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Effects of feeding frequency of live prey on larval growth, survival, resistance to hyposalinity stress, Na^+/K^+ ATPase activity, and fatty acid profiles in black sea bass *Centropristis striata*

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ABSTRACT

Feeding live prey organisms in a commercial marine fish hatchery requires significant labor and resources. The effects of feeding frequency of live rotifers and *Artemia* on growth, survival, hyposalinity stress resistance, and Na^+/K^+ ATPase activity in black sea bass larvae *Centropristis striata* were evaluated. Newly hatched larvae (day 0 post-hatching = d0ph) were stocked (53 larvae/L) into twenty-four 30-L tanks supported by a recirculating seawater (35 g/L) system in a controlled-environment laboratory. Temperature (22 °C), aeration (225 mL/min), light intensity (~1000 lx), and photoperiod (18 L: 6 D) were constant. Beginning on d3ph, larvae were fed a prescribed daily ration of live prey (enriched s-type rotifers and/or *Artemia* nauplii), but distributed at different frequencies: (1) 1×/d at 0800 h, (2) 2×/d at 0800 and 1600 h, (3) 3×/d at 0800, 1200 and 1600 h, and (4) 4×/d at 0800, 1200, 1600 and 2000 h, with six replicate tanks per treatment. The daily ration of live prey (maximum of 750,000 rotifers and 90,000 *Artemia*) was divided equally among feedings; therefore, prey density at each feeding (i.e., meal size) decreased at higher frequencies. By d31ph, larvae fed 4×/d showed significantly ($P < 0.05$) greater notochord length and SGR (mean ± s.e. = 7.05 ± 0.20 mm and 20.1 ± 0.467%/d) compared to larvae fed 1×/d (6.02 ± 0.11 mm, and 18.1 ± 0.204%/d) or 2×/d (6.08 ± 0.12 mm and 18.4 ± 0.399%/d). Feeding frequency had no effect on larval survival (23.8–38.2%) or whole body fatty acid composition. Ability to withstand an acute hyposalinity (10 g/L) challenge on d32ph was significantly poorer in larvae fed 1×/d than in those fed 2×/d, 3×/d or 4×/d. Following transfer to a sub-lethal salinity (15 g/L) on d32ph, Na^+/K^+ ATPase activity (μmol ADP/mg protein/h) was higher in larvae fed 3×/d (5.61) than in those fed 4×/d (5.22) or 1×/d (5.20). The largest larvae (4×/d treatment) showed reduced Na^+/K^+ ATPase activity (vs 3×/d), likely due to accelerated development and a shifting of the Na^+/K^+ ATPase from a global distribution to specialized osmoregulatory sites. The results demonstrated that, for a prescribed total daily ration, feeding lower prey densities (smaller meals) at higher frequencies (3×/d or 4×/d) improved larval growth and resistance to hyposalinity stress and may increase osmoregulatory ability, without affecting survival. These findings delineate more judicious use of limited live prey resources to maximize larval growth and vitality during hatchery production of pre-metamorphic stage black sea bass.

Statement of relevance: Live prey (rotifers and *Artemia*) are expensive to produce and can limit production of juvenile fish in a commercial marine fish hatchery. The results show that given a prescribed (albeit moderate) total daily ration of prey, feeding lower prey concentrations at higher frequencies optimized larval growth performance and vitality in black sea bass raised from first-feeding through pre-metamorphic stages.

1. Introduction

1.1. Black sea bass

Black sea bass *Centropristis striata* (family Serranidae) is a commercially and recreationally important marine finfish that inhabits coastal waters of the eastern US from the Gulf of Maine to central Florida. They are generally sedentary and are associated with hard substrates, rough bottom and natural reefs (Sedberry, 1988). Black sea bass are protogynous hermaphrodites; most fish develop first as females and then mature into males at one to five years of age (Wenner et al., 1986). Stringent regulations on harvesting of wild populations and the potential for limited future supplies and higher market prices of ocean-caught black sea bass have stimulated studies on black sea bass aquaculture to help meet market demand. Techniques for spawning

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adult black sea bass in captivity (Watanabe et al., 2003; Berlinsky et al., 2005), raising larvae through juvenile stages in a hatchery (Berlinsky et al., 2000; Copeland and Watanabe, 2006; Rezek et al., 2010; Carrier et al., 2011; Watanabe et al., 2015), and for growing juvenile fish to market sizes (Copeland et al., 2003, 2005; Watanabe, 2011) have been developed in the eastern US for black sea bass, and there is now developed technology to support commercial-scale hatchery operations. Based on research funded in North Carolina by federal and state agencies, premium market size black sea bass are being produced in marine recirculating aquaculture systems at UNCW's Aquaculture Facility (Wrightsville Beach) as well as startup farms in North Carolina, Virginia and Maine. Ultra-fresh cultured black sea bass have been distributed to niche ("upscale") restaurants serving seafood throughout the state of NC as well as to US metropolitan markets (NY City, Philadelphia, Miami and San Francisco) (Wilde et al., 2008; Dumas and Wilde, 2009) to evaluate market potential. These studies have demonstrated a significant demand for farm-raised black sea bass in upscale niche markets at prices that may be profitable to startup growers.

1.2. Feeding strategies for marine finfish larvae

Feeding of the early larval stages is a bottleneck for large scale production of many commercially and recreationally important marine finfish species (Wittenrich et al., 2009; Dhert et al., 2001). This is related to significant larval mortality that is often related to a lack of prey of suitable quantity and quality. In nature, marine finfish larvae consume zooplankton and phytoplankton, including a wide range of organisms such as amphipods, decapods, and copepods (Sedberry, 1988). In aquaculture, it is more practical to feed rotifers *Brachionus rotundiformis* and brine shrimp nauplii *Artemia franciscana* to fish larvae because the techniques to culture these organisms have been standardized (Støttrup and Attramadal, 1992; Dhert et al., 2001; Bentley et al., 2008; Watanabe, 2011). However, these prey organisms are nutritionally deficient and must be enriched, particularly with essential fatty acids (EFAs), before feeding them to the fish larvae (Watanabe et al., 1983; Izquierdo et al., 1989; Koven et al., 2001; Sorgeloos et al., 2001; Rezek et al., 2010; Carrier et al., 2011; Watanabe et al., 2014, 2016). Live feed organisms are expensive to produce because they require intensive manpower to grow and to improve nutritional value and are challenging to produce on a consistent basis (Bentley et al., 2008; Slembrouck et al., 2009). Providing an optimal supply of nutritionally-balanced feed is therefore important, not only for the growth and survival of fish larvae, but to minimize the use of expensive zooplanktonic prey and potentially the costs of producing fingerlings in a hatchery (Watanabe et al., 2016). Studies to determine the optimum feeding frequency of live prey for growth of larval fish are of considerable importance both biologically and economically (Rabe and Brown, 2000; Cho et al., 2003).

Larval finfish are often fed at high prey densities to maximize feed consumption, growth rate and survival (Houde and Schekter, 1978; Rabe and Brown, 2000; Slembrouck et al., 2009). Although a number of studies have investigated the effects of feeding frequency on post-larval and juvenile finfish (Folkvord and Ottera, 1993; Dwyer et al., 2002; Cho et al., 2003; Biswas et al., 2010; Luo et al., 2015), definitive experimental data demonstrating optimal feeding frequency of live prey on the performance of the early larval and pre-metamorphic stages of marine finfish is limited. In yellowtail flounder *Pleuronectes ferrugineus* feeding a moderate ration 2×/d, 4×/d or continuously resulted in better growth and survival (vs 1×/d) (Rabe and Brown, 2000). These authors concluded that feeding 2×/d may be more cost-effective than feeding 4×/d or continuously and that prolonged feeding at high prey densities may reduce gut retention time and digestive efficiency, citing earlier work in Pacific herring *Clupea harengus pallasi* (Boehlert and Yoklavich, 1984) and larval walleye *Stizostedion vitreum* (Canino and Bailey, 1995; Johnston and Mathias, 1996). In addition to minimizing wastage of prey, optimizing feeding frequency may also improve water quality and decrease size variation and cannibalism (Dwyer et al., 2002; Biswas et al., 2010).

Optimum feeding frequency and rate varies with species, ration size, fish size and rearing system (Rabe and Brown, 2000; Biswas et al., 2010). Live prey is often a limiting resource in commercial fish hatcheries, this constraint becoming more severe as scale of operation increases. Experimental data on the effects of feeding frequency of live prey for black sea bass during the early larval and pre-metamorphic stages are needed to optimize larval performance, reduce juvenile production costs, and advance industrial scale hatchery production of this high value marine finfish species. The objectives of this study were to determine, under controlled laboratory conditions, the effects of feeding frequency of live prey, including s-type rotifers *Brachionus rotundiformis* and *Artemia franciscana* on growth, survival, fatty acid composition, resistance to hyposalinity stress, and whole body Na^+/K^+ ATPase activity in black sea bass larvae.

2. Materials and methods

2.1. Experimental animals

Eggs were collected following induced spawning of black sea bass broodstock held at the University of North Carolina Wilmington's Center for Marine Science Aquaculture Facility (Wrightsville Beach, NC, USA) (Watanabe, 2011). Broodstock were maintained outdoors in six 2.46-m diameter cylindrical tanks supplied with recirculating seawater (32–35 g/L) under an artificial photothermal regime simulating ambient offshore conditions in coastal southeastern North Carolina. Seawater was pumped from the Atlantic Intracoastal Waterway adjacent to Wrightsville Beach. Adult females at the post-vitellogenic stage of ovarian development were induced to spawn using luteinizing hormone releasing hormone-analogue (LHRHa) following established protocols (Watanabe, 2011). Following strip spawning, eggs were transferred to a separatory funnel in full strength seawater where floating (viable) eggs were separated from sinking (non-viable) eggs. Fertilized eggs were transferred to a 125-L incubator at 16 °C and 33 g/L salinity for 48–55 h.

