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Effects of salinity and temperature on the growth, survival, whole body osmolality, and expression of Na^+/K^+ ATPase mRNA in red porgy (*Pagrus pagrus*) larvae

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ABSTRACT

Atlantic red porgy, *Pagrus pagrus*, is an important reef fish species in the Mediterranean and the snapper–grouper complex off the southeastern United States. Red porgy is a viable candidate for aquaculture with high market value and the ability to spawn freely in captivity. The objective of this study was to examine the combined effects of temperature and salinity on eggs, yolk-sac larvae, and early feeding-stage larvae of red porgy to day 16 post-hatching (d16ph). To determine the optimal temperature and salinity conditions for culture, embryos were reared under four temperatures (17, 19, 21, and 23 °C) and two salinities (24 and 34 g/L) in a 4×2 factorial design. Significant effects of temperature and salinity on growth (notochord length, wet and dry weight), survival, whole body osmolality, and expression of Na^+/K^+ ATPase were observed with minimal interactive effects. Under both salinities, growth increased with increasing temperature. On d16ph, wet weights at 21 and 23 °C (2.03 and 2.91 mg, respectively) were significantly higher than at 17 and 19 °C (0.20 and 0.69 mg). Salinity had no effect on growth at any temperature, but had a significant effect on survival to d16ph, with greater survival at 24 g/L (18.4%) than at 34 g/L (6.77%). Salinity significantly affected whole body osmolality on d2ph and d11ph, with 24 g/L having lower whole body osmolality than 34 g/L on both days. Temperature significantly affected whole body osmolality on d6ph, with no clear trends and on d17ph, with 17 °C (534 mOsm/kg) higher than all the higher temperatures (396–411 mOsm/kg). After increasing each tank to 44 g/L to create a sublethal salinity challenge on d16ph, larvae from 34 g/L treatments did not show an increase in levels of Na^+/K^+ ATPase mRNA after 24 or 48 h post transfer. However, fish in the 24 g/L treatments showed a significant increase in expression of Na^+/K^+ ATPase mRNA after 24 h followed by a decrease after 48 h. Within the ranges tested, a temperature of 23 °C and a salinity of 24 g/L appear to be optimal for culture of red porgy embryos, yolk-sac, and first-feeding stage larvae to d16ph.

1. Introduction

Red porgy are demersal fishes (family Spariade) found over sandy and hard bottoms of the continental shelf of the eastern and western Atlantic Ocean (Manooch and Huntsman, 1977; Potts and Manooch, 2002) ranging in depths from 18 to 185 m (Manooch and Hassler, 1978). An excellent food fish with high market value, natural populations of red porgy along the southeast coast of the U.S. have declined precipitously (South Atlantic Fishery Management Council, 2006) due to overfishing, with spawning stock biomass falling from 3530 tonnes in 1979 to 397 tonnes in 1997 (Vaughan and Prager, 2002). Recent findings suggest that red porgy stocks are recovering as a result of recreational and commercial quotas implemented in 2000 (South Atlantic Fishery Management Council, 2000) with landings of 47 metric tonnes in 2004 compared to 30 metric tonnes in 2000 (South Atlantic Fishery Management Council, 2006).

Red porgy has been found to be a viable aquaculture candidate in the Mediterranean (Conides and Glamuzina, 2001; Hernandez-Cruz et al., 1999; Kentouri et al., 1995; Kolios et al., 1997; Machinandiarena et al., 2003; Mihelakakis et al., 2001), Argentina (Aristizabal, 2005; Aristizabal et al., 2009), and North America (Morris et al., 2008). Much of the available literature on red porgy aquaculture describes larval development (Aristizabal et al., 2009; Conides and Glamuzina, 2001; Kolios et al., 1997; Morris et al., 2008; Roo et al., 2009; Roo et al., 1999), or various aspects of juvenile culture (Cejas et al., 2003; Kalinowski et al., 2005; Rotllant et al., 2003; Van der Salm et al., 2006). While a significant amount

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of work has been conducted in the Mediterranean on red porgy culture, much of the literature is anecdotal when describing optimizing larval culture, and no experimental studies to date have assessed the influence of environmental factors on red porgy larval development or survival.

Temperature and salinity are dominant environmental factors affecting marine finfish larvae (Blaxter, 1969; Brett, 1970; Holliday, 1969) and can have a significant impact on development, growth, and survival of marine finfish embryos and early larval stages. For red porgy, water temperatures ranging from 14 to 22 °C were found to support embryonic development from fertilization to hatching (Radonic et al., 2005; Saka et al., 2005), with optimal temperatures for development between 16 and 18 °C (Saka et al., 2005). No experimental studies have assessed the influence of temperature on red porgy larval development, growth, or survival.

Salinity has clear effects on the internal structures of marine larvae (Holliday, 1988). Salinity affects larval growth rates, with better growth rates observed between 8 and 20 g/L in many species such as seabream *Sparus sarba* (Tandler et al., 1995), summer flounder *Paralichthys dentatus* (Specker et al., 1999), southern flounder *Paralichthys lethostigma* (Smith et al., 1999), milkfish *Chanos chanos* (Swanson, 1996), and red tilapia *Oreochromis sp* (Watanabe et al., 1989). Higher growth rates at lower salinities are thought to result from lower standard metabolic rates at near iso-osmotic salinities and reduced costs of osmoregulation, which can consume a large percentage (20–50+ %) of energy resources in fishes (see review by Boeuf and Payan, 2001; Fielder et al., 2005). Despite a high osmotic pressure of the external seawater environment (~1000 mOsmol/kg), whole body osmolality (WBO) in marine fish larvae is maintained within a relatively narrow range (320–400 mOsmol/kg). A WBO value outside this normal range can therefore be indicative of osmotic stress (Davenport et al., 1981; Hahnenkamp et al., 1993; Riis-Vestergaard, 1982). To date, no studies have reported the effects of salinity on larval development and osmoregulation of red porgy.

Osmoregulation in larval fish is accomplished by Na^+/K^+ ion-transport pumps within cells in the integument until the gills and intestinal osmoregulatory systems develop (Rombough, 2007). Na^+/K^+ ATPase mediates the active secretory functions of chloride cells in juvenile fish by generating a chemical gradient for ion transport. Osmoregulatory ability can be evaluated by measuring the messenger RNA (mRNA) expression of Na^+/K^+ ATPase enzyme, with a higher mRNA level likely indicating the elaboration of the ion transfer pump and better osmoregulatory ability (Carrier, 2006). Increases in Na^+/K^+ ATPase mRNA levels during the first 35 days of larval development have been reported for the silver sea bream *Sparus sarba*, but the effects of different rearing temperatures or salinities were not evaluated (Deane et al., 2003).

