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# The effect of dissolved carbon dioxide ($CO_2$) on white grouper (*Epinephelus aeneus*) performance, swimbladder inflation and skeletal deformities

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**Abstract**

The effect of three seawater $CO_2$ concentrations (0.8±0.18, 5.64±0.64 and 28.58±3 mg $l^{-1}$), was tested on white grouper (*Epinephelus aeneus*) post-larvae reared at two water temperatures (23 and 27 $^{o}$C) in a 30 aquarium (17 l each) experimental system. Forty-five dph fish were stocked per aquarium allowing the testing of the 6 $CO_2$ temperature treatment combinations in replicates of 5 aquaria treatment$^{-1}$ for 60 days. The final weight of fish reared under the high $CO_2$ treatment was 25% and 10% ($P<0.05$) lower than that of fish reared under the control conditions at 23° and 27 °C, respectively. A similar pattern in total length (TL) was demonstrated where fish exposed to the high $CO_2$ treatment grew 10 and 7.4% more slowly ($P<0.05$) than the control at 23° and 27 °C, respectively. A large percentage (78.8±6.1%) of fish reared at the control $CO_2$ concentration (0.8±0.18 mg $l^{-1}$) and exposed to 27 °C had inflated swimbladders compared to only 17.6±9.2% in fish exposed to the high $CO_2$ concentration (28.58±3 mg $l^{-1}$) at 23 °C. Following the 60 days of the $CO_2$ and temperature exposure period (45-105 dph), 87-100% of the fish from the six treatment combinations, independent of temperature and $CO_2$ exposure history, had inflated swimbladders at 150 dph. It was shown that, during the recovery phase (125 dph) at 23 °C, the ratio between the swimbladder volume and body weight ($mm^3$ $g^{-1}$) in fish exposed to the high $CO_2$ concentration was 4.65±0.79 $mm^3g^{-1}$, which was significantly smaller ($P<0.05$) than 10.12±0.72 $mm^3g^{-1}$in the control $CO_2$ treatment. In general, the swimbladder volume was directly correlated with the rearing temperature and was up to 50% larger in fish reared at 27 °C compared to those from 23 °C independent of $CO_2$ concentration. The relative presence of skeletal deformities in fish with non-inflated swimbladders was 6.49%, which was significantly ($P<0.05$) higher than the 0.93% in fish with a normally inflated swimbladder. In addition, fish with an uninflated swimbladder demonstrated a significantly ($P<0.05$) more severe lordosis deformity with a vertebra angle of 47.5±15° compared to cohorts with an inflated swimbladder that exhibited an angle of 27±6°. In conclusion, there was a clear effect of high aqueous levels of $CO_2$ and environmental temperature on growth and skeletal deformity which, in turn, affected juvenile quality in the white grouper.

## 1.0 Introduction

The white grouper, *Epinephelus aeneus* (Serranidae), is a highly prized fish in Israel and around the Mediterranean basin due to its excellent taste and rapid growth rate (Hassin et al., 1997; Glamuzina et al., 2000; Lupatsch and Kissil, 2005), and is a promising candidate for intensive aquaculture. Groupers are usually farmed in floating net-cages or ponds. However, due to the intensification of the aquaculture industry in recent decades, significant efforts have been made towards commercial production of grouper in recirculating aquaculture systems (RAS). Attempts to domesticate the grouper began about four decades ago (Ukawa et al., 1966) but were severely hindered by major bottlenecks in larval survival and grow out to market weight. Toledo (2000) reported a rapid decrease in survival of groupers from 29.8% at 35 days post-hatching (dph) to 3% at 55 dph. A lack of knowledge of the physiology and nutritional requirements during development, particularly at the onset of exogenous feeding, were major stumbling blocks to successful larval rearing and grow-out in *E. aeneus* (Koven et al., 2016) as well as other grouper species (Toledo, 2000). A high incidence of skeletal malformations is a major factor which reduces grouper juvenile quality, leading to increased production costs to manually remove deformed fish or reduced profits from selling a misshapen fish at lower market values (Koumoundourous et al., 1997). Skeletal deformities can affect fish performance in terms of swimming ability, food conversion efficiency, growth rate, survival, as well as their susceptibility to stress and pathogens (Andrades et al., 1996; Koumoundourous et al., 1997; Boglione et al., 2001). In sea bream (*Sparus aurata)*, the onset of deformity was observed early on in larval development, with as much as 27% of the population being potentially affected, leading to severely reduced survival (5%) in the juvenile and adult stages (Andrades et al., 1996).