2.2. Experimental system

The experimental units consisted of black, 30-L cylindrical (diam. = 38 cm, depth = 40 cm) larval rearing tanks (LRTs) with center draining standpipes and in-flow manifolds. The LRTs were supported by a common recirculating system, including a 113-L bubble bead filter (Aquaculture Systems Technology, New Orleans, LA); a 40-W UV sterilizer (Emperor Aquatics, Pottstown, PA); a foam fractionator (Top Fathom TF300A, 1/10 HP Foam Fractionator Pump) (Orangeburg, NY); 3/4 HP pump (Jacuzzi Stingray) (Chino Hills, CA); and a 25-μm cartridge filter. Full strength seawater was recirculated through each tank at an exchange rate of 200% daily from d0ph through d14ph and then increasing to 300% from d15 through d32ph. Water temperature was held constant at 22 °C using a 1-HP heat pump (Aqualogic, San Diego, CA) (Watanabe, 2011). Air was supplied to each tank by two air stones on either side of the center standpipe. To maximize feeding efficiency, illumination was provided at 1000 lx at the water surface by two 40-W full spectrum fluorescent light bulbs suspended over the tanks (Copeland and Watanabe, 2006). To enable larvae to feed for a prolonged time each day, photoperiod was 18 L: 6D starting at 0800 h and ending at 0200 h (Moustakas et al., 2004; Copeland and Watanabe, 2006).

2.3. Experimental design

Newly hatched yolk sac larvae were transferred to twenty-four, 30-L larval rearing tanks at a density of 53 larvae/L. This marked the beginning of the experimental trials and is hereafter referred to as d0ph (day 0 post-hatch). Background microalgae *Nannochlorpsis oculata*

was added twice daily to maintain a density of 1×10^6 cells/mL. From d1ph to d2ph, all larval rearing tanks were fed rotifers at a density of 5 prey/mL to initiate larval feeding. During this time, rotifer density was maintained by quantifying rotifers in each tank twice daily and adding the appropriate number of enriched rotifers to make up the difference. From d2ph to d4ph, rotifer density was increased 2 prey/mL per day.

To determine the effects of feeding frequency on larval performance, beginning on d5ph larvae were fed a prescribed amount of live prey, enriched rotifers and/or *Artemia* nauplii, under different feeding regimes. Rotifers *Brachionus rotundiformis* were enriched for 16 h with "N-Rich" Algae paste (72 billion cells/mL, EPA: 18.1 mg/g dwt, DHA: 41.9 mg/g dwt) (Reed Mariculture, Campbell, CA, USA). *Artemia* nauplii were enriched for 24 h with *A*1-DHA Selco (Total HUFA's: minimum 200 mg/g dwt, DHA/EPA minimum ratio: 2.5) (INVE Aquaculture, Salt Lake City, Utah, USA). To maintain nutritional quality of enriched rotifers and *Artemia*, prey were harvested at 0800 h and a portion fed to the larvae, with the remainder stored at reduced temperatures (i.e. "cold-banked") of 4 °C and 12 °C, respectively, until the last feeding at 2000 h (Sorgeloos et al., 2001).

Beginning on d5ph, larvae in all experimental tanks received the same prescribed amount of rotifers (10/mL = 300,000 total prey per tank per day), with the daily ration increasing each day until a maximum density of 25/mL (750,000 total prey per tank per day) was reached on d13ph. Rotifers were fed using four different feeding regimes: (1) one time/d at 0800 h, (2) two times/d at 0800 and 1600 h, (3) three times/d at 0800, 1200 and 1600 h, and (4) four times/d at 0800, 1200, 1600 and 2000 h. For each treatment, the total daily ration of rotifers (300,000–750,000) was equally divided among the feedings. Six replicate tanks were assigned to each feeding regime.

On d15ph, newly hatched *Artemia* Instar 1 nauplii were added at a density of 0.5 prey/mL, increasing to 1 prey/mL by d17ph. Beginning on d18ph and continuing to d34ph, all experimental tanks received the same prescribed density of *Artemia* nauplii starting at 1 prey/mL (30,000 prey per day) and then increasing to 3 prey/mL (90,000 per day) by d27ph, using the same four feeding regimes described above for rotifers. By d20ph, rotifer feeding was stopped, and larvae were transitioned from Artemia Instar I nauplii to *Artemia* Instar II metanauplii enriched with A1-DHA Selco until d34ph.

2.4. Experimental conditions

Temperature at stocking was 16 °C and was increased 1 °C per day until an optimum rearing temperature of 22 °C was reached on d4ph (Watanabe, 2011). Temperature, salinity (35 g/L), dissolved oxygen, and pH were measured daily in each rearing unit using a multi-parameter probe (YSI 556 MPS, YSI Inc., Yellow Springs, OH, USA). Light intensity (1000 lx) was measured at the water surface of each tank using a light meter (Extech Instruments, Waltham, MA, USA). Aeration was supplied at approximately 226 mL/min (Watanabe, 2011). Exchange rates were kept at 200% (41 mL/min) until d12ph when it was increased to 300% (Watanabe, 2011).

2.5. Sampling and data collection

2.5.1. Growth and survival

To measure growth and survival, 10 larvae were randomly sampled from each replicate tank using volumetric methods on d0, d4, d10, d17, d25 and d31ph at approximately 0830 h (Moustakas et al., 2004). Before sampling, airflow was increased to uniformly distribute the larvae in the tanks. Larvae were sampled by plunging a 2-cm PVC pipe, 46 cm in length, into the water column and then transferring the water and larvae collected with each sample to a graduated cylinder until a total volume of approximately 600 mL and 10 larvae were collected. The number of fish were counted and divided by the water volume removed during sampling to determine sample density. Survival for each tank was calculated by dividing the sample density by the initial density. Larvae were anesthetized (0.5 mg/L of 2-phenoxyethanol) and placed in a 10-well depression slide and photographs were taken using a Nikon camera attached to a dissection microscope. A stage micrometer was also photographed as a calibration reference. Notochord lengths (NL) of larvae were measured by the computer program Image J (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). Wet weight was determined by placing 10 larvae on a pre-weighed microscope slide after they were poured onto a 23-μm mesh screen, which was blotted with a sponge from underneath to remove excess water. The slides were then placed into a laboratory oven at 60 °C for 72 h and then reweighed to determine dry weights of larvae. Specific growth rates were determined for d31ph from mean wet weight using this equation:

$$\mathrm{SGR} = \mathrm{K}' = \frac{1\mathrm{n}(\mathrm{m}_{t2}/\mathrm{m}_{t1})}{\mathrm{t}_2 - \mathrm{t}_1}$$

where m_{t2} is the mean wet weight on d31ph and m_{t1} is the initial mean wet weight on d4ph, and $t_2 - t_1$ represents the number of days (27).

2.6. Diurnal prey (rotifer and Artemia) concentration-time profiles in larval rearing tanks

To elucidate the prey concentration-time profiles in the larval rearing tanks under the different feeding frequency treatments, prey concentrations were monitored in situ during the photophase on d12ph (Fig. 5) and on d14ph for rotifers and on d27ph for *Artemia* (Fig. 6). Rotifers and *Artemia* were sampled every 4 h for a total of 12 h using volumetric methods as described above.

2.7. Fatty acid analysis

To determine changes in fatty acid composition of live prey (rotifers and *Artemia*) during storage at reduced temperature, prey were sampled at 0, 4, 8 and 12 h post-enrichment ($N = 3$ per sampling time) to correspond with the times (0800, 1200, 1600 and 2000 h) that prey were fed to the fish larvae. To determine fatty acid composition of whole body tissue of black sea bass larvae, larvae fed enriched rotifers and *Artemia* at four different feeding frequencies were sampled from replicate tanks ($N = 4$–5 per treatment) on d32ph.

Samples of live prey and larvae for fatty acid analysis were prepared from a homogenate created from approximately 10 larvae or ~300 mg of rotifers or ~135 mg of *Artemia* sampled on d31ph (Watanabe et al., 1989). Samples were poured onto a 23-μm mesh screen, which was blotted with a sponge to remove excess water. Samples (approximately 100–150 mg) were then transferred to a 1.2-mL cryogenic vial (Fisher Scientific, Pittsburg, PA, USA), filled with nitrogen gas, and then frozen (−25 °C) before lipid extraction and fatty acid analysis. Total lipid and fatty acid compositions of the prey and larvae were determined by first extracting total lipids into chloroform: methanol (Folch et al., 1957). One milliliter of a 0.001 g mL^{-1} solution of C19:0 fatty acid was added to each sample as an internal standard. Lipid fatty acids were converted to their methyl esters (FAMEs) for GC analysis as previously described in Watanabe et al., 2016.

2.8. Resistance to acute hyposalinity challenge

At the end of the experiment (d32ph), a stress resistance test was performed on a sub-sample of larvae to measure the ability of the larvae to withstand an acute decrease in salinity from 32 to 10 g/L under zero feeding, conditions lethal to the larvae. Ten larvae were randomly sampled from each replicate tank using volumetric methods and pooled. Twenty larvae from each treatment were stocked in a 15-L tank, with

three replicate tanks per treatment. To evaluate larval quality, survival activity index (SAI) was calculated using the following equation:

$$SAI = \frac{1}{N}\sum_{i:1}^{k}(N - h_i)x\ \ i$$

where N is the total number of initial larvae, h_i is the cumulative mortality by the ith hour, and k is the number of hours that elapsed until all larvae have died (Matsuo et al., 2006). Using volumetric methods, survival was monitored every 12 h for the duration of the experiment.