The combined effects of temperature and salinity on marine finfish embryo and larval development and survival are species specific. In snapper *Pagrus auratus*, the effects of temperature (15–33 °C) and salinity (5–45 g/L) on larval growth (total length, wet and dry weight) were independent (Fielder et al., 2005). In summer flounder, an interaction between temperature and salinity on hatching rate was observed, where development was accelerated by higher temperatures and salinities, but higher temperatures and lower salinities inhibited development and hatching (Watanabe et al., 1999). Interactive effects of temperatures and lower salinities on growth in yellowtail flounder *Limanda ferruginea* (Howell, 1980), and winter flounder *Psuedopleuronectes americanus* (Rogers, 1976), have also been reported. The objectives of this study were to determine, under controlled laboratory conditions, the combined effects of temperature and salinity on growth, survival, osmoregulatory ability, and mRNA expression of Na^+/K^+ ATPase in red porgy from embryo to day 16 post-hatch (d16ph), as a basis for recommending optimum conditions for commercial larviculture.

2. Methods

2.1. Experimental animals

Eggs were collected from red porgy broodstock held at the Center for Coastal Fisheries and Habitat Research, National Oceanic and Atmospheric Administration (Beaufort, NC, U.S.A.). The broodstock were collected by hook and line off the coast of North Carolina during March 2004 and maintained in a recirculating 31-m^3 round tank under conditions simulating seasonal offshore bottom temperatures off North Carolina from 27 to 33 m (Parker and Dixon, 1998) and under ambient photoperiod. Red porgy broodstock spawned volitionally in captivity (Morris et al., 2008), and embryos were collected by filtering surface water from the spawning tank through a 505-μm mesh nitex mesh net. Embryos were transferred to the University of North Carolina Wilmington Center for Marine Science (UNCW-CMS) Aquaculture Facility, Wrightsville Beach, NC, USA, in oxygen-filled plastic bags. Embryos were placed in a conical plexiglass tank (15 L volume) in 32 g/L seawater where viable (buoyant) embryos for use in the experiment were separated from nonviable embryos.

2.2. Experimental system

Red porgy larvae were reared in 15-L cylindrical, black polyethylene tanks held in four temperature-controlled water baths (213 L) in a controlled-environmental laboratory. Air was supplied to each tank through an air diffuser positioned at the bottom center of each tank and maintained at 90 mL/min. Light intensity at the surface of each tank was 500 lx (492.3 ± 3.9) supplied by two 40-W full-spectrum fluorescent light bulbs suspended over each table. Photoperiod was controlled at 16L:8D starting at 0600 h, with extraneous light excluded by black curtains surrounding the water bath.

2.3. Experimental design

A 4×2 factorial design was used to compare the effects of four temperature [17 (17.3 ± 0.10 SE), 19 (18.9 ± 0.02), 21 (21.4 ± 0.09), or 23 °C (22.9 ± 0.19)] and two salinity [24 (24.3 ± 0.04) or 34 g/L (33.5 ± 0.03)] levels on the growth, survival, and whole body osmolality of larval red porgy from d1ph to d16ph. Each treatment (n = 8) was replicated using four replicates per treatment. High salinity (34 g/L) seawater was pumped from the adjacent Atlantic Intracoastal Waterway, while brackish water (24 g/L) was prepared by mixing full strength seawater with dechlorinated tap water.

Embryos were initially stocked into each tank at a density of 60/L at 19 °C. Temperature was adjusted 2 °C per day after hatching until treatment temperatures were reached. To maintain embryos in suspension, airflow was adjusted and maintained at appropriate levels and decreased to 90 mL/min after hatching (48 h post fertilization) for the remainder of the experiment to avoid injury from excessive turbulence (Mangino and Watanabe, 2006).

2.4. Larval feeding

Water was exchanged daily (33%) by siphoning 5 L from each tank and then replacing with water of appropriate salinity. At d1ph, a non-viable, condensed microalgae, *Nannochloropsis oculata*, paste (Reed Mariculture, Campbell, California, USA) was added to each tank at a concentration of 750,000 cells/mL as background. Beginning d2ph, larvae were fed s-type rotifers, *Brachionus rotundiformis* (5–20 rotifers/mL), cultured in a continuous culture system (Bentley et al., 2008). To increase docosahexaenoic acid (DHA), rotifers were enriched with Algamac 2000 (Aquafauna, Bio-Marine Inc. Hawthorne, California, USA) at a concentration of 300 mg per million rotifers at 16 and 6 h before being fed to the larvae.

2.5. Water quality

Temperature (±0.1 °C), salinity (±0.1 g/L), D.O. (7.21±0.08 mg/L), pH (8.31±0.001) (YSI 560, Yellow Springs, Ohio, USA), and light intensity (492±4 lx) were measured twice daily (0900 and 1600 h) from alternating replicate tanks per treatment. Ammonia was tested twice a week (HACH DR 850, Loveland, Colorado, USA; 0.02±0.005 mg/L) and airflow to each tank was tested daily using a Gilmont flow meter (Gilmont Instruments Inc., Barrington, Illinois, USA; ±1 ml/min) and adjusted as necessary.

2.6. Growth, survival, and osmoregulation

To measure growth and survival, 10 larvae were randomly sampled using a vertical sampling rod from each replicate tank on days 1, 4, 10, and 16 ph at approximately 0730 h. Prior to sampling, airflow was increased to uniformly distribute the larvae in tanks. The number of fish were counted, then divided by water volume removed during sampling to determine density. Survival for each tank was calculated by dividing sample density by initial density. After anesthetizing the larvae with 0.5 mg/L of 2-phenoxyethanol, notochord lengths (NL) were measured using an ocular micrometer on a dissecting microscope (Meiji Techno Co., Ltd., Japan). Wet weight was determined using an electrobalance (±0.01 mg) (Sartorius, Goettingen, Germany) by placing 10 larvae on a pre-weighed microscope slide after they were blotted dry. The slides were then placed into a laboratory oven at 60 °C for 72 h and then reweighed to determine dry weights of larvae.

