking in some individuals (Figure 2-1). The lorica length and width were 85.6±3.1 µm and 75.4±3.6 µm, respectively. These measurements have been compared with those of other known *B. angularis* strains from elsewhere (Table 2-1).

Life table demography

The age-specific survivorship and fecundity curves in relation to food density and temperature are presented in Figure 2-2. The age-specific survivorship was not affected by temperature (χ^2=4.60, df=2, p=0.10) or food density (χ^2=0.40, df=2, p=0.83), while the fecundity was affected by temperature (F=11.38, p=0.00) but not food density (F=2.03 p=0.13). The highest age-specific fecundity (2.11±0.07 offspring female $^{-1}$ day $^{-1}$) was obtained at 25°C with 2.5×10^{6} algal cells ml^{-1}. The rotifers older than 8 days continued to propagate at 25°C but not at 20 or 30°C. The age-specific fecundity peaked on day 4 at 20 and 25°C but one day earlier (day 3) at 30°C regardless of food density (Figure 2-2).

The values of the life demographic parameters in relation to different food densities and temperatures are summarized in Table 2-2. There was significant effect of temperature and food density on the life table demographic parameters (p<0.05). Life expectancy at hatching (e_o) was affected by temperature but not food density. The longest e_o (12.41±0.28 days) was realized at 25°C with 2.5×10^{6} algal cells ml^{-1}, while the shortest e_o (8.91±1.28 days) was obtained at 30°C with 2.5×10^{7} algal cells ml^{-1}. Meanwhile, there was no significant difference in e_o between 20 and 25°C (p=0.40). The duration of hatching to first egg spawning (D_j) significantly decreased with increasing temperature and food density. The longest D_j was 8.83±0.39 h at 20°C with 2.5×10^{5} algal cells ml^{-1}, while the shortest D_j was 2.86±0.21 h at 30°C with 2.5×10^{7} algal cells ml^{-1}. The highest net reproductive rate (R_o) (8.43±0.24 offspring female^{-1}) was obtained at 25°C with 2.5×10^{6} algal cells ml^{-1}, while the lowest R_o (3.01±0.05 offspring female^{-1}) was recorded at 30°C with 2.5×10^{5} cells ml^{-1} of the algae. The generation time (T) was longer at 20 and 30°C compared to 25°C. The shortest T (2.87±0.03 d) was observed at 25°C with 2.5×10^{6} algal cells ml^{-1}, while the longest T

(4.96±0.11 d) was realized at 30°C with 2.5×10^{5} algal cells ml^{-1}. The highest intrinsic rate of natural population increase (*r*) (0.74±0.02 d^{-1}) was obtained at 25°C with 2.5×10^{6} algal cells ml$^{-1,}$ while the lowest *r* (0.22±0.01 d^{-1}) was recorded at 30°C with 2.5×10^{5} algal cells ml^{-1}.

Population growth in the batch cultures

The population growth curves in relation to different temperatures and food densities are presented in Figure 2-3. The rotifer population density was significantly affected by temperature (*F*=5.28, *p*=0.01) and food density (*F*=5.89, *p*=0.00). Regardless of temperature, there was an earlier peak in the rotifer population densities at 2.5×10^{7} algal cells ml^{-1} but with lower population densities compared to the rest (Figure 2-3A, B and C). The highest population density (255.6±12.6 ind ml^{-1}) was obtained at 25°C with 2.5×10^{6} algal cells ml^{-1}(Figure 2-3D). The specific population growth rate (*r*) was significantly influenced by temperature (*F*=76.13, *p*=0.00), food density (*F*=109.02, *p*=0.00) and the interaction between them (*F*=26.32, *p*=0.00). The highest (0.49±0.01 d^{-1}) and the least (0.39±0.01 d^{-1}) *r* values were obtained at 25°C with 2.5×10^{6} algal cells ml^{-1} and at 20°C with 2.5×10^{5} algal cells ml^{-1}, respectively (Figure 2-4).

Table 2-1: Comparison of lorica length and width of the Kenyan strain of *B. angularis* with those of other *B. angularis* strains; the values are mean ± SD µm for (*n*) samples (in parenthesis)

Origin	Lorica length (*n*)	Lorica width (*n*)	Reference
Kenya	85.6±3.1 (20)	75.4±3.6 (20)	Present study
Laos	86.0±4.9 (20)	75.6±5.7 (20)	Ogata et al. 2011
China	130±7.0	115±7.0	Yin & Niu 2008
Germany	120 – 140	-	Leutbecher 2000
New Zealand	122	-	Gilbert & Burns 1999
France	127.8±5.9	-	Pourriot & Rougier 1997

SD, standard deviation

Table 2-2: The life table parameters of the Kenyan rotifer strain, *B. angularis* in relation to different food densities and temperatures. Shown are the life expectancy at hatching (e_o), duration of first spawning (D_j), net reproductive rate (R_o), generation time (*T*) and intrinsic rate of natural increase (*r*). Values are mean ± SD. Different superscripts in the same column indicate significant differences at $p<0.05$, Two-way ANOVA; Tukey HSD test, $n=3$, a>b>c>d>e>f>g>h>i

Temperature (°C)	Food density (cells ml^{-1})	Life table parameters				
		e_o (d)	D_j (h)	R_o Offspring/female	*T* (d)	*r*
20	2.5×10^5	$11.33±0.57^a$	$8.83±0.39^a$	$3.71±0.01^g$	$4.80±0.15^{abc}$	$0.27±0.09^g$
	2.5×10^6	$12.08±0.14^a$	$6.90±0.10^a$	$6.25±0.04^d$	$3.49±0.02^{fg}$	$0.52±0.05^d$
	2.5×10^7	$11.08±0.14^a$	$6.69±0.94^a$	$3.87±0.05^{fg}$	$4.49±0.05^d$	$0.30±0.01^f$
25	2.5×10^5	$11.33±0.57^a$	$5.21±0.99^b$	$7.80±0.09^b$	$2.91±0.05^{hi}$	$0.70±0.01^b$
	2.5×10^6	$12.41±0.28^a$	$5.04±0.54^b$	$8.43±0.24^a$	$2.87±0.03^i$	$0.74±0.02^a$
	2.5×10^7	$12.08±0.14^a$	$4.44±0.82^b$	$6.71±0.06^c$	$3.42±0.03^g$	$0.55±0.01^c$
30	2.5×10^5	$9.33±0.57^b$	$4.16±0.28^c$	$3.01±0.05^i$	$4.96±0.11^{ac}$	$0.22±0.00^i$
	2.5×10^6	$9.33±0.57^b$	$3.75±0.07^c$	$4.73±0.05^e$	$3.78±0.07^e$	$0.41±0.01^e$
	2.5×10^7	$8.91±1.28^b$	$2.86±0.21^c$	$3.15±0.21^{hi}$	$4.76±0.18^{cd}$	$0.24±0.01^{hi}$

SD, standard deviation

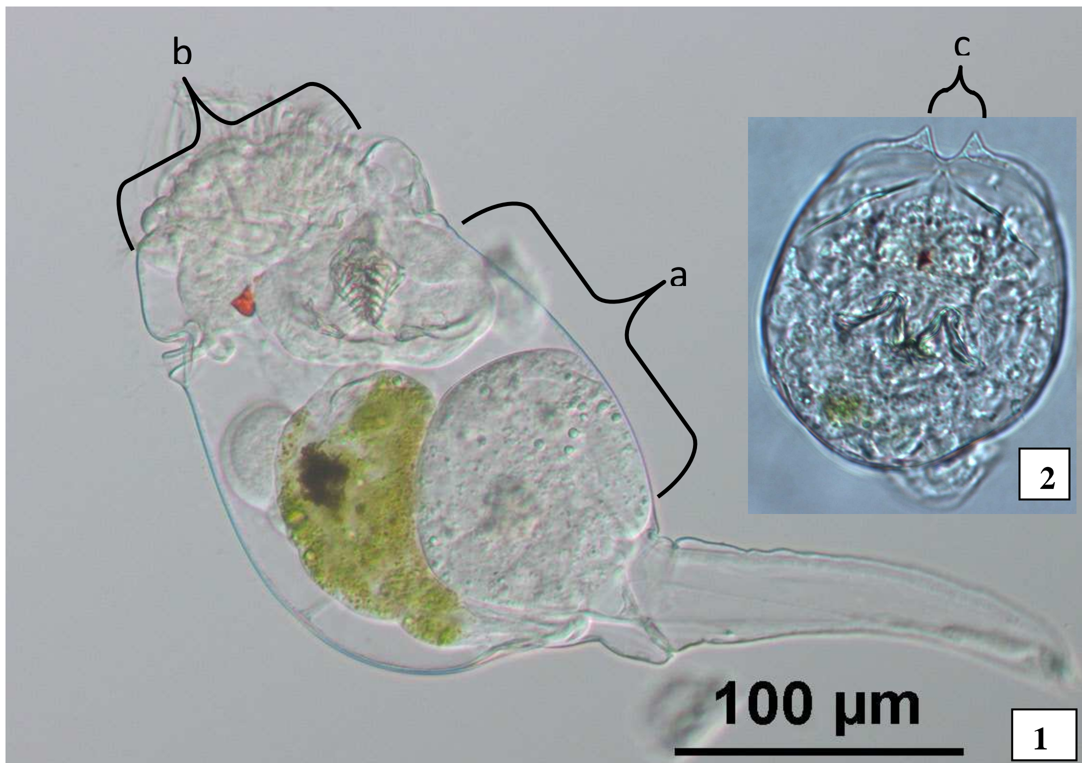

Figure 2-1: The image of live adult (1) and dead (2) *Brachionus angularis* isolated from a freshwater fish pond of Kenya Marine and Fisheries Research Institute (Axioskop, Zeiss microscope, Germany, ×40 magnification), a: lorica length, b: lorica width. The median occipital spines (c) are shown in the coronal area of the dead rotifer (image 2).

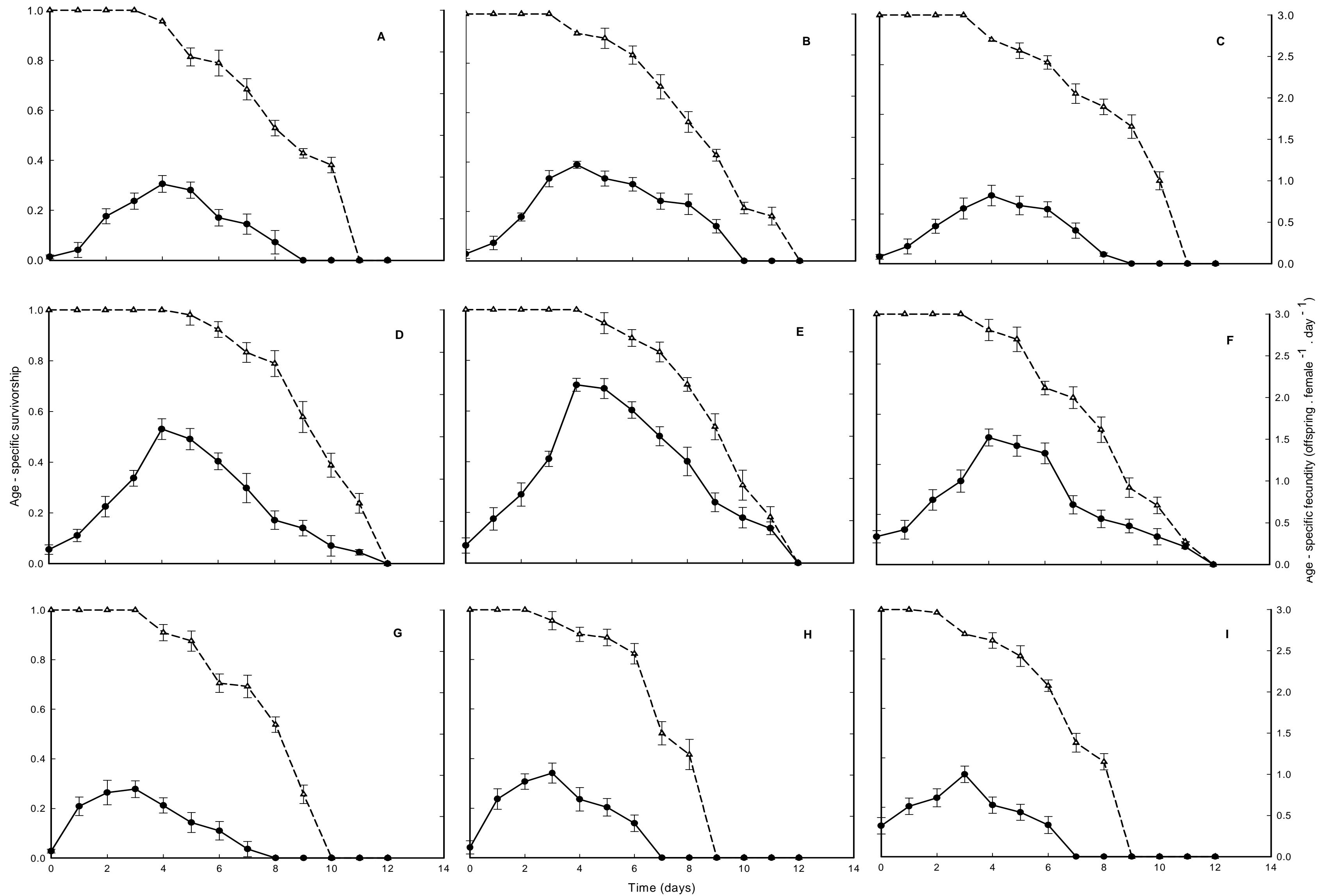

Figure 2-2: Age-specific survivorships (dotted lines) and fecundities (solid lines) of populations of the rotifer *B. angularis* cultured at three different temperatures and algal densities. Values represent mean ± SD. A: 20°C; 2.5×10^5, B: 20°C; 2.5×10^6, C: 20°C; 2.5×10^7, D: 25°C; 2.5×10^5, E: 25°C; 2.5×10^6, F: 25°C; 2.5×10^7, G: 30°C; 2.5×10^5, H: 30°C; 2.5×10^6, I: 30°C; 2.5×10^7 cells ml^{-1} of *C. vulgaris.* Survival: Log-rank test, $p>0.05$, $n=24$; Fecundity: 2-way ANOVA; $p<0.01$, $n=24$

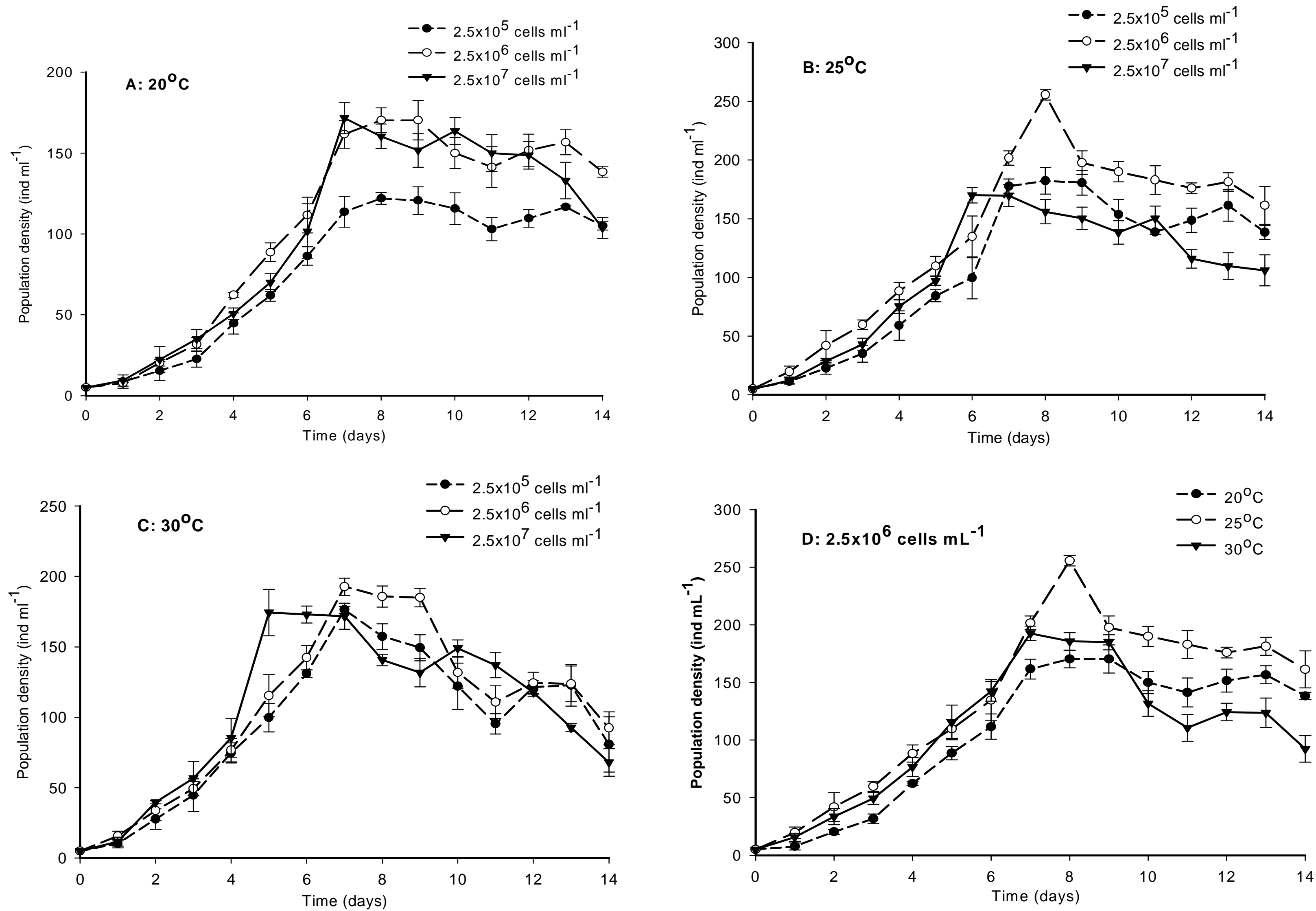

Figure 2-3: Population density growth curves of the rotifer *B. angularis* in relation to different temperatures and *C. vulgaris* densities. Values are means ± SD. 2-way ANOVA, Tukey HSD test, n=3

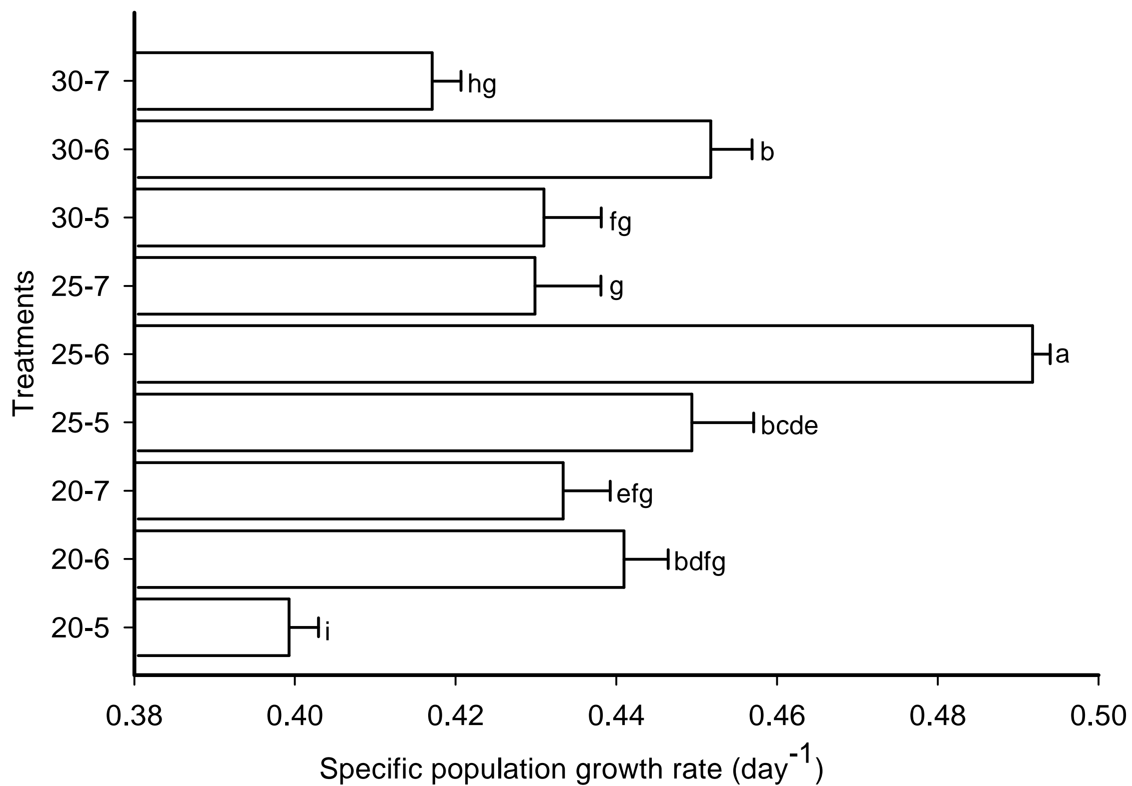

Figure 2-4: Population specific growth rate day $^{-1}$ for *B. angularis* in relation to temperature and food densities. Shown are the means ± SD, 2-way ANOVA, Tukeys HSD test, $p<0.001$, $n=3$. Different letters indicate significant differences at $p<0.05$; a>b>c>d>e>f>g>h>i; Treatments: 20, 25 and 30°C with 5=2.5×10^5, 6=2.5×10^6 and 7= 2.5×10^7 algal cells ml^{-1}

Discussion

Environmental factors such as changing food density and temperature have been found to influence the biological structures of the aquatic zooplankton communities (Edmondson 1964, 1965; Pejler 1995; Ma et al. 2010). However, the influence of these factors on the life table demography of the rotifers from African freshwater ecosystems has not been reported in literature. This study identified the Kenyan rotifer sample as *B. angularis*, and reported the effects of changing temperature and food density on its life table demography and biological characteristics. The morphological observations e.g. two median occipital spines with either reduced or lacking sub-median spines are consistent with descriptions of the rotifer, *B. angularis* by Shiel (1995). The rotifer strain has a smaller body size (Table 2-1) compared to other known *B. angularis* such as the Laos strain, which is so far considered suitable for small-mouth freshwater fish larvae (Ogata et al. 2011). Based on this finding, the Kenyan strain of *B. angularis* also qualifies as an appropriate live food for small-mouth freshwater larval fishes. The suitability of small rotifers for aquaculture has been extensively discussed in literature (e.g. Hagiwara et al. 1995a; Wullur et al. 2009). The size variations of the Kenyan rotifer, *B. angularis* compared to similar species from elsewhere (Table 2-1) could be linked to the ecological adaptations to the local geographical niches as reported in literature (Hu et al. 2003; Xi & Hu 2008).

The temperature and food density variations did not affect the age-specific survivorship, suggesting that rotifer survival was affected by aging. The longer life expectancy at lower temperatures (20 and 25°C) could have been due to decreased metabolic rate while the shorter life expectancy at 30°C could have been due to the accumulated thermo-physiological stress. Sarma & Rao (1990) observed a decrease in life expectancy of brachionid rotifers under increased temperature and food density. At 20 and 30°C, rotifers older than 8 days were not fecund (Figure 2-2), thus explain the low fecundity at such

conditions. However, rotifers older than 8 days were fecund at 25°C. Other studies have reported that the fecundity of rotifers can be affected by the ciliate epibiont-zooplankton interactions that occur in the cultures (Gilbert & Schroder 2003). However, the ciliate epibiont-rotifer interactions were not determined in this study. There was higher age-specific fecundity at 25°C, which was also reported for the Laos's strain of *B. angularis* by Ogata et al. (2011).

The duration of first egg spawning might have been delayed at 20°C by slower ontogenic phases necessary to hasten reproduction (Galkovskaya 1987; Walz 1987), while faster ontogenetic development phases under high temperature (Athibai & Sanoamuang 2008) could explain the shorter duration of first egg spawning observed at 30°C. Other studies have reported longer pre-reproductive phases at 20°C for rotifers (Ogata et al. 2011), and shorter duration of embryonic development at 25 and 30°C (Walz 1987; Hu et al. 2003; Xi & Hu 2008). Baker (1979) reported 8 to 12 h as the duration of first egg spawning for freshwater rotifers *B. angularis* and *B. calyciflorus* cultured at 20°C, which is comparable to the findings of the current study. The higher net reproductive rate at 25°C with 2.5×10^{6} algal cells ml^{-1} could have been due to the continuous reproduction of the older rotifers unlike in the other culture conditions. The findings resembled those of Ma et al. (2010) who found a range of net reproductive rates of up to 5 to 23 offspring female $^{-1}$ for the freshwater *B. calyciflorus* cultured between 18 and 28°C in different geographic populations. According to Edmondson (1964, 1965), the interactions of temperature and food densities affect the reproductive rate of rotifers. Pourriot & Rougier (1997) reported that ecological adaptations may cause different reproductive rates among species. The prolonged generation time at 20°C could have been caused by the longer duration of fist egg spawning. There was longer generation time at 30°C perhaps due to preference of survival to reproduction. According to Chen & Cuijuan (2015), a tradeoff exists between the energy required for maintenance and

for reproduction and growth. Sarma & Nandini (2001) also reported that generation time of rotifers decreases with increased food density and temperature. The findings of the current study are comparable to those of Galkovskaya (1987) and Ma et al. (2010) who reported generation time of 2 to 3 days for *B. calyciflorus* cultured at 27°C with 3.0×10^6 algal cells ml^{-1} of *C. vulgaris*. The higher intrinsic rate of population increase at 25°C with 2.5×10^6 algal cells ml^{-1} could be attributed to higher reproductive rates and shorter generation time of rotifers at that condition. Warmer temperatures cause shorter periods of embryonic development and thus enhance intrinsic rate of population increase at optimal food conditions (Xi & Hu 2008). Other studies suggest that genetic adaptations to local environmental pressures could affect the rotifer intrinsic rate of increase (Gilbert 2003; Ma et al. 2010). In general, temperature affects many parameters such as dissolved oxygen and biochemical reactions, which may individually, or in combination affect the rotifer life histories in any habitat (Edmondson 1965; Walz 1987).

The highest population density observed on day 8 at 25°C with 2.5×10^6 algal cells ml^{-1} of *C. vulgaris* (Figure 2-3B) suggested an occurrence of simultaneous reproduction of the old and new rotifer cohorts. The earlier peaks noted at 30°C (Figure 2-3C) were probably thermal-regulated and could have been due to the shift of the reproduction maxima to the earliest stages of maturity, and shorter duration of first egg spawning. This coincided with earlier peaks observed in the individual culture experiments under similar conditions. The population density quickly declined at 30°C, suggesting that the rotifers may have switched to mixis phase at this stressing condition. Mixis investment is likely to reduce the short-term fitness of rotifer clones (Chen & Cuijuan 2015), as more energy is used to fertilize mictic female to lay resting egg than for an amictic female to produce a daughter (Gilbert 2010). At such situations, life expectancy and fecundity are usually reduced (Snell & King 1977), hence lowers population density. Faster deterioration of culture medium in this condition

may have also contributed to the observed results because there was no regular water exchange.

The higher rate of specific population growth at 25°C with 2.5×10^{6} algal cells ml^{-1} was attributed to the high reproductive rate, longer life expectancy, shorter duration of juvenile phase and shorter generation time. Even though warmer temperatures with optimal food conditions enhance rotifer growth rates, exceeded thermal tolerance can cause rotifer culture crash (Stelzer 1998). The growth rates for most brachionid rotifers range from 0.2 to 2.0 (Sarma & Nandini 2001). The specific growth rate value (0.39-0.49 d^{-1}) reported in this study is within the known range reported in literature. The Kenyan rotifer strain of *B. angularis* has smaller size (i.e. lorica length: 85.6±3.1 µm and width 75.4±3.6 µm), making it convenient for freshwater fish larval rearing, especially those with smaller mouth gaps such as the larvae of gold fish, *Carassius auratus* (Lim et al. 2003). The rotifer reproduces optimally at 25°C with 2.5×10^{6} algal cells ml^{-1} of *C. vulgaris*. Since microalgal pastes are expensive and laborious to produce, cheaper rotifer food production technologies should be investigated to support the mass culture of this rotifer strain, for local aquaculture initiatives.