The effect of environmental temperature during different developmental stages is a major factor influencing skeletogenesis and induced deformity. During the first few weeks of growth in golden pompano (*Trachinotus ovatus*), the highest proportion of fish larvae exhibiting jaw deformities occurred at 33 $^{o}$C compared to 26 $^{o}$C (Ma et al., 2016). Georgakopoulou et al. (2010) found in gilthead sea bream (*Sparus aurata*) a significant effect of water temperature on skeletal deformities of the gill cover, caudal and dorsal fin as well as the vertebral column, which varied according to the ontogeny at which these anatomical features developed. These authors reported that post-metamorphic fish exposed to 22 $^{o}$C gave the lowest and less variable incidence of haemal lordosis, while Sfakianakis et al. (2006) concluded that 20 $^{o}$C, compared to 15 $^{o}$C, increased haemal lordosis only during the embryonic and larval stages of the gilthead sea bream (*Sparus aurata*).

Intensification of the production of *E. aeneus* in recent years has been based on elevated rearing densities at the juvenile stage, which are sustained by increased supplementation of pure oxygen. However, concurrent with maintaining a high oxygen concentration, is a significant increase in hypercarbic conditions, which can reach concentrations of up to 40 mg $CO_2$ $l^{-1}$ in the rearing ponds. This is orders of magnitude greater than natural sea surface $CO_2$ concentrations.

Dissolved inorganic carbon (DIC) exists in seawater in three main forms; bicarbonate ion ($HCO^-_3$), carbonate ion ($CO_3^{-2}$), and carbon dioxide ($CO_2$), which also includes carbonic acid ($H_2CO_3$) (Fabry et al., 2008). $CO_2$ concentration in the rearing water can be controlled via the rate of water exchange and is also dependent on fish metabolic rate and biomass (Fivelstad et al., 2004). At high environmental concentrations $CO_2$ can potentially diffuse through the gill epithelium into the body according to the $CO_2$ concentration gradient (Vandenberg et al., 1994). Following diffusion into the blood stream, $CO_2$ hydrates to form carbonic acid ($H_2CO_3$) which further dissociates into $H^+$ and $HCO^-_3$. Therefore, when fish are exposed to high $CO_2$ concentrations for long periods of time, blood $CO_2$ concentration will increase (hypercapania) with the resulting decrease in blood pH and respiratory acidosis (Eddy et al., 1977; Ultsch, 1996). Chronic respiratory acidosis in mice resulted in a compensatory release of skeletal calcium (Ca) and phosphorus (P) to maintain the homeostatic blood pH (Meghji et al., 2001). It is conceivable that these compensatory mechanisms of bone demineralization also exist in fish exposed to hypercarbic conditions, which might lead to skeletal deformities. Furthermore, this situation might be exasperated further in fish that fail to inflate their swimbladder. The association between the lack of swimbladder inflation and skeletal deformities in sea bream (*Sparus aurata*) has been reported (Chatain, 1994; Tandler and Koven, 2009) as well as in Japanese sea bass (*Lateolabrax japanicus*; Kitajima et al., 1994). However, the physiological mechanism underlying this correlation has not been thoroughly addressed.

White grouper display a physioclistous swimbladder. This means, that the swimbladder inflation is based on $O_2$, carried in the blood circulation, where it is transferred from the hemoglobin (Hgb) molecule into the swimbladder space, whose volume is regulated via a counter current exchange mechanism called the rete mirabile (Umezawa et al., 2012). A high blood $CO_2$ concentration at the respiratory surfaces of the gills causes a markedly reduced capacity of fish Hgb to bind $O_2$ (Ito et al., 1995; Mylvaganam et al., 1996) due to Root and Bohr effects. The Bohr effect, refers to the effect of high blood $CO_2$ which lowers the affinity of Hgb for oxygen as to shift the dissociation curve to the right. In active tissues which generate large amount of $CO_2$, with a consequent lowering of pH, the Bohr effect facilitates

the release of $O_2$ from Hgb and assures a steady and sufficient supply of $O_2$ to the metabolically active tissues. While, Root effect refers to the effect of $CO_2$ on the maximum oxygen carrying capacity of the Hgb (Fromm, 1980). This decreased affinity of Hgb to oxygen results in a reduced capacity to normally inflate the swimbladder, potentially leading to skeletal deformities.