To measure osmoregulatory ability, whole body osmolality (mOsm/kg) was measured on d2–3, 5–6, 11–12, and 17–18 ph using a vapor pressure osmometer designed for very small samples (2–10 uL) including tissue samples (Wescor Vapor Pressure Osmometer 5520, Logan, Utah, USA). Three to five larvae were removed from each tank, gently rinsed with deionized water on a nitex screen, and transferred to a sample disc before being placed into the osmometer chamber. Measurements were taken after a 25 min equilibration period (Henne and Watanabe, 2003; Mangino and Watanabe, 2006; Moustakas et al., 2004).

2.7. mRNA expression analysis

Fish on d16ph were subjected to a sublethal hyperosmotic salinity challenge of 44 g/L to measure the change in expression of Na^+/K^+ ATPase mRNA. Fish were sampled prior to the increase in salinity (time 0 h) and every 12 h thereafter for a total of 48 h. Fish were pooled at each sample period and stored in RNAlater (Ambion, Woodward Texas, USA) at −20 °C to preserve the integrity of the RNA within the larval tissues. RNA extraction and reverse transcription followed previously published methods (Faircloth and Shafer, 2007). Briefly, total RNA was obtained using the RNeasy Mini Kit (Qiagen, Valencia, California, USA) modified by homogenizing in TRIzol (Invitrogen, Carlsbad, California, USA). Then, the quality and quantity of each RNA extract was assessed using the RNA 6000 Nano chip on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, California, USA). Complimentary DNA (cDNA) synthesis was completed from 200 ng of total RNA using SuperScript III reverse transcriptase (Invitrogen) primed with olio(dT). Betaine was added to the reverse transcription reaction and a slightly higher temperature was employed than suggested by the manufacturer in order to more effectively denature the RNA.

Fragments of the alpha subunit of red porgy Na^+/K^+ ATPase mRNA were amplified from the cDNA by PCR using degenerate primers designed from an alignment of Na^+/K^+ ATPase sequences from other fish species. This PCR product was purified using a Wizard SV Cleanup Kit (Promega, Madison, Wisconsin, USA) and cloned in the pGEM-T Easy vector (Promega). Colonies were selected from carbenicillin-containing agar plates and the inserts were sequenced using Big-Dye Terminator Ready Reaction Mix and an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Partial sequences of two different isoforms of Na^+/K^+ ATPase were obtained from the red porgy cDNA and were deposited in NCBI. One (GenBank accession no. EU884411) is homologous with the isoform 1 complex reported in other fish species and the other (EU884412) is homologous with isoform 3 (Rajarao et al., 2001; Richards et al., 2003). Primer Express software (Applied Biosystems) was used to design a primer set from the isoform 1 sequence for quantitative real-time PCR (qPCR). The forward primer is 5′-CATCACCGGTTGCTTCTCCTA-3′, and the reverse primer is 5′-GGGACCAGGTTCTTGAAGGAA-3′). All cDNA samples were then analyzed by qPCR in the presence of SYBR green fluorescent dye using an ABI 7500 quantitative PCR system (Applied Biosystems) and the reaction conditions and standards previously described (Faircloth and Shafer, 2007). To insure that the values obtained for the Na^+/K^+ ATPase α-subunit isoform 1 sequence were comparable across all sample periods (0, 12, 24, and 48 h post hypersalinity transfer), identical standards were used throughout.

2.8. Analytical methods

Quantitative values were expressed as treatment means ± standard error. Prior to analysis, percentage data were arcsine transformed. To meet the assumptions of ANOVA, all data were analyzed using Levene's homogeneity of variance test prior to a two-way ANOVA. If the data violated the assumptions of the two-way ANOVA, a Kruskal–Wallis test was run to compare the independent effects of temperature and salinity. The effects of temperature, salinity, and their interaction on growth, survival, and osmoregulatory ability were analyzed using a two-way ANOVA. When no interactive effects were observed, temperature treatments were combined across salinities and salinity treatments were combined across temperatures. A Tukey–Kramer honesty significant difference test was used for multiple comparisons among means. All statistical analyses were performed using SAS statistical software (version 9.1.3, SAS Institute, Cary, North Carolina USA) and a P-value ≤0.05 was considered significant.

3. Results

3.1. Growth

Under both salinities, highly significant ($P<0.001$) effects of temperature on NL (mm) were observed on all sampling days (d1, 4, 10, and d16ph, Table 1A). On d1ph, notochord lengths ranged from 2.61 mm at 19 °C to 2.89 mm at 17 °C, which were not significantly different from each other but significantly smaller than 23 °C (3.15 mm) (Table 1A). On d4ph, mean NL at 17 °C (3.36 mm) was significantly higher than at 19–23 °C (3.08–3.15 mm). On d10ph, a clear departure among treatments was observed, with NL increasing with temperature from a minimum of 3.28 mm at 17 °C to a maximum of 4.68 mm at 23 °C, with significant differences among all temperature treatments. This trend was maintained on d16ph, when NL ranged from 3.65 mm at 17 °C to a maximum of 5.8 mm at 23 °C (Table 1A). NL at 21 and 23 °C were significantly higher than at 17 and 19 °C (Table 1A). No significant interactive effects between temperature and salinity on NL were observed on any sampling day throughout the study.

Under all temperatures, no significant salinity effects on NL were observed on d1ph (Table 1B). NL were significantly greater at 24 g/L than at 34 g/L on d4ph (3.24 mm versus 3.12 mm) ($P<0.01$) and d10ph (3.91 mm versus 3.78 mm) ($P<0.05$). By d16ph, however, mean NL was 4.77 mm for both salinities (Table 1B).

No significant interactive effects between temperature and salinity on wet weight were observed, except on d10ph (Table 2A). Significant effects of temperatures on wet weight (mg) were observed starting on d4ph (Table 2A). On d1ph, wet weights ranged between 0.17 mg and

Table 1
Growth in notochord length (mm) (mean ± s.e.) of larval red porgy at different temperatures (17, 19, 21, and 23 °C, n = 8) and salinities (24 and 34 g/L, n = 16). In A, both salinities were combined under each temperature. In B, all temperatures were combined under each salinity. At each age, probability values are shown (ANOVA). Means in the same row followed by the same letter are not significantly different (Tukey's, P>0.05). NS = not significant (P>0.05).