Chapter III

CHICKEN MANURE EXTRACT (CME) ENHANCES REPRODUCTION OF THE ROTIFER, *Brachionus angularis*

Effects of chicken manure extract on the population growth, mixis induction and body size of the Kenyan rotifer strain, *Brachionus angularis*

Ogello & Hagiwara 2015: Asian Fisheries Science Journal

Introduction

The monogonont rotifer, *Brachionus angularis* is potentially an excellent live food for small-mouth freshwater fish larvae due to their small size and high reproduction rate (Ogata et al. 2011; Ogello et al. 2016). The reproductive characteristics of *B. angularis* are comparable to those of the rotifer, *Brachionus plicatilis* complex, which is an important zooplankton widely used as initial food in marine larviculture (Hirayama & Hagiwara 1995). However, production of sufficient quantities of *B. angularis* for the larviculture of commercially important fishes is a major problem. Locally available materials can be used to culture sufficient quantities of rotifers for local aquaculture. For example, chicken manure has been used to augment the pond biological productivity in semi-intensive fish farming practices (Kang'ombe et al. 2006; Elsaidy et al. 2015). However, the optimal quantity and mode of application to achieve pond maximum productivity is poorly understood especially

in African countries where semi-intensive fish farming is commonly practiced. Also, raw chicken manure contains additional organic loads and litters, which quicken the depletion of dissolved oxygen in ponds, thus compromises fish health and increases cost of production. This study explores on the use of chicken manure extract (CME) technique, which eliminates organic debris or loads and other pathogenic contaminants that can compromise fish quality. Meanwhile, the CME retains essential nutrients that can be useful for live food production.

The effects of the chicken manure are linked to the actions of different sex hormones present in them (Finlay-Moore et al. 2000). For example, egg-laying chicken excrete about 50 and 250 ng g^{-1} dry manure day^{-1} of 17β-estradiol and testosterone respectively (Shemesh & Shore, 1994; Hakk et al. 2005), while chicken litter contains between 1 to 904 ng g^{-1} (Shore et al. 1993; Bevacqua et al. 2011) of 17β-estradiol and 0.05 to 254 ng g^{-1} of testosterone (Jenkins et al. 2006). Such chemicals are used by zooplankton to regulate their reproduction, induce predator defenses and accomplish selective foraging in their respective habitats (Gilbert 1966). There are scientific evidences that sex hormones influence the population growth, mixis induction and body size of zooplankton species (Preston et al. 2000; Yang & Snell 2010). Gallardo et al. (1997) reported a 2.3 fold increase in mictic female production of *B. plicatilis* exposed to 50 mg l^{-1} of 17β-estradiol. Up to 7.8 fold enhancement of resting egg production was reported for *Brachionus manjavacas* exposed to 5 mg l^{-1}, and complete inhibition at 14 mg l^{-1} of progesterone (Snell & DesRosiers 2008). Huang et al. (2012) reported that 1 µg l^{-1} of 17β-estradiol produced optimal demographic parameters for the freshwater rotifer *Brachionus calyciflorus*. A significant increase in amictic population growth rate of *B. calyciflorus* was observed under the influence of combined progesterone and estradiol hormones (Yang & Snell 2010). Apparently, such studies are limited for the rotifer *B. angularis*, which are potential live food for small-mouth

freshwater larval fishes. Therefore, a hypothesis whether CME can enhance mass reproduction of the rotifer *B. angularis* was formulated. To test this hypothesis, a CME was prepared and its effects on the population growth, mixis induction and body size of the Kenyan rotifer strain, *B. angularis* was determined.

Materials and Methods

Rotifers

The stock culture of the Kenyan strain rotifer *B. angularis* was available in the laboratory. From this stock, about 200 rotifers were isolated and acclimatized for 4 days in 100 ml culture medium at 25±1°C using ad libitum amount of *Chlorella vulgaris*. The culture medium (pond water) was GF/C filtered (Whatman) prior to autoclave sterilization at 121°C for 15 min. From this population, egg bearing rotifers were shaken in a screw-capped bottle, and the detached amictic eggs were collected and hatched in a petri dish (50 mm diameter) with similar *C. vulgaris* suspensions as stated previously. Twenty hours old hatched rotifer clones were employed in this study.

Preparations of chicken manure extract (CME)

One kilogram of fermented chicken manure (Shitama Inc., Fukuoka, Japan) was mixed with 10 g of fossil coral powder (Coral international Co. Ltd., Okinawa, Japan). This mixture was boiled in 5 l of sterilized pond water for 40 - 50 min and then kept overnight at room temperature. The supernatant liquid was filtered off the sludge using nylon net of pore size 100 μm. The sludge was re-filtered and the liquid was mixed with the previous

supernatant. The CME was preserved at 4°C for subsequent use during the experiment. The quantities of sex hormones present in the CME are summarized in Table 3-1.

Experimental design

Bioassay experiment was conducted at nominal concentrations of CME i.e. 0.0 (control), 0.5, 1.0, 2.0 and 3 ml l^{-1}, which were obtained after a series of prior range finding tests in the laboratory. Each CME concentration and the control were triplicated. Thirty single egg bearing amictic females were placed in different 100 ml glass jars containing 20 ml of culture medium. The CME concentrations were introduced into the glass jars once at the start of experiment without exchanging water. All the rotifers were incubated at 25±1°C under total darkness with daily feeding on *C. vulgaris* at 2.5×10^{6} cells ml^{-1} (Ogello et al. 2016) for 7 days. The density of *C. vulgaris* was monitored twice daily and adjusted accordingly to maintain a constant density of 2.5×10^{6} algal cells ml^{-1} in the culture medium. From the first day, the population density of rotifers was daily defined by counting all live rotifers in three-1 ml from each replicate jar using a graduated counting plate with lugol fixation, under stereo microscope at × 25 magnification. In the same sample, the number of amictic females and fertilized mictic females were counted based on the type of egg they carried (Hagiwara et al. 1988). At the end of experiment (day 7), the total number of resting eggs were counted in each replicate jar. The population density of all live rotifers and amictic females were reported as ind ml^{-1} ± standard deviation of the three replicates in each treatment. The rate of fertilized mictic females (%) was calculated using the formula:

$$= \left[\frac{fertilized\ mictic\ females}{unfertlized\ mictic\ females + fertilized\ mictic\ females} \right] \text{X}\,100$$

The specific population growth rate of rotifers was calculated as $r = [\ln N_t - \ln N_o] / t$, where N_t= number of individuals in culture after t days, N_o = initial number of individuals, and t = time in days (7 days). Upon termination of the experiment, the lorica length and width of 10 rotifers randomly sampled from the replicate treatments were measured using microscope (Axioskop, Zeiss, Germany) with an ocular micrometer at $\times$ 40 magnifications. The rotifers were fixed with 10% formalin before taking the measurements.

Data analysis

The data were analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015) after test for homogeneity of variance using the Bartlett test. Repeated ANOVA measures were performed to determine the effects of CME the on the rotifer population density and growth rate. Log_{10} transformation was performed on the proportions of ovigerous fertilized mictic females, and the number of resting eggs before performing the ANOVA tests to identify significant differences among the treatment means. Multiple comparisons were conducted using Tukey HSD test to determine where the differences were situated at $p<0.05$.

Results

The total rotifer population density was significantly affected by the days of culture (F=40.02, p=0.00), CME (F=99.72, p=0.00) and their interaction (F=10.94, p=0.00). The density of amictic females were also affected by days of culture (F=42.17, p=0.00), CME (F=78.51, p=0.00) and their interaction (F=7.81, p=0.00). Both the total population density (Figure 3-1) and that of amictic females (Figure 3-2) were significantly higher ($p<0.05$) at

2.0 ml l^{-1} of CME than other CME concentrations and the control from day 4 – 7. However, there was no significant difference in the population density ($p>0.05$) among the control, 0.5 and 1.0 ml $l^{-1}$of CME each day, from day 4 – 7. The CME did not affect the total population density and that of amictic females from day 1–3 ($p>0.05$). The total population density and amictic females reduced significantly from day 4 – 7 at 3.0 ml l^{-1} of CME. The highest total population density (248.7±16.4 ind ml^{-1}) (Figure 3-1) and amictic females (183.3±8.5 ind ml^{-1}) (Figure 3-2) were obtained on day 5 at 2.0 ml l^{-1} of CME. The fertilization rate of mictic females significantly increased with days of culture ($F=26.65$, $p=0.00$), where up to 32.9±2.3 % of mictic females were fertilized on day 7 in the control experiment (Figure 3-3). However, CME significantly ($p<0.05$) reduced the fertilization rate of mictic females from day 5 – 7 (Figure 3-3).

The population specific growth rate (SGR) was significantly affected by CME ($F=27.85$, $p=0.00$). The SGR was higher (0.71±0.01 day $^{-1}$) at 2.0 ml l^{-1} than the control, 0.5 and 1.0 ml l^{-1} of CME ($p<0.02$) but reduced significantly to 0.51±0.03 day $^{-1}$ at 3.0 ml l^{-1} of CME ($p=0.00$) (Figure 3-4). CME significantly affected the mean number of resting eggs ($F=37.14$, $p=0.00$), where the number of resting eggs reduced with the increasing CME concentrations. At 2.0 and 3.0 ml $l^{-1}$of CME, the resting eggs were ×1.7 and ×3.1 lower than in the control, respectively (Figure 3-4). The CME significantly increased the lorica length ($F=7.042$, $p=0.00$) but not the width ($F=1.55$, $p=0.20$) At day 7, the lorica length of the rotifers exposed to 3.0 ml l $^{-1}$ of CME was significantly ($p<0.03$) higher than those of other treatments, which were similar to each other (Figure 3-5).

Table 3-1: Sex hormones ($\mu g \cdot kg^{-1}$) in chicken manure extract (CME); ND = not determined. Adapted from Hagiwara et al. 2014

Sex hormones	CME	Limit of detection
17 α-estradiol	0.16 ± 0.04	0.05
17 β-estradiol	0.53 ± 0.35	0.05
Estron	2.20 ± 1.65	0.05
Estriol	ND	0.05
Progesterone	ND	0.1
Testosterone	ND	0.5
Methyltestosterone	ND	0.5

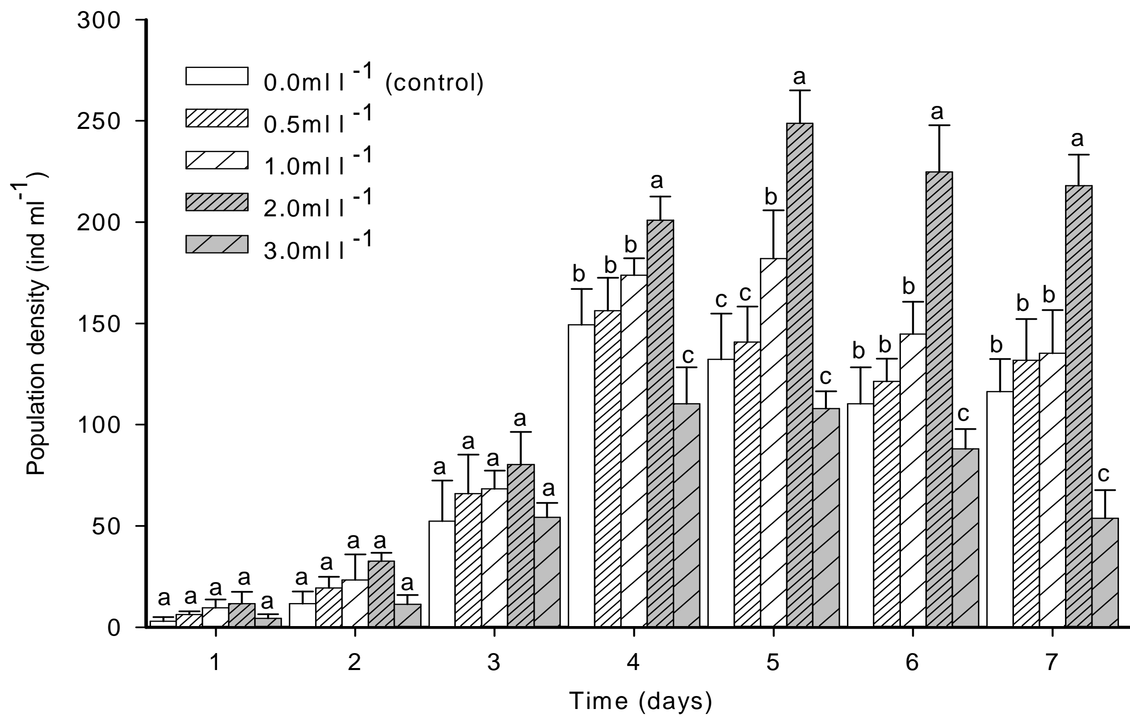

Figure 3-1: Effects of CME and days of culture on the total population density of *B. angularis*. Data shown are means ± SD; two-way ANOVA, Tukey HSD test, n=3. Different letters on top of the bars each day denote significant differences at p<0.05; a>b>c

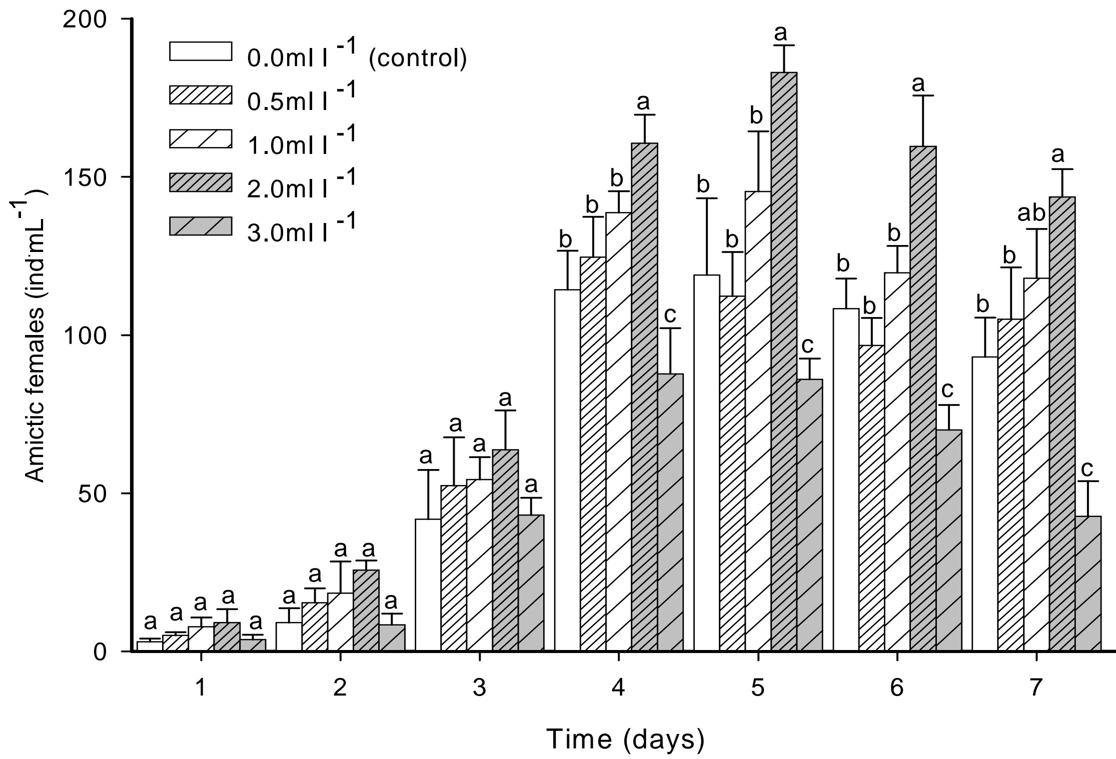

Figure 3-2: Effects of CME and days of culture on the population density of amictic females of the rotifer *B. angularis*. Data shown are means ± SD; two-way ANOVA, Tukey HSD test, n=3. Different letters on top of the bars each day denote significant differences at $p<0.05$; a>b>c

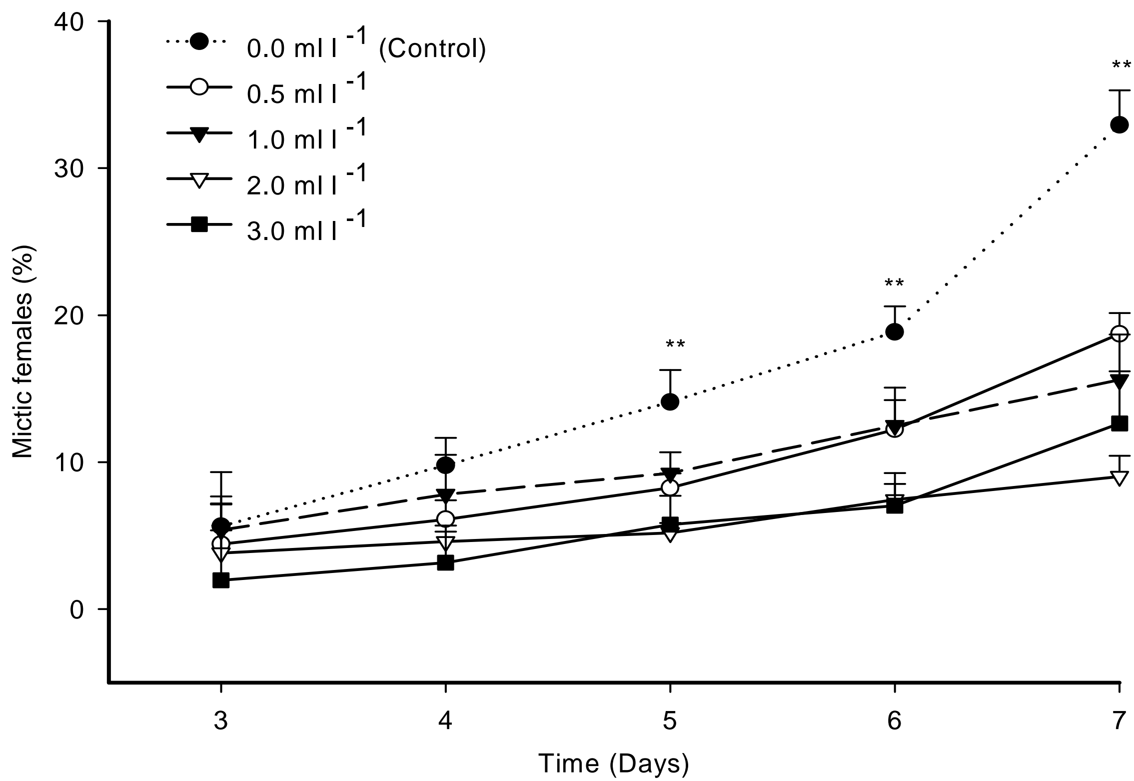

Figure 3-3: Effects of CME and days of culture on the fertilized mictic female production rate of the rotifer *B. angularis*. Data shown are means ± SD; two-way ANOVA, Tukey HSD test, n=3. **each day denotes significant difference at $p<0.05$

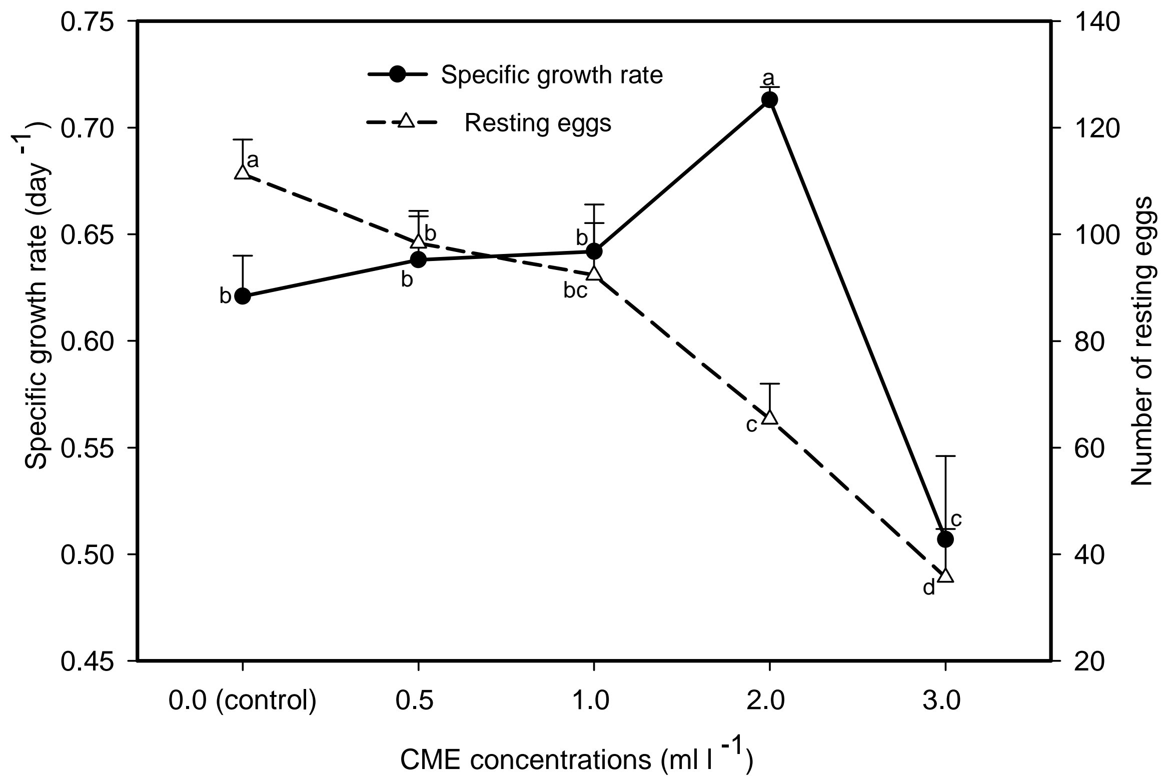

Figure 3-4: Effects of CME on specific population growth rate and resting egg production of the rotifer *B. angularis* as at day 7 of culture. Data shown are means ± SD; one-way ANOVA, different letters in each curve denote significant differences at $p<0.05$; Tukey HSD test, $n=3$; a>b>c>d

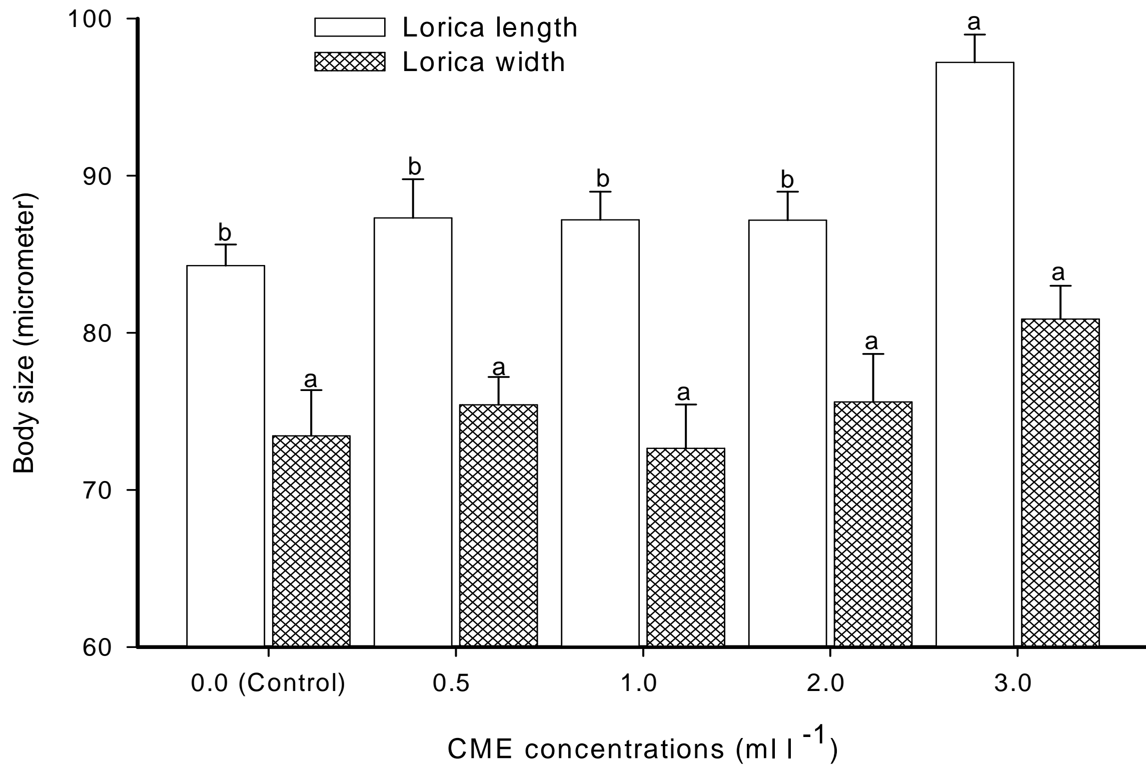

Figure 3-5: Effect of different concentrations of CME on the body size of the rotifer *B. angularis.* The values represent mean lorica length and width (µm) ± SD as at day 7 of culture; one-way ANOVA, Tukey HSD test. Different letters for each lorica size denote significant difference at $p<0.05$, a>b, $n=10$

Discussion

Application of organic manure enhances the biological productivity in the fishponds (Elsaidy et al. 2015). The current study has demonstrated the influence of CME on the population density, mictic induction, and specific growth rate and body size of the rotifer, *B. angularis*. Application of 2.0 ml l^{-1} of CME prolonged the rotifer population density and amictic female population, suggesting that only specific amount of CME is effective to increase parthenogenetic reproduction of this rotifer species. This observation is consistent with the persistent nature of growth promoting compounds contained in the CME (Shemesh & Shore 1994). According to Pentikainen et al. (2006), the estradiol compounds act as growth hormone for the female reproductive tissues, hence resulting in high viability of oocytes. The biomolecules in the CME can be recovered and applied in the production of the rotifer *B. angularis*, where harvesting can be done on day 5. In another study, Hagiwara et al. (2016) reported that 2 ml l^{-1} of CME was optimum for active population growth of *Tigriopus japonicus* and *Diaphanosoma celebensis* regardless of their developmental stages. The active population growth of the zooplankton is linked to the water-soluble natural 17β-estradiol contained in the chicken manure extract (Hakk et al. 2005). It is probable to suggest that CME acts like a capsule of hormones that synergistically augments each other to produce stunning growth and reproduction effects on rotifers. Furthermore, there are literature evidences indicating that chicken manure as an excellent substrate for the heterotrophic production of probiotic bacteria e.g. *Bacillus* sp. that can be utilized by rotifers to improve their reproduction and survival (Rapatsa & Moyo 2013; Elsaidy et al. 2015). However, this study did not characterize the bacterial flora present in the culture media, thus require further investigation.

CME suppressed rotifer mixis stimulus but favored parthenogenetic propagation. Mixis investment reduces rotifer life expectancy, fecundity and population density (Snell &

King 1977). Preston et al. (2000) identified estrogen agonist chemicals such as nonylphenol in the freshwater *B. calyciflorus*, which have no effect on amictic reproduction but reduces mictic induction and stops fertilization rate at 50 µg l^{-1}. Similar results i.e. reduction of fertilization rate of females was reported for *B. plicatilis* under the influence of 30 - 50 µg l^{-1} of nonylphenol (Marcial 2004). Even though these hormones were not tested in their pure form, it is suspected that their presence could probably explain the reduced mixis under the influence of CME in the current study. However, further investigations should unravel this speculation. According to Gilbert (1963), it is the environmental stimuli, and not internal rhythmic cycle that causes mictic female production in the rotifer *B. calyciflorus*. Mictic reproduction is an inherently more complicated process, which depends on the fertility of both male and female and, on their successful mating behavior (Sugumar & Munuswamy 2006). Thus, rotifer mictic reproduction is likely to be a factor of signals rather than amictic reproduction (Preston et al. 2000).

The reduced production of resting eggs (Figure 3-4) could have been a direct consequence of suppressed fertilization of mictic females. Rotifer resting egg production is believed to be the most sensitive endpoint for a number of chemicals (Preston et al. 2000) probably due to the complexity of rotifer mictic reproduction, which integrates toxicant effects over the full life cycle (Preston & Snell 2001). The higher specific population growth rate (0.71±0.01 day $^{-1}$) obtained at 2.0 ml l^{-1} of CME (Figure 3-4) was probably due to higher parthenogenetic reproduction at that condition. This value is comparable to the findings of Yang &Snell (2010) for freshwater *B. calyciflorus* exposed to 1,000 µg l^{-1} of progesterone and estradiol hormones. The increase in lorica length at 3.0 ml l^{-1} of CME (Figure 3-5) coincided with reduced population density, therefore reduced competition for food could have enabled the rotifers to achieve their full growth potentials, and not directly influenced by CME. However, it is not clear why the lorica width was unaffected by CME.