Therefore, the aim of the present study was to study the effect of dissolved $CO_2$ concentration at two temperatures on growth performance, swimbladder inflation, and skeletal deformities in the white grouper (*Epinephelus aeneus*).

**2.0 Material and methods**

2.1 Fish rearing

In the present study we used the larval rearing protocol of the National Center for Mariculture, (NCM, IOLR, Eilat, Israel) as follows: White grouper (*Epinephelus aeneus*) eggs were obtained from natural spawning of brood stock maintained at the NCM. Fertilized eggs were stocked (100 eggs $l^{-1}$) in 6,000 l cylindrical rearing tanks. These tanks were supplied continuously with filtered (8 µm), UV treated, ambient seawater (40‰) at 27 °C, which was well aerated (>95% saturation). Seawater in the tanks remained stagnant from 1-5 dph, followed by a daily 20% exchange rate until 15 dph, which was then increased to 100% $day^{-1}$ until 30 dph. When larvae were 2 dph, unicellular microalgae (*Nanochloropsis oculata*) were added ($0.5x10^6$ cells/ml) to the rearing tanks twice daily prior to the feeding of 2-30 dph larvae with enriched rotifers, *Brachionus rotundiformis* (10 $ml^{-1}$) two times per day. From 15 to 30 dph, the larvae were also fed *Artemia* nauplii. All live food organisms were prior enriched with a 5% DHA enrichment preparation (Red Pepper, BernaAqua, Belgium) for 18-24 h. At 30 dph, 1200 grouper larvae were randomly distributed into a 30 aquaria (17 l each) system at a density of 40 larvae per aquarium. During the following two weeks, the larvae were gradually acclimated to the target $CO_2$ concentrations and temperatures.

2.2 Experimental conditions

The experiment was conducted at a salinity of 25‰, simultaneously at two environmental temperatures (23 and 27 °C) and three free $CO_2$ concentrations (0.8±0.2, 5.64±0.6 and 28.58±3 mg $l^{-1}$) allowing the testing of the 6 temperature-$CO_2$ treatments in 5 replicate aquaria each. The rationale behind those temperatures and $CO_2$ concentrations was to cover the range of the rearing conditions which are common in commercial production of this species. 0.5 mg $l^{-1}$ is common in seawater $CO_2$ concentrations, while in our study the natural seawater $CO_2$ concentration (control treatment) was 0.8±0.2 mg $l^{-1}$. The concentration of 5 mg

$l^{-1}$ $CO_2$ is normally found in semi-intensive farming, while 30 mg $l^{-1}$ $CO_2$ is frequently measured in Recirculating Aquaculture Systems (RAS).

In two separate tanks (200 l), the controlled addition of HCL (32%), through a dosing pump, provided two specific pH levels (7.1 and 6.2), that shifted the water carbonate system equilibrium, resulting in two $CO_2$ levels (5.64±0.6 and 28.58±3 mg $l^{-1}$, respectively). Seawater containing these $CO_2$ levels then flowed into their respective test aquaria at 23 or 27 °C. Ambient seawater with a pH of 7.9 and a $CO_2$ concentration of 0.8±0.2 mg $l^{-1}$ flowed separately into the control aquaria. The water exchange rate in the entire aquaria system was 17 l $aquarium^{-1}$ $h^{-1}$, equal to one exchange $h^{-1}$. All aquaria in the system were exposed to a photoperiod regime of 12 h light/12 h dark throughout the 60 day (45-105 dph) experimental period. During the trial, fish were fed 3 meals a day to satiation with a commercial diet (BioMar, www.biomar.com) of 0.8-3 mm pellets, according to fish age. At the age of 105 dph, the groupers juveniles from each aquarium were transferred to 65 l plastic perforated containers, which were placed into a 6,000 l conical cylindrical tank in a flow through system with ambient 40 ‰ seawater for another 45 days (105-150 dph). This was done in order to determine any long term effect from the earlier $CO_2$ and temperature treatments.