A

	Temperature (°C)				
Age (dph)	17	19	21	23	P-value
1	2.89 ± 0.08^{ac}	2.61 ± 0.05^{b}	3.12 ± 0.09^{cd}	3.15 ± 0.03^{d}	<0.0001
4	3.36 ± 0.03^{a}	3.13 ± 0.07^{b}	3.08 ± 0.06^{b}	3.15 ± 0.02^{b}	<0.001
10	3.28 ± 0.05^{a}	3.48 ± 0.05^{b}	3.94 ± 0.05^{c}	4.68 ± 0.06^{d}	<0.0001
16	3.65 ± 0.04^{a}	4.32 ± 0.12^{b}	5.31 ± 0.12^{c}	5.80 ± 0.20^{c}	<0.0001

B

	Salinity (g/L)		
Age (dph)	24	34	P-value
1	2.99 ± 0.08	2.90 ± 0.07	NS
4	3.24 ± 0.02	3.12 ± 0.05	<0.01
10	3.91 ± 0.14	3.78 ± 0.14	<0.05
16	4.78 ± 0.23	4.75 ± 0.24	NS

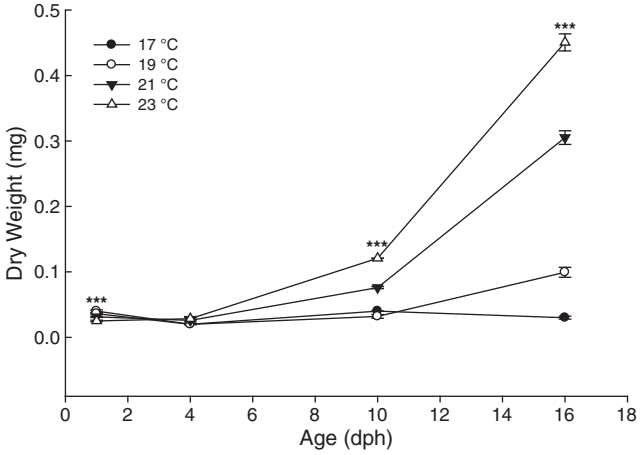

Fig. 1. Growth in dry weight (mg) (mean ± s.e.) of larval red porgy at different temperatures (17, 19, 21, and 23 °C, n = 8). Salinities were combined under each temperature. Asterisks indicate significant treatment differences and probability levels (*0.05, **0.01, ***0.001, ANOVA).

0.20 mg (Table 2A). On d4ph, wet weights remained constant for 17 °C, but decreased for all other temperatures to between 0.11 mg (21 and 23 °C) and 0.15 mg (19 °C), with a significant (P<0.0001) difference between 17 and 21–23 °C (Table 2A). There was a clear divergence in wet weight on d10ph (Table 2A). At 17 °C, fish decreased in wet weight to 0.12 mg, while at 19, 21, and 23 °C, fish grew to 0.21 mg, 0.43 mg, and 0.84 mg, respectively (Table 2A). A significant (P<0.05) interaction between temperature and salinity was observed on d10ph (Table 2A). Wet weight was markedly higher at 24 g/L than at 34 g/L at 23 °C, but this difference was progressively reduced as temperature decreased. On d16ph, fish at 21 and 23 °C continued to show an increase in wet weight (2.03 mg and 2.91 mg, respectively) that was significantly (P<0.0001) higher than 17 °C (0.20 mg) and 19 °C (0.69 mg) and between 21 and 23 °C (Table 2A).

When temperatures were combined across salinities, there were no significant effects on wet weight observed on d1 or d4ph (Table 2B). On d10ph, wet weights were significantly (P<0.001) heavier at 24 g/L (0.45 mg) than at 34 g/L (0.34 mg). On d16ph, wet weights increased to 1.58 mg in 24 g/L and 1.34 mg in 34 g/L, but with no significant difference (Table 2B). On d10ph, an interaction between temperature and salinity was observed. Wet weight increased with increasing temperatures at 24 g/L with fish at 23 °C being significantly (P<0.05) larger than at 34 g/L at the same temperature (0.97 mg versus 0.71 mg).

There were no significant interactive effects between temperature and salinity on dry weight on any sampling day throughout the study. Under both salinities, highly significant (P<0.001) temperature effects on dry weight (mg) were observed on d1, 10, and 16 ph (Fig. 1). On d1ph, dry weight ranged from 0.025 mg to 0.038 mg among treatments and was significantly lower at 23 °C (0.025 mg) than at 17 °C (0.038 mg) and 19 °C (0.036 mg) (Fig. 1). On d4ph, dry weights ranged from 0.022 mg to 0.028 mg, with no significant differences. On d10ph, dry weight at 23 °C (0.121 mg) was significantly higher than at 17 °C (0.036 mg), 19 °C (0.032 mg), and 21 °C (0.076 mg); dry weight at 21 °C was also significantly greater than 17 and 19 °C (Fig. 3) on d10ph. A clear trend toward faster growth with increasing temperature was observed on d16ph, with dry weights increasing from 0.026 mg at 17 °C to 0.451 mg at 23 °C (Fig. 1). Under all temperatures, there were no significant salinity effects on dry weight for the duration of the experiment (Table 3).

3.2. Survival

Under both salinities, 23 °C showed the highest ending survival (%). Initial survival fell abruptly to 42.2%–54.0% on d1ph and then more gradually to 35.7%–41.9 % on d4ph, but had no significant differences between temperatures on either day (Table 4). Significant (P<0.05) temperature effects on survival were first observed on d10ph, with higher survival at 23 °C (23.9%) than at 17–21 °C (12.1–14.9%) (Table 4). On d16ph, survival was significantly higher (P<0.01) at 23 °C (22.0%) than at 17–21 °C (8.11–11.2%) (Table 4). No significant

Table 2
Growth in wet weight (mg) (mean ± s.e.) of larval red porgy at different temperatures (17, 19, 21, and 23 °C, n = 8) and salinities (24 and 34 g/L, n = 16). In A, both salinities were combined under each temperature. In B, all temperatures were combined under each salinity. At each age, probability values are shown (ANOVA). Means in the same row followed by the same letter are not significantly different (Tukey's, P>0.05). NS = not significant (P>0.05). Asterisk (*) indicates interaction.