Gallardo et al. (1997) observed reduced body size of the rotifer *B. plicatilis* with different hormonal treatments and attributed the results to high competition for food caused by increased population densities.

Even though the observed reproductive effects of CME in this study seem consistent with the influence of sex hormones e.g.17β-estradiol, testosterone, oestrogens and androgens that are significantly represented in chicken manure (Shemesh & Shore 1994; Hakk et al. 2005), it remains speculative that such effects were related to endocrine disruption mechanisms because no molecular essay was done to authenticate these observations. According to Preston et al. (2000), in the absence of a molecular assay for interactions with a rotifer endocrine receptor, the observed effects should be considered as consistent with those of endocrine disruption mechanisms but not as proof of such mechanisms. Future studies should investigate presence of the specific hormone receptor molecules in the rotifers.

The CME technique eliminates organic loads, litters and perhaps disease causing pathogens in the raw chicken manure, while retaining essential compounds, making it more effective and socially attractive for improving biological productivity of the culture facilities. CME is cheap and blends varieties of hormones (Hakk et al. 2005; Hagiwara et al. 2014) hence eliminates the need to purchase commercial growth promoting compounds. The results of this study show that 2.0 ml l^{-1} of CME is optimal for enhanced population density, amictic female population and specific growth rate of the Kenyan rotifer strain *B. angularis*. The optimal CME concentration is most effective on day 5 of culture and does not increase the rotifer size, hence applicable in mass culture of the rotifer, *B. angularis* for freshwater aquaculture. However, CME still requires addition of algae for optimal growth. Further studies should focus on low-cost rotifer culture techniques to spur aquaculture growth especially in the developing countries, where fish consumption is relatively low.

CHAPTER IV

USING DRIED ALGAL DIETS FOR MASS CULTURE OF ROTIFERS, WITH gamma-aminobutyric ACID (GABA) SUPPLIMENTATION

Culturing *Brachionus rotundiformis* Tschugunoff (S-type) using dried foods: application of gamma-aminobutyric acid (GABA)

Ogello et al. 2017: Hydrobiologia

Introduction

Live microalgal diets are commonly used to culture live foods such as rotifers. However, year round cultivation of sufficient live microalgae especially *Nannochloropsis oculata* is a heavy burden on many hatcheries (Maruyama et al. 1997). Besides, the live microalgae have short shelf life, thus limiting pre-planning of fish seedling production in microalgae-based hatcheries (Lubzens et al. 1995). Alternatively, frozen microalgae diets have been used to culture rotifers (Lubzens et al. 1995; Yufera & Navarro 1998) but high storage costs are incurred and also, stable supply of energy is not guaranteed everywhere to maintain freezer facilities. Dried *Nannochloropsis oculata* and *Chlorella vulgaris* are storable at room temperature for up to two years and can support population growth of rotifers (Dobberfuhl & Elser 1999). However, culture instabilities due to poor ambient conditions (high ammonia), clumping and low digestibility (food scarcity) have been reported (Yufera & Navarro 1998), hence requires techniques to stabilize.

Various chemicals have been used to stabilize the rotifer cultures (Gallardo et al. 1997, 1999). For example, growth hormone (GH) has been found to promote rotifer reproduction under optimal conditions while gamma-aminobutyric acid (GABA) enhances even stronger amictic reproduction in the progeny of the rotifer *Brachionus plicatilis* during high ammonia and food scarcity (Gallardo et al. 1997, 1999, 2000). Most aquatic habitats where rotifers inhabit are characterized by high water viscosity stress which affects the reproduction, swimming activity and ingestion rate of rotifers (Hagiwara et al. 1998). Fortunately, GABA can neutralize the effects of the combined stressors of high water viscosity, ammonia, food scarcity and protozoa (*Euplotes* sp.) contamination on rotifer reproductive characteristics and enzyme (glucosidase) activities (Araujo & Hagiwara 2005). GABA application is an effective mechanism for maintaining the viability of the physiological condition of the rotifers under low temperature as well as during population growth after preservation at low temperature (Assavaaree & Hagiwara 2011). In another study, 50 µg ml^{-1} of GABA and 0.025 IU ml^{-1} of porcine GH were found to enhance L-type rotifer population growth in individual cultures after 48 h of rotifer exposure to these chemicals prior to their transfer to new media (Assavaaree & Hagiwara 2011).

Most studies have focused on the biological manipulations of the rotifer *Brachionus plicatilis* using live *N. oculata* diet supplemented with GABA (Gallardo et al. 2000a; Araujo & Hagiwara 2005; Assavaaree & Hagiwara 2011). The rotifer *B. rotundiformis* is an indispensable larval fish food, whose stable production using dried algae with GABA supplementation could be a positive step towards achieving pre-planned hatchery seedling production and improving the reproduction of target species. The population growth response of the rotifer *B. rotundiformis* to dried *N. oculata* and *C. vulgaris* with GABA supplementation is unclear. To advance on this aspect, this study first determined the effectiveness of GABA application at lag phase growth stage of the rotifers and every 2

days on the population density and egg/female ratio of the rotifers in small batches before up-scaling to larger cultures.

Materials and methods

Algae and rotifers

Firstly, sufficient rotifer population was established using 7.0×10^6 cells ml^{-1} of either live *C. vulgaris* or *N. oculata* (Araujo & Hagiwara 2005) daily for 7 days at 25±1°C. The liquid paste of live *C. vulgaris* was supplied by Chlorella Company in Fukuoka, Japan. The stock cultures of *N. oculata* and the rotifer *B. rotundiformis* (S-type, Perth strain) were available in the laboratory. *N. oculata* was cultured using modified Erd-Schreiber medium (Hagiwara et al. 1994) at 25 °C under constant illumination (115.5 µmols $^{-1}$ m $^{-2}$) with gentle aeration. The alga was harvested during the log phase period by centrifugation (2100 × g for 8 min) and the algal pellet was re-suspended in sterilized seawater (22 ppt). The algal densities in diluted aliquots of suspended concentrates of the microalgae were determined using haemocytometer. Dried *N. oculata* (cell diameter, 2.5 µm) and *C. vulgaris* (cell diameter, 3.0 µm) were obtained from AlgaSpring (The Netherlands) and Daesang EMERALD (South Korea), respectively. The dried microalgae were stored at room temperature in the laboratory.

Experimental design

Before starting the experiments, the rotifers were preconditioned to the respective dried foods at 800 mg dry weight 10 $^{-6}$ rotifers day $^{-1}$ for 2 days. In the small trials, the rotifers were cultured in 1 l jars containing 300 ml of seawater at an initial density of 50 ind

ml^{-1} with gentle shaking by a triple shaker machine (NR-80, Taitec Co. Japan) at 61 rpm to keep the dried microalgae suspended. The rotifers were divided into three treatment groups with three replicates each: 1) no GABA addition (control), 2) GABA (50 mg l^{-1}) addition once at lag phase, and 3) GABA addition every 2 days. The cultures were incubated at 25±1°C in total darkness with daily feeding on either dried *N. oculata* or *C. vulgaris* at the rate of 800 mg dry weight 10 $^{-6}$ rotifers day $^{-1}$ for 5 days. The dried algae was first re-suspended in 3 - 5 ml of seawater then subjected to ultrasonic agitation for 5 - 10 min to break down aggregates before adding to the rotifer cultures. The diet quantities were adjusted daily depending on the rotifer population density, which was estimated by counting the rotifers in 1 ml sampled from each replicate. In the same samples, the number of amictic females and amictic eggs they carried or detached were counted and recorded.

In the mass cultures, the optimal results of the small scale trials were employed. Firstly, the rotifers were concentrated in 1 l culture medium at 200 ind ml^{-1} and applied 50 mg l^{-1} of GABA once at lag phase 24 and 48 h before up-scaling to 20 l cultures with aeration at room temperature. For each treatment, a corresponding control was prepared. The rotifer population density monitoring and feeding was done daily as explained previously. The specific growth rate (SGR) was calculated during the exponential growth phase using the formula: $r = [\ln N_t - \ln N_o] / t$, where, N_o = initial population density, N_t = population density after the time (t) and t = 6 days. Every 2 days, 30 ml of water samples were obtained from each replicate tank of the 48 h of GABA experiment for ammonia (NH_3 – N) (mg l^{-1}) determination (Palintest® 8000 Ltd, USA).

Statistical analysis

The data were analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test of homogeneity of variances was used to test homogeneity of variances. Two-way ANOVA was used to test the effects of culture period, food type and GABA on the rotifer population density, specific population growth rate and ammonia levels. Where significant differences were detected, the Tukey's HSD Post Hoc test was performed to locate them. Wilcoxon rank sum test was used to compare the rotifer population densities between 24 h and 48 h GABA culture experiments. All statistical differences were accepted at $p<0.05$.

Results

In the small cultures, the population density of the rotifers fed with *N. oculata* was significantly affected by culture period (F=988.05; p=0.00), GABA (F=77.82; p=0.00) and their interaction (F=16.91; p=0.00). GABA supplementation at lag phase and every 2 days caused significantly higher population densities on day 3 – 5 than the control (Figure 4-1A). Similarly, with *C. vulgaris* diet, there was significant effect of culture period (F=810.34; p=0.00), GABA (F=33.20; p=0.00) and their interaction (F=4.96; p=0.00) on the rotifer population density. Here, GABA supplementation at lag phase and every 2 days caused significantly higher population densities on days 2, 3 and 5 than the control (Figure 4-1B).

With *N. oculata* diet, the egg/female ratio was significantly affected by culture period (F=424.62; p=0.00), GABA (F=700.51; p=0.00) and their interaction (F=13.86; p=0.00). Higher egg/female ratio was observed on day 1 - 5 than the control ($p<0.02$) (Figure 4-2A). Similarly, with *C. vulgaris* diet, the egg/female ratio was significantly affected by culture

period (*F*=905.33; *p*=0.00), GABA (*F*=1760.08; *p*=0.00) and their interaction (*F*=68.81; *p*=0.00) with higher egg/female ratio than the control observed on day 2 - 5 (*p*<0.02) (Figure 4-2B). Highest egg/female ratio of 0.59±0.02 was obtained in the *N. oculata* diet on day 3 with GABA supplementation at lag phase.

In the mass cultures, there was significant effect of culture period, feed type and GABA on the rotifer population densities (*p*<0.01), but without significant interaction effects in the 24 h of prior GABA supplementation (*p*>0.05). No significant differences occurred on the daily rotifer population densities among the treatments in the 24 h GABA mass cultures (*p*>0.05) (Figure 4-3). However, 48 h of pre GABA supplementation caused significantly higher rotifer population densities on days 5 and 6 (with both feeds) and 8 and 10 (with *C. vulgaris*) than their respective controls (*p*<0.01) (Figure 4-4). Highest rotifer population densities of 301.3±22.2 and 246.3±10.1 ind ml^{-1} were obtained with *N. oculata* + GABA and *C. vulgaris* + GABA on day 6 and 8 respectively (Figure 4-4). GABA treatment for 48 h prior to mass culture produced significantly higher rotifer population density than the 24 h of GABA treatment (Wilcoxon rank sum test; *W*=1725.5, *p*=0.03).

The SGR was affected by feed type (*F*=8.34; *p*=0.02) in the 24 h GABA experiment where the rotifers fed with *N. oculata* had significantly higher SGR than those fed with *C. vulgaris* (*p*=0.03) (Figure 4-5A). However, GABA affected the SGR after 48 h of prior GABA incubation (*F*=31.97; *p*=0.00) where the GABA-treated rotifers had higher SGR than those in non-GABA treatments (*p*=0.00) (Figure 4-5B). There was no significant difference in SGR between 24 and 48 h GABA mass cultures as at day 6 (student t test, t=- 1.71; df=11, *p*=0.11). Highest SGR of 0.42 ± 0.03 day^{-1} was realized with *N. oculata* + GABA in the 48 h of prior GABA culture. The ammonia concentration was significantly affected by the culture period (*p*<0.01), but not food type or GABA (*p*>0.05). Each day, ammonia levels increased equally in all the treatments to about 0.7 mg l^{-1} on day 10 (Figure 4-6).

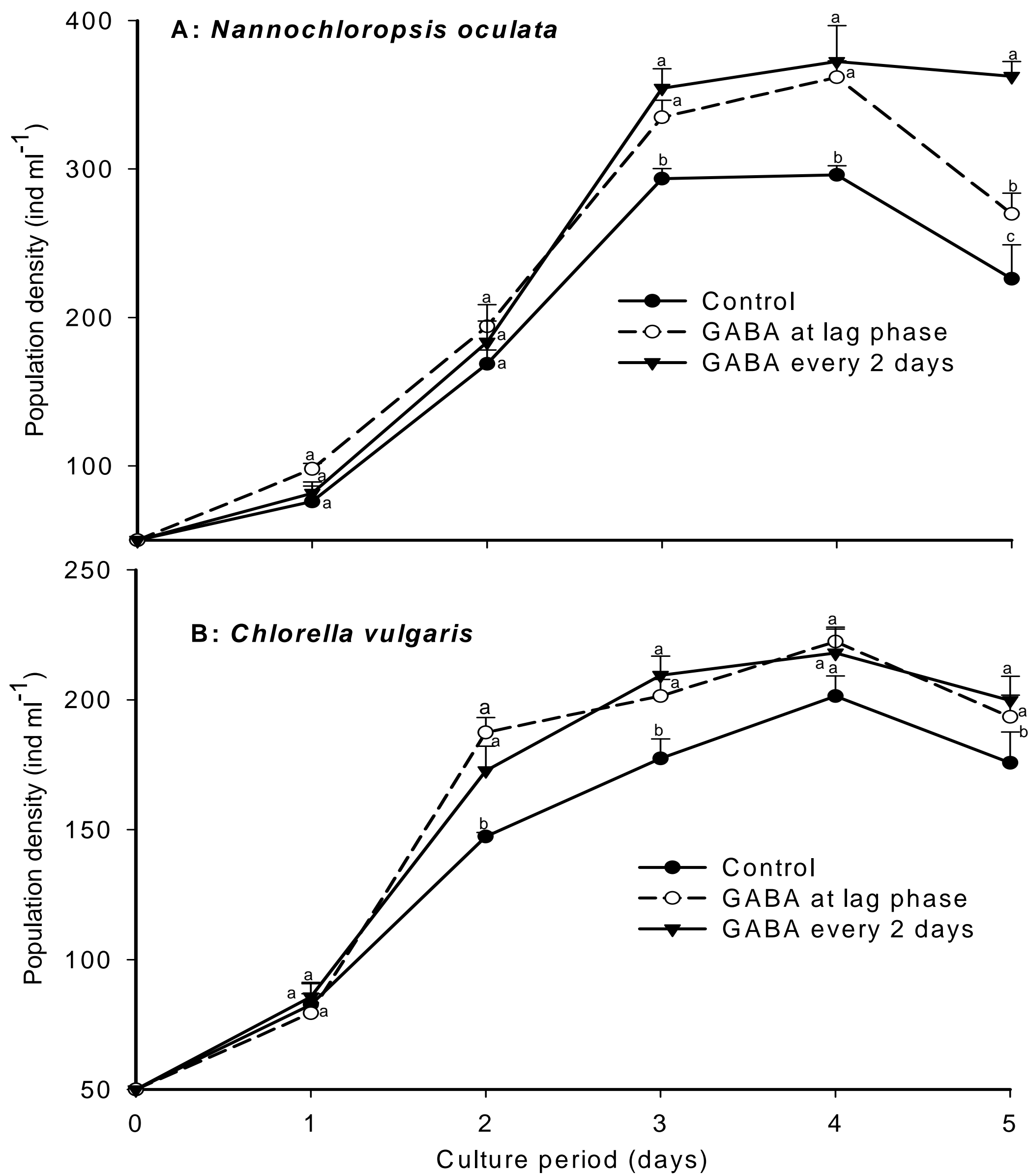

Figure 4-1: The population growth curves of the rotifers fed with dried *N. oculata* (A) and *C. vulgaris* (B) with GABA supplementation at lag phase and every 2 days. Each plot represents mean value of 3 replicates ± SD. Two-way ANOVA, Tukey HSD test, n=3; different letters in each day represent significant differences at $p<0.02$, a>b>c

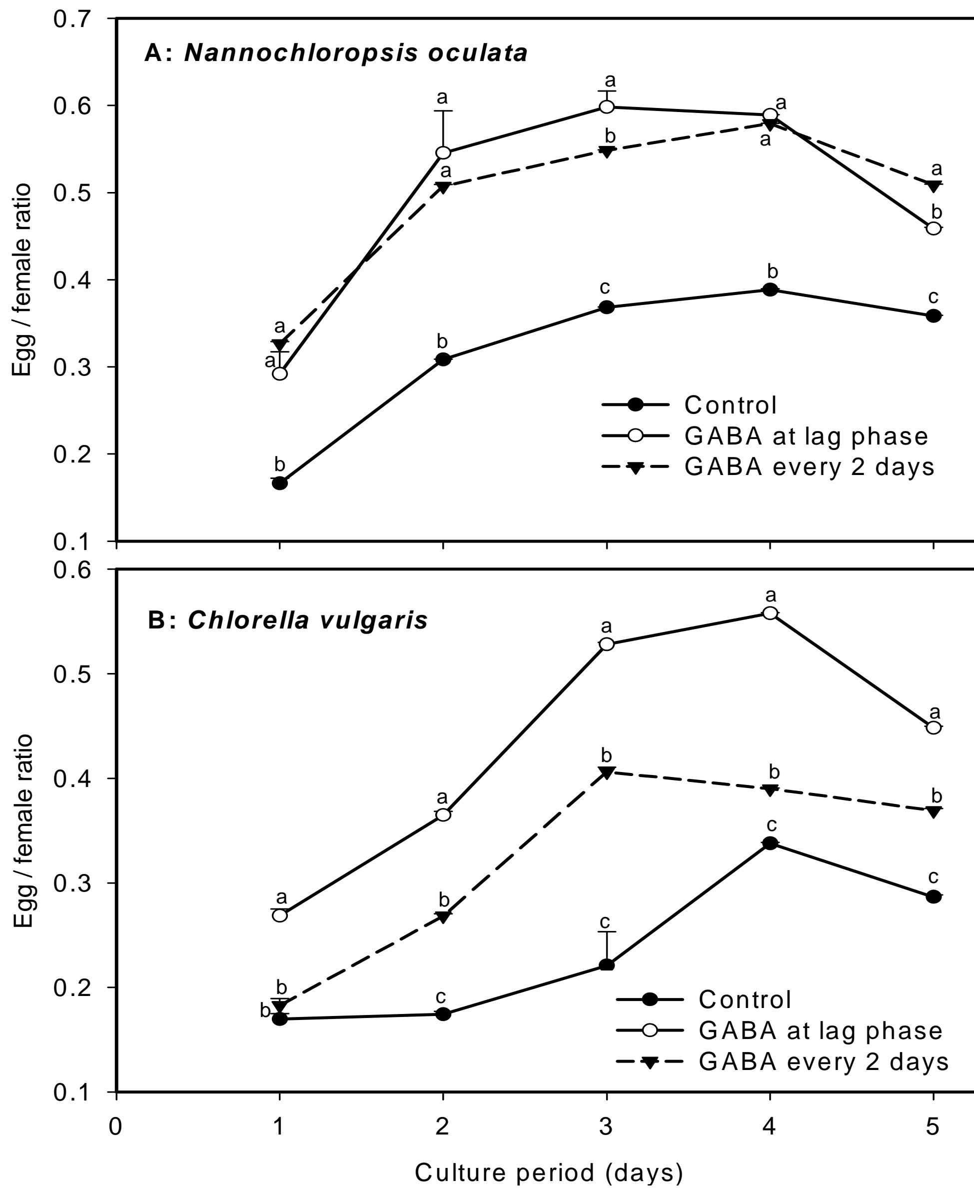

Figure 4-2: The egg / female ratio curves of the rotifers fed with dried *N. oculata* (A) and *C. vulgaris* (B) with GABA supplementation at lag phase and every 2 days. Each plot represents mean value of 3 replicates ± SD. Two-way ANOVA, Tukey HSD test, n=3; different letters in each day represent significant differences at p<0.001, a>b>c

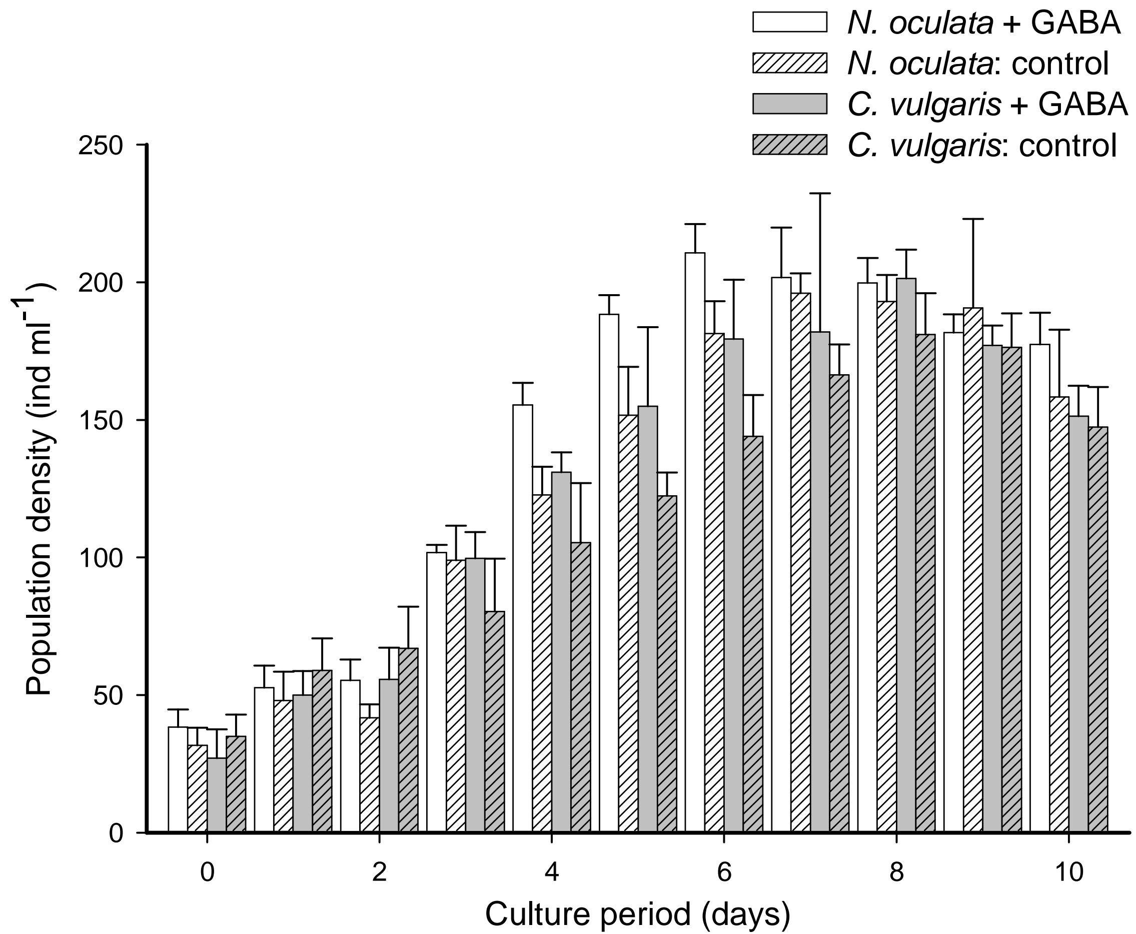

Figure 4-3: Population density of the rotifers fed with dried *N. oculata* and *C. vulgaris* with GABA supplementation for 24 h before mass culture. The bars are means ± SD of 3 replicates. Two-way ANOVA, n=3; No significant differences occurred on daily population densities among treatments p>0.05

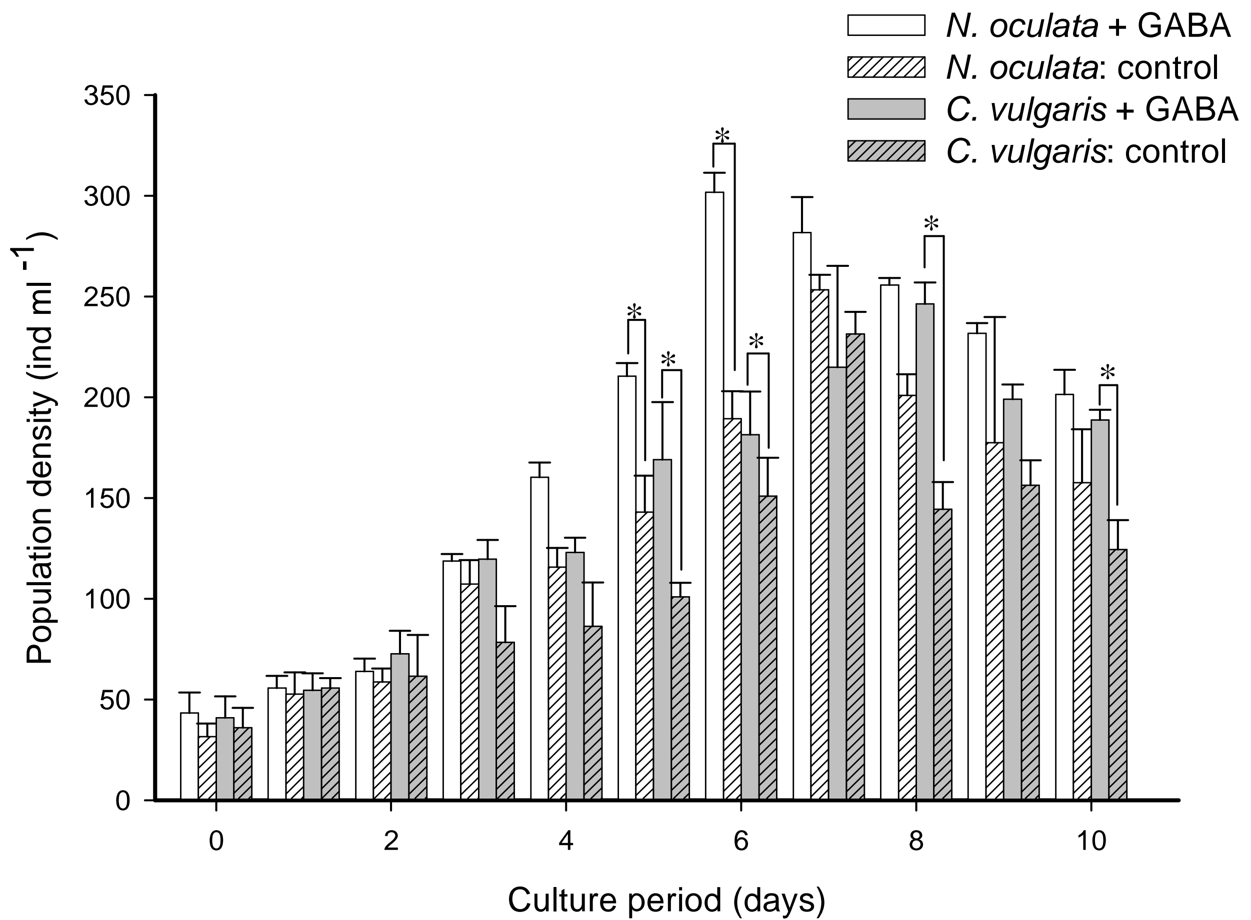

Figure 4-4: Population density of the rotifers fed with dried *N. oculata* and *C. vulgaris* with GABA supplementation for 48 hours before mass culture. The bars are means ± SD of 3 replicates. Two-way ANOVA, Tukey HSD test, $n=3$; * in each day denote significant differences for each feed at $p<0.01$