2.9. Measurement of whole body Na^+/K^+ ATPase activity following sub-lethal salinity challenge

At the end of the experiment (d32ph), the remaining larvae in each replicate tank were abruptly transferred to a lower, but sub-lethal salinity of 15 g/L. Using volumetric methods, 50 larvae were sampled volumetrically from each replicate tank at 0 h (immediately before the hyposalinity challenge) and subsequently at 12, 24, and 48 h post-transfer. Sampled larvae were placed in 10 microcentrifuge cryo-vials (5 larvae/vial) and immediately frozen. Samples were stored at −80 °C.

Measurement of Na^+/K^+ ATPase activity in larval whole body samples was similar to that described by Penefsky and Bruist (1984) and McCormick and Bern (1989, 1993), modified for 96-well microplates (McCormick, 1993). In this kinetic assay, the ouabain-sensitive hydrolysis of adenosine triphosphate (ATP) is enzymatically coupled to the oxidation of nicotinamide adenine dinucleotide (reduced form, NADH), which was directly measured in a microplate reader. An assay mixture (solution A) containing 4 U lactate dehydrogenase (LDH)/mL, 5 U pyruvate kinase (PK)/mL, 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole (pH 7.5) was made just prior to the assay (all biochemical reagents from Sigma, St. Louis, MO, USA, or VWR, Radnor, PA, USA). Assay solution B was as above, but also contained 0.5 mM ouabain. A salt solution containing 189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, and 50 mM imidazole (pH 7.5) was prepared in advance and was stable for several weeks at 4 °C. Assay solutions A and B and salt solutions were mixed separately in a 3:1 ratio, and kept on ice.

Whole body larval black sea bass were homogenized for 10–15 s in the presence of 25 μL of a solution (SEI) (pH 7.4) containing 300 mM sucrose, 20 mM disodium ethylenediamine tetraacetate (Na_2EDTA) and 100 mM imidazole (pH 7.1) (Zaugg, 1982) using a motorized pestle (Kontes, Vineland, NJ, USA). Samples were centrifuged at 5000 g for 30 s and then a 1:10 dilution of the supernatant from centrifuge to SEI was created. For every sample, ten microliters of diluted homogenate was added to each of four wells. Two-hundred microliters of the A-plus-salt mixture was added to two wells per sample, and 200 μL of the B-plus-salt mixture was added to the other two wells per sample. The plate was then placed in a temperature-controlled plate reader (Flex Station 3, Molecular Devices Corp., Menlo Park, CA, USA) and the linear rate of NADH disappearance was measured at 340 nm for 10 min. The linear rate from 2 to 10 min in each pair of duplicate wells was determined, and Na^+, K^+-ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, expressed as micromoles of ADP per milligram of protein per hour.

2.10. Analytical methods

The effects of feeding frequency on notochord length, wet weight, dry weight, SGR, fatty acid and lipid concentrations, SAI and Na^+/K^+ ATPase activity were expressed as treatment means ± standard error of the mean (SEM) and compared by a one-way analysis of variance (ANOVA). A two-way ANOVA was used to investigate the interactive effects of sampling time and feeding frequency on Na^+, K^+-ATPase activity of whole-body black sea bass larvae. To test the assumptions of ANOVA, all data were analyzed using Levene's homogeneity of variance test prior to the ANOVA. A Tukey-Kramer analysis ($P < 0.05$) was used for multiple comparisons among means. If data did not meet the assumptions for an ANOVA, a non-parametric Wilcoxon/Kruskal-Wallis test was used. Percentage data was arcsine transformed before analysis. Analyses were performed using the JMP IN version 7 statistical software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Growth

3.1.1. Notochord length

Larvae were stocked on d3ph and initial larval length and weight were determined beginning on d4ph. Mean initial notochord length on d4ph was 2.51 mm ± 0.08 (Fig. 1a). Notochord length increased steadily in all treatments to d10ph (mean = 3.65 mm) and d17ph (mean = 4.99 mm), with no significant effects of feeding frequency observed. The increase in notochord length among treatments slowed from d17ph, and there was a departure in growth rates among treatments on d25ph (Fig. 1a) when mean notochord length generally increased with higher feeding frequency and was significantly ($P < 0.05$) higher in fish fed 4×/d (5.39 mm) than in fish fed 1×/d (5.05 mm) (Fig. 1a). This trend toward higher notochord length with increasing feeding frequency was maintained on d31ph, when notochord length of fish fed 4×/d (mean = 7.05 mm) was significantly higher than at 1×/d (mean = 6.02 mm) or 2×/d (mean = 6.08 mm) (Fig. 2a).

3.1.2. Wet and dry weight

On d4ph, wet weights (mean = 0.022 mg) did not differ among treatments (Fig. 1b). Wet weight increased steadily in all treatments on d10ph (mean = 0.29 mg), d17ph (mean = 0.91 mg), and on d25ph (mean = 1.40 mg), with no significant differences. However, there was a clear increase in wet weight and a departure in growth rates among treatments on d31ph (Figs. 1b, 2b) when wet weight increased with increasing feeding frequency and was markedly higher ($P < 0.05$) at 4×/d (5.10 mg) compared to 1×/d (2.86 mg). There were no significant differences between the 2×/d (3.16 mg) and the 3×/d (4.16 mg) treatments (Fig. 2b).

Due to small larval size, accurate measurements of dry weight on d4ph could not be obtained (Table 1). Dry weights increased steadily from d10ph (mean = 0.06 mg) to d17ph (mean = 0.17 mg) and to d25ph (mean = 0.26 mg) in all treatments, with no significant differences. On d31ph, there was an apparent trend toward higher dry weights with increasing feeding frequency from lowest values in larvae fed 1×/d (mean = 0.55 mg) to maximum values in larvae fed 4×/d (mean = 0.96 mg) (Table 1). Due to high inter-replicate variability, no statistical differences were observed.

3.1.3. Specific growth rate (SGR)

A trend toward higher SGR of body weight (wet) with increasing feeding frequency was observed, from a minimum (18.1%/d) in larvae fed 1×/d to a maximum (20.1%/d) in larvae fed 4×/d (Table 2). SGR was significantly higher in larvae fed 4×/d than in those fed 1× or 2×/d (Table 2).

3.2. Survival

No significant effects of feeding frequency on larval survival were observed for the duration of the study. From d4ph, survival decreased slowly to d10ph (mean = 87.9%) and to d17ph (mean = 85.8%) (Table 3). Survival decreased sharply in all treatments by d25ph (mean = 50.2%), remained relatively stable through d31ph (mean = 52.6%), and then fell to 31.4% by d35ph (Table 3).

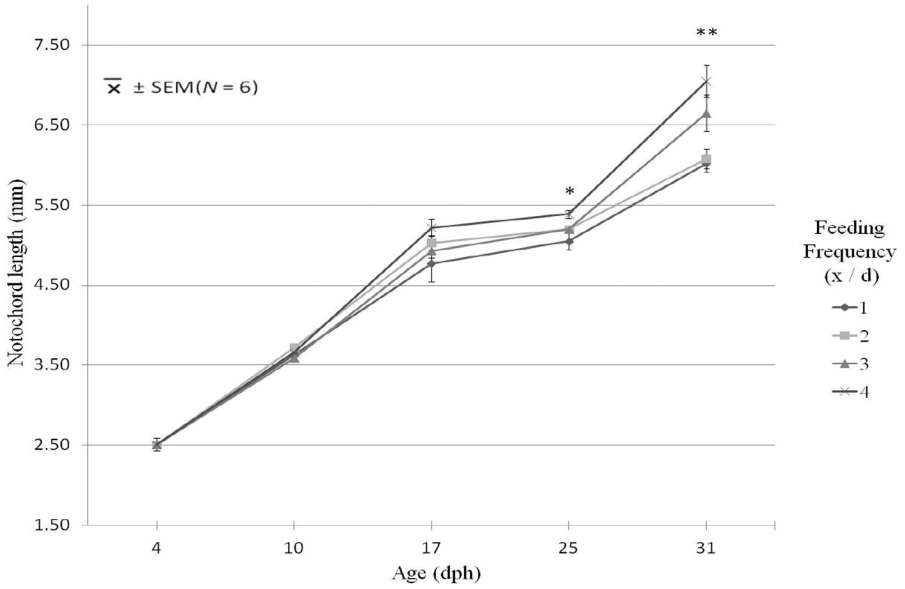

Fig. 1. Growth in notochord length (mm) (mean ± SEM, $N = 6$, except on d4ph when $N = 4$) of larval black sea bass fed enriched rotifers and *Artemia* at different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) from d4ph to d31ph. Asterisks indicate significant (* $P < 0.05$, ** $P < 0.01$) treatment differences for that sampling date.

3.3. Resistance to acute hyposalinity challenge

Following acute hyposalinity challenge (from 32 to 10 g/L) on d32ph, larvae fed 2×/d, 3×/d, and 4×/d had significantly ($P < 0.05$) higher survival activity indices (SAI) (526.5, 498.0, 491.9, respectively) than larvae fed 1×/d (244.5) (Table 2).