2.3 Water quality analysis

According to Ben Asher et al. (2013) dissolved $CO_2$ concentrations can be determined from calculations based on measured alkalinity, salinity and pH values. In the present study, $CO_2$ concentrations were measured daily at 8:00 before the first feeding by sampling of 1l from each aquarium and calculated from alkalinity, salinity, temperature and pH measurements using the computer program PHREEQC and applying the Pitzer approach to account for ion interactions (Parkhurst and Appelo, 2013). Water temperature and salinity were automatically controlled and recorded daily (Gavish control systems, www.gavish.org.il). The average temperatures during the experiment were close to their target values of 23 and 27 $^{o}$C (23.3±0.7 °C and 26.7±0.6 °C, respectively). Oxygen was measured twice daily using an Oxygen meter (OxyGaurd®, USA) and kept above 85% saturation at the outlet of the aquaria (Table 1). The pH was controlled automatically with a pH controller (GonDO 4801, Taiwan), and verified twice a day in the aquaria with a manual pH meter (IQ 140 scientific instruments, USA). Alkalinity was manually measured daily by the Gran titration method (Table 1; Gran, 1952). Water for TAN determination was performed daily and measured by an auto-analyzer ($SAN^{++}$ continuous flow analyzer, Skalar, the Netherlands; Table 1).

2.4 Growth determination

Body weight (0.4±0.12g) and total length (2.2±0.2 cm) were individually determined at the start of the experiment (45 dph) and after 60 days at the end of the $CO_2$ period (105 dph; **Fig.1; table 2**).

2.5 Determination of deformities

Since the skeleton of young groupers was still cartilaginous and had not yet mineralized into bone, X-raying for deformities at this stage was not possible. Therefore, before the onset of the experimental period, when the fish were transferred to the aquaria system at 30 dph, 90 larvae (15 per treatment) were euthanized (20% clove oil in ethanol, 0.5 ml $l^{-1}$), fixed in buffered formalin (BNF 10%) and stained for cartilage with Alcian blue (**Fig. 5**; Pottoff, 1984). At, 125 and 150 dph, 90 fish were X-rayed (Media 65 CT-H, Philips, the Netherland) at the Eilat "Yoseftal" hospital and the analysis for skeletal deformity was carried out using a medical image viewer software (DiagNet R2.3, Philips, the Netherland). The severity of lordosis was measured according to Chatain (1994) based on the angle formed between the interpolated line from the first vertebra through the curvature point and a second interpolated line from the curvature point through the last vertebra (**Fig. 8**).

2.6 Swimbladder development

A non-lethal approach to measure swimbladder inflation was employed at 45 and 105 dph by the "floatation test" based on Chatain and Corrao (1992). Briefly, fish were lightly anaesthetized and placed in an 80 ‰ hypersaline solution prepared by dissolving 80 g $l^{-1}$ of NaCl into fresh water. Under these conditions the number of groupers with inflated swimbladders, which floated or were neutrally buoyant, were counted. Groupers with uninflated swimbladders sank to the bottom of the container. At 125 and 150 dph, 15 fish per treatment were sampled euthanized and X–rayed in order to determine the presence of inflated swimbladders and to calculate their volumes. This calculation was based on the assumption that the swimbladder approximates a prolate sphere whose volume can be calculated by the formula; $Vs=\pi/6LH^2$, where "L" is swimbladder length, "H" is the height of the swimbladder and $\pi$ is equal to 3.14 (Trotter et al., 2001).

2.7 Statistical analysis

Statistical analysis of the results was conducted with SPSS (ver. 20; SPSS Inc.). Growth parameters were analyzed using one way ANOVA and Barlett's test for equal variances followed by Newman-Keuls test, as a multiple range test. The relative presence (%) of swimbladders was analyzed using the logistic regression procedure. The Chi-square test was used to analyze the relationship between lack of swimbladder and skeletal deformities. It

should be noted that *n* in tables and figures corresponds to the number of sampled fish per treatment.

**3.0 Results**

3.1 Water quality

Water quality parameters were measured daily in the culture water and were found to be stable during the experimental period (**Table 1**). The pH measurements reflected the $CO_2$ levels of 0.8±0.2, 5.64±0.6, 28.58±3 mg $l^{-1}$. In the control group, pH remained stable at 7.93±0.1, while in the medium and high $CO_2$ treatments the pH decreased to 7.1±0.1 and 6.2±0.1, respectively. The alkalinity also decreased from 1.78±0.15 meq $l^{-1}$ in the control group to 1.49±0.12 meq $l^{-1}$ and 0.98±0.18 mg $l^{-1}$ in the medium and high groups, respectively.