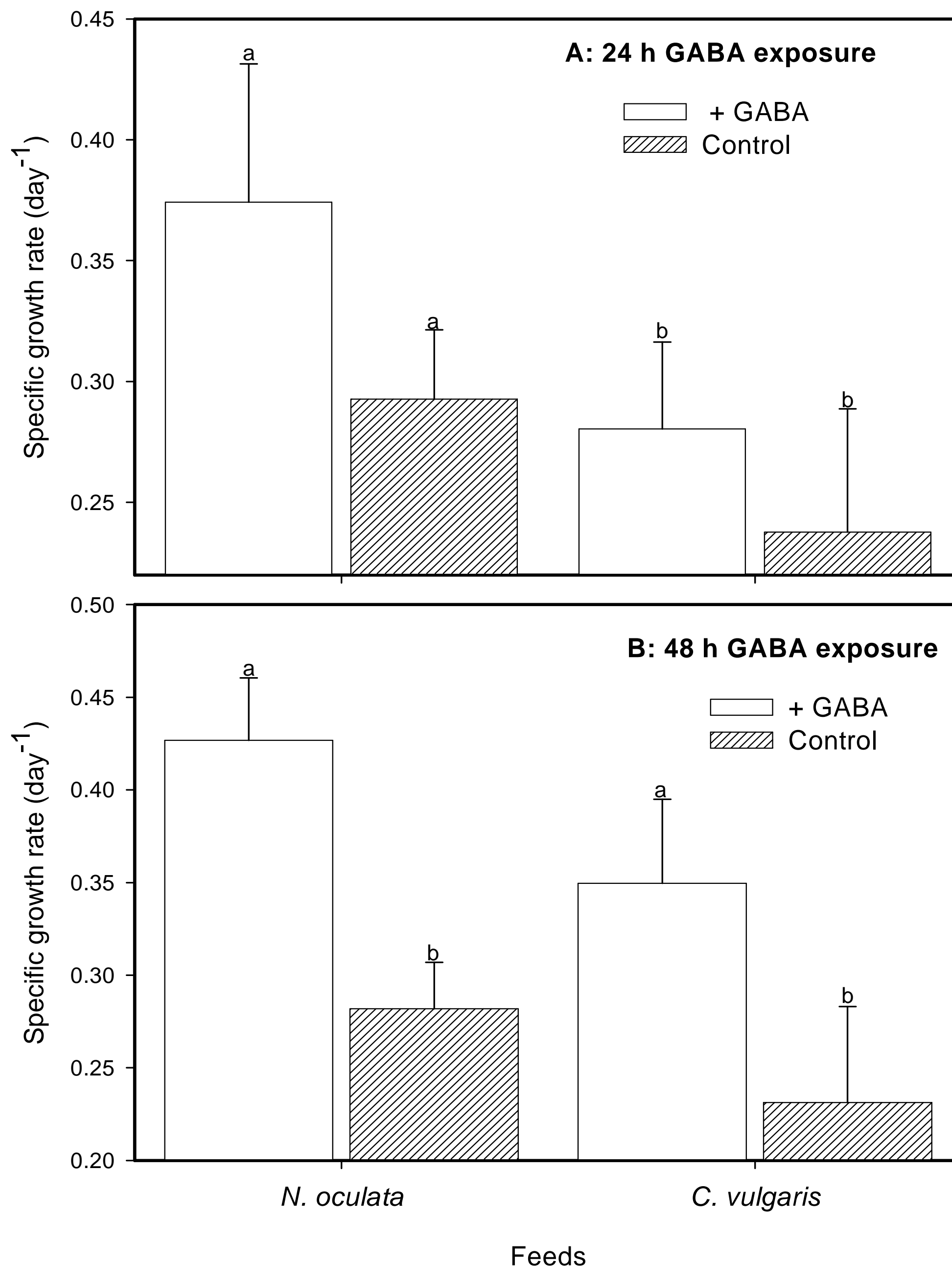

Figure 4-5: The specific growth rate (SGR) of the rotifers fed with *N. oculata* and *C. vulgaris* with GABA supplementation for 24 h (A) and 48 h (B) before mass culture. Two-way ANOVA, Tukey HSD test, n=3; Different letters in each feed denote significant differences at $p<0.05$; a>b

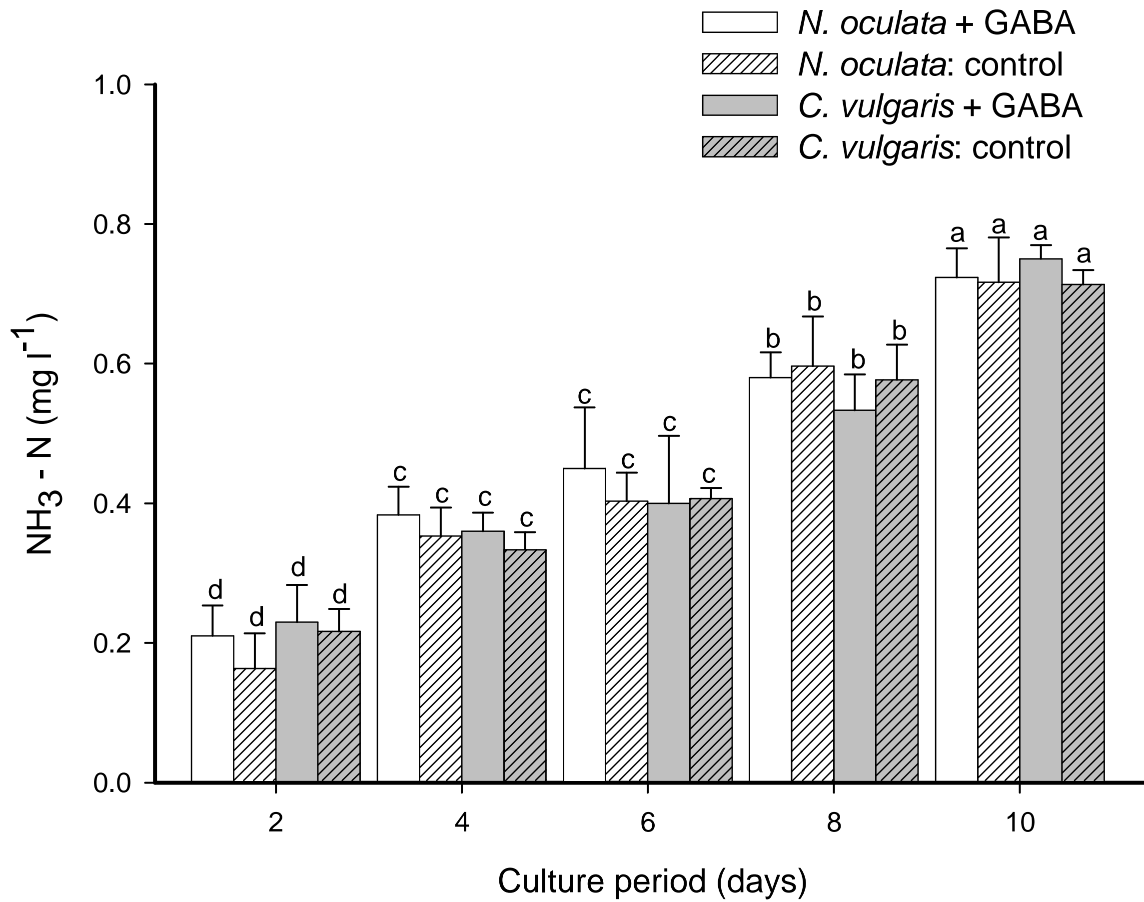

Figure 4-6: The daily NH_3-N fluctuations in culture tanks for the rotifers fed with *N. oculata* and *C. vulgaris* with 48 h of GABA supplementation before mass culture. The bars are means ± SD of 3 replicates. Two-way ANOVA, Tukey HSD test, n=3. Different letters on each day denote significant differences at among the treatments at p<0.05, a>b>c

Discussion

The difficulties in maintaining artificial food chain of microalgae and rotifers in hatcheries often hinders pre-planning of fish seedling production programs (Lubzens et al. 1995; Hagiwara et al. 2001). This study shows that production of *B. rotundiformis* using dried *N. oculata* and *C. vulgaris* can be significantly improved by GABA supplementation, thus providing an opportunity to eliminate direct dependence on the immediately cultured live alga. GABA can be applied at the lag phase growth stage of the rotifers 48 h before up-scaling to mass cultures to enhance the rotifer population density within 8 and 6 days of culture with dried *N. oculata* and *C. vulgaris*, respectively. In the small cultures, GABA application at lag phase and every 2 days produced superior results (Figure 4-1) but GABA application at lag phase was preferred during mass cultures due to cost implications. The results mirror the findings of Gallardo et al. (2000), who reported the effectiveness of GABA treatment at lag phase and every 2 days for the reproduction of the rotifer *B. plicatilis* supplied with fresh *N. oculata* diet in small and mass batch cultures. Gallardo et al. (2000) further observed that continuous GABA supply (every 2 days) is detrimental to the health of rotifers as it caused culture crash beyond 6 days of culture.

The higher egg/female ratio and population densities observed with GABA treatment suggested a continued rotifer reproduction even after passing their exponential growth phases (Figure 4-2). This phenomenon is attributable to the influence of GABA on the subsequent rotifer progenies. This observation corroborates previous studies that GABA causes even stronger growth effects on the F_1 and F_2 generations of *B. plicatilis*, which were not initially exposed to GABA (Gallardo et al. 1997, 1999). In this study, GABA exposure at lag phase for 48 h prior to mass culture was more effective compared to the 24 h. This observation suggests that longer GABA absorption time is necessary to trigger higher rotifer population growth. Similar observations have been reported in literature for the rotifer *B.*

plicatilis fed with live *N. oculata* diet (Gallardo et al. 2000a; Assavaaree & Hagiwara 2011). However, Gallardo et al. (2000) cautioned that holding rotifers for longer hours at high density may be counterproductive. A time lag exists for GABA entry and utilization before its effectiveness against stress can be realized and, it takes about 4 days at 25°C to observe positive effect in the rotifer mass cultures (Gallardo et al. 2000). In the current study, GABA produced better population growth results on day 6 and 8 with *N. oculata* and *C. vulgaris* respectively (Figure 4-4). By using dried *C. vulgaris* in a 12 l batch culture, Hirayama & Nakamura (1976) obtained about 434 ind ml^{-1} of *B. plcatilis* on day 16 from an initial inoculation of 13.2 ind ml^{-1}. This study reported 301.3 ± 22.2 ind ml^{-1} with *N. oculata* on day 6 and 246.3 ± 10.1 ind ml^{-1} with *C. vulgaris* on day 8 from an initial inoculation of 10 ind ml^{-1}, thus suggesting the significance of GABA supplementation. Nonetheless, other studies have reported 600-1000 rotifers ml^{-1} using fresh microalgal diets in batch cultures after about 4 days starting with 200 to 250 rotifers ml^{-1} (Dhert 1996). However, the problem with using fresh microalgal diets is instability and high cost of maintaining the cultures.

In this study, it was theoretically expected that GABA would be able to reduce the physiological stress caused by deteriorating culture conditions (e.g. high ammonia) as reported in other rotifer cultures with dried microalgal diets (Yufera & Navarro 1998). Indeed, despite increasing NH_3-N concentration in all the treatments (up to 0.7 mg^{-1} on day 10) (Figure 4-6), the GABA treated cultures maintained higher rotifer densities and SGR than the controls. The observation suggests that GABA probably enhanced the physiological condition of the stressed rotifers, confirming previous studies of Gallardo et al. (1999) in which GABA enhanced rotifer reproduction under un-optimal conditions. This observation further compliments the sentiments of Araujo & Hagiwara (2005) that GABA mitigates the effects of the environmental stressors and stabilizes the quality of rotifer cultures. GABA is an amino acid derivative with a direct effect on rotifer growth when consumed directly as a

nutrient (Morse et al. 1979) and is also important for maintaining the viability of the rotifer physiological condition under low temperatures (Assavaaree & Hagiwara 2011). GABA induces the production of endorphins hormone, which brings calming and stress reduction effects in higher animals (Abe et al. 1977). It is possible that this observation also holds for rotifers. GABA also induces secretion of growth hormone in *B. rotundiformis* as it has been demonstrated in rats (Abe et al. 1977). The endogenous presence of GABA and other important vertebrate and invertebrate neurotransmitters has been confirmed in the rotifers *B. plicatilis* and *B. rotundiformis* (Gallardo et al., 1999). Recently, GABA receptor type A-associated protein (GABARAP), which are known to be involved in intracellular membrane trafficking of $GABA_A$ receptors and autophagy was discovered to be ubiquitously distributed in the coronal area of neonates, males, and females of the rotifer *B. plicatilis* (Marcial et al. 2014).

In other ecological studies, GABA has been found to induce settlement and metamorphosis of the larvae of many marine invertebrates (Rumrill & Cameron 1983; Garcia-Lavandeira et al. 2005). GABA dose of between 0.5 -10 μM for 48 h has been reported as optimum for induction of settlement and metamorphosis in some larvae of marine invertebrates (Garcia-Lavandeira et al. 2005). Even though the mechanism by which GABA induces metamorphosis is poorly understood, there is evidence suggesting that the algae recognizing receptors are the same that recognize GABA for settlement and metamorphosis (Morse et al. 1979). The processes of settlement and metamorphosis are two crucial stages in the commercial culture of bivalve mollusks.

GABA is also suspected to control the level of quorum-sensing signal in some pathogenic bacteria e.g. *Agrobacterium tumefaciens* (Chevrot et al. 2006) and *Pseudomonas aeruginosa* (Dagorn et al. 2013). This opens up opportunities to develop strategies for controlling the virulence of pathogenic bacteria in rotifer cultures. This argument partially

compliments the findings of Araujo & Hagiwara (2005) that GABA can neutralize the effects of protozoa (*Euplotes* sp.) contamination in rotifer cultures. Even though we did not determine the bacterial loads in our cultures, it is speculated that GABA might have neutralized effects of pathogenic bacteria on the rotifer growth. More studies should focus on this aspect to unravel specific information. GABA is biodegradable (Saskiawan 2008), cheap ($3.00 per gram; Sigma Chemical) and requires low doses (50 mg l^{-1}) making it economically attractive across the board. It is speculated that GABA will be practically beneficial to fish larvae feeding on rotifers, but this speculation requires further scientific proof.

This study has demonstrated that mass production of the rotifer *B. rotundiformis* using dried *N. oculata* and *C. vulgaris* can be significantly enhanced through GABA supplementation to reach maximum densities of about 301.3±22.2 and 246.3±10.1 ind ml^{-1} respectively within 6 and 8 days respectively. This can be achieved by pre-incubating rotifers with 50 mg l^{-1} of GABA for 48 h before up-scaling to 20 l mass cultures. This study is relevant for promoting mass rotifer cultures smooth for pre-planning fish seedling production in aquaculture facilities where it is difficult to maintain live algal cultures. However, the use of dried algae could be limited by other factors e.g. nutrient leach, low digestibility and money exchange rate issues during importations. For this reason, further studies should focus on microalgae-less rotifer culture techniques. Such techniques will provide major leaps toward aquaculture growth especially in most developing countries without high density algae-production infrastructure.

Chapter V

DEVELOPING FISHWASTE DIET (FWD) FOR PLANKTONIC LIVE FOOD CULTURE: PROTOCOL AND APPLICATIONS

V.1: FWD FOR CULTURING *Brachionus rotundiformis* Tschugunoff (ROTIFERA): A LABORATORY STUDY

Composting fishwastes as low-cost and stable diet for mass culture of the euryhaline rotifer, *Brachionus rotundiformis* Tschugunoff: influence on water quality and microbiota

Ogello EO, Stenly W, Sakakura Y. Hagiwara A. Submitted to Aquaculture

Introduction

Successful marine fish larviculture depends on the availability of high quality live food resources such as rotifers and *Artemia* (Sorgeloos et. al. 2001). Among the live food resources, rotifers, especially of the genus *Brachionus* are essentially preferred as first exogenous food for most marine fish larvae due to their favorable morphological and biological traits (Akazawa et al. 2008). The demand for *Brachionus rotundiformis*, which is considered indispensable in marine larviculture, is high in hatcheries (Lubzens et al. 1989). For example, in Japan alone, an average hatchery requires about 20 billion rotifers day $^{-1}$, where 20,000 to 100,000 rotifers per fish larvae are needed to raise marine fish during its

first 20 to 30 days (Fushimi 1989; Fu et al. 1997). Regular supply of such a high rotifer demand is indeed stressful to many hatcheries, primarily due to the high cost of microalgae, which is the preferred diet for the rotifers. Alternatively, cheaper diets e.g. baker's yeast (Hirayama & Funamoto 1983) has been used, but culture instabilities are common. These instabilities are attributed to imbalances of bacterial flora in the cultures (Watanabe 1983). Other products e.g. condensed microalgae and artificial diets such as Selco (Inve-Co. Ltd., Thailand) are also commercially available (Lavens & Sorgeloos 1996) but costly for most fish farmers, especially in the developing countries, which are potential future leaders in marine larviculture production. Therefore, research studies are needed to develop low-cost, nutritious and stable live food diets as a relevant priority for profitable aquaculture in the less developed areas.

Due to the increasing global demand for fish and processed fish products, about 64 million tons of fishwastes e.g. heads, viscera, skin, bones, fins, air bladder, scales, blood, liver, gonads and guts are generated annually (Rai et al. 2010). These wastes are a source of environmental pollution if poorly disposed. The fishwastes are substrates for microbial growth, some of which have probiotic properties such as *Bacillus* sp. and lactic acid bacteria (LAB) (Balcazar et al. 2008). Probiotics are known to enhance growth and immunity of rotifers and fish larvae either individually or in combination (Yasuda & Taga 1980; Gatesoupe et al. 1991; Verschuere et al. 2000; Nayak 2010). Even though the decomposing fishwastes may produce other pathogenic microflora, probiotics are known for suppression of pathogenic bacterial strains through release of bactericidal or bacteriostatic compounds (Verschuere et al. 2000). In addition, the fishwastes also contain appreciable amounts of recoverable essential bio-molecules including the *n*-3 series of the long chain polyunsaturated fatty acids (PUFAs) e.g. eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) (Khoddami et al. 2009; Cho et al. 2014). These

elements are critical for growth and survival of the fish larvae feeding on the rotifers (Watanabe et al. 1983; Harel et al. 2002). Being filter feeders and top predators in the microbial web, it is hypothesized that rotifers can transfer the essential bio-molecules from the fishwastes to the fish larvae through bacteria-rotifer-fish trophic pathway, or through direct ingestion of micro-fishwaste particles. This hypothesis hints to a major leap towards eliminating the need for fresh algae for rotifer culture or, the expensive emulsions for rotifer enrichment.

Composing organic matter yields huge densities of bacterial cells under optimum carbon/nitrogen (C/N) ratio (Avnimelech 2003). Bacteria, and by extension the microbial loop are known to play important roles as recycling pathways for C and N in the ecological food webs (Azam et al. 1983). Usually, optimum C/N ratio ensures immobilization of inorganic nitrogen into huge bacterial proteins (biomass) and restores good water quality by removing toxic ammonia (Azim & Little 2008). However, the use of bacteria for live food culture has been confined at experimental scales, and is mainly added as supplements to microalgae or baker's yeast. This study explores the feasibility of promoting simultaneous growth of high dense bacterial biomass and rotifers in the same culture facilities, where rotifers feed directly on the microbial flora and /or micro-fishwaste particles as source of nutrition.

In the recent past, various protocols have been developed for converting specific fishwastes into other useful products such as fertilizers (López-Mosquera et al. 2011), silage (Ferraz et al. 2007), collagen (Nagai & Suzuki 2000), pharmaceutical products (Vanquez et al. 2004) and essential enzymes and minerals (Rebah & Miled 2013). However, there is no protocol for converting fishwastes into a rotifer diet, thus merits research priority. The objectives of this study were: 1) to develop a protocol for a producing fishwaste diet (FWD) for rotifer culture, 2) to determine the population growth, sexual reproduction and nutritive

value of the rotifer, *B. rotundiformis* fed with the FWD and 3), to analyze total fatty acid content of the rotifers and bacterial flora involved in the processes.

Materials and methods

FWD preparation protocol

Fishwastes (heads) of the chub mackerel; *Scomber japonicus* Houttuyn 1782 were obtained from Nagasaki fish market and frozen at -80°C in the laboratory for further use. *S. japonicus* is a pelagic marine fish with appreciable amounts of PUFAs (Cho et al. 2014). Fish heads were preferred because they are mostly inedible but nutritious (Khoddami et al. 2009). The fish heads were crushed using mortar and pestle (to increase surface area for microbial growth) before weighing 0.5 g of the fishwaste per litre of culture medium using a digital scale (Mettler Toledo – AG204, Japan). The fishwaste was wrapped in a piece of plankton net (200 µm mesh size) and inserted in a small container (about 150 cm^{3}) made of fine texliner plastic mesh. A 10 g metallic sinker was inserted in the container to keep it under the culture media. This technique reduces the intensity of stench. Wheat flour was added in the culture as carbon source to maintain an optimum C/N ratio of 16 (Ebeling et al. 2006; Crab et al. 2009). The crude protein of *S. japonica* is about 15.2% (Cho et al. 2014). The wheat flour was first re-suspended in 150 ml of seawater then subjected to ultrasonic agitation for 5 - 10 min to avoid aggregates before adding to the rotifer cultures. The fishwaste, referred to as the FWD, was investigated as food source for the rotifer *B. rotundiformis*. During range finding tests in the laboratory, fishwastes of common carp, *Cyprinus carpio* Linnaeus 1758 was used to develop FWD and used to culture the Kenyan rotifer strain *B. angularis*, but the cultures were unsuccessful and, the proposal was dropped in favour of *B. rotundiformis*.

Rotifer population growth

Stock culture of the rotifer, *B. rotundiformis* (SS-type, Perth strain) was available in the Laboratory. Each of the three diets (Table 5-1.1) was triplicated in polycarbonate tanks each containing 30 l of artificial seawater (22 ppt), where the rotifers were semi-continuously cultured without aeration for 18 days. The temperature of the culture media was maintained at 28±1°C in a water bath with heater (Figure 5-1.1). Each tank was initially inoculated with 20 rotifers ml^{-1} on the same day as diet application (day 0). The tanks were covered to minimize evaporation. Each day, three-1 ml of culture water was sampled at top 10 cm from each tank, fixed with lugol and then rotifer numbers were counted under stereo microscope at ×25 magnification to estimate rotifer population density. At every exponential growth phase, partial harvesting was done by replacing 50% of the culture medium with new artificial seawater and diet. The specific growth rate (SGR) was calculated as: $r = [\ln N_t - \ln N_o] / t$, where, N_o= initial population density, N_t= population density after the time (t) and t = time in days (i.e day 4, 9 and 13). The coefficient of variation (CV) of the mean SGR was computed as standard deviation / mean SGR, to determine the stability of the cultures. The CV was calculated on day 4, 9 and 13 for each diet.

Table 5-1.1: The composition and quantities of the diets applied in 30 l polycarbonate rotifer culture tanks

Diets	Composition	Quantity applied
FWD_1	Fishwastes (heads) only	15 g (i.e. 0.5 g l^{-1})
FWD_2	FWD_1 + starch (wheat flour)	FWD_1 + 6 g of starch (i.e. 0.2 g l^{-1})
Control	*Chlorella vulgaris*	7.0×10^6 cells ml^{-1} daily (Hagiwara et al. 1994)

Water analysis

Dissolved oxygen (DO) (mg l^{-1}) was measured in the culture tanks using a multi-parameter water meter (YSI-model 85/10 FT, Yellow Springs, Ohio USA). The pH was measured by pH meter (HM-30G DKK TOA, Japan) while unionized ammonia (NH_3-N) (mg l^{-1}) was determined by a photometer system for water analysis (Palintest® 8000 Ltd, USA) according to the company's manual.

Mictic reproduction

Sexual reproduction of the rotifers was monitored using batch cultures in glass jars containing 400 ml of seawater (22 ppt.) with initial density of 20 rotifers ml^{-1}. The rotifers were incubated at 28±1°C under total darkness with each diet (i.e. FWD_1, FWD_2 and control) for 10 days without aeration or water exchange. Rotifer population density was daily counted in three-1 ml from each diet group. In the same sample, the number of mictic

and amictic females were counted based on the type of egg they carried (Hagiwara et al. 1988). Then, the rotifer mixis rate was calculated as follows:

$$\text{Mixis (\%)} = \left[\frac{\textit{mictic females}}{\textit{amictic females} + \textit{mictic females}}\right] \text{X}\,100$$

Microbiology

The rotifer gut and culture medium were separately screened for bacteria under sterile conditions. For gut microbial analysis, rotifers were sampled from FWD_2 and control tank, and separately rinsed thoroughly with distilled water to remove external flora. The rotifers were dried using filter paper from beneath plankton sieve (45µm) and transferred into a sterile 1.5 ml Eppendorf tube and homogenized using sterile pellet pestle (Sigma-Aldrich Z35997-1EA) to expose the internal gut microflora. The homogenized samples were re-suspended in 1 ml of sterile (Mili-Q) water, vortexed at high speed for 10 s and filtered through a 10 µm net using a pipette, to remove the rotifer tissues. The homogenate was serially diluted up to 10^{-6}. Similar dilutions were done for the culture medium (after filtering out rotifers and residues using the 10 µm net). For platting, the Zobell marine agar (Difco™ 2216, Becton, Dickinson & Co. France) was used. Then, 0.1 ml of the respective diluted samples was inoculated over the surface of the solidified agar in triplicates. The plates were incubated upside down (to avoid vapor condensation on the agar) at 32°C for 48 h. The bacterial colony forming units (CFU) were calculated as CFU ml^{-1} = (No. of colonies × dilution factor) / inoculated volume (ml).

Fatty acid analysis

The harvested rotifers were thoroughly rinsed with distilled water to remove salts and residues, dried as mentioned previously, and kept frozen at -80°C until chemical analysis. Visible dense microbial suspensions (bioflocs) were filtered from the FWD tanks and centrifuged at 8,000 × g for 5 min. The biofloc pellet was aseptically collected and kept at -80°C for lipid analysis. Some bacterial colonies isolated from FWD culture medium as described previously, were grown in rotating tubes containing marine broth (Pearcore Trypto-soy, Eiken, Japan) for 24 h at 32°C. The broth-cultured bacteria were also centrifuged and preserved at -80°C for lipid analysis. Total lipid and fatty acid composition analysis were conducted at Chlorella Industry Co., LTD, Fukuoka, Japan. The sample methanolysates were prepared at 100°C for 2 h after the addition of 2 M hydrogen chloride methanol. Fatty acid methyl esters (FAME) were extracted by n-hexane. Gas chromatography analysis was performed using a GC-2010 (Shimadzu Scientific Instruments, Inc.) equipped with a HR-SS-10 column (Shinwa Chemical Industries, Ltd.). The column temperature was regulated at 150 to 220 °C. Individual fatty acids were quantified by means of the response factor to 15:0 fatty acids as the internal standard (Folch et al., 1957).

Microparticle distribution

The experiment on micro-particle size and concentration in the culture medium was conducted to determine whether the rotifers also ingested the micro-particles. FWD_1 (no wheat flour) was employed to avoid interference from wheat flour particles. Two treatments i.e. FWD_1 only, and FWD_1 + rotifers (20 rotifers ml^{-1} initial density) were each triplicated in tanks containing 30 l of artificial seawater (22 ppt) without water exchange for 18 days. The experimental set up was similar to the previous one used for rotifer population density study.

From day 8 to 17, about 10 ml of culture water was sampled daily from each tank and filtered through 10 µm net to remove residues and/or rotifers. The microparticle concentration and their mean sizes in each treatment were determined using the particle distribution analyzer (Sysmex PDA-500, USA) according to company's protocol.

Data analysis

The data was analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test was used to test for the homogeneity of variances. Two-way analysis of variance (ANOVA) was used to test the effects of FWD and culture days on water quality, rotifer population densities, SGR and mixis rate. Wilcoxon rank sum test and student t-test were used to compare the mean particle concentration and sizes in each treatment, respectively. Where significant differences were detected, Tukey's HSD Post Hoc test was performed to locate them at $p<0.05$.