A

	Temperature (°C)				
Age (dph)	17	19	21	23	P-Value
1	0.17 ± 0.01	0.19 ± 0.01	0.18 ± 0.002	0.20 ± 0.01	NS
4	0.18 ± 0.01^{a}	0.15 ± 0.002^{ab}	0.11 ± 0.003^{b}	0.11 ± 0.01^{b}	<0.0001
10	0.12 ± 0.01^{a}	0.21 ± 0.02^{a}	0.43 ± 0.03^{b}	0.84 ± 0.07^{c}	<0.0001*
16	0.20 ± 0.01^{a}	0.69 ± 0.05^{a}	2.03 ± 0.07^{b}	2.91 ± 0.11^{c}	<0.0001

B

	Salinity (g/L)		
Age (dph)	24	34	P-value
1	0.19 ± 0.01	0.19 ± 0.002	NS
4	0.15 ± 0.01	0.13 ± 0.01	NS
10	0.45 ± 0.09	0.34 ± 0.07	<0.001
16	1.58 ± 0.33	1.34 ± 0.29	NS

Table 3
Growth in dry weight (mg) (mean ± s.e.) of larval red porgy at different salinities (24 and 34 g/L, n = 16). All temperatures were combined under each salinity. At each age, probability values are shown (ANOVA). NS = not significant (P>0.05).

	Salinity (g/L)		
Age (dph)	24	34	P-value
1	0.03 ± 0.002	0.03 ± 0.002	NS
4	0.02 ± 0.008	0.02 ± 0.002	NS
10	0.07 ± 0.01	0.07 ± 0.01	NS
16	0.23 ± 0.05	0.21 ± 0.05	NS

Table 4
Survival (%) (mean ± s.e.) of larval red porgy at different temperatures (17, 19, 21, and 23 °C, n = 8). Both salinities were combined under each temperature. At each age, probability values are shown (ANOVA). Means in the same row followed by the same letter are not significantly different (Tukey's, P>0.05). NS = not significant (P>0.05).

	Temperature (°C)				
Age (dph)	17	19	21	23	P-value
1	48.6 ± 5.01	53.2 ± 6.24	42.2 ± 0.07	54.0 ± 4.29	NS
4	35.7 ± 7.23	40.1 ± 11.24	36.5 ± 6.77	41.9 ± 7.18	NS
10	12.1 ± 1.86^{a}	15.7 ± 3.99^{ab}	14.9 ± 2.65^{ab}	23.9 ± 3.15^{b}	<0.05
16	8.99 ± 1.87^{a}	11.2 ± 3.42^{a}	8.11 ± 1.98^{a}	22.0 ± 4.31^{b}	<0.01

Table 5
Whole body osmolality (mOsm/kg) (mean ± s.e.) of larval red porgy at different temperatures (17, 19, 21, and 23 °C, n = 8). Both salinities were combined under each temperature. At each age, probability values are shown (ANOVA). Means in the same row followed by the same letter are not significantly different (Tukey's, P>0.05). NS = not significant (P>0.05).

	Temperature (°C)				
Age (dph)	17	19	21	23	P-value
2	390 ± 48	457 ± 51.6	427 ± 39.1	457 ± 40.8	NS
6	446 ± 6.56^{ab}	667 ± 95.3^{ab}	683 ± 42.8^{a}	402 ± 4.1^{b}	<0.05
11	398 ± 38.3	349 ± 8.63	385 ± 5	383 ± 11.9	NS
17	524 ± 47^{a}	408 ± 3.31^{b}	411 ± 8.31^{b}	396 ± 10.8^{b}	<0.05

interactive effects between temperature and salinity on survival were observed throughout this study.

When the effects of salinity were compared across all temperatures, highly significant effects were observed from d1ph (Fig. 2), with larvae reared at the lower salinity (24 g/L) showing higher survival than the larvae reared at 34 g/L for the duration of the experiment. On d1ph, survival at 24 g/L was 57.2% compared to 41.7% at 34 g/L. Survival remained stable at 54.7% at 24 g/L on d4ph, compared to a large decline to 21.3% at 34 g/L (Fig. 2). On d10ph, survival in 24 g/L (22.4%) continued to be more than double that of the 34 g/L treatment (10.8%). On d16ph, survival was 18.4% at 24 g/L versus 6.77% at 34 g/L (Fig. 2).

3.3. Whole body osmolality

Under both salinities, there were no significant temperature effects on whole body osmolality (WBO, mOsm/kg) observed on d2ph, with WBO ranging between 390 at 17 °C and 457 at 19 and 23 °C (Table 5). WBO was significantly (P<0.05) different on d6ph, with a marked increase at 19 and 21 °C, (to 667 mOsm/kg and 683 mOsm/kg, respectively), which was not observed in 17 or 23 °C. WBO was significantly higher at 21 °C than at 23 °C (Table 5). On d11ph, WBO was near initial levels for all temperature treatments, with no significant treatment differences. On d17ph, there was an increase at 17 °C to 524 mOsm/kg, with significant differences (P<0.05) observed compared to all other temperatures which remained around 400 mOsm/kg (Table 5). There were no significant interactive effects between temperature and salinity on WBO any sampling day throughout this study.

When salinities were compared across all temperatures, mean osmolality values were generally higher at 34 g/L (441–613 mOsm/kg) than at 24 g/L (342–486 mOsm/kg) for the duration of the study. On d2ph, WBO was significantly (P<0.05) higher at 34 g/L (522 mOsm/kg) than at 24 g/L (342 mOsm/kg) (Fig. 3). On d6ph, WBO increased in 34 g/L from 522 mOsm/kg to 613 mOsm/kg and from 342 mOsm/kg to 486 mOsm/kg in 24 g/L, but with no significant differences. WBO at 24 g/L was however, significantly (P<0.05) higher than on d2ph. On d10ph, WBO decreased under both 24 g/L (347 mOsm/kg) and 34 g/L (411 mOsm/kg), with a significant difference (P<0.01) observed (Fig. 3). At 34 g/L, WBO was significantly (P<0.05) lower than on d2ph. On d17ph WBO was not significantly higher at 34 g/L (481 mOsm/kg) than at 24 g/L (412 mOsm/kg).