3.2 Growth

$CO_2$ concentrations significantly affected ($P<0.05$) the groupers' growth rate compared to the control (**Fig. 2; table 2**). The average weight of 105 dph fish reared at 23 °C and exposed to the high $CO_2$ concentration (28.58±3 mg $l^{-1}$), was 11.24±0.9 g, which was significantly lower ($P<0.05$) than the moderate (5.64±0.6 mg $l^{-1}$) and the control (0.8±0.2 mg $l^{-1}$) $CO_2$ levels (14.5±0.16 g and 14.91±0.4 g, respectively) . Similarly, the high $CO_2$ exposed fish at 27 °C weighed significantly less (16.42±0.2 g; $P<0.05$) than the moderate and control $CO_2$ concentrations (17.26±0.2 and 18.15±0.7 g, respectively). The total length (TL) reflected similar patterns where fish at 23 °C and exposed to high $CO_2$ concentration exhibited a TL of 10.25±0.2 cm, which was 10% shorter ($P<0.05$) than the 11.38±0.1 cm of the control group. This pattern continued at 27 °C, where fish exposed to the high $CO_2$ concentration exhibited a TL of 11.83±0.2 which was significantly shorter ($P<0.05$) than the 12.78±0.2 cm in the control group.

3.3 Swimbladder inflation

$CO_2$ concentration within the range of 5.64±0.6-28.58±3 mg $l^{-1}$ had a significant effect ($P<0.05$) on the proportion of 105 dph groupers with inflated swimbladders reared at 23 or 27 °C (**Fig. 3; table 3**). A large percentage (78.8±6.1%) of fish reared at the control $CO_2$ concentration (0.8±0.2 mg $l^{-1}$) and exposed to 27 °C had inflated swimbladders compared to only 17.6±9.2% in fish exposed to the high $CO_2$ concentration (28.58±3 mg $l^{-1}$) at 23 °C. Following the 60 days of the $CO_2$ and temperature exposure period (45-105 dph), all the fish from all six treatment combinations were transferred to net-cages for another 45 days in ambient seawater and exhibited at the end of this period, independently of the environmental temperature and $CO_2$ exposure history, 87-100% swimbladder inflation rates in the remaining 150 dph fish (**Fig. 3 a, b; table 3**).

In addition to affecting the proportion of fish having inflated swimbladders, $CO_2$ was associated with a 30-50% reduction in the swimbladder volume (**Fig. 4; table 4**). During the recovery phase (125 dph) at 23 °C, the ratio between swimbladder volume and body weight ($mm^3$ $g^{-1}$) in fish exposed to the high $CO_2$ concentration was 4.65±0.79 $mm^3g^{-1}$, which was significantly smaller ($P<0.05$) than 10.12±0.72 $mm^3g^{-1}$in the control $CO_2$ treatment. Generally, the swimbladder volume was directly correlated with the rearing temperature and was approximately 50% larger in fish reared at 27 °C as compared to groupers reared at 23 °C at any $CO_2$ concentration (**Fig. 4; table 4**).

3.4 Skeletal deformities

Groupers were checked for skeletal deformities before the onset of the experiment, when the fish transferred to the aquaria system at 30 dph, based on their staining with Alcian blue. It was confirmed that all the fish at that point had a normal vertebral column (**Fig. 5**). During the $CO_2$ exposure period (105 dph) skeletal deformities could be easily identified externally, while minor deformities could be analyzed through x-ray examination (125 dph; **Fig. 6**). Malformations observed in the present study were lordosis, kyphosis and caudal deformity. Lordosis is an abnormal ventral curvature that always appeared in the mid-posterior region of the vertebral column in fish with non-inflated or incompletely inflated swimbladders (**Fig. 6b**). Kyphosis is an abnormal dorsal curvature that appeared in the mid region of the vertebral column in fish with an asymmetrical swimbladder (**Fig. 6d**). Caudal deformity appeared at vertebra no. 3-4 from the tail, and mostly in fish with lordosis or kyphosis. The relative presence of skeletal deformities in fish with non-inflated swimbladders was 6.49%, which was significantly ($P<0.05$) higher than the 0.93% in fish with a normally inflated swimbladder (**Fig. 7a**). In addition, fish with a non-inflated swimbladder demonstrated a significantly ($P<0.05$) more severe lordosis deformity with a vertebra angle of 47.5±15° as compared to fish from the same cohort with an inflated swimbladder, exhibiting an angle of 27±6° **(Fig. 7b)**