Results

Rotifer population growth

There was significant effect of FWD ($F=18.81$, $p<0.05$), culture days ($F=102.59$, $p<0.05$) and their interaction ($F=5.94$, $p<0.05$) on the rotifer population density. The rotifers fed with FWD_2 had significantly higher density than those fed with FWD_1 and control diet on day 9, 12 and 13 (Tukey HSD, $p<0.05$), but declined significantly on day 17 and 18 (Tukey HSD, $p=0.00$) (Figure 5-1.2). There was no significant difference between population density of the rotifers fed with FWD_1 and control diet except on day 17 and 18,

where density of the control-rotifers significantly exceeded those in given FWD (Tukey HSD, p=0.00) (Figure 5-1.2). Highest population densities were 883.6±84.6, 1,188±69.7 and 830.3±109.7 ind ml^{-1} on day 13 for the rotifers fed with FWD_1, FWD_2 and control diet, respectively (Figure 5-1.2). The SGR was significantly influenced by the FWD (F=13.63, p=0.00) and culture days (F=28.16, p=0.00), where rotifers fed with FWD_2 had significantly higher SGR than the control-rotifers on day 9 (Tukey HSD, p=0.00). There was no significant difference in SGR between the rotifers fed with FWD_1 and the control-rotifers (Tukey HSD, p=0.11), and between the FWD treatments (Tukey HSD, p>0.05) (Table 5-1.2). There was no significant effect of FWD on the coefficient of variation (CV) of the mean SGR in the diets (One-way ANOVA, F=0.94, p=0.43). The CV was 0.11±0.05, 0.07±0.02 and 0.08±0.03 for FWD_1, FWD_2 and control tanks, respectively.

Mictic reproduction

The rotifer mictic induction was significantly affected by FWD (F=114.89, p=0.00), culture days (F=15.13, p=0.00) and their interaction (F=4.53, p=0.00). The control-rotifers had significantly higher mixis rate than those given FWD on day 4 and 7-10 (Tukey HSD, p=0.00) (Figure 5-1.3). There was no significant difference in mixis rate between the two FWD treatments (Tukey HSD, p=0.53).

Water analysis and microbiology

The range and mean values of water quality parameters are presented in Table 5-1.3 while the daily fluctuation curves in the culture tanks are shown in Figure 5-1.4. The bacterial colonies were counted at $\times 10^{-4}$ dilutions. The FWD significantly affected the CFU

(ml^{-1}) (One-way ANOVA, F=15.11, p=0.01), where the FWD-fed rotifers ingested significantly higher amounts of CFU (ml^{-1}) than the control-rotifers (p=0.03). However, there was no significant difference of CFU in the culture medium of FWD and that of control, and between the rotifer gut and the culture medium in each diet (Figure 5-1.5).

Fatty acids analysis

About 0.35 and 0.39 mg g^{-1} of DHA and EPA, respectively was obtained in the rotifers fed with FWD_2 and both were under detectable limit in the control-rotifers. The total lipid analysis was not done for the rotifers fed with FWD_1, bioflocs and broth-cultured bacteria due to insufficient sample quantities (i.e. < 10 g). The DHA/EPA ratios were as follows: bioflocs, 2.7; broth-cultured bacteria, 0.0; FWD_1-rotifers, 0.9; FWD_2-rotifers, 0.9 and control-rotifers, 0.0. The DHA/ARA (20:4 n-6) ratio was 1.3 and 90.0 for the broth-cultured bacteria and bioflocs, respectively, but was under detectable limit in the FWD- and control-rotifers (Table 5-1.4).

Microparticles distribution

Only particle sizes between 0.61 - 6.0 μm were recognized by the particle counter machine. There was significantly higher concentration of particles in the FWD tanks without rotifers ($2.56\pm0.19\times10^7 ml^{-1}$) than in the FWD tanks with rotifers ($3.46\pm1.75\times10^5$ ml^{-1}) (Wilcoxon rank sum test, W=886, p=0.00). Meanwhile, the mean particle sizes were significantly larger (2.71±0.27 μm) in the FWD tanks with rotifers than in the FWD tanks without rotifers (2.01±0.33 μm) (Student t-test, t = -8.58, df=29, p=0.00). Visually, the FWD tanks without rotifers were cloudier than the FWD tanks with rotifers.

Table 5-1.2: The specific growth rate (SGR) at day 4, 9 and 13 of the rotifers cultured in the FWD and control tanks; the values are means ± SD. Two-way ANOVA, Tukey HSD test, Different superscripts each day in a row indicate significant differences at $p<0.05$; $n=3$, a>b

Day	Treatments		
	FWD_1	FWD_2	Control
4	0.78 ± 0.04^{a}	0.81 ± 0.04^{a}	0.78 ± 0.04^{a}
9	0.60 ± 0.09^{ab}	0.69 ± 0.06^{a}	0.44 ± 0.02^{b}
13	0.61 ± 0.07^{a}	0.76 ± 0.05^{a}	0.58 ± 0.07^{a}

Table 5-1.3: The ranges and means ± SD of three replicates daily for each water quality parameter for 18 days in the FWD and control tanks; Two-way ANOVA, Tukey HSD test, Different superscripts in a row indicate significant differences at p<0.05; n=54, a>b>c

Water parameters	Treatments					
	FWD_1		FWD_2		Control	
	Range	Mean	Range	Mean	Range	Mean
DO (mg l^{-1})	0.61-5.22	2.09 ± 1.32^b	0.49-5.23	1.97 ± 1.41^c	1.51-5.31	3.09 ± 1.05^a
NH_3-N (mg l^{-1})	0.00-2.31	0.88 ± 0.45^a	0.00-1.69	0.81 ± 0.34^b	0.00-0.89	0.60 ± 0.24^c
pH	7.51-7.77	7.63 ± 0.07^a	7.44-7.73	7.61 ± 0.07^a	7.52-7.79	7.64 ± 0.07^a

Table 5-1.4: Total lipids (mg g $^{-1}$ dry weight) and fatty acid composition (%) in total lipids of raw fishwaste, broth-cultured bacteria and bioflocs and, FWD- and control-rotifers; Note: DHA, Docosahexaenoic acid (22:6 *n*–3); EPA, Eicosapentaenoic acid (20:5 *n*–3); FWD, Fishwaste diet. - Means total lipids not measured due to limited sample quantity. * means total lipids not measured due to limited sample quantity

	Raw	Bacteria	Bioflocs	Rotifer samples from		
	Fishwaste	(cultured)	(in medium)	FWD_1	FWD_2	Control
Total lipids	28.7	*	*	*	20.5	11.1
Fatty acids						
C14:0	3.7	4.5	3.5	4.5	5.4	1.8
C14:1	0.2	0.5	0.2	1.5	2.3	1.0
C16:0	18.7	21.6	18.9	17.7	15.2	15.1
C16:1	2.1	3.6	2.2	2.1	3.0	0.2
C16:2	1.3	1.0	1.3	0.1	0.6	0.3
C18:0	6.9	7.0	7.0	3.0	4.3	4.8
C18:1	14.5	12.8	14.9	6.3	4.8	2.0
C18:2 n-6	1.4	0.8	1.4	4.3	4.1	30.8
C18:3 n-3	0.8	0.4	0.8	0.3	0.5	5.7
C20:0	0.8	0.7	0.8	0.2	0.3	0.2
C20:1	0.3	1.5	0.3	0.6	0.6	1.4
C20:4 n-6	2.4	2.4	0.2	0.0	0.0	0.4
C20:5 n-3	6.5	0.0	6.6	1.6	1.9	0.0
C22:0	0.3	0.0	0.3	0.0	0.0	0.0
C22:1	0.4	1.5	0.4	14.2	15.1	0.6
C24:0	1.3	0.3	1.3	0.6	1.4	0.0
C24:1	0.4	0.9	0.4	1.2	0.8	0.4
C22:5 n-3	2.3	0.3	2.2	0.4	0.5	0.0
C22:6 n-3	18.7	3.1	18.0	1.5	1.7	0.0
Others	16.8	39.2	17.1	40.6	37.4	35.0
DHA / EPA	2.9	0.0	2.7	0.9	0.9	0.0
DHA/ARA	7.8	1.3	90.0	0.0	0.0	0.0
Total	100	100.0	100.0	100.0	100.0	100.0

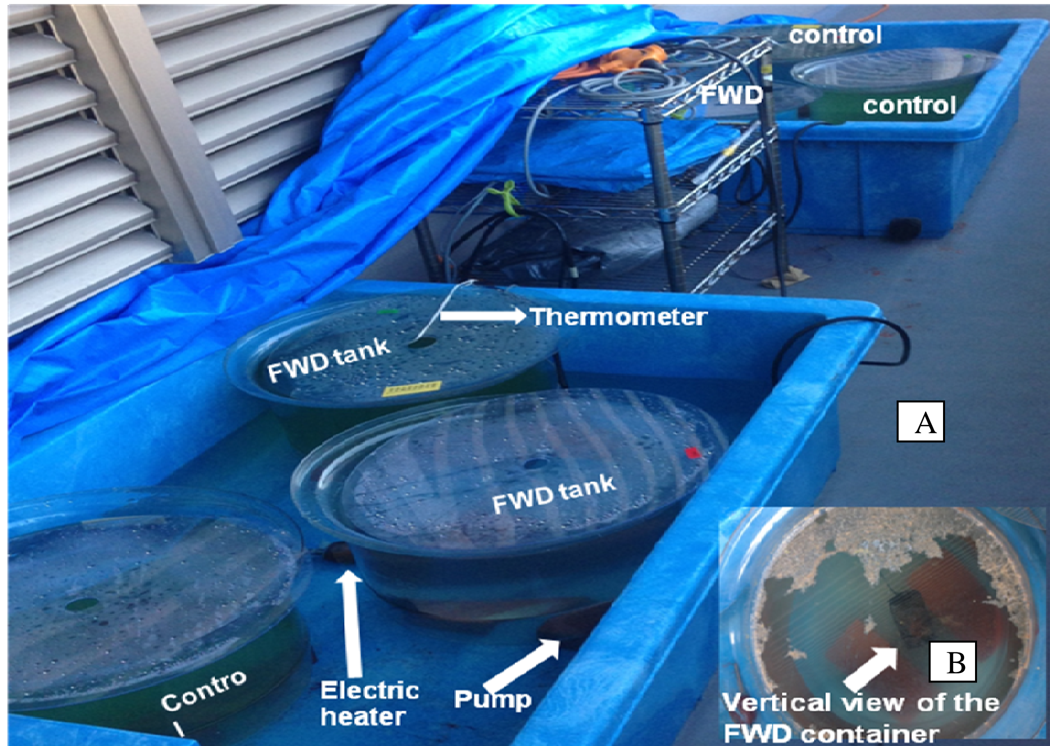

Figure 5-1.1: The experimental design of the rotifer culture tanks (FWD and control treatments) with an electrically heated and thermally regulated water bath system; water pump (to circulate the water currents) and thermometer (to measure water temperature), B: the vertical view of the FWD culture tank showing biofloc film and the FWD at tank bottom after 7 days.

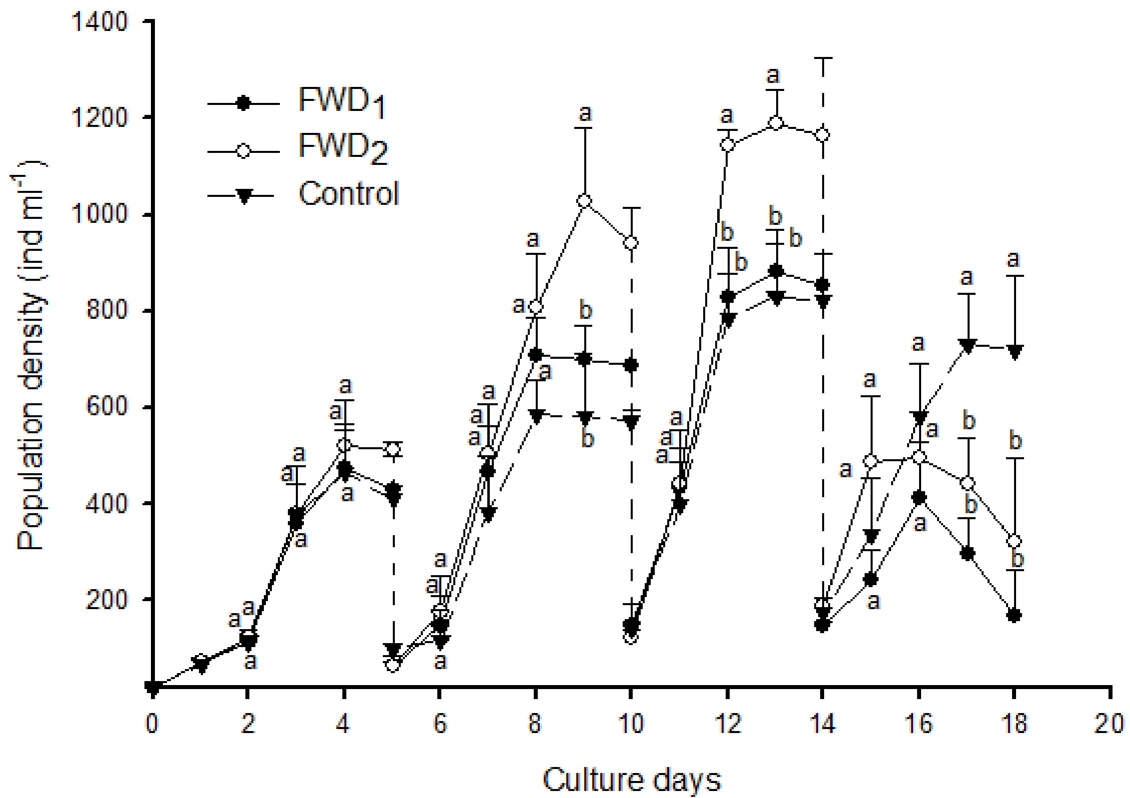

Figure 5-1.2: Population density of the rotifers cultured with FWD and control diets. Half of culture medium was replaced with new media on day 5, 10 and 14 as shown by the dotted lines. The values represent mean ± SD. Different letters each day denote significant differences at $p<0.05$. Two-way ANOVA, Tukey HSD test, $n=3$, a>b

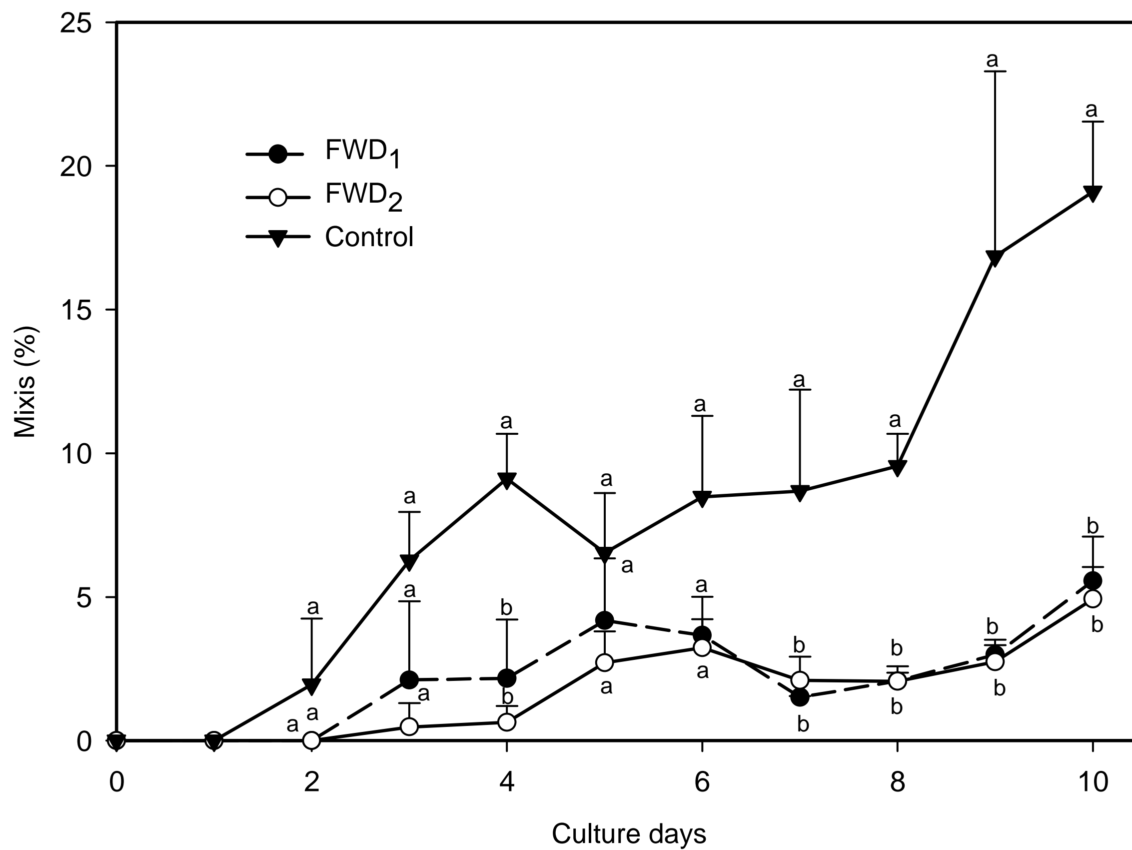

Figure 5-1.3: The percent mixis rate of rotifers batch cultured in 400 ml glass jars. The values represent means ± SD. Different letters each day denote significant difference at $p<0.05$; Two-way ANOVA, TukeyHSD, $n=3$, a>b

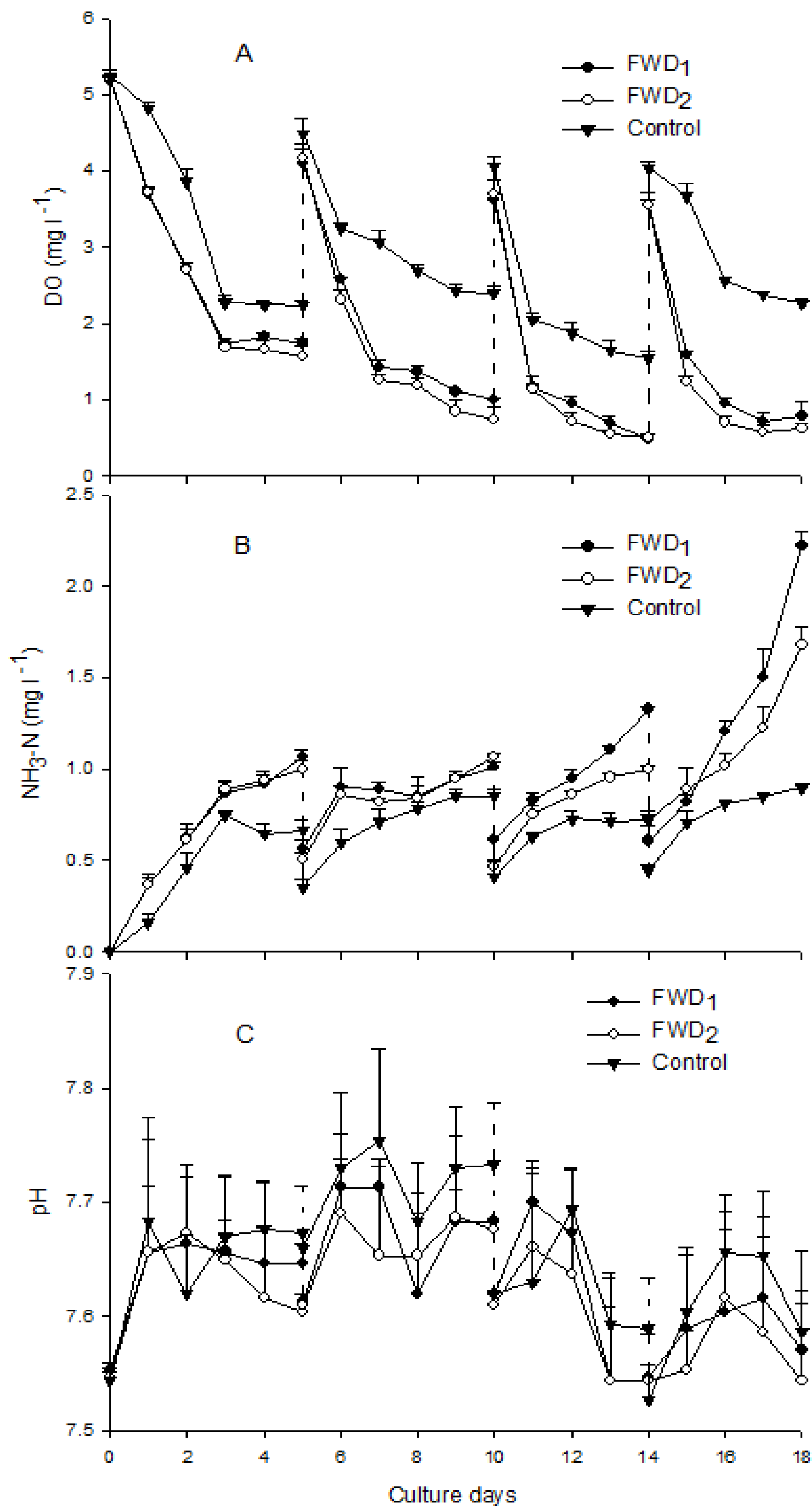

Figure 5-1.4: Daily fluctuations of the water quality parameters in the culture tanks. The values are mean ± SD. Two-way ANOVA, Tukey HSD test, n=3, p<0.05; Half of culture water was replaced with new media on day 5, 10 and 14 as shown by the dotted lines

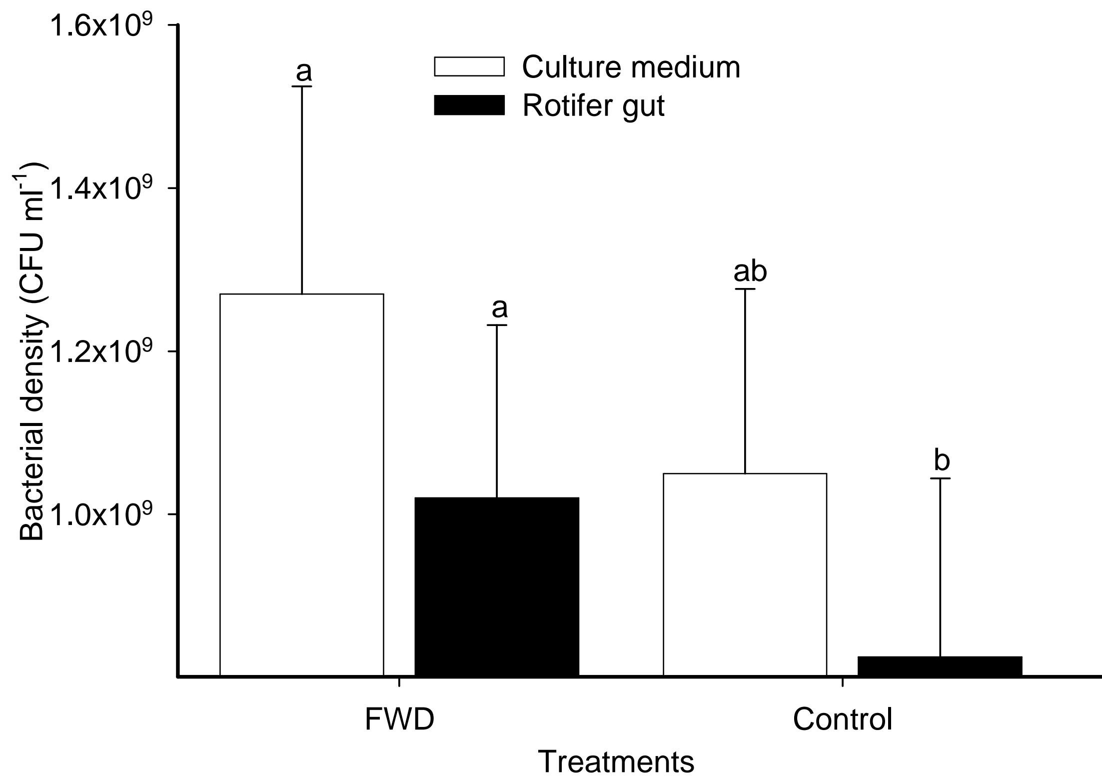

Figure 5-1.5: The bacterial colony forming units (CFU /ml) in the culture medium and rotifer gut in FWD and control tanks at $\times 10^4$ dilution rate. Two-way ANOVA, Tukey HSD test, n=3, p<0.03, a>b

Discussion

In the recent past, biosynthesis and utilization of microbial proteins has attracted the attention of aquaculture nutritionists (Avnimelech 2003; De Schryver et al. 2008; Ogello et al. 2014). The microbial proteins are generated through heterotrophic processes that are stimulated when nutrients from organic materials are recycled at optimal C/N ratios (Eberling et al. 2006; Azim & Little 2008; Crab et al. 2009). To date, scientific evidence of converting fishwastes into microbial proteins as food source for rotifers are scanty. This study has provided a protocol for converting fishwastes into a cheap and stable diet and, determined its usefulness as a food source for the rotifer, *B. rotundiformis*.

Higher SGR (Table 5-1.2) and population density of the rotifers cultured with FWD_2 than the control-rotifers in day 9 corresponded to a high microbial biomass in the rotifer gut (Figure 5-1.5) and, a low mixis rate (Figure 5-1.3). Based on these results, the FWD must have favored parthenogenetic reproduction of rotifers but suppressed mixis induction. Rotifer densities and/or egg/female ratio dynamics in natural eutrophic water are positively correlated with bacterial load (Ooms-Wilms 1997). However, Hagiwara et al. (1994) reported that addition of certain bacteria or rotifer extracts increases rotifer mixis rates. The FWD_2 produced up to 1,200 rotifers ml^{-1} bi-weekly, beyond which, the system became exhausted and, required a complete restart. A generally lower CV (~ 0.1) in all the diets suggested that the FWD cultures were just as stable as those fed with normal *C. vulgaris* diet.

The water quality aspects may have also influenced rotifer growth. For example, lower NH_3-N level recorded in FWD_2 probably favored faster rotifer growth rate, thus depicting carbon source addition (i.e. maintaining high C/N ratio) as an improvement of the FWD technology. Literature reports that optimal C/N ratio facilitates biochemical transformation

of excess NH_3-N into microbial biomass and restores good water quality (Avnimelech 2003; Azim & Little 2008). C/N ratio of 16 is optimal for efficient functioning of a biofloc aided system (Crab et al. 2009). Whereas the pH and DO values in this study are comparable to those of standard rotifer cultures (Yu et al. 1989), the NH_3-N values in FWD tanks appeared to be slightly higher than in normal cultures, perhaps due to the deamination process of FWD. However, the values are still below the lethal concentration level (LC_{50}) for normal rotifer mass cultures (Yu & Hirayama 1986). In addition, the pH was relatively stable in all cultures (7.51-7.77). Unstable pH is detrimental to rotifers as higher pH (above 8.0) favors production of toxic ammonia in the cultures (Yu & Hirayama 1986). The FWD-fed rotifers appeared to have crashed after 2 weeks of culture, during which the NH_3-N level was 2.0 mg l^{-1}. Studies have shown that accumulation of NH_3-N in culture tanks significantly impedes mass production of rotifers (Yu et al. 1986; Dhert et al. 2001). Further technologies should be investigated to stabilize FWD-rotifer cultures beyond 2 weeks. It may have been possible to prolong the life of the FWD cultures through frequent harvests or large quantity harvests (i.e. > 50%). It may also be interesting to investigate whether supplementation of FWD cultures with chemicals e.g. GABA or commercial probiotics can improve the productivity of the cultures beyond the 2 weeks.