3.4. mRNA expression of Na^+/K^+ ATPase

All the quantitative real-time PCR data for Na^+/K^+ ATPase mRNA in this study are based on the same standards and so are relative to each other. The arbitrary units used do not, however, represent absolute RNA concentrations. For both initial salinities, there were no significant temperature effects observed at any sampling period after the stress was initiated (P>0.05) (Table 6). At time 0 h, Na^+/K^+ ATPase mRNA ranged from 7.48 to 14.7 among temperatures. There was a decrease in expression of Na^+/K^+ ATPase mRNA after 12 h, ranging from 2.47 to 5.59 among temperatures, followed by an increase at 24 h to levels (13.4–24.5) that were not significantly different from the time zero levels. After 48 h, expression of Na^+/K^+ ATPase mRNA ranged from 18.1 to 26, again with no significant temperature effects (Table 6). There were no significant interactive effects between temperature and initial salinity on the expression of Na^+/K^+ ATPase mRNA at any sampling time after the increase of salinity on d16ph to a sublethal hyperaline challenge of 44 g/L.

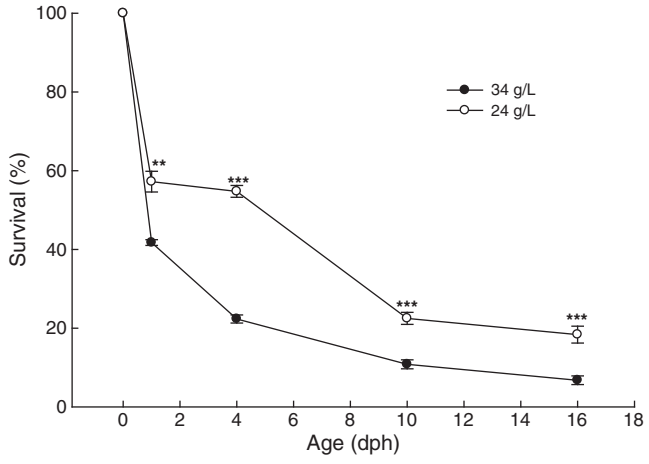

Fig. 2. Survival (%) (mean ± s.e.) of larval red porgy at different salinities (24 and 34 g/L, n = 16). Temperatures were combined under each salinity. Asterisks indicate significant treatment differences and probability levels (*0.05, **0.01, ***0.001, ANOVA).

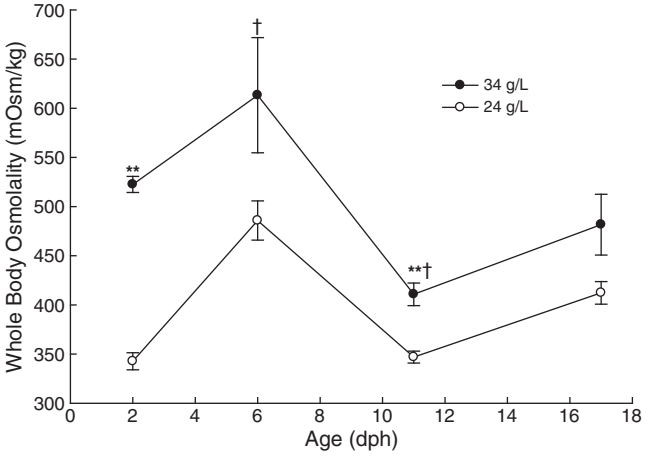

Fig. 3. Whole body osmolality (mOsm/kg) (mean ± s.e.) of larval red porgy at different salinities (24 and 34 g/L, n = 16). Temperatures were combined under each salinity. Asterisks indicate significant treatment differences and probability levels (*0.05, **0.01, ***0.001, ANOVA). † indicates a significant (P<0.05) difference from the d2ph value.

Table 6
Relative expression Na^+/K^+ ATPase mRNA (arbitrary units; mean ± s.e.) of d16ph larval red porgy after a salinity challenge to 44 g/L at different temperatures (17, 19, 21, and 23 °C, n = 8). Both salinities were combined under each temperature. At each time period, probability values are shown (ANOVA). NS = not significant (P>0.05). † indicates a significant (P<0.05) difference from the time 0 value.

	Temperature (°C)				
Time	17	19	21	23	P-value
0	10.85 ± 2.44	7.48 ± 2.03	14.69 ± 3.00	9.12 ± 2.33	NS
12	5.59 ± 1.00	4.15 ± 0.73	2.47 ± 0.38 †	3.84 ± 1.41	NS
24	24.55 ± 10.08	13.36 ± 3.95	18.11 ± 4.40	15.39 ± 2.66	NS
48	15.76 ± 5.03	12.14 ± 5.06	26.03 ± 12.22	12.84 ± 4.16	NS

The salinity treatment expressions of Na^+/K^+ ATPase mRNA were similar in the initial time periods (0 and 12 h) but diverged after 24 and 48 h. When initial salinities were compared across all temperatures, there were no significant differences at any sampling time, except for 24 h (Fig. 4). At time 0 h, expression of Na^+/K^+ ATPase mRNA for both initial salinities was just over 10. A decrease to about four was observed at 12 h initial salinities. Then, the expression of Na^+/K^+ ATPase mRNA increased at 24 h to 24.3 for larvae raised at 24 g/L and 11.5 in those raised at 34 g/L. These values are significantly different (P<0.05) (Fig. 4). A similar trend was observed at 48 h for both salinities, (10.8 and 22, respectively) but the difference was not significant (Fig. 4).

4. Discussion

Early larval growth of red porgy was markedly affected by the temperatures examined in this study. At the higher temperatures (19, 21, and 23 °C), yolk-sac larvae exhibited an initial rapid increase in wet weight and a subsequent decrease on d4ph when the yolk reserves were exhausted, a pattern typical of marine finfish larvae (Blaxter, 1969). In contrast, yolk-sac larvae at 17 °C maintained initial weight on d4ph, indicating that rate of yolk-sac absorption was negligible at this temperature. Similar results of higher temperatures causing faster exhaustion of yolk reserves were reported in newly hatched larval summer flounder (Johns and Howell, 1980; Watanabe et al., 1999), southern hake (Bustos et al., 2007) and Nassau grouper (Watanabe et al., 1995). Morphologically, red porgy in this study were ready to begin exogenous feeding (eyes formed and mouth open) by d4ph at temperatures 19, 21, and 23 °C, but few fish in this study had food in the digestive tract, most likely due to learning to capture living prey. Larval Australian snapper, which belong to the same genus as red porgy, showed comparable patterns in the utilization of yolk reserves, with an initial rapid length increase (2.8 mm to 3.2 mm SL) during yolk-sac absorption and an intermediate phase of no growth during transition from endogenous to exogenous feeding (Battaglene and Talbot, 1992; Pankhurst et al., 1991).