**4.0 Discussion**

4.1 Water parameters

The equilibrium between carbonate, bicarbonate and carbon dioxide in seawater is determined by water pH. It is dominated by free $CO_2$ at lower pH values then 6 and by carbonate at higher pH values (Sanni and Forsberg, 1996). Höglund (1961) and Fromm (1980) reported that the main directive factor in the combined pH/$Pco_2$ gradients is $CO_2$, particularly within the pH range of 7.4 to about 5.5. Since the range of pH values in the present study were within this range we considered the changes in dissolved $CO_2$ as the chief factor that affected

the groupers in the present study. The change in water quality parameters (**Table 1**), in terms of dissolved $CO_2$ concentration and temperature, were altered gradually over the first 14 days prior to the experiment, to allow fish to acclimate to the target dissolved $CO_2$ and temperature values.

4.2 Growth

To the best of our knowledge, this is the first study to investigate the problem of $CO_2$ in the rearing of white grouper under intensive and super intensive conditions. Exposure of post-larval groupers (45 dph) to the $CO_2$ concentrations of 5.64±0.6-28.58±3 mg $l^{-1}$, which typify intensive and super intensive recirculating aquaculture systems (RAS) (Colt, 2006; Stiller et al., 2015), demonstrated a clear impact on juvenile fish growth in terms of final weight and total length. The negative effect of a higher concentration of $CO_2$ on fish weight gain was observed in juvenile sea bream (*Sparus aurata*), whose growth decreased by 15% when $CO_2$ was higher than 20.5 mg $l^{-1}$ (Ben Asher et al., 2013). Similarly, exposing Atlantic salmon (*Salmo salar*) to 10 mg $CO_2$ $l^{-1}$ significantly lowered their growth by 10.3% compared to a $CO_2$ free condition (Martens et al., 2006). The mechanism by which chronic dissolved $CO_2$ exposure reduces growth rates is not well understood. However, a review by Ishimatsu et al. (2008) reported that the metabolic rate of fish at high dissolved $CO_2$ environment would likely be markedly higher due to elevated energetic costs of acid-base regulation, increased swimming speeds and higher ventilation for gill irrigation. Fish use their swimbladder as a hydrostatic organ to control their position at a chosen water depth, which minimizes energy expenditure (Harden, 1952; Blake, 1979; Harden and Scholes, 1985; Arnold and Greer, 1992; Pelster, 2004). Fish at 105 dph from the high dissolved $CO_2$ treatment, which was the end of the exposure to the $CO_2$ and temperature treatments, had a lower proportion of inflated swimbladders together with a 10% lower body weight compared to those from the control treatment. This suggests a positive correlation between the success of swimbladder inflation and growth rate in white grouper juveniles. In support of this, Chatain (1989) reported that gilthead sea bream larvae with uninflated swimbladders grew slower and weighed 23-33% less than larvae with inflated swimbladders. The average growth rate of yellow perch (*Perca flavescens*) larvae with inflated swimbladders was 0.50 mm $d^{-1}$ while larvae without a functional swimbladder grew at rate of 0.32 mm $d^{-1}$ during the initial 2 weeks after hatching (Czesny et al., 2005). Marty et al. (1995) found that Japanese medaka (*Oryzias latipes*) larvae with uninflated swimbladders had a 36% - 90% greater oxygen consumption rate than those with inflated swimbladders. Overall, this suggests that a functional swimbladder minimizes the metabolic rate associated with vertical positioning and for station holding, leading to improved fish growth. This trend agrees with the Strand et al. (2005) model, which simulates

the energy cost of buoyancy for 1-3 kg cod (*Gadus morhua*) that migrate daily from the surface to a depth of 100 m. Their model predicts that buoyancy compensation through swimming can increase energy costs by over 300%, suggesting why fish lacking a functional swimbladder would have a lower food conversion efficiency and reduced growth rate.