Meanwhile, the FWD caused proliferation of microflora, which formed rotifer diet as shown by ingestion of higher CFU ml^{-1} of bacterial by the FWD-fed rotifers than the control-rotifers (Figure 5-1.5). Indeed, lower rotifer reproduction has been reported in bacteria-free cultures than those in the cultures supplemented with microbial flora (Tinh et al. 2006). Under optimal conditions, bacteria form flocs or attach to detritus to reach a size large enough to be eaten by rotifers (Hino & Hirano 1980; Rothhaupt 1990). In this study, a preliminary biochemical characterization of the microbial flora (results not shown due to the ongoing microbial studies), revealed the presence of probiotic bacterial strains such as

Bacillus sp., *Shewanella* sp. and *Thiocapsa* sp. in the FWD culture medium and rotifer gut. The *Bacillus* sp. that are commonly found in rotifer cultures are known to have probiotic properties to the rotifers (Jamali et al. 2015; Duy et al., 2017). Probiotic bacteria produce vital chemicals e.g. protease, lipase and vitamins that promote growth, reduce the stress, aid in the digestive processes and increase the reproduction of cultured animals (Yu et al. 1988, 1989; Parker 1974; Gatesoupe 1991). Probiotics are also known to improve water quality, reduce occurrence of pathogenic bacteria and quorum sensing (Vazquez et al. 2005), and thus increases rotifers growth rate (Yu et al. 1988, 1989; Yu et al. 1994; Planas et al. 2004). In addition, some probiotic trains of *Bacillus* sp. produce chemical metabolites e.g. gamma aminobutyric acid (GABA) (Li & Cao 2010), an organic acid that stabilizes rotifer cultures and enhances parthenogenetic reproduction of rotifer progenies during sub-optimal conditions (Gallardo et al. 1999, 2000a; Assavaaree & Hagiwara 2011). Despite the positive effects of bacteria in rotifer cultures (Yasuda & Taga 1980; Ushiro et al. 1980; Hagiwara et al. 1994), certain bacteria e.g. *Vibrio anguillarum*, that is also commonly found in rotifer cultures could be problematic for successful culture of marine fish larvae (Frans et al. 2011). Therefore, further studies should focus on complete characterization of the bacteria flora including their succession patterns over time in the FWD cultures.

The suitability of marine fish larval diet is determined by the content and dietary balance of the essential fatty acids e.g. DHA, EPA and ARA (Sargent et al. 1997, 1999; Boglino et al. 2014). The presence of these essential fatty acids at optimal amount is indeed critical for proper growth, development and survival of the fish larvae (Watanabe et al.1983; Watanabe 1993). This study showed that FWD_1 and FWD_2 enhanced the DHA/EPA ratio of the rotifers by 0.9 while DHA/ARA for the bioflocs was 90.0 (Table 5-1.4). However, it was difficult to explain why the ARA was undetected in FWD- and control-rotifers. It is speculated that the rotifers may have obtained the EPA and DHA via FWD-bacteria trophic

pathway, thus portraying bacteria as conduits for transporting the bio-molecules. Nutritionally, bioflocs have been found to contain up to 40-50% crude protein (Widanarni et al. 2012), 3% lipid and 19 k J g $^{-1}$ energy on dry matter basis (Azim & Little 2008). These values are much better than most manufactured fish feeds. According to Simon and Azam (1989), 63% of marine bacteria dry weight is protein, making bacteria a substantial source of protein. Depending on the mixture of available food particles, 10- 40% of the rotifer's diet consists of bacteria (Arndt, 1993). On the other hand, the rotifers may have also obtained the EPA and DHA through direct ingestion of microparticles as shown by the higher concentration of particles in the FWD only tanks than in the FWD + rotifer tanks. This observation reaffirms the findings of Hino & Hirano (1980) and Agasild & Nõges (2005) that rotifers can ingest bacteria-sized particles in the absence of microalgae or at low microalgae density. However, these particles may have also included bacteria and rotifer feaces. Hino et al. (1997) reported that rotifers can ingest their own feaces, thus increasing their food conversion ratio.

The DHA/EPA ratio of 0.9 reported for the FWD-fed rotifers in this study falls below the recommended threshold ratio of 2.0 required for effective marine fish larval nutrition (Sargent et al. 1997). Sargent et al. (1999) reported that optimum ratios for the HUFAs are species specific, and are about 10:5:1, for DHA/EPA/ARA, respectively for most marine fishes. However, Darias et al. (2013) reported that high ARA levels could cause malpigmentation in flatfishes. The FWD developed in this study promises as a major leap towards developing a nutritionally rich diet for the rotifers. Based on the food value of the FWD-rotifers and bioflocs, the rotifers can be used to feed larval fishes while the FWD culture fluid can be considered as cheap enrichment medium for low quality diets e.g. rice or wheat bran for grow-out fishes. Consequently, FWD promises to reduce the need for the

expensive enrichment emulsions. However further studies are needed to determine the feasibility of this recommendation.

The protocol for fish waste FWD is simple, cheap and easily adjustable and, has been considered for patenting under the registration number P00201609066 in Indonesia. The FWD can produce up to 1,200 rotifers ml^{-1} bi-weekly, thus presents an opportunity to develop a self-sustaining biotechnology for stable production of high density and nutritious rotifers without algae, for aquaculture. The potential advantages of the FWD technique are three-fold: it promises to 1), reduce environmental pollution sources by reusing poorly discarded fishwastes; 2) reduce or eliminate direct dependence on the immediately cultured or the expensive on-site microalgae production and, 3) to lower the cost of rotifer enrichment, thus making it convenient for profitable aquaculture production, especially in the less developed countries. Further studies are needed to improve the efficiency of the FWD, and to investigate its usefulness for mass production other potential planktonic live food resources in aquaculture production systems that are popular in the developing countries.

V.2: FWD FOR CULTURING FRESHWATER ZOOPLANKTON IN OUTDOOR TANK CULTURE SYSTEM

Blending fishwastes and chicken manure extract as low-cost and stable diet for outdoor mass culture of freshwater zooplankton: optimized for aquaculture production in Kenya

Ogello EO, Wullur S, Sakakura Y, Hagiwara A. in preparation

Introduction

In the recent past, aquaculture activities have increased considerably in the tropical countries, leading to high demand for the zooplankton species such as rotifers, copepods, cladocerans and *Artemia* nauplii as suitable starter foods for fish larviculture (Lavens & Sorgeloos 1996; Conceicao et al. 2010). The live foods are highly digestible, more palatable, contain essential nutrients and, preserve water quality unlike inert diets (Kitajima & Fugita 1983). However, inconsistent supply of the live food resources continues to limit the intensive culture of economically valuable fishes. In nature, the zooplankton communities form a significant component of aquatic ecosystems, and are the primary prey for most larval fishes and crustaceans. The zooplankters have relatively high reproductive rates, thus making them attractive for use in the semi-intensive culture systems, which are popular in the developing countries.

Fresh microalgae are commonly used as diet to culture various zooplankton species in hatcheries, for aquaculture (Maruyama et al. 1997). However, the challenges of high density microalgal culture protocols have caused inconsistencies in the production of sufficient quantities of the zooplankton to match the increasing demand of the live foods for

aquaculture development, almost everywhere. These inconsistencies are more severe in the developing countries and therefore, organic manures and inorganic fertilizers are commonly applied in the ponds to naturally boost the pond biological productivity (Kang'ombe et al. 2006). However, inorganic fertilizers are expensive, and the direct use of animal manures is not only poorly understood but often hindered by the diverse socio-cultural aspects (Ogello et al. 2013). Recently, freshwater fish farming received greater attention in Kenya, thanks to the government incentives to enhance aquaculture production for national food security and rural livelihoods (Munguti et al. 2014; Ogello & Munguti 2016). However, these efforts did not address live food production mechanisms, which is the most frustrating obstacle to aquaculture development in Kenya. For this reason, massive larval fish mortalities in most hatcheries are still being reported. Therefore, economically viable and socially attractive live food production technologies are necessary to spur the growth of the Kenyan aquaculture sector.

In the previous chapters, application of 2.0 ml of chicken manure extract (CME) per litre of culture medium increased the parthenogenetic reproduction of the freshwater rotifer, *B. angularis* (Ogello & Hagiwara 2015) (Chapter III), while 0.5 g of fishwaste diet (FWD) combined with 0.2 g of wheat flour per litre of culture medium provided optimum conditions for higher growth rate and population density of the euryhaline rotifer, *B. rotundiformis* (Chapter V.1). The chicken manure contains sex hormones e.g. estradiol (Hagiwara et al. 2014) that enhances rotifer reproduction (Rapatsa & Moyo 2013), while the fishwastes contain essential PUFAs that can be reused in larviculture. The fishwastes also provide excellent substrates for proliferation of probiotic bacteria as food source for the rotifers (Chapter V.1). However, earlier attempts to culture single freshwater rotifer strain, *B. angularis* from Kenya using the FWD were unsuccessful. In this chapter, it was hypothesized whether a blend of the CME and the FWD can provide positive synergy to

enhance growth and reproduction of combined freshwater zooplankton (i.e. rotifers, copepods and cladocerans). The zooplankton obtain a substantial proportion of their carbon from detritus pathways via bacteria, and previous studies have reported the role of certain bacterial species in improving population growth and nutrition status of various zooplankton species (Pace et al. 1983; Bjørnsen et al. 1986; Yu et al. 1988, 1989, 1994; Turner &Tester 1992; Hagiwara et al. 1994).

Heterotrophic production of bacteria using fish defecates for *in situ* production of zooplankton communities has been exploited in the polyculture of shrimp, catfish and tilapia (Bouvy et al. 1994) with adequate carbon source (De Schryver et al. 2008). In the previous chapters, the CME and FWD technologies were independently used to culture single rotifer species i.e. *B. angularis* and *B. rotundiformis*, respectively under controlled laboratory conditions. However, the feasibility of the combined CME and FWD for *in situ* zooplankton production is not yet clear. This chapter explored the applicability of the two technologies (FWD and CME) for mass culture of freshwater zooplankton communities in an outdoor tank culture system, optimized for aquaculture production.

Materials and methods

The study area

This study was conducted at Kenya Marine & Fisheries Research Institute (KMFRI), Kegati Aquaculture Research Centre, located at 00°42'S; 034°47'E, from September, 2015 to January, 2016. The inoculant zooplankton i.e. rotifers, cladocerans and copepods were obtained from one of the fish production ponds that was previously fertilized with diammonium phosphate (DAP) and urea. One litre of the pond water was randomly sampled

3 times and filtered through a kitchen sieve to remove physical debris before determining the zooplankton densities in 1 ml sub-samples from each filtrate using a graduated plate with lugol fixation under stereo microscope, at × 25 magnifications. The densities of the zooplankton were used to determine the quantity of pond water needed to inoculate the experimental tanks. Plankton net of pore size 45 μm was used to filter the pond water to concentrate the zooplankton.

Fishwaste diet (FWD)

Barbus altinalis Linnaeus 1758, which is a low-valued bi-catch freshwater fish, were caught from river Mara, Kenya, using an electro-fisher (Smith-Root GPP USA) and transported to the laboratory in a cooler box. The FWD preparation protocol was similar to the one used in Chapter V.1, but this time, the volume of culture medium was 500 l of well water (plankton free). The FWD was modified by adding 2 ml l^{-1} of CME (Chapter III) and 0.2 g l^{-1} of maize flour as carbon source (Chapter V.1) into the tanks. The chicken manure was obtained from a local chicken farmer while the maize flour was sourced from a nearby maize miller. The CME was prepared as described in Chapter III.

Experimental design

The experiment was conducted in 9 asbestos tanks each containing 500 l of well water. After determination of the density of each zooplankton in the pond water, different plankton nets of mesh sizes 250 μm, 500 μm and 1000 μm were used to adjust the inoculated density of rotifers, copepods and cladocerans, respectively, in the experimental tanks. Three treatments i.e. FWD_A, FWD_B, and control (see Table 5-2.1) were each triplicated in the

culture tanks, 3 days prior to inoculation with a combination of 5, 2 and 0.4 ind ml^{-1} of rotifers, copepods and cladocerans, respectively in each culture tank on day 0. The control tanks were supplied with CME only. The tanks were covered with mosquito nets to keep off insects and birds. Every day, 5 ml of water was randomly sampled three times at the top 10 cm of each tank from which, rotifers, copepods and cladocerans were counted with lugol fixation. At the end of the first exponential phase, partial harvesting was done by replacing 50% of all the tank water with new well water and fresh FWD. The experiment lasted for 16 days.

The percent relative abundance (% RA) of each zooplankton species was calculated as [mean abundance (MA)] / total zooplankton taxa (N)] $\times$ 100. The specific growth rate (SGR) and the coefficient of variation (CV) of the mean SGR were calculated as shown in Chapter V.1 For qualitative composition, the zooplankters were identified up to the genus level according to Shiel (1995) while the zooplankton diversity was calculated using the Shannon-Weiner diversity index: H' = - SUM [(pi)*ln (pi)], where pi = number of individuals of species i/ total number of samples (Shannon & Weaver 1948).

Water quality

Temperature (^{o}C), dissolved oxygen (DO; mg l^{-1}), pH and electrical conductivity (µS cm^{-1}) were measured *in situ* using a multi-probe water checker (U-10 model, Horiba, Tokyo, Japan). The water turbidity (cm) was also measured *in situ* using a secchi disk. The water quality parameter measurements were taken daily at 1200 hours.

Table 5-2.1: The summary of the diets showing the composition and quantities of the fishwastes and CME applied in the culture tanks

Diets	Composition	Quantity applied per litre of culture medium
FWD_A	Fishwastes + starch	0.5 g of fishwastes + 0.2 g of starch (Chapter V.1)
FWD_B	FWD_A + CME	FWD_A + 2 ml of CME
Control	CME only	2 ml of CME (Ogello & Hagiwara 2015)

Data analysis

The data was analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test was used to determine the homogeneity of variances. Two-way analysis of variance (ANOVA) was used to test the effects of the FWD and culture days on the water quality parameters and zooplankton densities. One-way ANOVA was used to test the effect of FWD on the zooplankton mean abundance, SGR and diversity (H'). The Tukey's HSD Post Hoc test was performed to locate any significant differences at $p<0.05$.

Results

Water quality analysis

Measurements of the water quality parameters are presented in Table 5-2.2. The water temperature varied significantly during the culture days ($F=2.31$, $p=0.01$) but was not

affected by the FWD (F=0.45, p=0.63). Similarly, conductivity varied significantly during the culture days (F=1.76, p=0.04) with no significant effects of FWD (F=0.56, p=0.57). The DO was significantly affected by the FWD (F=4906.74, p<0.05) and culture days (F=200.44, p<0.05), with higher DO being recorded in the control than in the FWD-treated tanks (p=0.00). FWD_A tanks had significantly higher DO than FWD_B tanks (p=0.00). Turbidity varied significantly within the culture days (F=12.83, p<0.05), and between the FWD (F=841.80, p<0.05), with higher turbidity being recorded in the FWD tanks than in the control tanks (p=0.00). FWD_B tanks were more turbid compared to FWD_A tanks (p=0.00). The pH was neither affected by the FWD (F=1.18, p=0.31) nor culture days (F=1.07, p=0.38).

Zooplankton population dynamics

The population density of each zooplankton taxa was significantly affected by the FWD, culture days and the interaction between them (p<0.05). There was significantly higher rotifer density in FWD_A than in control tanks from day 4 - 6 (Tukey HSD, p<0.05), and higher density in FWD_B than in control tanks from day 3 - 7 and 11 - 12 (p<0.05). Meanwhile, there was significantly higher rotifer density in FWD_B than in FWD_A tanks on day 7 and 11 (p<0.05). The rotifer densities were 100.6±14.8, 146.3±7.0, and 60.0±7.9 ind ml^{-1} in FWD_A, FWD_B and the control tanks, respectively on day 7 (Figure 5-2.1). The population density of copepods was significantly higher in FWD_A than in control tanks on day 5 and 8 (p<0.05), and higher in FWD_B than in control tanks from day 5 - 8 and 12 (p<0.05). There was no significant difference between the FWD tanks (p>0.05). The copepods' densities were 7.8±2.5, 12.1±2.7 and 3.6±0.4 ind ml^{-1} in FWD_A, FWD_B and control tanks, respectively on day 7 (Figure 5-2.1). For the cladocerans, there was no

significant difference in the population density between FWD_A and control tanks (p=0.08). However, there was significantly higher density in FWD_B than in control tanks from day 6 - 8 and 11 - 13 (p<0.05), and higher density in FWD_B than in FWD_A tanks on day 7 and 11-13 (p<0.05). The densities of the cladocerans were 3.1±1.2, 7.7±1.7 and 2.3±0.5 ind ml^{-1} in FWD_A, FWD_B and control tanks, respectively on day 7 (Figure 5-2.1). The summary of the composition of zooplankton taxa, mean and percent abundance is presented in Table 5-2.3. The most abundant genera were *Brachionus* sp., *Cyclops* sp. and *Daphnia* sp. for rotifers, copepods and cladocerans, respectively.

The SGR of each zooplankton taxa was significantly affected by the FWD (One-way ANOVA, p<0.05) with higher SGR for the rotifers in FWD_B than in FWD_A (p=0.05) and control tanks (p=0.00), and higher SGR in FWD_A than in control tanks (p=0.00) (Table 5-2.4). The SGR for the copepods was significantly higher in the two FWD than in the control tanks (p<0.05), but not significantly different between the FWD tanks (p=0.11). There was significantly higher SGR for the cladocerans in FWD_B than in FWD_A (p=0.02) and control tanks (p<0.05), but not significantly different between FWD_A and control tanks (p=0.66) (Table 5-2.4). The FWD did not affect the diversity (H') of the rotifers (One-way ANOVA; F=0.06, p=0.93), copepods (One-way ANOVA; F=0.09, p=0.91) and cladocerans (Kruskal-Wallis test; χ^2=1.67, df=2, p=0.43) (Table 5-2.4). Similarly, there was no significant difference in coefficient of variation (CV) among the treatments (One-way ANOVA, F=1.97, p=0.22) (Table 5-2.4).

Table 5-2.2: The mean values of water quality parameters ± SD in the culture tanks for 16 days; Values with different superscript in each row are significantly different at $p<0.05$; Two-way ANOVA; Tukey HSD test, a>b>c, $n=48$

	Treatments			
Water quality parameters	Before experiment	FWD_A	FWD_B	Control
Temperature (^oC)	25.1 ± 1.0	25.6 ± 1.0^a	25.7 ± 1.0^a	25.8 ± 1.2^a
Conductivity ($\mu S\ cm^{-1}$)	113.7 ± 2.2	115.9 ± 2.8^a	116.2 ± 2.4^a	115.7 ± 2.1^a
Dissolved oxygen (mgl^{-1})	5.73 ± 0.19	3.94 ± 0.77^b	3.84 ± 0.68^c	5.61 ± 0.21^a
pH	7.51 ± 0.01	6.63 ± 0.03^a	6.65 ± 0.04^a	6.66 ± 0.12^a
Turbidity (cm)	39.6 ± 1.5	35.7 ± 1.3^c	23.4 ± 3.1^a	27.6 ± 3.1^b

Table 5-2.3: Zooplankton taxa composition, mean abundance (MA) and percent relative abundance (% RA) of the zooplankton species in each treatment at day 16; the values are mean ind/ml ± SD. Different superscripts in a row denote significant differences for each zooplankton species at $p<0.05$, One-way ANOVA, Tukey HSD test, n=6, a>b>c; -, absent

Zooplankton taxa (ind/ml)	Treatments					
	FWD_A		FWD_B		Control	
	MA	% RA	MA	% RA	MA	% RA
Rotifers						
Brachionus sp	38.6 ± 8.6^{ab}	25.3	51.8 ± 12.1^{a}	25.5	33.6 ± 3.7^{b}	23.7
Filinia sp	-	-	9.6 ±1.2	4.9	-	-
Lecanea sp	13.8 ± 4.6^{a}	9.1	10.6 ± 6.1^{a}	4.6	12.0 ± 3.2^{a}	8.4
Keratella sp	17.3 ± 3.0^{a}	11.0	12.2 ± 2.9^{b}	5.4	7.5 ± 2.8^{c}	5.6
Asplanchnia sp	19.0 ± 2.2^{b}	12.3	25.5 ± 6.2^{a}	12.7	24.3 ± 2.1^{ab}	16.7
Others	4.6 ± 3.0	3.2	5.5 ± 2.2	2.9	3.3 ± 2.1	2.1
Copepods						
Cyclops sp	16.5 ± 2.6^{b}	11.0	21.3 ± 2.8^{a}	10.3	18.3 ± 1.6^{ab}	12.6
Diaptomus sp	10.8 ± 3.4^{b}	7.1	20.1 ± 5.7^{a}	9.8	14.5 ± 2.0^{ab}	10.5
Cladocerans						
Diaphanosoma sp	9.3 ± 2.2	5.8	16.3 ± 3.2	7.1	-	-
Daphnia sp	15.6 ± 3.7^{a}	10.4	16.0 ± 3.1^{a}	7.8	15.0 ± 5.1^{a}	10.5
Moina sp	7.6 ± 1.8^{a}	5.2	10.0 ± 7.1^{a}	4.9	13.6 ± 3.7^{a}	9.8
Ceriodapnia sp	-	-	4.5 ± 2.8	2.5	-	-
Total (N)	**154**		**204**		**143**	

Table 5-2.4: Specific growth rate (SGR) and diversity (*H'*) of each zooplankton taxa in every treatment, and the coefficient of variation (CV) of each treatment. The values are mean ± SD. One-way ANOVA, Tukey HSD test, different letters in each row represent significantly differences at $p<0.05$; a>b>c

		Treatments		
Parameters	Zooplankton taxa	FWD_A	FWD_B	Control
SGR (day^{-1}) (n=3)	Rotifera	0.42 ± 0.02^b	0.48 ± 0.01^a	0.35 ± 0.02^c
	Copepoda	0.42 ± 0.04^a	0.48 ± 0.03^a	0.32 ± 0.02^b
	cladocera	0.51 ± 0.06^b	0.65 ± 0.03^a	0.48 ± 0.03^b
Diversity (*H'*) (n=6)	Rotifera	1.44 ± 0.14^a	1.51 ± 0.08^a	1.36 ± 0.14^a
	Copepoda	0.67 ± 0.05^a	0.69 ± 0.01^a	0.68 ± 0.02^a
	cladocera	1.05 ± 0.17^a	1.30 ± 0.06^a	0.69 ± 0.20^a
CV (n=3)		0.09 ± 0.03^a	0.05 ± 0.02^a	0.06 ± 0.01^a

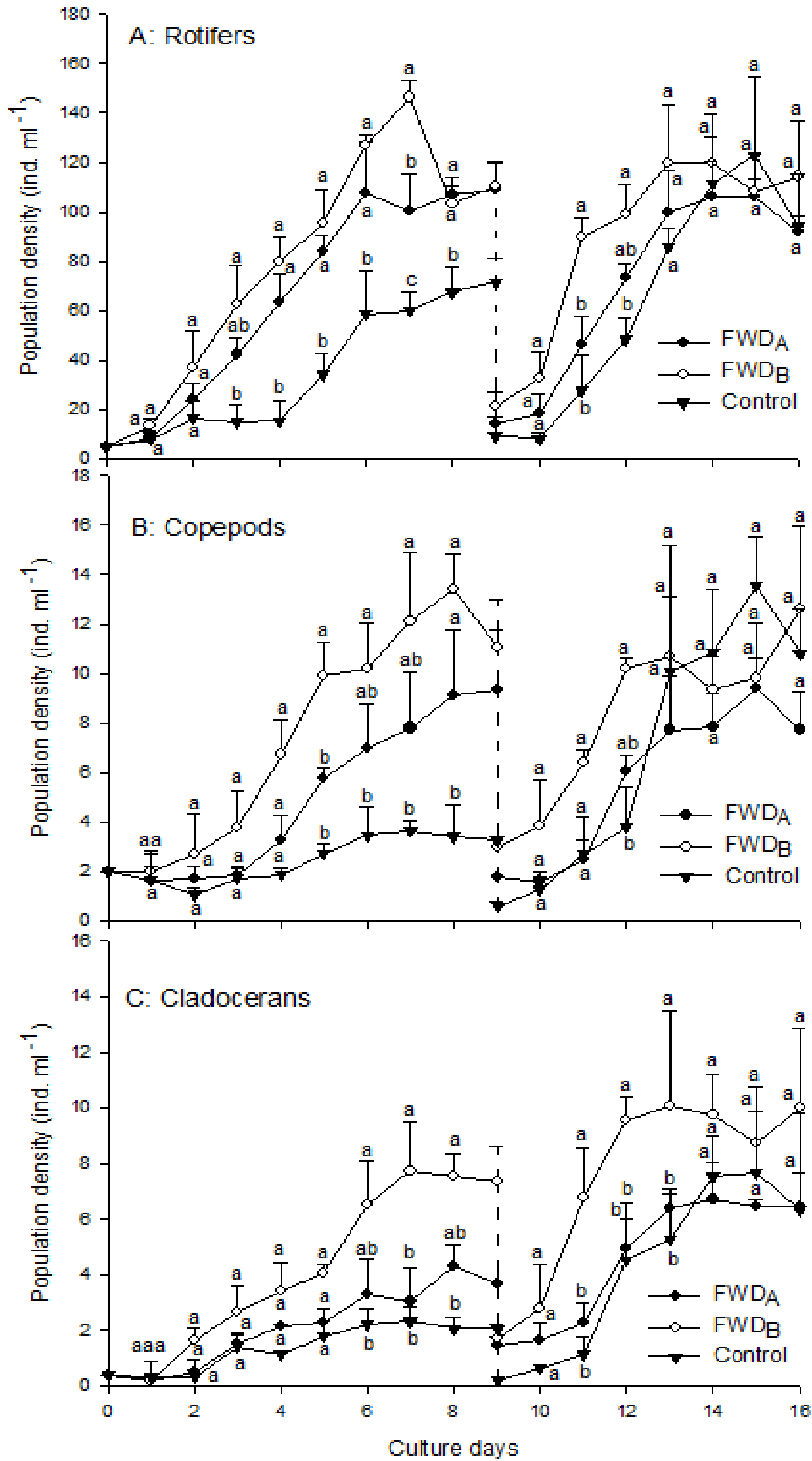

Figure 5-2.1: The population density curves of each zooplankton taxa. Each plot and vertical bar represents mean ± SD. Half of the culture water was replaced with new media on day 9 in each treatment as shown by the dotted lines. Different superscripts on each day represent significantly different mean population densities at $p<0.05$; Two-way ANOVA, Tukey HSD test; $n=3$, a>b>c

Discussion

In larviculture, management of the zooplankton forage base is a critical phase for the successful transition of the fish larvae to fingerling stage. The dynamic characteristics of zooplankton populations have led researchers to apply different techniques to produce high densities of the desired zooplankton species until fish are either harvested, or able to consume inert feeds (Geiger 1983). Despite the significant progress in zooplankton cultivation techniques, cost-effective and stable protocols for producing sufficient quantities of the desired zooplankton species without microalgae are scanty. This study demonstrated the feasibility of FWD made by blending CME and fishwastes for culturing zooplankton species in an outdoor mass culture system.

Despite changes in the water quality parameters during the experimentation period, the values remained within the acceptable limits for freshwater pond aquaculture (Boyd 1982). The water conductivity increased slightly, perhaps due to leached ions from decaying fish tissues or zooplankton metabolites. Low DO in the FWD tanks could have been caused by high zooplankton abundance, microbial biomass and decomposing organic matter. Oxygen depletion in culture facilities is commonly experienced during decomposition of organic matter (Boyd 1982), but can be improved by frequent water exchange or aeration. The poor secchi disk visibility (high turbidity) in FWD_B tanks could be explained in terms of high zooplankton organic matter, bacteria and plankton biomass.

More than half of zooplankton species in every sample consisted of rotifers (Table 5-2.3). Rotifers, especially the *Brachionus* spp. are normally the first zooplankters to reach large numbers in newly colonized habitats, thus taking competitive advantage (Shandhu et al. 1985; Louette et al. 2008). With the shortest lifespan of 5 - 12 days, rotifers reach peak reproductive levels much earlier (about 3.5 days) than other zooplankters (Allan 1976).

Nonetheless, large cladocerans (> 1.2 mm) e.g. daphnia, can suppress rotifer abundance through physical interference and predation (Gilbert 2003). Copepods and cladocerans have similar life span (about 50 days) but have different peak reproductive periods (Filenko et al. 2011), which take about 24 and 15 days to reach, for copepods and cladoceans, respectively (Allan 1976). The copepods only reproduce sexually and therefore require longer periods to increase their population levels (Allan 1976). In addition, overcrowding decreases fecundity of the calanoid copepods and the reproduction of the harpacticoid copepod, e.g. *Tigriopus japonicus* is more successful when mixed-cultured with rotifers (Hagiwara et al. 1995b). Previously, the culture for single species of *B. angularis* was unsuccessful with the FWD. In this chapter, many species of freshwater zooplankton were successfully cultured together and, it is probable that inter- and intra-specific interactions were important for their growth and reproduction. However, the zooplankton species interactions were not studied, thus requiring further study.