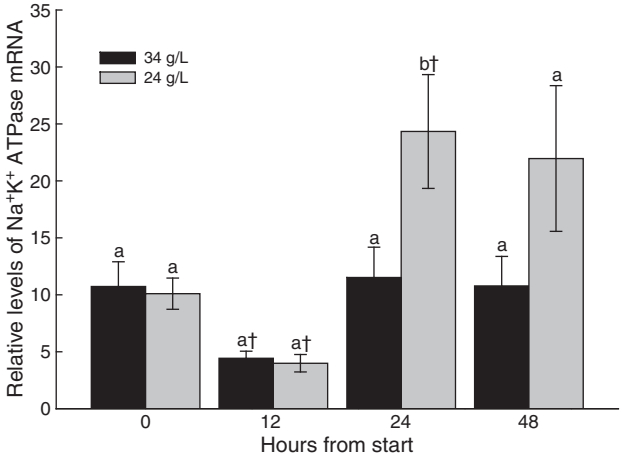

Fig. 4. Relative expression of Na^+/K^+ ATPase mRNA (arbitrary units; mean ± s.e.) of d16ph larval red porgy after a salinity challenge to 44 g/L at different salinities (24 and 34 g/L, n = 16) under zero feeding. Temperatures were combined under each salinity. Bars within a cluster with different letters are significantly (P<0.05, Tukey's test) different. † indicates a significant (P<0.05) difference from the time 0 value.

Growth of larval red porgy during the exogenous feeding period in this study exhibited a clear trend toward faster growth with increasing temperature in the range of 17–23 °C. This was likely related to accelerated metabolism and feeding at higher temperatures as described in other marine species including summer flounder (Johns and Howell, 1980; Watanabe et al., 1999), southern hake *Merluccius australis* (Bustos et al., 2007), brown sole *Pseudopleuronectes herzensteini* (Aritaki and Seikai, 2004), Nassau grouper *Epinephelus striatus* (Watanabe et al., 1995), Malabar grouper *Epinephelus malabaricus* (Yoseda et al., 2006), greenback flounder (Hart and Purser, 1995), Australian snapper (Fielder et al., 2005), and black sea bass (Berlinsky et al., 2004). These authors attributed faster growth at higher temperatures to increases in metabolism, feed conversion, nutrient absorption, and similarity to natural temperatures within the range tested.

The significant interaction between temperature and salinity on wet weight on d10ph was related to a marked advantage in growth for larvae reared at 24 g/L compared to 34 g/L at 23 °C that diminished with decreasing temperature so that no growth difference between salinities was observed at 17 °C. This was likely related to higher metabolism and feeding at the higher temperature coupled with lower osmoregulatory stress at 24 g/L, allowing more energy to be used for growth than for osmoregulation. As the temperature decreased to 17 °C, however, the metabolic differences were reduced so that differences in feeding and growth were negligible.

There are upper and lower temperature limits for growth in marine fish larvae. When compared to fish at 19 °C, which showed a clear increase in growth, red porgy larvae reared at 17 °C showed minimal growth in terms of NL (Table 1A), wet weight (Table 2A), and dry weight (Fig. 1). This suggested that 17 °C was near the lower limit for red porgy larval rearing in the laboratory. Red porgy spawn at temperatures ranging from 16 to 22 °C (Manooch, 1976), so 16 °C may be the lower temperature limit of their ecological range (Manooch, 1975). The upper rearing temperature for larval red porgy was not determined since maximum growth was obtained at the highest temperature treatment (23 °C). In brown sole larvae, growth rates increased from 6 to 15 °C, but were similar to 15 °C at 21 °C, indicating that larvae do not need to be raised above 15 °C to achieve optimal growth (Aritaki and Seikai, 2004). In this study, larvae grew fastest at 23 °C, however it is possible that they can be raised at even higher temperatures since wild larval red porgy may experience temperatures of 23–25 °C in warmer areas of their geographical range. Further studies over higher temperature ranges are needed to determine the optimum and maximum temperature for rearing red porgy larvae.

This is the first study to compare the effects of temperature on growth of larval red porgy. Other studies have reported performance of larval red porgy at specific temperatures, but many different rearing conditions (e.g., feed rate, tank size, and rearing density) were used in those studies, making comparisons difficult. Mean NL, wet and dry weights of red porgy in this study at 19 and 21 °C were lower than reported for this species in the Mediterranean at these temperatures at yolk exhaustion (d3–d5ph), early feeding larvae (d10ph), and late feeding larvae (d16ph) (Hernandez-Cruz et al., 1999; Mihelakakis et al., 2001; Papandroulakis et al., 2004), indicating genetic differences among Atlantic red porgy and Mediterranean strains of red porgy, as previously suggested by Morris et al. (2008). Large-scale genetic differences between Eastern (Mediterranean) and Western (North and South American) Atlantic strains of red porgy have been reported based on microsatellite and mitochondrial DNA markers (Ball et al., 2003; Ball et al., 2007). No studies have reported growth of red porgy at 17 °C or 23 °C.

Temperature effects on survival were similar during the yolk-sac stage (36–42%), but survival fell conspicuously (40–8%) during the early feeding stage in the lower temperature treatments (17–21 °C) while remaining significantly higher (42–22%) at 23 °C. Survival similar to that observed in this study (17 and 19 °C) was described by Conides and Glamuzina (2001) for red porgy reared at 18 °C (<20%). Lower survival at the lower temperature range in this study was possibly due to slower development of foraging capabilities (Büke et al., 2005), or slower transition to exogenous sources of food (Kolios et al., 1997).

Salinity had a greater impact on red porgy larval survival throughout this study than temperature. During the yolk-sac through the early feeding stages, survival was higher at 24 g/L than at 34 g/L (Fig. 4). This is similar to what was reported in larval gilthead seabream in which survival increased as salinity decreased from 40 to 15 g/L, allowing larvae to divert more energy into growth rather than metabolism (Tandler et al., 1995). In contrast, southern flounder could not tolerate lower salinities until post metamorphosis, with early survival higher in 34 g/L than 24 g/L (Henne and Watanabe, 2003; Moustakas et al., 2004) possibly due to full strength seawater optimizing buoyancy, vertical positioning, and feeding (Moustakas et al., 2004). On the other hand, survival was not affected in Atlantic halibut larvae (Lein et al., 1997) or Australian snapper (Fielder et al., 2005) at salinities of 20–42 g/L or 5–45 g/L, respectively. While this study has demonstrated that red porgy can be raised in 24 g/L brackish water during the early larval periods, the effects of salinity on growth and survival of juveniles are unknown and warrant further investigation.