3.4. Diurnal prey concentration-time profiles in larval rearing tanks

Mean rotifer density on d12ph under the different feeding frequency treatments are shown in Fig. 5. Under the 1×/d feeding treatment, maximum concentration (20 rotifers/mL) was observed at 0800 h, abruptly decreased to 7.6 rotifers/mL within 1 h, and then gradually declined to zero by 9 h post-feeding (Fig. 5). Under the 2×/d feeding treatment, maximum concentration (10 rotifers/mL) was observed at 0800 h, abruptly decreased to 2.3 rotifers/mL within 1 h post-feeding, and then gradually declined to zero by 7 h post-feeding. Rotifer concentration increased to 10 rotifers/mL at the second feeding at 1600 h, decreased abruptly to 2.1 rotifers/mL by 1700 h, and then gradually declined to zero by 2000 h. The 3×/d feeding treatment showed similar trends with a maximum concentration of 6.6 rotifers/mL at 0800 h, 1200 h, and 1600 h, decreasing abruptly within 1 h of each feeding to an average of 0.66 rotifers/mL, then declining to zero over the next 3 h. Under the 4×/d feeding treatment, maximum concentrations (5 rotifers/mL) were observed at 0800 h, 1200 h, 1600 h, and 2000 h with each feeding. After each feeding, rotifer density decreased abruptly within 1 h to an average of 0.26 rotifers/mL and then declined to zero within 1–3 h after each feeding.

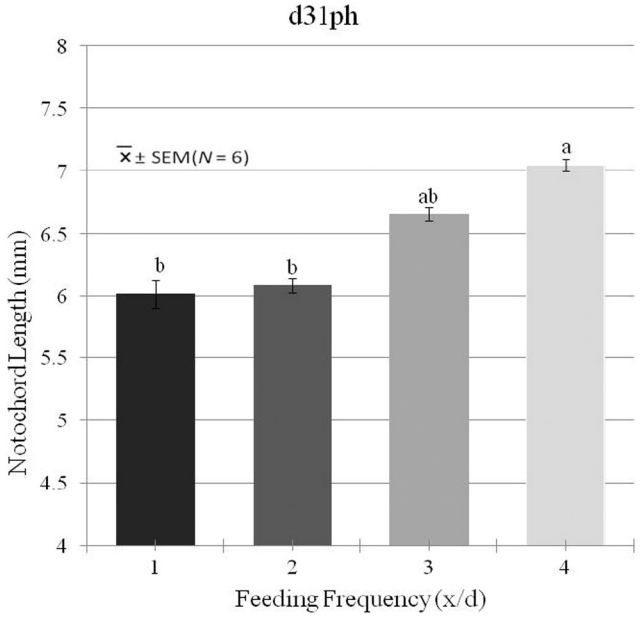

Fig. 2. Notochord length (mm) (mean ± SEM, $N = 6$) of black sea bass larvae fed enriched rotifers and *Artemia* at different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) on d31ph. Means not sharing the same letter are significantly ($P < 0.05$) different.

On d14ph, total prey concentration was increased from 20 rotifers/mL to 25 rotifers/mL. Similar trends to those found on d12ph were observed in rotifer concentrations across treatments and times, except that peak rotifer concentrations increased to 25, 12.5, 8.33 and 6.25 rotifers/mL at feeding frequencies of 1, 2, 3 and 4×/d, respectively.

Artemia concentration profiles on d27ph (Fig. 6) were similar to those observed for rotifers on d12ph (Fig. 5). Under the 1×/d treatment, maximum concentration (3 *Artemia*/mL) was observed at first feeding (0800 h), abruptly decreased to 1.28 *Artemia*/mL within 1 h, and then gradually decreased to zero by 9 h post-feeding (Fig. 6). Under the 2×/d treatment, maximum concentration (1.5 *Artemia*/mL) was observed at first feeding (0800 h), abruptly decreased to 0.02 *Artemia*/

Table 1
Dry weights (mg) (mean ± SEM, $N = 6$) of black sea bass larvae fed enriched rotifers and *Artemia* at four different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) from d4ph to d31ph.

	Feeding frequency (x/d)				
Age (dph)	1	2	3	4	P^*
4	ND	ND	ND	ND	
10	0.05 ± 0.01	0.06 ± 0.05	0.06 ± 0.01	0.07 ± 0.01	NS
17	0.13 ± 0.02	0.26 ± 0.13	0.13 ± 0.08	0.15 ± 0.01	NS
25	0.22 ± 0.03	0.26 ± 0.01	0.28 ± 0.02	0.29 ± 0.02	NS
31	0.55 ± 0.03	0.69 ± 0.16	0.80 ± 0.12	0.96 ± 0.12	NS

ND = no data.
* Probability level of overall ANOVA. NS = not significant.

Table 2
Specific growth rate of wet weight (SGR, %/d) and survival activity index (SAI) (mean ± SEM, $N = 6$) for black sea bass larvae fed enriched rotifers and *Artemia* at four different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) from d4ph to d31ph.

	Feeding frequency (x/d)				
	1	2	3	4	P^*
SGR[x] (%/d)	18.1 ± 0.204[b]	18.4 ± 0.399[b]	19.3 ± 0.507[ab]	20.1 ± 0.467[a]	0.01
SAI[y]	244.5 ± 55.3[b]	526.5 ± 48.32[a]	498.0 ± 40.23[a]	491.9 ± 102.27[a]	0.05

[x] SGR calculated from d4ph to d31ph.
[y] SAI calculated following an acute hyposalinity (10 g/L) challenge on d32ph.
* Probability level of overall ANOVA. NS = not significant. Means in the same row not sharing the same letter are significantly different (Tukey-Kramer HSD Test).

mL within 1 h, and then decreased to zero after 3 h. *Artemia* concentration increased to 1.5 *Artemia*/mL again at the 1600 h feeding, and then abruptly decreased to zero within 1 h (Fig. 6). Under the 3×/d treatment, maximum concentration (1 *Artemia*/mL) was observed at 0800 h and then decreased to zero within 1 h. This pattern was repeated at 1200 and 1600 h. Under the 4×/d treatment, maximum concentration (0.75 *Artemia*/mL) was observed at 0800, 1200, 1600 and 2000 h, decreasing to zero within 1 h of feeding.

3.5. Fatty acid composition

3.5.1. Fatty acid composition of enriched rotifers

Following enrichment of rotifers, no significant effects of storage time (from 0800 h to 2000 h) at 4 °C were observed on total lipid (mean = 312 mg/g dwt) (Table 4). There were no significant differences among storage times in concentrations of any of the fatty acids, including the total saturates, monounsaturates, n-3 or n-6 PUFAs, or ratios of n-3 to n-6 PUFAs (Table 6).

3.5.2. Fatty acid composition of enriched Artemia

Following enrichment of *Artemia*, no significant effects of storage time (from 0800 h to 2000 h) at 4 °C were observed on total lipid (mean = 431 mg/g dwt) (Table 5). There were no significant differences among storage times in concentrations of any of the fatty acids, including the total saturates, monounsaturates, n-3 or n-6 PUFAs, or ratios of n-3 to n-6 PUFAs (Table 5).

3.5.3. Fatty acid composition of larval black sea bass

The fatty acid composition whole body tissue of black sea bass larvae fed at the different feeding frequencies is shown in Table 6. No significant ($P > 0.05$) treatment effects were observed in total lipid (mean = 221.5 mg/g dwt) or any of the fatty acids, including ARA (range = 2.29–2.49%TFA), EPA (7.50–7.79%TFA), DHA (9.27–9.57%TFA), total saturates, monounsaturates, n-3 or n-6 PUFAs, or ratios of n-3 to n-6 PUFAs, DHA to EPA, EPA to ARA, or DHA to ARA (Table 6).

Table 3
Survival (%) (mean ± SEM, $N = 6$) for black sea bass larvae fed enriched rotifers and *Artemia* at four different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) from d0ph to d31ph.

	Feeding frequency (x/d)				
Age (dph)	1	2	3	4	P^*
10	80.7 ± 7.87	87.5 ± 5.82	92.8 ± 4.67	90.5 ± 6.12	NS
17	81.1 ± 6.10	83.7 ± 6.37	88.7 ± 5.47	89.6 ± 5.20	NS
25	40.1 ± 5.09	51.4 ± 4.60	59.4 ± 8.77	52.9 ± 6.47	NS
31	55.3 ± 8.14	52.1 ± 5.77	53.5 ± 7.82	49.8 ± 5.70	NS
35	32.5 ± 3.31	36.8 ± 6.23	32.7 ± 5.70	23.4 ± 4.69	NS

* Probability level of overall ANOVA. NS = not significant. Means in the same row not sharing the same letter are significantly different (Tukey-Kramer HSD Test).

Table 4
Fatty acid composition (% TFA, mean ± SEM, $N = 3$) of enriched rotifers *Brachionus rotundiformis* harvested at 08:00 h and then stored at 4 °C ("cold-banked") until 20:00 h. No significant differences among storage times were observed.