Addition of CME to the fish wastes caused significant increase in SGR and population densities of the zooplankton species and therefore appears as an improvement to the FWD. In chapter V.1, it was determined that decomposing fish tissues provided substrates for the proliferation of diverse microbial flora, some of which have probiotic properties. The CME probably facilitated phytoplankton growth in the tanks, thus expanding forage base (i.e. bacteria and phytoplankton) for the zooplankton growth and reproduction. The synergy between FWD and CME could be important for zooplankton growth and reproduction. Chicken manure is also an excellent substrate for probiotic bacteria (Rapatsa & Moyo 2013; Elsaidy et al. 2015), and contains growth promoting compounds e.g. 17α and β-estradiol that can positively influence zooplankton reproduction history (Shemesh & Shore 1994; Hagiwara et al. 2014) either individually or in combination (Verschuere et al. 2000). The efficacy of CME in enhancing the production of *Diaphanosoma celebensis* and *Tigriopus*

japonicus has been reported where up to 14 ind ml^{-1} was produced within 12 days (Hagiwara et al. 2016). Omnivorous zooplankton feed on detritus-bacteria complex in the absence of live prey (Hart & Jarvis 1993). Morris & Mischke (1999) reported that phytoplankton alone do not necessarily increase zooplankton populations and thus explains the relatively low zooplankton density in the control experiment. Fungi and bacteria associated with decaying organic substances supplement phytoplankton foraging with essential proteins, lipids and vitamins to cause high growth effects for zooplankton (Morris & Mischke 1999). The high *Brachionus* spp. abundance observed in this study reflects observations in chapter III, which demonstrated the effectiveness of CME in enhancing parthenogenetic reproduction of *B. angularis*. After the first harvest, the zooplankton population densities increased to new peaks, which were comparable to the previous ones, suggesting that the system can be self sustaining, perhaps with frequent harvesting to reduce organic loads. Generally, lower coefficient of variation (CV) of the treatments suggests stability of the FWD technology. With application of FWD_B, it is possible to obtain about 150, 12, and 8 ind ml^{-1} of rotifers, copepods and cladocerans, respectively, on weekly basis. Comparatively, Kyewalyanga & Mwandya (2002) obtained about 76 and 100 rotifers ml^{-1} within 5 days using conventional chicken manure methods in ponds and tanks, respectively. The results of the present study demonstrate the superiority of FWD technique compared to the normal traditional live food culture methods using chicken manure only. Further studies are recommended to determine the suitability of the FWD-fed zooplankton species for larviculture of the local fish species.

V.3: DIETARY VALUE OF THE FWD-FED ROTIFERS FOR LARVICULTURE: A PRELIMINARY STUDY

Dietary value of the rotifer *Brachionus rotundiformis* (sensu stricto) fed with fishwaste diet (FWD) for larval growth and development of Japanese whiting, *Sillago japonica* Temminck & Schlegel 1843

Ogello EO, Wullur S, Sakakura Y, Hagiwara A. in preparation

Introduction

At the close of endogenous feeding phase, most marine larval fishes still have small mouth gap and rudimentary digestive track and therefore, cannot ingest and digest inert feeds (Lavens & Sorgeloos 1996; Yufera & Darias 2007). At this critical phase, the larval fishes require timely and adequate supply of appropriate live foods that are readily ingested, efficiently digested and, that contain essential nutrition for their healthy growth and development (Watanabe et al. 1983). Depending on the rotifer culture condition, essential nutrients may be deficient, sometimes lower than the required threshold for healthy fish development (Hamre et al. 2008). Therefore, due to the possibility of nutritional deficiencies, rotifers are often provided with supplements to improve their nutritional value for larviculture. The freshly cultured microalgae, sometimes enriched with essential nutrients such as EPA, DHA and vitamin B_{12}, have been commonly preferred as first choice diet for the rotifer cultures (Maruyama et al. 1997). However, year round cultivation of sufficient and nutritious microalgae is a costly and laborious task for many hatcheries and, thus limits

the continuous production of sufficient live food and, sometimes disrupts the fish seedling production programs in the microalgae-based hatcheries (Lubzens et al. 1995). In chapter V.1, about 1,200 individuals ml^{-1} of the rotifer, *B. rotundiformis* were obtained using FWD only. In addition, the rotifers had relatively higher EPA and DHA contents than those rotifers cultured using normal (non-enriched) *C. vulgaris* diet. This finding demonstrates a possibility of obtaining nutritious rotifers without necessarily feeding them on microalgae or enriching them with the expensive commercial emulsions. However, the suitability of the FWD-fed rotifers for larval fish rearing still remains unclear. To explore on this aspect, a study was conducted to determine the effects of the rotifer, *B. rotundiformis* (SS-type) fed with FWD, on the larval growth and development of the Japanese whiting *Sillago japonica* (Chapter V.3). *S. japonica* is a valuable fish resource distributed along the coasts of Japan, South Korea, Taiwan and Philippines (Kashiwagi et al. 2000).

Materials and methods

Rotifer preparation

The inoculant rotifer, *B. rotundiformis* (SS-type; Thai strain) was obtained from Nagasaki Prefectural Institute of Fisheries, Japan. The SS-type rotifer is small in size (i.e. 70 – 160 μm), and is considered appropriate for the small-mouth fish larvae such as of *S. japonica* (Tsukashima et al. 1983). The rotifers employed in the test experiment were cultured using the FWD as described in chapter V.1. Meanwhile, the rotifers used for the control experiment were batch cultured in 50 l of artificial seawater (22 ppt) and fed with HUFA enriched *C. vulgaris* diet (Super Fresh Chlorella-V12, Chlorella Industry Co. Ltd., Fukuoka, Japan) at 25°C with aeration. The *C. vulgaris* diet was daily maintained at 7.0×10^6 cells ml^{-1} in the rotifer cultures (Araujo & Hagiwara 2005).

Rotifer swimming speed

To determine the viability of the rotifers, swimming speed was recorded for 30 seconds under a stereomicroscope at 10× (SteREO Discovery V8, Zeiss, Germany) equipped with a digital camera (AxioCam, HSm) and image-analysis software (AxioVision 4.8). Five rotifers were sampled from each treatment and placed in 20 µl water drop, in which the swimming speed was measured using Dipp Motion Pro version 8.0 (DITECT Co. Ltd., Japan) in millimeters per second (mm s^{-1}).

Larviculture experiment

Fertilized eggs of *S. japonica* were obtained from the same institute as rotifers. The eggs were carefully transferred into six polycarbonate tanks each containing 100 l of artificial sea water (33 ppt) at 10 eggs l^{-1} with aeration fixed at 50 ml min^{-1} (Kim et al. 2014). After hatching, larval fish development was monitored every 6 hours until mouth opening and then, the feeding process began (Figure 5-3.1). Two feeding regimes i.e. the test (FWD-fed rotifers) and control-rotifers were each triplicated in the culture tanks. The fish larvae were reared at 25°C with 12-h diurnal photoperiod (900–2100), for 10 days. The density of the rotifers (diet) in each larval fish rearing tank was maintained at 10 ind. ml^{-1} throughout the rearing period (Oozeki et al. 1992) from 2 days post hatching (dph). Ten larval fish were randomly sampled every 2 days from 2 dph for morphometric and gut content analysis. The fish samples were kept in small screw-cap bottles anesthetized with 2-3 drops of MS 222 followed by 5% formalin fixation (Kim et al. 2014).

The hatching rate and survival activity index (SAI) experiments were performed to determine the quality of the fish eggs and the fish larval survivability during starvation,

respectively (Shima & Tsujigado 1981). Here, 20 eggs were placed in 500 ml beaker containing 300 ml of artificial sea water (33 ppt) at 25°C in total darkness, without aeration and feeding. Dead larvae were counted and removed every 24 h until total larval mortality was reached. Triplicate observations were used to calculate the hatching rate and SAI. The percentage of eggs that hatched normally was calculated after 24 h by the formula: [Normally hatched larvae / total number of eggs] $\times$ 100%, while SAI was calculated using the equation:

$\text{SAI} = \frac{1}{N}\sum_{i=1}^{K}(N - hi)\times i$ (Shimma & Tsujigado 1981), where N = total number of examined larvae, hi = cumulated mortality by i-th day and K = number of days elapsed until all larvae died due to starvation. Morphological characteristics of the larval fish samples i.e. total and standard length, eye diameter, body depth and head length (Figure 5-3.2), were measured using a microscopic measurement system that included a stereomicroscope (Discovery V8, Zeiss, Germany) equipped with a digital camera (AxioCam, HSm) and image-analysis software (AxioVision 4.8). At 10 dph, the percent survival of the fish larvae was calculated from the average number of surviving fish larvae in each culture tank. Also, the viability test of the fish larvae was determined at 10 dph by subjecting 10 fish larvae from each replicate tank to 30 s of air exposure before determining the rate of their survival thereafter. The dry weight of the fish larvae was measured in pre-weighed aluminum boats dried at 60°C for 24 h and weighed using ultra-micro chemical balance (Mettler Toledo, USA).

Fatty acid analysis

At 10 dph, all the fish larvae were harvested from each treatment tank, dried using filter paper from beneath the harvesting mesh and kept at -80°C until biochemical analysis.

The total lipid and fatty acid composition of the fish larvae and rotifer samples were performed by Chlorella Industry Co., Fukuoka, Japan as explained in Chapter V.1.

Statistical analysis

Statistical analysis was carried out using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015). The Bartlett test for homogeneity was used to test the equality of variances. Repeated ANOVA measures were used to test the effects of the diets on fish larval morphological parameters, gut content and swimming speed. The larval fish survival and viability tests were analyzed using Kruskal-Wallis test and Log-Rank test for groups, respectively. Where significant differences were detected, the Tukey HSD post hoc test was performed to locate them at $p<0.05$.

Results

Larviculture

The hatching rate of *S. japonica* eggs was 93.3±2.9%. The hatched fish larvae survived up to 5 days of starvation, with a SAI of 5.8±1.1. The rotifer swimming speed was affected by the diets (One way ANOVA, $F=14.51$, $p=0.01$), where the control-rotifers had significantly higher speed (0.83 mm s^{-1}) than FWD-rotifers (0.65 mm s^{-1}) (Tukey HSD, $p=0.01$) The number of ingested rotifers by individual fish larvae was significantly affected by age of fish larvae (Two-way ANOVA, $F=128.04$, $p=0.00$), but not the diets ($F=0.24$, $p=0.62$). The fish larvae from the two treatments ingested similar amounts of rotifers each day and, at 10 dph, about 10.5±3.1 and 11.6±2.7 rotifers were ingested by the FWD-fish and control-fish, respectively. At 10 dph, there was no significant differences in morphological

parameters e.g. head length (One-way ANOVA, F=2.46, p=0.13), eye diameter (One-way ANOVA, F=2.46, p=0.12) and body depth (One-way ANOVA, F=0.16, p=0.69) between FWD-fish and control-fish. However, there was a significantly higher total and standard length for the FWD-fish than the control-fish (p=0.00) (Table 5-3.1). There was no significant differences in fish larval survival rate (Kruskal-Wallis test, χ^2=0.19, df=1, p=0.66), viability (Log-rank test for groups, χ^2 = 0.4, df=1, p=0.55) and dry weight (One-way ANOVA, F=6.41, p=0.06) between the two diets (Table 5-3.2). The nutritional value of rotifers and fish larvae is summarized in Table 5-3.3. Due to high fish mortality experienced during the experiment, the quantities of fish sample were insufficient for total lipid analysis. Nonetheless, percent compositions of DHA were 5.2 and 18.2 for FWD- and control- fish, respectively. The EPA was under detectable limit in both samples. The ratio of DHA: ARA was 2.4 and 8.3 for the FWD- and control- fish, respectively (Table 5-3.3).

Table 5-3.1: Morphological parameters of the fish larvae as at 10 dph, the values are mean length (mm) ± SD, with different numbers of fish larvae per replicate. One-way ANOVA, Tukey HSD, different superscripts in each column denote significant differences at $p<0.05$ between the diets, a>b

Diet	Total length (n=30)	Standard length (n=30)	Head length (n=12)	Eye diameter (n=30)	Body depth (n=12)
FWD	3.65 ± 0.30^{a}	3.33 ± 0.33^{a}	0.76 ± 0.03	0.25 ± 0.02	0.69 ± 0.11
Control	3.42 ± 0.22^{b}	2.94 ± 0.33^{b}	0.74 ± 0.05	0.24 ± 0.02	0.68 ± 0.11

Table 5-3.2: Means ± SD of fish larval characteristics at 10 dph. The fish dry weight at 2 dph was 0.08 ± 0.01mg/ind in both diets, n=3

Diets	Dry weight (mg/ind)	Survival rate (%)	Viability (%)
FWD	0.26 ± 0.03	9.70 ± 9.06	58.8 ± 9.3
Control	0.21 ± 0.01	4.93 ± 0.83	64.4 ±7.9

Table 5-3.3: Total lipids (Total, mg/g dry weight) and fatty acid composition (% of total lipids) of the rotifers fed with FWD and control diet, and of the fish (*S. japonica*) larvae cultured with the FWD- and control-rotifers for 10 days. * = total lipids not measured due to limited sample quantity

	Rotifers fed with		*S. japonica* larvae fed with	
	FWD	Control diet	FWD-fed rotifers	Control-rotifers
Total lipids	20.5	23.3	*	*
Fatty acids				
14:0	5.4	3.6	1.1	2.8
14:1	2.3	0.2	0.4	0.1
16:0	15.2	18.7	14.5	17.4
16:1	3.0	2.1	1.1	5.1
16:2	0.6	1.2	1.3	0.5
18:0	4.3	7.0	10.0	5.6
18:1	4.8	14.7	4.9	13.6
18:2*n*-6	4.1	1.3	21.8	9.5
18:2*n*-3	0.5	0.7	2.4	1.0
20:0	0.3	0.8	0.0	0.4
20:1	0.6	0.3	0.3	0.1
20:4*n*-6	0.0	2.5	2.2	2.2
20:5*n*-3	1.9	6.6	0.0	0.0
22:0	0.0	0.3	1.0	0.3
22:1	15.1	0.4	2.7	5.0
24:0	1.4	1.3	0.4	0.6
24:1	0.8	0.5	1.1	0.3
22:5*n*-3	0.5	2.2	3.0	2.1
22:6*n*-3	1.7	18.8	5.2	18.2
Others	37.4	16.8	26.6	15.3
DHA/EPA	0.9	2.8	0.0	0.0
DHA/ARA	0.0	7.5	2.4	8.3
Total	100	100	100	100

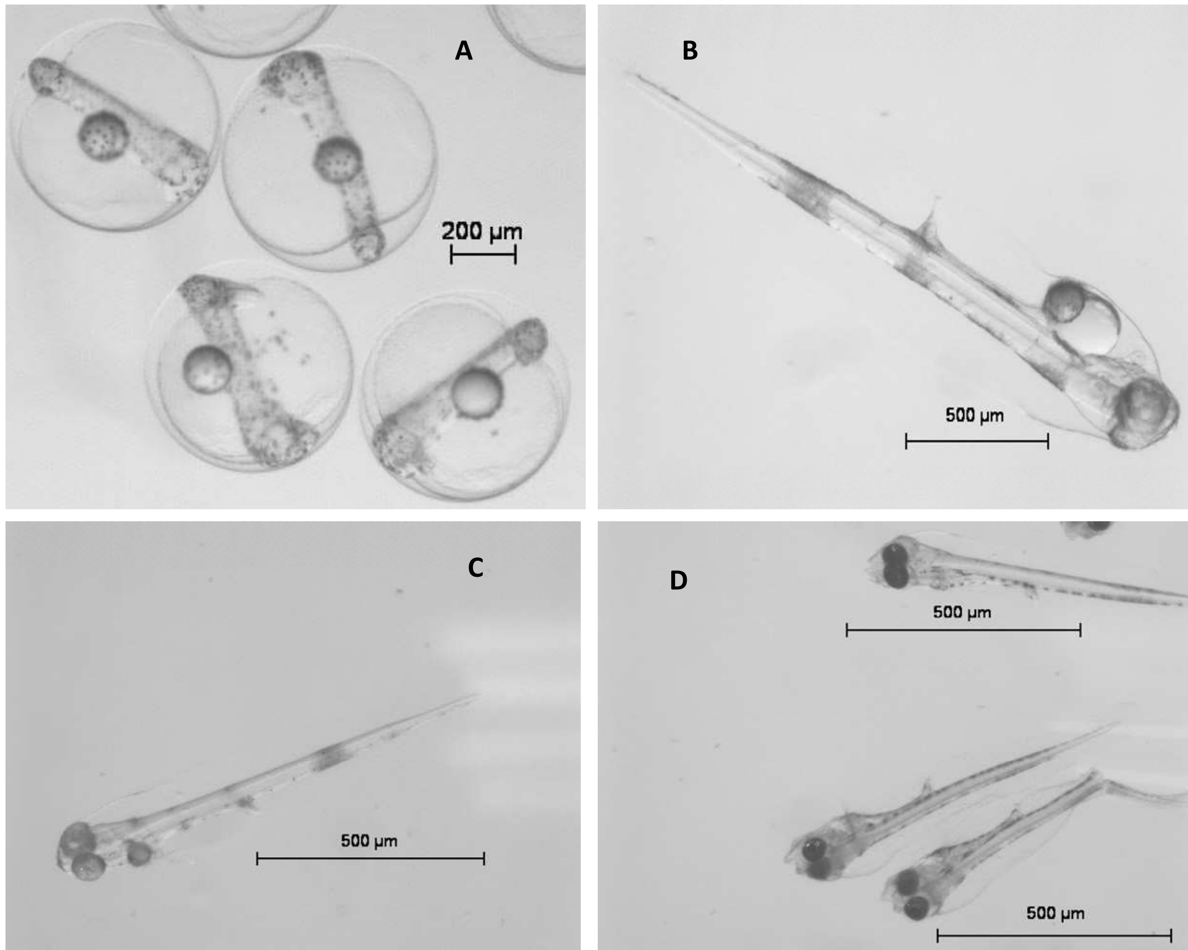

Figure 5-3.1: Larval developmental stages of *Sillago japonica*; A) egg stage, B) newly hatched larvae with a bigger egg yolk, C) 1 dph showing reduced egg yolk size, D) 2 dph showing mouth opening (end of exogenous feeding)

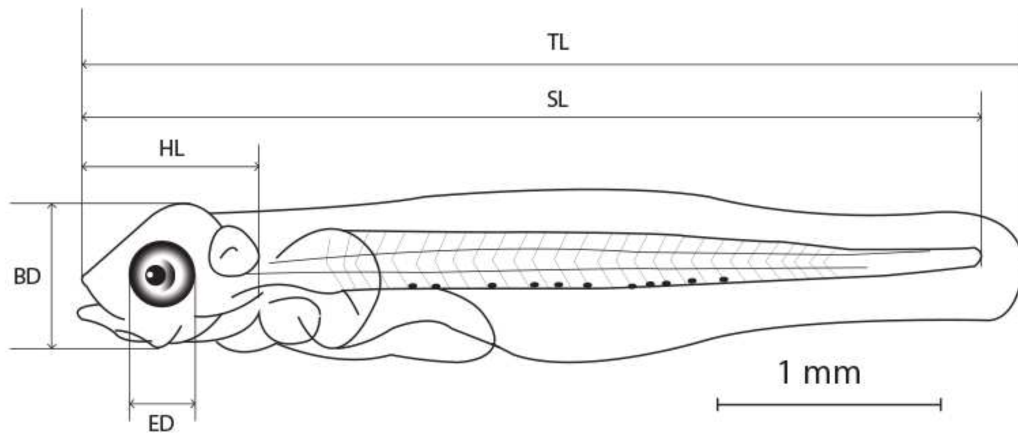

Figure 5-3.2: The five morphological characteristics to estimate larval growth and development of *S. japonica*. TL, total length; SL, standard length; HL, head length; ED, eye diameter; BD, body depth

Discussion

This chapter presents a preliminary study on the dietary value of FWD-fed rotifer *B. rotundiformis* (SS-type) for larval rearing of *S. japonica*. The fish eggs were of high quality (93.3±2.9% hatching rate) and, the fish larvae showed high survivability during starvation (SAI: 5.8±1.1). These results are comparable to those of Oozeki & Hirano (1986) who reported 95% and 4.1 – 8.4 hatching rate and SAI, respectively for *S. japonica* larvae. There was unprecedented high fish mortality and the reason was not clearly determined. However, it was suspected that aeration rate employed in the study was probably not optimal for this fish species. Further study especially on the optimum rate of aeration for *S. japonica* larvae should be considered. The viability of the FWD-fed rotifers as determined by the swimming speed may have been weaker than control-rotifers perhaps due to high bacterial turbidity in the FWD culture medium. More active rotifers normally improve larval fish development because of the ability to stimulate fish raptorial behaviour (Personal communication, Prof. Atsushi Hagiwara).

Previous larval fish rearing data confirm that survivability of fish larvae strongly depends on the amount of DHA and ARA present in the diet (Sargent et al. 1999; Furuita et al. 2000). Since most marine fish larvae cannot synthesize DHA from precursor molecules e.g. EPA or α-linolenic acid (ALA), supplying DHA-rich feeds to fish larvae is important (Masuda et al. 1998). The DHA/ARA ratio of 2.4 obtained in this study is less than the optimal ratio of 10.0 required for proper larval fish development (Sargent et al. 1999). Nonetheless, the similarity of larval fish development parameters between the test and control diet suggests a possibility of FWD for the larviculture of *S. japonica*. However, due to insufficient data, it is difficult to conclusively discuss the total amounts of DHA and EPA of *S. japonica* larvae in this study and therefore, further experiments are recommended to draw concrete conclusions on the dietary value of FWD for larviculture.

Chapter VI

GENERAL DISCUSSION

The history of agricultural programs in most developing countries have primarily focused on the provision of cereal crops and staple food commodities, which cannot not completely address the persistent malnutrition challenges (Brummett & Williams 2000). Today, aquaculture is an integral pillar in the diversification of crop production and micronutrient intake especially in the developing countries where consumption of fish protein is comparatively low (Brummett et al. 2008; FAO 2016). However, achieving absolute sustainable aquaculture productivity is subject to fixing some technical, social and, sometimes, administrative challenges that have staggered aquaculture growth for decades. Technically, the major impediment to aquaculture development is the scarcity of nutritionally complete and cost-effective larval fish foods for the production of high quality fish seedlings (Lavens & Sorgeloos 1996). In order to spur considerable socio-economic benefits of aquaculture, and to make fish more accessible and affordable, capacity building on local aquaculture technologies is a critical priority. The supply of adequate live food resources e.g. rotifers, copepods and cladocerans chiefly depends on the availability of high density microalgae, which require expensive investments to produce. Consequently, inadequate supply of appropriate live food resources consistently disrupts fish seedling production programs in the hatcheries (Lubzens et al. 1995). Rotifers, especially the brachionid spp. are the most valuable larval fish food and therefore, production of sufficient quantity and timely supply is a prerequisite for successful larviculture (Hagiwara et al. 2001). However, intensive larviculture of economically important fishes (mostly marine) depends on the sufficient supply of nutrient-rich zooplankton species (usually rotifers), which have traditionally been raised on enriched microalgal diet, but maintaining a constant

supply of enriched microalgal diet is an economic burden on many hatcheries. Besides, algal cultures are unstable, prone to risks of contamination, have seasonal quality variations, and, have limited shelf life (Laing 1991). For that reason, studies are recommended to investigate cost-effective and stable rotifer production techniques, preferably, without algae to enhance sustainable and profitable aquaculture, especially in tropical countries without modern infrastructure for microalgae production.

In order to develop cost-effective live food production methods for local aquaculture, it is imperative to establish a local library of the live food resources. On review of literature, it is evident that knowledge on the biological aspects of local rotifers as live food candidates for aquaculture in most developing countries is fragmentary and scanty (De-Ridder 1987; Murray 2011; Sutherland et al. 2013). For example, in Kenya, there is dearth of information with respect to the suitability of local rotifer strains for aquaculture activities and, their ecological stability during changing climatic conditions. Culturing rotifers under microcosmic conditions enables investigations on the reproductive capacities, ecological behavior and mass culture potential. For this reason, I studied the morphological and reproductive ecology of a common freshwater rotifer species, *Brachionus angularis* in Kenya and, the effects of varying temperatures and food concentrations on its reproductive biology (Chapter II). As expected, temperature and food concentration affected the reproductive traits of the rotifer directly, thus confirming the studies of Edmondson (1964, 1965) that temperature-food interactions affect the rotifer reproductive history. The rotifer's life table demographics and population ecology were optimal at 25°C with 2.5×10^{6} cells ml^{-1} of *C. vulgaris.* An expanded rotifer life expectancy, high fecundity, high intrinsic rate of natural increase and specific growth rate leads to quicker and longer rotifer reproduction. This is probably good news for hatchery managers who always require high rotifer densities within shortest time, for larviculture activities. Miracle & Serra (1989) reported that warm

water-adapted rotifer species show highest or maximum growth rate values at temperatures above 27°C, but Walz (1987) reported 25°C as the best temperature for maximum growth rate for *B. angularis*, same as the study in chapter II. Nonetheless, varying rotifer reproductive traits has been linked to genetic adaptations to local environmental pressures (Gilbert 2003; Ma et al. 2010), clone selection at the environmental temperatures of origin and the influence of temperature-food interactions (Edmondson 1964, 1965; Galkovskaya 1987). With a relatively small lorica size (Table 2-1); the Kenyan *B. angularis* appears to be a convenient diet for larviculture of small-mouth freshwater fishes such as gold fish, *Carassius auratus* (Lim et al. 2003). Indeed, this study lays a platform to identify other potential local live food resources, perhaps in some isolated biotopes, to provide a local live food library for spurring aquaculture growth in Kenya. This study also opens up opportunities to understand the potential effects of the changing ecological conditions (i.e. temperature and food availability) on the reproductive ecology of the Kenyan strain *B. angularis.* Further studies should develop technologies for producing cheaper diets for mass culture of this rotifer strain.

To advance on the above studies, I formulated a research hypothesis that investigated whether chicken manure extract (CME) would affect the population growth, sexual reproduction and body size of the Kenyan rotifer strain *B. angularis* (chapter III). It is imperative that larval fish food should be of convenient size for easy ingestion by the fish larvae. Generally, chicken manure has commonly been used to enhance biological productivity in fishponds in most tropical countries (Elsaidy et al. 2015). However, the optimal quantity and mode of application of the animal manure is poorly understood and, could lead to stunted fish productivity (Kang'ombe et al. 2006). For example, large amounts of manure generate high organic loads that raise biological oxygen demand (BOD), creating anoxic zones in the culture facilities. On the contrary, less manure application limits

proliferation of natural live foods, leading to low natural productivity (Elsaidy et al. 2015). Whereas some animal manures e.g. of pigs are restricted by social and religious concerns, there are no such restrictions to chicken manure, making it socially attractive across the board. The study in chapter III showed that 2.0 ml l^{-1} of CME is optimum for enhancing population density, parthenogenetic reproduction and specific growth rate of the rotifer *B. angularis*, without affecting the lorica size. The observation is consistent with the persistent nature of growth promoting compounds contained in the CME (Shemesh & Shore 1994; Hagiwara et al. 2014). According to Pentikainen et al. (2006), estradiol compounds, which are found in chicken manure act as growth hormone for the female reproductive tissues, hence resulting in high viability of oocytes. CME suppresses rotifer mixis stimulus but favors parthenogenetic propagation, which is the desired result in daily hatchery operations. Literature evidences indicate that chicken manure is also an excellent substrate for the heterotrophic production of probiotic bacteria e.g. *Bacillus* sp. that can be utilized by rotifers to improve their survival and reproduction (Gatesoupe 1991; Moriarty 1997; Rapatsa & Moyo 2013; Elsaidy et al. 2015). CME blends varieties of hormones (Hakk et al. 2005; Hagiwara et al. 2014), and thus provides an opportunity to reduce purchases of commercial growth promoting compounds. The common practice is the spreading of raw chicken manure on pond, or stuffing them in sacks at a particular spot in the pond. The former method causes quicker depletion of dissolved oxygen while the latter causes non-uniform distribution of nutrients and creates anoxic spots during poor water circulations in the pond. Such technologies are outdated and are generally characterized by low pond productivity (Kangombe et al. 2006). The CME technique developed in chapter III eliminates organic loads and pathogens from raw chicken manure but retains essential compounds, making it more effective and socially attractive for improving pond biological productivity. The CME mixes uniformly with the water and avoids anoxic spots. Application of CME can reduce the purchase of costly feeds and inorganic fertilizer, which accounts for >50% of the total

cost of aquaculture inputs (Tacon & Metian 2008). However, the CME technology still requires application of minimum microalgae for better rotifer growth and reproduction. Further studies should focus on techniques that can reduce or eliminate the need for the expensive on-site microalgae production to spur profitable aquaculture in the developing countries.