The increase in survival at the lower salinity (614 mOsm/kg) was likely due to reduced osmoregulatory stress. In this study, larvae reared at 24 g/L had whole body osmolality values (WBO = 342–486 mOsm/kg) that were closer to iso-osmotic conditions than larvae reared at the high salinity of 34 g/L (WBO = 522–613 mOsm/kg). Assuming that WBO of marine finfish body fluids are generally within the range of 320–400 mOsm/kg (Alderdice et al., 1979; Davenport et al., 1981; Hahnenkamp et al., 1993; Riis-Vestergaard, 1982), red porgy in 34 g/L were under osmotic stress throughout the experiment.

Temperature did not have clear effects on WBO of red porgy larvae. On d6ph, WBO of larvae reared at the intermediate temperatures of 19 and 21 °C were relatively high (WBO>660 mOsm/kg) compared to larvae at 17 and 19 °C which showed WBO values closer to a normal range (WBO = 427–457 mOsm/kg). The higher WBO values at the intermediate temperatures were possibly related to the timing of sampling in relation to the osmotic adjustment period at each temperature, which was likely accelerated at higher temperature. On d6ph, larvae at 19 and 21 °C were undergoing osmoregulatory adjustment, larvae at 23 °C had completed this adjustment, while larvae at 17 °C had not yet reached this adjustment period. On d17ph, the increase in WBO at 17 °C was likewise related to the slower development of these fish and thus a slower osmoregulatory response than in the other treatments.

Lethal salinity challenges may abruptly kill larvae before they can physiologically adapt to salinity stress (Rezek et al., 2010). Following a sublethal hypersaline challenge, larval red porgy experienced a time course of adjustments in whole body levels of mRNA for the 1α subunit of Na^+/K^+ ATPase. On the assumption that salinity-related enzyme induction is at the gene level and also assuming coordinate transcription of all Na^+/K^+ ATPase subunits, this is taken as evidence for an osmoregulatory response. The initial decrease and subsequent increase of Na^+/K^+ ATPase mRNA was likely due to chloride cell death, causing a decrease in expression of Na^+/K^+ ATPase mRNA, followed by regrowth of new chloride cells, and subsequent increase in expression of Na^+/K^+ ATPase mRNA. Caberoy and Quinitio (2000) suggested that following salinity transfer from 30 to 40 g/L, Na^+/K^+ ATPase enzyme activity in d20ph orange-spotted grouper *Epinephelus coidoides,* reached a threshold and resulted in the death of Na^+/K^+ ATPase enzyme units within 12–24 h. This period was followed by an increase in fractional area of chloride cells, where Na^+/K^+ ATPase are expressed within 48 h. While Na^+/K^+ ATPase enzyme activity was not measured in this study, the present results with larval red porgy Na^+/K^+ ATPase mRNA are consistent with their findings. Carrier (2006) also found an increase in relative expression of Na^+/K^+ ATPase mRNA in black sea bass larvae fed high dietary arachidonic acid levels (>6.7% TFA) 24 h after exposure to a sublethal hypersaline challenge (43 g/L). However, Na^+/K^+ ATPase mRNA level was not measured at 12 h post transfer.

Two osmoregulatory periods have been described: an adjustment period, where changes in osmotic parameters occur and a chronic regulatory period when these levels return to basal levels (Arjona et al., 2007; Mancera et al., 2002). Within the sampling period of this study, larval red porgy in 24 g/L were in the adjustment period during the time after salinity transfer because the relative expression of Na^+/K^+ ATPase never returned to initial (0 h) levels. In contrast, Na^+/K^+ ATPase mRNA levels in fish in the 34 g/L returned to initial (0 h) levels by 24 h post transfer (i.e., regulatory period). These differences in relative expression of Na^+/K^+ ATPase mRNA levels after transfer to 44 g/L are likely due to the relatively large salinity increase for fish transferred from 24 g/L (Δ salinity = 20 g/L) compared to those transferred from 34 g/L (Δ salinity = 10 g/L).

Temperature did not affect the synthesis of the osmoregulatory machinery (i.e., expression Na^+/K^+ ATPase mRNA) in larval red porgy at any time following a sublethal salinity challenge, suggesting that temperature had a larger effect on larval metabolism, feeding, and growth than on osmoregulation. Although no studies have examined temperature effects on expression of Na^+/K^+ ATPase mRNA in larval fish, temperature has been shown to affect osmoregulatory ability in juvenile marine finfish. For example, a cold-tolerant strain of northern killifish *Fundulus heteroclitus* showed more rapid expression of alpha subunit Na^+/K^+ ATPase mRNA and Na^+/K^+ ATPase enzyme activity following abrupt transfer to seawater than a warm-tolerant southern strain (Scott and Schulte, 2005). Within the range of 10–22 °C, the highest gill enzyme (Na^+/K^+ ATPase) activity in juvenile turbot was at 22 °C, and there was a positive correlation between temperature and enzyme activity (Imsland et al., 2003). On the other hand, Na^+/K^+ ATPase activity was unaffected by rearing temperatures between 20 and 35 °C in juvenile tilapia *Oreochromis mossambicus* (Fiess et al., 2007).

5. Conclusions

Within the range of 17–23 °C and at salinities of 24 and 34 g/L, larval red porgy growth to d16ph improved with increasing temperature. Survival was affected by temperature and salinity with the greatest survival to d16ph at the highest temperature of 23 °C in both salinities and at the lower salinity of 24 g/L under all temperatures. Larval whole body osmolality values indicated less osmotic stress throughout the experiment in 24 g/L than at 34 g/L. Following a sublethal (44 g/L) hypersaline challenge, expression of Na^+/K^+ ATPase mRNA decreased at 12 h and then increased at 24 h to a value that was higher for larvae previously reared at 24 g/L than at 34 g/L. This suggested that greater chloride cell activity and elaboration of the Na^+/K^+ transport pump at 24 g/L, probably because of the greater salinity challenge. Based on maximum growth, survival, and osmoregulatory abilities in this study, 23 °C and 24 g/L are recommended for larval red porgy culture, but additional studies to evaluate performance at higher temperatures are warranted.

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