	Time of sampling after storage			
Fatty acids	08:00 h	12:00 h	16:00 h	20:00 h
14:0	3.48 ± 0.01	3.50 ± 0.12	3.30 ± 0.07	3.56 ± 0.07
16:0	22.0 ± 0.30	22.1 ± 0.29	21.8 ± 0.21	21.8 ± 0.20
16:1n-7	11.8 ± 0.38	12.1 ± 0.44	11.6 ± 0.65	12.1 ± 0.67
18:0	5.49 ± 0.20	5.68 ± 0.17	5.62 ± 0.32	5.69 ± 0.12
18:1n-9	4.70 ± 0.24	4.84 ± 0.28	4.58 ± 0.33	5.58 ± 0.39
18:2n-6	5.57 ± 0.66	5.43 ± 0.80	5.29 ± 0.73	4.11 ± 0.54
18:3n-3	12.4 ± 0.75	11.4 ± 0.42	12.0 ± 0.42	11.2 ± 0.28
18:4n-3	1.95 ± 0.55	2.00 ± 0.68	1.54 ± 0.37	2.44 ± 0.73
20:1	3.34 ± 0.44	2.83 ± 0.10	2.79 ± 0.06	2.68 ± 0.08
20:4n-6	2.81 ± 0.12	2.65 ± 0.04	2.40 ± 0.52	2.95 ± 0.21
20:5n-3	15.4 ± 0.61	15.5 ± 0.41	15.4 ± 0.56	14.7 ± 0.69
22:5n-3	5.02 ± 0.18	5.83 ± 0.71	5.94 ± 0.81	6.81 ± 0.22
22:6n-3	5.00 ± 0.35	6.08 ± 0.98	8.18 ± 1.72	6.29 ± 1.15
Σ saturates	31.0 ± 0.51	31.3 ± 0.35	30.73 ± 0.36	31.0 ± 0.20
Σ monounsaturates	19.9 ± 0.40	19.8 ± 0.25	18.9 ± 0.42	20.4 ± 0.61
Σ n-3 PUFA	40.8 ± 0.56	40.9 ± 1.39	42.6 ± 1.40	41.5 ± 1.11
Σ n-6 PUFA	8.39 ± 0.66	8.08 ± 0.84	7.69 ± 0.94	7.06 ± 0.74
n-3/n-6 PUFA	4.93 ± 0.40	5.28 ± 0.76	5.73 ± 0.77	6.05 ± 0.83
DHA/EPA	0.32 ± 0.01	0.40 ± 0.07	0.54 ± 0.12	0.44 ± 0.09
EPA/ARA	5.50 ± 0.33	5.85 ± 0.13	7.19 ± 1.85	5.02 ± 0.28
DHA/ARA	1.80 ± 0.17	2.30 ± 0.41	4.25 ± 1.96	2.20 ± 0.51
Total lipid (mg/g dwt)	309.8 ± 17.6	375.0 ± 48.8	271.7 ± 26.7	291.2 ± 18.1

3.6. Na^+/K^+ ATPase activity following sub-lethal salinity challenge

Black sea bass larvae abruptly transferred on d32ph from 32 g/L to a sub-lethal salinity of 15 g/L showed significant effects of feeding frequency on whole body Na^+/K^+ ATPase activity, but there were no significant effects related to time post-transfer and there were no interactive effects. Hence, the effects of feeding frequency on Na^+/K^+ ATPase activity were compared by combining data for all sampling

Table 5
Fatty acid composition (% TFA, mean ± SEM, $N = 3$) of enriched *Artemia* cold-banked at ~12 °C from 08:00 h until 20:00 h. No significant differences among storage times were observed.

	Time of sampling after storage			
Fatty acids	08:00 h	12:00 h	16:00 h	20:00
14:0	1.13 ± 0.01	1.15 ± 0.06	1.04 ± 0.13	1.12 ± 0.04
16:0	10.7 ± 0.31	10.4 ± 0.28	10.2 ± 0.79	11.312 ± 0.54
16:1n-7	4.30 ± 0.03	4.33 ± 0.08	4.2 ± 0.20	4.12 ± 0.19
17:0	0.56 ± 0.03	0.54 ± 0.04	0.83 ± 0.26	0.67 ± 0.08
18:0	4.98 ± 0.18	4.54 ± 0.27	4.8 ± 0.25	4.95 ± 0.08
18:1n-9	22.4 ± 2.89	27.1 ± 3.88	22.4 ± 3.19	26.4 ± 4.04
18:1n-11	5.01 ± 0.05	4.48 ± 0.28	6.02 ± 1.84	5.02 ± 0.63
18:2n-6	7.28 ± 0.15	6.9 ± 0.18	6.72 ± 0.42	6.22 ± 0.35
18:3n-3	16.7 ± 3.87	14.5 ± 2.28	19.0 ± 1.88	16.2 ± 2.56
18:4n-3	3.32 ± 0.14	3.06 ± 0.22	3.00 ± 0.18	3.29 ± 0.17
20:1	2.44 ± 0.13	2.55 ± 0.13	2.89 ± 0.13	2.65 ± 0.13
20:4n-6	1.34 ± 0.09	1.06 ± 0.18	1.03 ± 0.05	0.96 ± 0.13
20:5n-3	8.26 ± 0.47	7.64 ± 0.44	7.73 ± 0.24	7.1 ± 0.33
22:1	0.84 ± 0.07	0.95 ± 0.08	1.00 ± 0.05	0.91 ± 0.04
22:5n-3	1.18 ± 0.42	0.95 ± 0.07	1.10 ± 0.18	0.86 ± 0.14
22:6n-3	9.56 ± 0.34	8.14 ± 0.66	9.27 ± 0.11	8.29 ± 0.56
DHA/EPA	1.16 ± 0.03	1.08 ± 0.12	1.20 ± 0.03	1.17 ± 0.03
EPA/ARA	6.19 ± 0.11	7.51 ± 0.81	7.73 ± 0.57	7.80 ± 1.59
DHA/ARA	7.19 ± 0.31	8.29 ± 1.77	9.12 ± 0.48	9.15 ± 2.00
Σ saturates	17.4 ± 0.46	16.6 ± 0.51	16.9 ± 0.74	18.1 ± 0.58
Σ monounsaturates	35.0 ± 2.86	39.5 ± 3.94	36.6 ± 2.05	39.1 ± 3.26
Σ n-3 PUFA	39.0 ± 3.07	34.2 ± 1.75	39.1 ± 1.65	35.7 ± 3.25
Σ n-6 PUFA	25.0 ± 3.40	22.1 ± 2.53	25.7 ± 1.64	23.3 ± 2.77
n-3/n-6 PUFA	1.59 ± 0.09	1.58 ± 0.11	1.53 ± 0.04	1.55 ± 0.05
Total lipid (mg/g dwt)	570.6 ± 132.1	500.5 ± 77.5	303.6 ± 77.3	352.7 ± 20.1

Table 6
Fatty acid profile (% total fatty acid) (mean ± SEM, $N = 5$, except treatment 3, $N = 4$) of whole body tissue of black sea bass *Centropristis striata* larvae (d32ph) fed enriched rotifers *Brachionus rotundiformis* and *Artemia* at four different feeding frequency frequencies (1×/d, 2×/d, 3×/d, 4×/d).

	Feeding frequency (x/d)			
Fatty acids	1	2	3	4
16:0	16.7 ± 0.48	15.3 ± 0.99	15.9 ± 0.33	14.7 ± 0.94
16:1n-7	2.27 ± 0.08	2.63 ± 0.21	2.54 ± 0.13	2.51 ± 0.06
17:0	0.91 ± 0.05	0.89 ± 0.04	0.85 ± 0.02	0.83 ± 0.03
18:0	10.7 ± 0.54	10.1 ± 0.22	9.49 ± 0.23	9.43 ± 0.26
18:1n-9	19.1 ± 0.55	19.4 ± 1.03	19.2 ± 1.37	19.6 ± 0.99
18:1n-11	8.05 ± 0.56	7.71 ± 0.68	6.90 ± 0.51	7.05 ± 0.29
18:2n-6	5.29 ± 0.25	5.61 ± 0.38	6.32 ± 0.47	6.22 ± 0.68
18:3n-3	12.5 ± 0.89	13.1 ± 0.66	13.6 ± 1.10	14.2 ± 0.42
18:4n-3	1.54 ± 0.90	1.73 ± 0.10	1.68 ± 0.12	1.96 ± 0.16
20:1	1.99 ± 0.06	2.11 ± 0.05	2.15 ± 0.03	2.25 ± 0.14
20:4n-6	2.40 ± 0.09	2.49 ± 0.08	2.29 ± 0.07	2.24 ± 0.15
20:5n-3	7.50 ± 0.10	7.79 ± 0.07	7.75 ± 0.103	7.78 ± 0.11
22:5n-3	1.86 ± 0.21	1.94 ± 0.06	1.81 ± 0.05	1.73 ± 0.08
22:6n-3	9.27 ± 0.57	9.30 ± 0.20	9.57 ± 0.29	9.45 ± 0.41
Σ saturates	28.2 ± 0.95	26.2 ± 0.98	26.2 ± 0.54	24.9 ± 0.89
Σ monounsaturates	33.3 ± 0.98	33.8 ± 1.21	32.6 ± 1.65	33.1 ± 0.96
Σ n-3 PUFA	36.8 ± 0.47	36.03 ± 0.90	36.7 ± 0.23	35.7 ± 1.07
Σ n-6 PUFA	7.69 ± 0.30	8.1 ± 0.39	8.61 ± 0.53	8.46 ± 0.63
n-3/n-6 PUFA	4.81 ± 0.17	4.48 ± 0.16	4.301 ± 0.22	4.29 ± 0.31
DHA/EPA	1.23 ± 0.06	1.19 ± 0.03	1.24 ± 0.02	1.21 ± 0.04
EPA/ARA	3.14 ± 0.12	3.15 ± 0.08	3.39 ± 0.07	3.53 ± 0.21
DHA/ARA	3.87 ± 0.24	3.76 ± 0.19	4.18 ± 0.04	4.30 ± 0.35
Total lipid (mg/g dwt)	211.0 ± 21.1	224.7 ± 19.1	250.6 ± 22.5	199.6 ± 36.9

times (Fig. 7). Na^+/K^+-ATPase activity (μmol ADP/mg protein/h) was significantly ($P < 0.05$, Student's *t*-test) higher in the larvae fed 3×/d (5.61 ± 0.13) than in larvae fed 1×/d (5.20 ± 0.10) or 4×/d (5.22 ± 0.11).