In order to reduce overdependence on the expensive freshly cultured- or onsite grown-microalgae for live food production, previous studies have reported the viability of dried algae as rotifer diet, but frequent culture instabilities still occur (Lubzens et al. 1995; Yufera & Navarro 1998), thus require techniques to stabilize. Certain chemicals such as gamma-aminobutyric acid (GABA) have been employed to stabilize rotifer cultures with fresh algal diet (Gallardo et al. 1997, 1999, 2000). Based on this information, I formulated a hypothesis to explore whether GABA supplementation can stabilize and/or enhance rotifer cultures using dried algal diet. Dried algae are considered as convenient alternatives to the freshly cultured microalgae because dried algae are storable at room temperature for long time and, thus eliminating the need for constant energy supply to maintain freezer facilities. Firstly, I employed dried *Nannochloropsis oculata* and *Chlorella vulgaris* to culture the Kenyan rotifer strain *B. angularis* with GABA supplementation as an antidote for culture instabilities but the cultures were not successful due to contamination by ciliates. Instead, I investigated the mass production potential of the euryhaline rotifer *Brachionus rotundiformis* (SS-type) (perth strain) as a representative of tropical rotifers (Chapter IV). GABA, if applied at the lag phase growth stage of the rotifers 48 h before up-scaling, enhanced the population of the rotifer *B. rotundiformis* to a maximum density within 6 and 8 days of culture with dried *N. oculata* and *C. vulgaris*, respectively. This phenomenon is attributable to the influence of GABA on subsequent rotifer progenies. Gallardo et al. (2000) reported similar results for the reproduction of the rotifer *B. plicatilis* supplied with a

fresh *N. oculata* diet. However, continuous GABA application is detrimental to rotifer cultures after 8 days (Gallardo et al. 2000).

Despite the gradual increase of NH_3-N in all the culture tanks (Figure 4-6), GABA treated cultures produced higher rotifer densities than the control. Thus confirming the hypothesis that GABA reduces physiological stress caused by deteriorating culture conditions (e.g. high ammonia and rotifer density) as reported in literature (Gallardo et al. 1997, 1999, 2000; Araujo & Hagiwara 2005; Assavaaree & Hagiwara 2011; Ogello et al. 2017). In practice, GABA can be used to reduce physiological stresses caused by oil additives and high population densities during rotifer enrichment with marine oils. GABA is important for increasing the fecundity of asexual females of the S- and SS-type rotifers, which are highly susceptible to low temperature stress (Araujo & Hagiwara 2005). The physiological stressors are known to influence mictic induction, egg hatchability, lifespan, fecundity and mixis investment in rotifers (Snell & King 1977). Since mixis investment causes short-term fitness of rotifer clones (Chen & Cuijuan 2015), as more energy is invested in fertilization (Gilbert 2010), GABA can reduce the effects of the physiological stressors and facilitate rapid colonization (Gallardo et al. 1999). GABA has also been found to improve visual capacity in animals (Sandberg et al. 2014), which is important in feeding, mating and predator avoidance.

The endogenous presence of GABA has been confirmed in the rotifers *B. plicatilis* and *B. rotundiformis* (Gallardo et al. 2000). GABA receptor type A-associated protein (GABARAP), which control intracellular membrane trafficking of $GABA_A$ receptors and autophagy is ubiquitously distributed in the coronal area of the rotifer *B. plicatilis* (Marcial et al. 2014). GABA also induces settlement and metamorphosis in larvae of some species of marine invertebrates (Morse et al. 1979; Rumrill & Cameron 1983; Gapasin & Polohan 2004; Garcia-Lavandeira et al. 2005). GABA has been linked to quorum-sensing signal

suppression in some pathogenic bacteria (Chevrot et al., 2006), thus making it useful in controlling virulence of pathogenic bacteria in the rotifer cultures. GABA is biodegradable (Saskiawan 2008), cheap (US$3.00 per gram; Sigma Chemical) and requires low doses (50 mg l^{-1}).

The production protocols for dried microalgal diets are already patented by different commercial companies e.g. AlgaSpring (The Netherlands) and Daesang EMERALD (South Korea). Dried *N. oculata* costs about US$ 2.0 per kg (AlgaSpring) and is readily available for export. The convenient transportation and storage makes dried algae important in regions where algal cultures are either restricted by seasonal conditions or by the lack infrastructure for high density algal production. Dried algae can also be used to maintain stock cultures in the hatcheries. With dried algae, it is possible to pre-determine the nutritional content of food by supplementing it with essential elements that are crucial for the proper development of targeted fish larvae (Camacho-Rodriguez et al. 2013). Mass production of the rotifer *B. rotundiformis* using dried *N. oculata* and *C. vulgaris* can be significantly enhanced by pre-incubation of the rotifers with 50 mg l^{-1} of GABA for 48 h prior to mass cultures to reach maximum densities within a week. The results are relevant where mass culturing of rotifers is necessary for the pre-planning of fish larval production in aquaculture facilities. In addition to aquaculture, the larval fishes can be used to re-stock depleted natural stocks. Despite the usability of dried algae, they still have problems related to nutrient leach and low digestibility, and, the importation costs may be affected by fluctuating foreign exchange rates. For this reason, studies are needed to discover live food production techniques without microalgae.

The freshly cultured microalgae are still the supreme diet for various zooplankton species in aquaculture hatcheries (Maruyama et al. 1997). Although commercial diets e.g. baker's yeast, condensed microalgae and Selco (Inve-Co. Ltd., Thailand) have been used as

alternatives to microalgae, these diets have various shortcomings including high cost and culture instabilities, making them problematic for most fish farmers, especially in the developing countries, which are potential future leaders in marine larviculture production. So far, availability of low-cost and stable replacement diet for microalgae is still debatable, hence the studies in chapter V. Here, I developed a protocol for producing a fishwaste diet (FWD) as a low-cost and stable live food diet and, determined its efficacy for rotifer production under controlled laboratory conditions (Chapter V.1). I then applied the laboratory results for mass culture of zooplankton in an outdoor culture facility in a tropical country (Kenya) (chapter V.2) and, determined the dietary value of the FWD-fed rotifers for larviculture (Chapter V.3).

In the laboratory experiments, the FWD favored proliferation of microbial biomass as food for the rotifer, *B. rotundiformis*. The microbial biomass is a product of heterotrophic processes at optimal C/N ratios (Eberling et al. 2006; Azim & Little 2008; Crab et al. 2009). The rotifers fed with FWD ingested larger amounts of bacteria that are believed to have suppressed the rotifer's mixis rate but favoured the parthenogenetic reproduction. The bioflocs are rich source of proteins, lipids and vitamins (Simon and Azam 1989; Yu et al. 1994; Widanarni et al. 2012; Ogello et al. 2014) and the rotifers may have converted these nutrients into biomass. In normal cultures, the gut of live diets contain between 10^7 - 10^{10} while the culture media contains between 10^4 - 10^7 CFU ml^{-1} of bacteria (Miyakawa & Muroga 1988; Skjermo &Vadstein 1993). These data are comparable to the results obtained in Chapter V.1. In the preliminary biochemical characterization of the microbial flora (results not shown due to the ongoing studies on microbial aspects of FWD), some probiotic bacterial strains such as *Bacillus* sp., *Shewanella* sp. and *Thiocapsa* sp. were identified in the FWD culture medium and rotifer gut. Although certain bacteria are known to provide important nutritional supplementation to rotifers (Yasuda & Taga 1980; Ushiro et al. 1980;

Hagiwara et al. 1994; Hagiwara et al. 1995a), some bacterial species could be problematic for successful culture of marine fish larvae. For example, *Vibrio anguillarum* that is common in rotifer cultures is a pathogenic bacterium that causes rotifer culture crashes, but *Bacillus* sp., *Thiocapsa* sp. and *Shewanella* sp. have probiotic properties (Bhaskar et al. 2011). Such properties include suppression of pathogenic bacterial strains through quorum quenching mechanisms and, release of bactericidal or bacteriostatic compounds, to maintain biosecurity (Verschuere et al. 2000). However, the world's demand for high quality aquaculture products insist for strict biosecurity measures e.g. absence of pathogenic bacteria in aquaculture products. Nonetheless, techniques have been developed to limit bacterial contamination of larval fishes (Skjermo et al., 1997, Skjermo and Vadstein, 1999). For this reason, I recommend further studies to document a comprehensive account of the microbial flora associated with the FWD, including their successive colonization overtime.

Usually, the fatty acid composition accumulated by marine fishes is dependent on the marine phytoplankton that has the capacity to synthesize long chain PUFAs through series of desaturation and elongation reactions (Berge et al. 2005). Studies have found that some specific microbes can also manufacture PUFAs through polyketide synthase-like reactions (Strobel 2012). In chapter V.1, about 0.35 and 0.39 mg g^{-1} of DHA and EPA respectively was obtained from the FWD-fed rotifers, while the same were under detectable limit in the control-rotifers. It is believed that the rotifers may have bio-accumulated the essential bio-molecules via FWD-bacteria trophic pathway, thus portraying bacteria as conduits for transporting the bio-molecules. In addition, some bacteria species e.g. *Shewanella* sp., are known to contain sufficient EPA and DHA, and can be used to enrich rotifer cultures (Lewis et al. 1998). The rotifers may have also obtained these elements through the direct ingestion of microparticles as shown in Chapter V.1. Hino & Hirano (1980) reported that rotifers can ingest bacteria-sized particles ranging between 0.5 – 6.0 μm. However, these particles may

have included bacteria and rotifer feaces. Rotifer cultures are synonymous with high food conversion ratios because they ingest their own feaces (Hino et al. 1997). Even though zooplankton contains their own enzymes that make them easily digestible by fish, rotifers are usually deficient in essential nutrients required by the fish larvae. To be nutritionally complete, rotifers are often enriched with commercial emulsions before giving them to the fish. In chapter V.1, the FWD-fed rotifers had DHA/EPA ratio of 0.9. This value still falls below the recommended threshold ratio of 2.0 required for effective marine fish larval nutrition (Sargent et al. 1997). Nonetheless, FWD offers a promising start to the reduction or elimination of expensive commercial enrichment emulsions. Other home-made emulsion products have been produced using fish oil and egg yolk as cheap ways of enriching rotifer cultures (Hirayama & Funamoto 1983), but these products have short shelf life that limits their application in aquaculture. The bacterial PUFAs are more protected against oxidation, and provide a variety of other natural nutrients that meet the species-specific nutritional requirements of the cultured fishes (Harel et al. 2002). The protocol for FWD is simple, cheap and appears to be insensitive to frequent population crash with proper management. With a production of up to 1,200 rotifers ml^{-1} bi-weekly, the FWD presents a promising self-sustaining biotechnology for stable production of high density rotifers, for aquaculture.

It was necessary to apply the FWD technique in local aquaculture initiatives. Traditional aquaculture depends solely on the algal activity as the pivot that drives food chains / webs in presence of light energy, to feed the biotic communities in ponds. In the outdoor culture experiments, the FWD coupled with light energy promoted primary production in presence of limiting nutrients within the system (Chapter V.2). Addition of CME to the fishwastes seems to be an improvement to the FWD because the CME probably facilitated phytoplankton growth that perhaps expanded the zooplankton forage base in addition to the microbiota. Chicken manure is also a substrate for probiotic bacteria

(Rapatsa & Moyo 2013; Elsaidy et al. 2015), and contains growth promoting compounds e.g. 17 α and β-estradiol (Hagiwara et al. 2014) that positively influence the growth and reproduction of the zooplankton (Shemesh & Shore 1994). Literature affirms that phytoplankton alone do not necessarily increase zooplankton populations but additional micro-flora associated with decaying organic substances supplements the phytoplankton foraging with essential proteins, lipids and vitamins to cause high growth effects for zooplankton (Morris & Mischke 1999). Previously, the FWD failed to support mass culture of the rotifer, *B. angularis* in the laboratory, but supported mixed culture of diverse freshwater zooplankters in outdoor tanks, thus showing the significance of inter- and intra-specific relations in the zooplankton cultures. Studies have shown that reproduction of the harpacticoid copepod, *Tigriopus japonicus* is more successful when mixed-cultured with rotifers (Hagiwara et al. 1995b).

The FWD technology embodies a complex microbial control system that leads to degradation of waste materials, facilitates the recycling of nutrients and proliferation of microflora that flourishes under optimum C/N ratio. The FWD technology represents a biofloc microcosm where the decomposing fish tissues produce diverse microbial flora, which also form zooplankton diet. The system also attracts opportunistic benthic community that feed on the detritus and bioflocs. In addition, the dissolved nutrients from FWD, in presence of sunlight, facilitate primary production of phytoplanton that is grazed upon by the zooplankters. The complex food web that exists in FWD aided culture system is summarized in Figure 6.

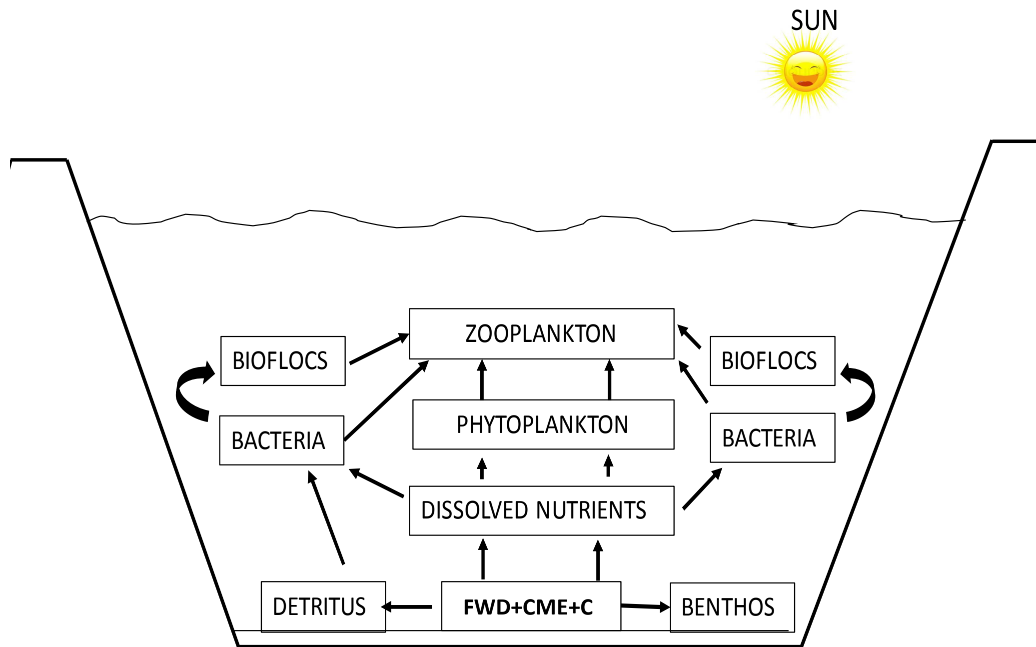

Figure 6-1: Schematic flow of nutrients from FWD, CME, detritus and carbon source (C) to biotic communities in the tank culture system. FWD = fishwaste diet; CME = chicken manure extract

Initiatives geared towards developing nutrition strategies such as bioflocs and periphyton that maximizes the contribution of natural and supplemental feeds in culture facilities would help to expand aquaculture production. Studies have proposed the use of aeration devices and PUFA-rich carbon sources e.g. glycerol (Ekasari et al. 2010) to improve the efficiency of biofloc aided systems, but these proposals may increase production cost. Also, aeration may facilitate distribution of toxic ammonia accumulated at the bottom of culture facility, and this may cause rotifer culture crash. A combination of fishwastes and CME can be used as cheap and stable diet to obtain up to 150, 12, and 8 ind ml^{-1} of rotifers, copepods and cladocerans, respectively on weekly basis in outdoor cultures. However, the success of the FWD technology could be limited by presence of pathogenic microbes, which may affect the cultured animals. Copepods produced extensively may cause mass mortalities in cultured fishes through transmission of viruses and parasites (Su et

al. 2005). Further studies are needed to investigate the dietary value of the FWD-fed zooplankton species for larviculture activities.

In terms of production costs, successful microalgal culture in conventional larviculture systems basically depends on availability of optimal parameters such as nutrients, light, pH range, aeration, temperature and salinity (Stein 1973). In addition to these parameters, high ratio of algal biomass to target species (usually about 5-10:1) elevates the cost of microalgae production (Treece 1995). On average, mono-specific algal cultures cost up to USD 120-200 kg^{-1} DW, where labour accounts for 50-85%, pumping; 4-24%, nutrients; 4-20% and mixing; 5-8% of the total production costs (Treece 1995). Guillard & Ryther (1962) estimated the cost of algae production using Guillard's f/2 medium at USD 0.002 l^{-1}. Consequently, the high microalgae production costs influence the zooplankton production costs. For example, the estimated cost of rotifer mass production using large scale batches is USD 4.5/ million rotifers where feeds, live algae and yeast account for 72, 50 and 22% of the production costs, respectively (Treece 1995). Comparatively, the FWD (Chapter V) is significantly cheaper because fishwaste, which is the main ingredient in the diet, can be obtained for free. In addition, the efficacy of the FWD can be improved by addition of cheap carbon source. In general, the estimated cost of rotifer production in 500 l culture tanks using FWD is approximately USD 0.01/ million rotifers.

Fish larval rearing is the most critical stage that determines the success of aquaculture. At the time of hatching, most marine fish larvae lack functional organs and have relatively small yolk reserves. Even after the endogenous feeding, the onset of exogenous feeding is still synchronized with a primitive digestive system and a small mouth gap that limits their successful first feeding (Lavens & Sorgeloos 1996). At this stage, adequate and timely supply of appropriate nutrition is critical. Rotifers especially Brachionid spp. are considered excellent first nutrition for most marine fish larvae, but difficulties in production of

sufficient quantities continue to frustrate the pre-planning of fish seedling production in microalgae-based hatcheries (Lubzens et al. 1995). In Chapter V.3, I presented a preliminary data on the food value of FWD-fed rotifer, *B. rotundiformis* SS-type, for larviculture of Japanese whitting, *S. japonica*. The significant increase in total and standard length at 10 dph for the fish larvae fed with FWD-cultured rotifers compared with those given control diet, and similar growth and development parameters of fish larvae in all the diets suggests the usability of the FWD-fed rotifers for larviculture. Also, there were no significant differences in the dry weight, survival rate, viability and quantity of ingested rotifers between the diets. The quality of FWD can be improved perhaps by several screenings to eliminate organic particles that may compromise water quality faster. It is also possible that FWD could contain some pathogenic microflora, which can be detrimental to the fish larvae. These factors may have caused the low survival rate of fish larvae that was observed during experiment. The survivability of fish larvae strongly depends on the amount of essential bio-molecules e.g. DHA and EPA present in their food (Furuita et al. 2000). Due to high larval fish mortality, amounts of fish samples were not sufficient for total lipid analysis. Therefore, it is difficult to conclusively discuss the total amounts of DHA and EPA in the *S. japonica* larvae fed with the two diets. The DHA/ARA ratio of 2.4 obtained for the fish larvae fed with FWD-rotifers (Table 5-3.3) still falls below the recommended threshold (about 10) for larviculture (Sargent et al. 1999). Further experiments are recommended to draw more concrete conclusions on the food value of the FWD for larviculture initiatives.

Conclusions and recommendations

The Kenyan rotifer, *B. angularis* has a small lorica size (length: 85.6 µm, width: 75.4 µm) and optimally reproduces at 25°C with 2.5×10^6 algal cells ml^{-1} of *C. vulgaris* making it appropriate for enhancing local aquaculture. Mass production of *B. angularis* can be enhanced with 2.0 ml l^{-1} of CME. In order to reduce overdependence on fresh algae, euryhaline rotifers can be pre-incubated with 50 mg l^{-1} of GABA for 48 h to achieve maximum production within a week using dried diets e.g. *N. oculata* and *C. vulgaris*. To eliminate microalgae from aquaculture production chain, FWD is a promising alternative. The production flow-chart is summarized in Figure 6-2 below. FWD can produce up to 1,200 rotifers ml^{-1} bi-weekly, thus presents a self sustaining biotechnology for stable production of DHA- and EPA-rich rotifers, for aquaculture. FWD causes proliferation of micro-flora, suppresses rotifer mictic cycle and favors parthenogenetic reproduction of rotifers. FWD combined with CME produced about 150, 12, and 8 ind ml^{-1} of rotifers, copepods and cladocerans, respectively, on weekly basis in outdoor culture tanks. FWD production protocol is cheap and costs about USD 0.01/ million rotifers. Therefore, FWD is convenient for profitable aquaculture production particularly in the countries without sophisticated infrastructural investments for high dense microalgal production. However, further studies should document a comprehensive account of the microbial flora associated with the FWD, including their successive colonization overtime. Even though the preliminary data showed that FWD-fed rotifers can be used for larviculture of *S. japonicas*, more studies are needed to authenticate these findings. FWD appears to be a major leap toward making pre-planning of fish seedling production in aquaculture facilities feasible. Further studies are recommended to investigate the suitability of the FWD for mass culture of other planktonic live food resources e.g. copepods, cladocerans and *Artemia*. There is

need to test the suitability of other environmental wastes e.g. from livestock abattoirs for live food production.

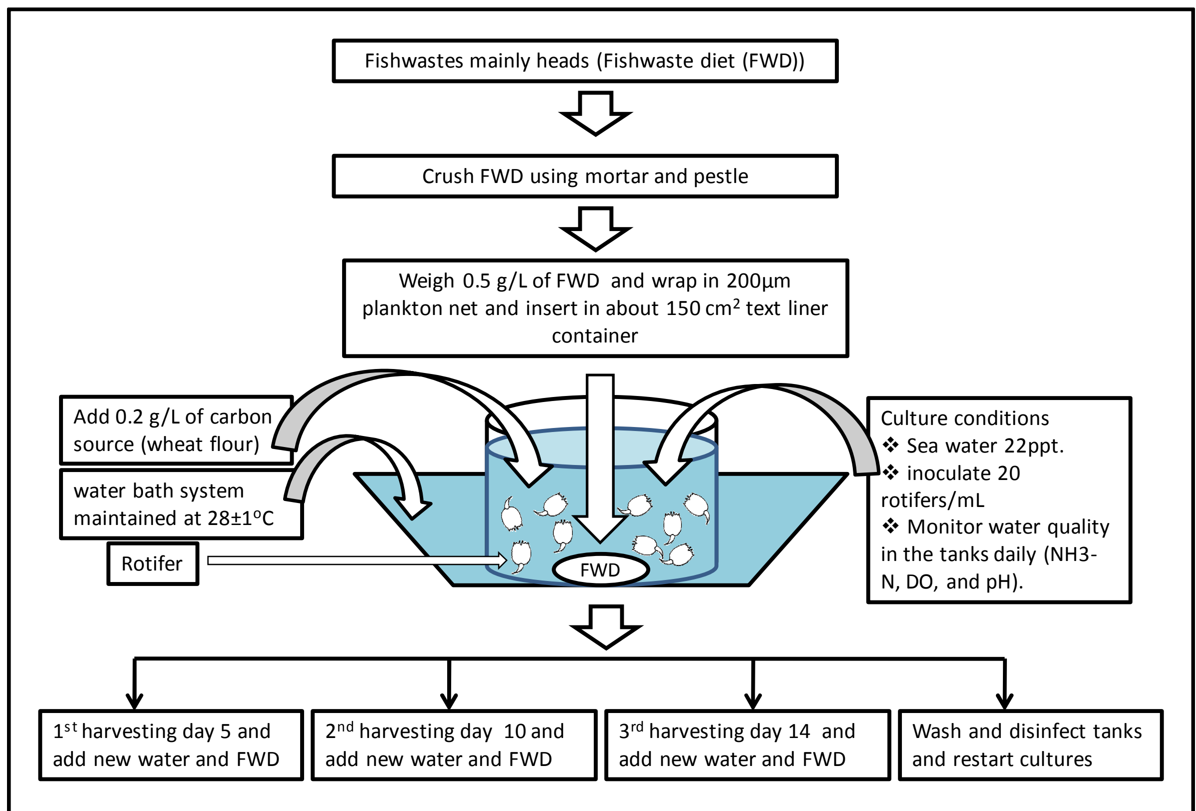

Figure 6-2: Flowchart for rotifer production using the low-cost and stable FWD technology

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In the same vein, I wish to enthusiastically convey my special accolade to Prof. Yoshitaka Sakakura for his generous and selfless support during experimentations in the laboratory. His brilliance in data analysis and manuscript edition is second to none.

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APPENDIX

A: Curriculum vitae

Personal data:

Name: Erick Ochieng Ogello

Nationality: Kenyan

Current employer: Kenya Marine & Fisheries Research Institute (KMFRI)

Education history:

September 2017: Doctor of Philosophy in Fisheries Science (Ph.D. Fisheries Science).
Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan. Monbukagakusho (MEXT) Scholar
Dissertation: "Studies on the Development of Low-cost and Stable Live Food Production Technologies for Tropical Aquaculture: A case study of Rotifera (Family: Brachioninae)"

September 2013: Master of Science in Aquaculture (Msc. Aquaculture – Great distinction)
Faculty of Bioscience Engineering, Gent University, Belgium
VLIRUOS-Scholar
Thesis: Genetic Differentiation of *Artemia franciscana* (Kellogg, 1906) in Kenyan Coastal Salt-works

December 2005: Bachelor of Science in Fisheries (Bsc. Fisheries – First class Honors)
Moi University, Kenya
Government sponsored scholar -Joint Admission Board (JAB).
Thesis: Use of Macroinveterbrate Diversity Index to Assess the Quality of Small Wetlands in the Lake Victoria Basin, Kenya

November 1999: Kenya Certificate of Secondary Education (K.C.S.E),
Kisumu Boys' High School, Kenya

Employment history:

2007 – 2009: The Cooperative Bank of Kenya (Customer service, cash & ATM custodian)

2010 – to date: Kenya Marine & Fisheries Research Institute (KMFRI) (Research scientist, Fisheries & Aquaculture).

B: International conferences attended during the PhD. study period

1. 22nd – 24th September 2017: The Japanese Society of Fisheries Science International Symposium. Fisheries Science for Future Generations. Tokyo University of Marine Science & Technology, Tokyo, Japan. Presented 'Composting fishwastes as a low-cost and stable diet for mass culture of *Brachionus rotundiformis* Tschugunoff (Rotifera)
2. 4th – 7th September 2017: The 7th Fish & Shellfish larviculture symposium, Gent University, Belgium. Presented 'Blending fishwastes and chicken manure extract as low-cost and stable diet for planktonic live food production'
3. 28th – 29th September 2016: The 1st International seminar on Tropical Aquatic Resources Science & Management. Theme: Enhancing Sustainable Environmental Resources Management; Poverty alleviation & Climate Adaptation Strategies. Sintesa Peninsula Hotel, Manado, Indonesia. Presented 'Morphological and molecular identification of a freshwater rotifer species isolated from Kenyan fish pond'.
4. 3rd – 7th August 2016: The 11th The ASEAN Fisheries and Aquaculture Conference and Exposition 2016 "ASEAN Seafood for the World" Bangkok, Thailand. Presented 'Developing low-cost and stable live food culture techniques for tropical aquaculture'.
5. 30th August – 4th September 2015: Rotifera XIV, International Rotifer Sympozium, Institute of Soil Biology, Biology Centre, České Budějovice, Czech Republic. Presented 'Culturing *Brachionus rotundiformis* Tschugunoff (Rotifera) using dried foods: application of gamma-aminobutyric acid (GABA)'.
6. 3rd – 6th August 2015: The United States Japan Natural Resource (UJNR) Aquaculture Workshop, Nagasaki University, Japan. Presented 'Life table demography of the rotifer *Brachionus angularis* Gosse: influence of temperature and food density'.