4. Discussion

4.1. Growth

The results of this study demonstrated that, given a fixed daily ration of live prey, the frequency of feeding of prey, which required lower prey concentrations (i.e., smaller meal sizes) with increasing feeding frequency, significantly influenced growth of black sea bass larvae from first-feeding through the pre-metamorphic stage. By d31ph, notochord length, wet weight and SGR increased with increasing feeding frequency from a minimum in larvae fed 1×/d to a maximum in larvae fed 4×/d (Figs. 1, 2, 3, and 4). Thus, black sea bass larvae showed slowest growth when fed a high concentration of live prey once per day, but growth improved when larvae were fed the same daily ration distributed over an increasing number of smaller meals. These results are not directly comparable to earlier studies of feeding frequency in larval finfish in which live prey was fed to satiation at each feeding (Mollah and Tan, 1988), ration size was the same at each feeding (Rabe and Brown, 2000), or fish were fed artificial microdiets at each feeding (Xie et al., 2011). For example, yellowtail flounder *Pleuronectes ferrugineus* larvae fed a moderate prey level (8000/L) 1×/d grew slower than fish fed 2×/d, 4×/d, or continuously (Rabe and Brown, 2000). However, since meal size was constant at each feeding, total ration in the 2×/d and 4×/d treatments were two and four times higher, respectively, than in the 1×/d treatment (Rabe and Brown, 2000). Growth of catfish *Clarias microcephalus* (Mollah and Tan, 1988) and yellow croaker *Pseudosciaena crocea* (Xie et al., 2011) larvae also increased at higher feeding frequencies when larvae were fed to satiety at each feeding. The present study is unique in that it reports higher growth rates in fish fed equivalent daily rations of prey, but at different frequencies and prey concentrations.

Improved growth at higher feeding frequencies in larval black sea bass may have been related to a number of factors. At the lowest feeding frequency (1×/d), prey concentrations were depleted after 8 h, with 10 h of daylight remaining and with 16 h until the next feeding (8 am) (Fig. 5). Likewise, for larvae fed 2×/d, prey concentrations were depleted 6 h after the first feeding, remained at zero for 1 h until the second feeding, and then were depleted again for 15 h, so that larvae were deprived of prey for a total of 16 h in a diurnal cycle (Fig. 5). For the larvae fed 3×/d, larvae were deprived of prey for 1 h after each of the first two feedings and then for 13 after the final feeding for a total of 15 h of total prey deprivation. Larvae fed 4×/d were deprived of prey for 1 h after the first feeding and second feedings, for 2 h after the third feeding, and then for 12 h after the fourth feeding for a total 16 h

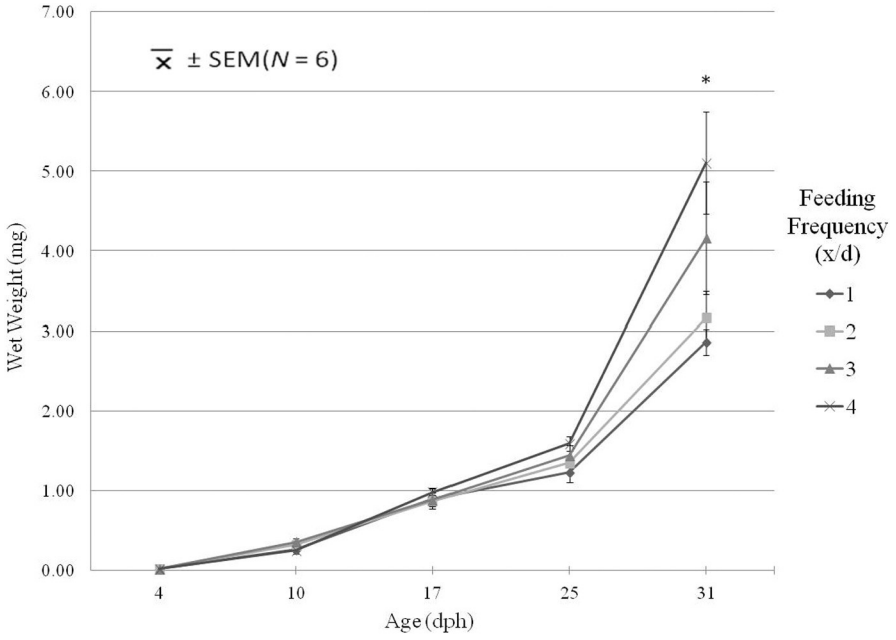

Fig. 3. Growth in wet weight (mg) (mean ± SEM, $N = 6$, except on d4ph when $N = 4$) of black sea bass larvae fed enriched rotifers and *Artemia* at different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) from d4ph to d31ph. Asterisks indicate significant ($^* P < 0.05$) treatment differences for that sampling date.

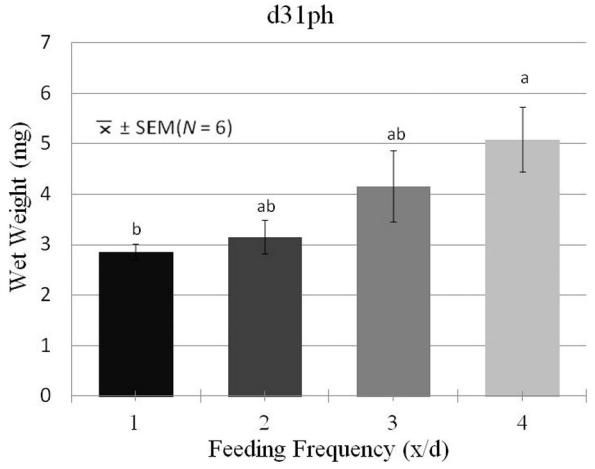

Fig. 4. Wet weight (mg) (mean ± SEM, $N = 6$) of black sea bass larvae fed enriched rotifers and *Artemia* at different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) on d31ph. Treatments not sharing the same letter are significantly ($P < 0.05$) different.

of prey deprivation (Fig. 5). Hence, in all treatments, the total duration of prey deprivation was comparable, ranging from 9 to 10 h during the photophase and 15–16 h over a 24-h diurnal cycle.

While total duration of feed deprivation among treatments was comparable, the length of continuous feed deprivation was disparate and likely affected growth. Whereas fish fed 1×/d experienced 10 h of continuous feed deprivation, those fed 2×/d, 3×/d and 4×/d experienced 8 h, 7 h and 6 h, respectively, of continuous feed deprivation. Hence, at lower feeding frequencies (Fig. 5) the longer durations of continuous feed deprivation may have increased competition for limited prey. In contrast, at the highest feeding frequency (4×/d), a shorter continuous duration of feed deprivation alleviated competition and improved growth. In larval yellow croaker *Pseudosciaena crocea*, increasing size variation and slower growth were observed with a decrease in feeding frequency (Xie et al., 2011). It is possible that size heterogeneity of black sea bass in the present study was increased at the lower feeding frequencies which inhibited growth.

The energy that is required to breakdown, absorb, transport and assimilate nutrients from a meal (specific dynamic action of the meal, or SDA) require up to 25% of daily energy expenditures in fish (Secor, 2011). At constant meal type, fish size, and water temperature, an increase in meal size results in a larger SDA (Secor, 2011). While the black sea bass larvae under all treatments in this experiment were fed the same number of prey (i.e. ration size) each day, the allocation per meal was reduced as feeding frequency was increased. Thus, fish fed smaller meals more frequently would have a lower SDA than fish fed a single large meal, consistent with the trend toward faster growth with increasing feeding frequency observed in the present study.

Higher growth at higher feeding frequencies may have also been related in part to water flow and exchange patterns in the larval rearing tank inherent to a recirculating aquaculture system. Daily water exchange ranged from 200 to 300% in each tank throughout the experiment. Although effluent standpipes in each tank were screened to prevent larvae from escaping, smaller prey were lost with water exchange, and recycled water was 1-um-filtered to exclude prey. At higher feeding frequencies (3×/d and 4×/d), a rapid and total depletion of prey was observed after each feeding (Figs. 5 and 6), suggesting that larval appetite and prey consumption were maximized and prey loss via water exchange was minimized. In contrast, at the lower feeding frequencies (1×/d and 2×/d), high prey concentrations at each feeding may have rapidly satiated the larvae as evidenced by a rapid decline in prey concentration within 1 h post-feeding (Figs. 5 and 6) followed by a more gradual decline. Thus, a higher percentage of prey was removed from the tank via system water exchange.

The lack of differences in growth related to feeding frequency during through d17ph (i.e., rotifer phase) and the signficant departure in growth from d25ph (i.e., *Artemia* phase) (Fig. 1) seem contradictory. As observed in larval red porgy, it is likely that the first 13-d period of feeding on rotifers was insufficient for treatment effects to be clearly demonstrated and that differences in growth rates were accentuated during the *Artemia* phase when larval feeding and specific growth rates (Fig. 3) were much higher (Andrade et al., 2012; Watanabe et al., 2016).

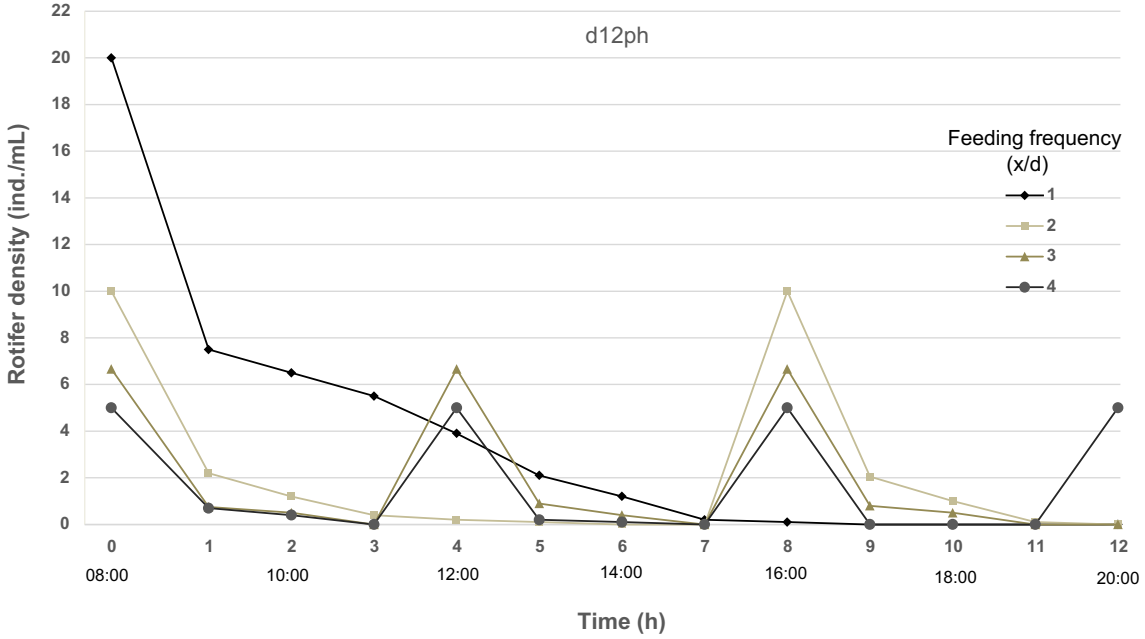

Fig. 5. Mean rotifer density (individuals/mL) ($N = 3$) sampled in situ on d12ph over a 12 h sampling period. Rotifers were fed at 08:00 (1×/d), 08:00 and 16:00 h (2×/d), 08:00, 12:00 and 16:00 h (3×/d), and 08:00, 12:00, 16:00 and 20:00 h (4×/d). The gradual increases in rotifer densities preceding feeding at 12:00, 16:00 and 20:00 h are artifacts of the time interval between samples.

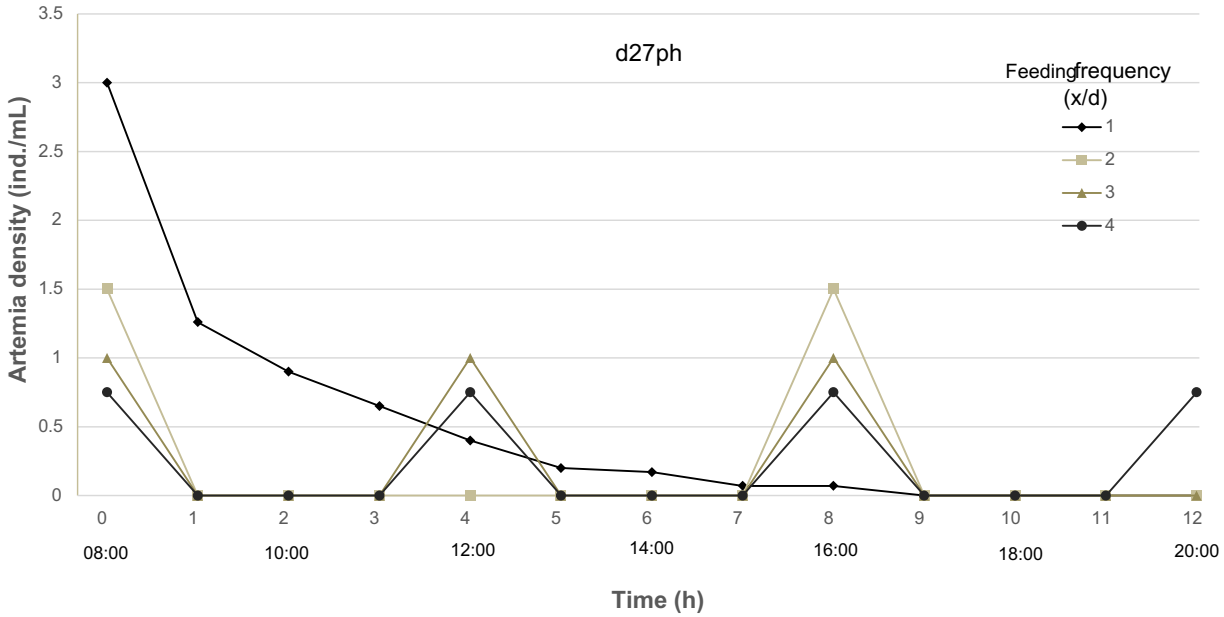

Fig. 6. Mean *Artemia* density (individuals/mL) ($N = 3$) sampled in situ on d27ph over a 12 h sampling period. *Artemia* were fed at 08:00 (1×/d), 08:00 and 16:00 h (2×/d), 08:00, 12:00 and 16:00 h (3×/d), and 08:00, 12:00, 16:00 and 20:00 h (4×/d). The gradual increases in rotifer densities preceding feeding at 12:00, 16:00 and 20:00 h are artifacts of the time interval between samples.

4.2. Survival

Final survival on d32ph in all treatments was high (23.4–32.5%) and was typical for hatchery rearing of black sea bass (Copeland and Watanabe, 2006; Rezek et al., 2010; Carrier et al., 2011; Watanabe, 2011) but was not influenced by feeding frequency (Table 5). Hence, while a constant daily ration fed under different feeding frequencies caused differential growth of the larvae, the level of nutrition in all treatments met the minimum maintenance requirements for survival. In gilthead sea bream *Sparus aurata* (Goldan et al., 1997) and in yellowtail flounder *Pleuronectes ferrugineus* (Rabe and Brown, 2000), no differences in larval survival were observed as daily ration increased proportionately with increasing feeding frequency.

Behavioral changes may help explain the relatively uniform survival of black sea bass larvae at the different feeding frequencies in the present study. Cod *Gadus morhua* larvae fed at low prey densities maintained good growth and survival by increasing swimming activity and attack rate and decreasing the size selectivity of prey, allowing them to capture more prey (Munk and Kiorboe, 1985; Munk, 1995). In yellowtail kingfish *Seriola lalandi Valenciennes*, 1833, rotifer consumption and larval survival over the first 12 days post-hatching were unaffected when prey were fed at high or low densities (10–30 or 5–8 individuals/mL, respectively), or at high density until 5 dph and then at a low density thereafter (Woolley and Partridge, 2015), presumably related to behavioral adaptations to prey concentrations. Black sea bass larvae may likewise increase hunting activity and prey consumption at lower prey densities.

During larval culture of black sea bass, artificial microdiets are typically co-fed with both rotifers and *Artemia* beginning on d15ph, when live prey is gradually reduced and artificial feeds increased through d35ph (Watanabe, 2011). Microdiets were not included in the present study in order that the effects of manipulating the feeding frequency of live prey could be delineated. Nonetheless, survival (23.4–32.5%) was typical for hatchery rearing of black sea bass. The co-feeding of microdiets with live prey at correspondingly high frequencies may further improve growth performance and the efficiency of utilizing live prey resources in a hatchery.

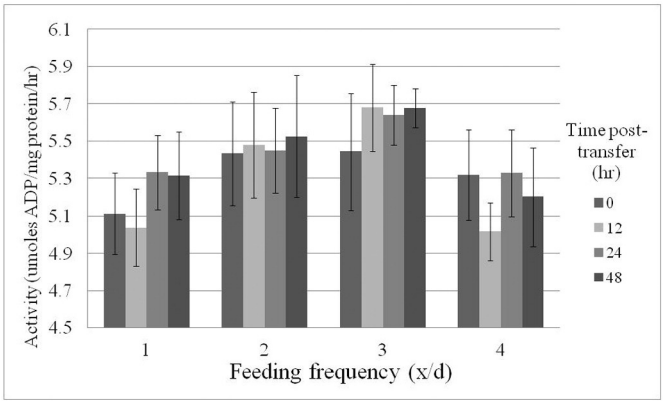

Fig. 7. Na^+/K^+ ATPase activity (μmol ADP/mg protein/h, mean ± SEM, $N = 6$) of whole body homogenates of d32ph black sea bass larvae fed live prey (rotifers and *Artemia*) at different feeding frequencies (1×/d, 2×/d, 3×/d, 4×/d) following abrupt transfer from 32 g/L to a sub-lethal salinity of 15 g/L. Data for all sampling times (0, 12, 24, and 48 h post-transfer) were combined.

4.3. Fatty acid concentrations of live prey and black sea bass larvae

4.3.1. Fatty acid composition of enriched rotifers

Starvation can cause a loss of essential fatty acids in enriched prey, which can be minimized by storing prey at low temperature (Estevez et al., 1998). Concentrations of DHA, EPA, ARA, and total lipids in enriched rotifers and *Artemia* did not change ($P < 0.05$) over time when held at ~12 °C for up to 16 h until feeding. This indicates that the effects of feeding frequency on larval performance in this study were not influenced by a reduction in prey nutritional quality post-enrichment. Rainuzzo et al. (1989) found that starvation of rotifers for 49 h at 16 °C after enrichment did not change the essential fatty acid (n-3 HUFA) profile of rotifers, although total amounts were decreased by 25%. On the other hand, Birkou et al. (2012) found that *Brachionus* spp. enriched with n-3 PUFAs were able to maintain their improved nutritional value for only several hours after being transferred to a low PUFA-containing media at 25 °C.

ARA levels in rotifers ranged from 2.40%TFA (2.69 mg/g dwt) to 2.95%TFA (5.59 mg/g dwt), whereas a previous study (Rezek et al., 2010) showed that growth and survival of black sea bass improved as ARA was increased from 0 to 6% TFA (4.37–18.0 mg/g dwt). Koven et al. (2001) found that increasing ARA levels in live feeds from 1.0 to 2.75 mg/g dwt did not affect larval growth, but increased tolerance to handling stress in larval gilthead sea bream *Sparus aurata*. In the present study, the ratio of DHA/ARA (1.80 to 4.25) was within the range of 2:1 recommended for many species of larval marine fish (Sargent et al., 1999). The ratio of DHA/EPA (0.324 to 0.538) was below the suggested dietary ratio (2:1) for European sea bass *Dicentrarchus labrax*, suggesting that the rotifer enrichment medium was deficient in DHA, or that the enrichment protocol was not optimized (Sargent et al., 1999). The ratio of EPA/ARA in rotifers (ranged from 1.79 to 4.25) was higher than the optimal dietary ratio of 1:1 for European sea bass (Sargent et al., 1999).

4.3.2. Fatty acid composition of enriched Artemia

Fatty acid composition of cold-banked *Artemia* did not change significantly over a 12 h period post-enrichment (0800 h–2000 h). Similar findings were reported by several workers (Coutteau and Mourente, 1997; Han et al., 2001; Koven et al., 2001). The ratio of DHA/EPA found in cold-banked *Artemia* (1.08–1.20) was lower than that in the enrichment medium (minimum DHA/EPA of 2.5). Mean DHA and EPA levels in enriched *Artemia* (38.1 and 33.2 mg/g dwt, respectively) were higher than those previously reported in DHA Selco-enriched *Artemia* nauplii (17.7 and 10.8 mg/g dwt) (Lavens and Sorgeloos, 1996). During starvation, *Artemia* selectively catabolize certain fatty acids such as 18:4n-3, 22:5n-3, and 22:6n-3, while retaining fatty acids such as 18:0, 20:4n-6, and 20:5n-3, but significant losses of fatty acids were found only after 72 h (Coutteau and Mourente, 1997). This is consistent with the stable fatty acid concentrations found in enriched *Artemia* cold-banked for 12 h post-enrichment in the current experiment. Furthermore, retro-conversion of DHA to EPA by *Artemia* via 22:5n-3 (docosapentaenoic acid, DPA) (Sargent et al., 1999; Rezek et al., 2010; Carrier et al., 2011) could explain the low DHA relative to the amount supplied by the enrichment. The EPA/ARA ratio (6.19–7.80) was above the 1:1 recommended dietary ratio for European sea bass larvae (Sargent et al., 1999). The DHA/ARA ratio (7.19–9.15) was also above the recommended 2:1 dietary ratio for European sea bass larvae (Sargent et al., 1999).

4.3.3. Fatty acid composition of black sea bass larvae

There were no significant differences in total lipid or fatty acid composition of whole body larval black sea bass fed enriched rotifers and *Artemia* at the four feeding frequencies (1×/d, 2×/d, 3×/d, or 4×/d). This may be explained by increased whole body protein content associated with greater muscle (rather than lipid) deposition in faster-growing fish in the 3×/d and 4×/d treatments. Different fish species use different strategies for meeting energetic demands during food deprivation, including selectively catabolizing carbohydrates, lipids and proteins (Czesny et al., 2003; Barcellos et al., 2010). Some species (eel *Anguilla anguilla*, goldfish *Carassius auratus*, and plaice *Pleuronectes platessa*) use muscle protein as their primary fuel source, whereas others (northern pike *Esox lucius*, roach *Rutilus rutilus*, and golden perch *Macquaria ambigua*) use lipids (see Czesny et al., 2003 for review). Proximate composition data in the present study suggest that lipid was utilized and deposited uniformly among feeding frequency treatments, while more protein was catabolized for energy at lower feeding frequencies, causing reduced muscle growth. This is consistent with what has been reported in larval Ayu (Yao et al., 1994; Cho et al., 2003; Xie et al., 2011), Trairao *Hoplias lacerdae* (Light and Portella, 2005), and yellow croaker *Pseudosciaena crocea* (Xie et al., 2011) where growth increased with increasing feeding frequency, but without correlation to total lipid or fatty acid profiles. In other species including Ayu (Cho et al., 2003), meager *Argyrosomus regius* (Roo et al., 2010), and red-spotted grouper *Epinephelus akaara* (Kayano et al., 1993; Yao et al., 1994), total lipid has been reported to increase with increasing feeding frequency.

4.4. Resistance to acute hyposalinity challenge (survival activity index, SAI)

The SAI test was designed to reveal differences in the ability of larvae from different feeding frequency treatments to withstand an acute salinity decrease under zero feeding, conditions prescribed to induce short-term, albeit gradual mortality. Despite uniformly high survival among feeding frequency treatments through the pre-metamorphic stage, stress resistance (i.e., SAI) of d32ph black sea bass larvae abruptly transferred from seawater (32 g/L) to a lethal hyposalinity of 10 g/L under conditions of feed deprivation was poorer in larvae fed only 1×/d than in larvae fed at higher frequencies (Table 2). In tilapia *Oreochromis mossambicus*, feed deprivation prior to salinity challenge impaired the salinity acclimation process, indicating that nutritional state affects osmoregulatory ability (Vijayan et al., 1996). In the present study, it is possible that black sea bass larvae under the lowest feeding frequency (1×/d) were exposed to longer periods of continuous feed deprivation during a diurnal cycle (Fig. 5), reducing their ability to meet the increased metabolic demands of the hyposalinity challenge. Hence, these slowest growing fish succumbed more rapidly (i.e., lower SAI) to the combined stresses of feed deprivation and hyposalinity challenge. Other workers have reported differences in larval stress resistance even when no obvious differences in survival were evident (Ako et al., 1994; Koven et al., 2001; Watanabe et al., 2016).

4.5. Na^+/K^+ ATPase activity following sub-lethal salinity challenge

In this study, exposure of 32dph black sea bass larvae to a sub-lethal hyposalinity challenge (i.e., from 32 to 15 g/L) elicited a greater osmoregulatory response (i.e., whole body Na^+/K^+ ATPase activity) in larvae fed 3×/d than in those fed 1×/d or 4×/d. Na^+/K^+ ATPase is a key enzyme involved in osmoregulation in larval fish and is a component of the plasma membranes of chloride cells which are the cellular sites for the excretion of monovalent ions (primarily Na^+, Cl^- in seawater) (Li et al., 1995; Varsamos et al., 2004, 2005). In most teleost fish larvae, Na^+/K^+ ATPase is concentrated in the chloride cells of the skin, but as larvae develop these cells become more localized and are found primarily in the integument, gills, urinary system, and digestive tract (Hiroi et al., 1998; Varsamos et al., 2002, 2005; Li et al., 2011).

In early larval marine finfish species, such as obscure puffer *Takifugu obscures* (Li et al., 2011) and European sea bass *Dicentrarchus labrax* (Varsamos et al., 2002; Varsamos et al., 2004), a hyper- or hypoosmotic challenge elicited an initial increase in whole body Na^+/K^+ ATPase activity within 12–48 h. In the present study, no increase in Na^+/K^+ ATPase in whole body tissues of black sea bass was observed from 12 to 48 h following hyposalinity challenge, possibly due to the magnitude of the salinity challenge conditions used. After transfer of day-old obscure puffer larvae hatched in freshwater to salinities of up to 16 g/L, Na^+/K^+ ATPase activity increased threefold after 48 h at higher salinities of 12–16 g/L, but not at the lower salinities of 0–10 g/L (Li et al., 2011). In European sea bass, early larvae cope more easily with hypoosmotic than with hyperosmotic challenges (Varsamos et al., 2004). This suggests that the modest hyposalinity challenge (i.e., from 32 to 15 g/L) used in the present experiment elicited a weak Na^+/K^+ ATPase response in black sea bass larvae. The selection of more acute salinity challenge conditions may accentuate the Na^+/K^+ ATPase response, but must be weighed against the possibility of overwhelming the larva's osmoregulatory capacity and causing rapid mortality (Varsamos et al., 2004; Carrier et al., 2011).

Developmental stage (i.e., ontogeny) can also influence the magnitude of the Na^+/K^+ ATPase response in larval fish. After European sea bass larvae reached 5 mm (~10dph), Na^+/K^+ ATPase activity increased due to chloride cell accumulation in the gills, and overall osmoregulatory ability

increased as the larvae developed the ability to regulate drinking rate (Varsamos et al., 2004, 2005). It is likely that near metamorphic stage (d32ph) black sea bass in the present study had well-developed osmoregulatory organs and could regulate drinking rate to adapt to the hyposalinity challenge without a need to boost Na^+/K^+ ATPase enzyme activity.

Across all sampling times post salinity challenge, Na^+/K^+ ATPase activity was higher in larvae fed 3×/d than in larvae fed 1× or 2×/d, possibly related to ontogenetic factors. In the present study, the development of gills and chloride cells in branchial filaments may likewise have been greater in larger black sea bass larvae fed 3×/d (vs 2× or 1×/d). On the other hand, the lower Na^+/K^+ ATPase specific activity response in largest fish fed 4×/d compared to fish fed 3×/d seems contradictory. As observed in tilapia *Oreochromis* sp. (Watanabe et al., 1985), increased osmoregulatory ability in black sea bass may be related to fish size rather than age. Although black sea bass in all feeding frequency treatments in the present study were the same age (32dph) at the time of hyposalinity challenge, larger larvae in the 4×/d treatment likely had more well-developed osmoregulatory organs (e.g. gills) and ability to regulate drinking rate, decreasing the need to boost specific Na^+/K^+ ATPase activity.

4.6. Conclusions

The results demonstrate that, given a prescribed total daily ration of live rotifers and *Artemia*, feeding lower prey concentrations (i.e., smaller meal sizes) at higher frequencies, within a range of 1×, 2×, 3×, and 4×/d, increases growth performance and stress resistance of black sea bass larvae through pre-metamorphic stages, with no diminution of survival. The results suggest that, when live prey resources are limiting in a marine fish hatchery, feeding lower concentrations of prey at higher frequencies maximizes growth, survival, and larval vitality and therefore optimizes the efficiency of utilization of expensive planktonic prey while maximizing fingerling yield and quality. These findings have important implications for improving the economics of marine finfish hatchery operations by minimizing the duration of the larval rearing period to produce healthy fingerlings and to maximize productivity of hatchery and grow out operations.

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