4.3 Swimbladder development

The present study demonstrated that an increase in $CO_2$ to 5.64±0.6 mg $l^{-1}$ or higher during the juvenile stage (45-105 dph) had a negative effect on the fish's ability to inflate their swimbladder (**Fig. 3; table 3**). In fact, there was a negative association between $CO_2$ increase and swimbladder volume decrease (**Fig. 4; table 4**). This is probably associated with the kinetics of loading and unloading of respiratory gases in the red blood cells (RBC). These kinetics are dependent on the provision of protons based on the dissociation of carbonic and lactic acids. The latter play a central role in controlling the affinity of fish hemoglobin to oxygen and its transfer into the swimbladder via the rete mirabile (Umezawa et al., 2012).

Fish farming in net-cages, which have stocking densities of 10-34 kg /$m^3$ (Turnbull et al., 2005), have very low $CO_2$ concentrations. In contrast, intensive land-based aquaculture systems stock fish at densities as high as 100 kg/$m^3$ (Blancheton, 2000) and, unless expelled, $CO_2$ may accumulate in the fish rearing water. Under such conditions, the equilibrium between the three carbonate forms ($CO_2$, $HCO_3^-$, $CO_3^{-2}$) would tend to produce an abundance of carbonic acid and protons resulting in increased $CO_2$ concentration. The higher concentration and reduced polarity of $CO_2$ would favor enhanced penetrability and diffusion of this gas into the blood via the gills, and interfere in the Hgb-$O_2$ affinity. This would reduce the capacity of the RBC to transfer oxygen to the swimbladder as well as to body tissues (Moran and Støttrup, 2011). Although, that the blood pH was not measured in the present study, our findings about low inflation rate of swimbladder in the fish from the high dissolved $CO_2$ treatment could be explained based on Pelster and Scheid (1992) who showed that gas deposition into the swimbladder of European eel (*Anguilla anguilla*) was correlated with the pH difference between dorsal arterial and venous blood, leaving the swimbladder tissue. Consequently, the high gas deposition rate coincided with a pH difference of 0.2 units or more, while a difference of 0.1 units was correlated with a low gas deposition rate. Further research is required and should focus in the biochemical mechanism of the swimbladder inflation in groupers

The rearing temperature also was found to affect swimbladder inflation in groupers. Fish reared at 27 °C had a significantly ($p<0.05$) higher proportion of inflated swimbladders then fish reared at 23 °C (**Fig. 3a, b; table 3**). A delay in the development of the swimbladder in

fish at 23 °C could be related to the effect of temperature on the activity of relevant enzymes that have a central role in the amplification of the blood $O_2$ concentration in the gas gland cells (Hochachka and Lewis, 1971). Mcnabb and Mecham (1971) reported that in bluegill sunfish (*Lepomis macrochirus*) the rate of gas secretion into the swimbladder increased as the temperature rose, with significantly lower rates of gas secretion into the swimbladder at 12 °C compared to 32 °C.

4.4 Skeletal deformities

We found a strong correlation between the rate of swimbladder dysfunction and an increase in skeletal malformations. The percentage of deformed fish lacking a swimbladder was 6.98 times higher than the deformed fish with normal swimbladders (**Fig. 7a**). In the present study, fish were sampled and skeletal malformations were not found at the onset of the experiment (**Fig. 5**). However, lordosis, kyphosis and caudal deformity were observed at the end of $CO_2$ exposure period (**Fig. 6**). Lordosis, which occupies the mid-posterior region of the vertebral column, was more associated with uninflated swimbladders. Chatain (1994) reported that sea bream (*Sparus aurata*) and sea bass (*Dicentrarchu. labrax*) larvae having functional swimbladders never developed lordosis while fish with uninflated swimbladders exhibited some degree of this deformity. On the other hand, Divanach et al., (1997) found that skeletal malformations occurred in juvenile sea bass (*Dicentrarchus labrax*) having a functional swimbladder that were exposed to a high current velocity (> 10 cm $s^{-2}$). In support of this, Backiel et al. (1984) demonstrated lordosis in the common carp (*Cyprinus carpio*) post-larvae that was induced by rearing in a strong water current of 12 cm $s^{-1}$. These carp larvae developed muscle hypertrophy and differentiated muscular fibers in the lateral muscle that may have produced an asymmetrical tension on the bone. Chatain, (1994) similarly argued that the mechanical forces, from tail beats while swimming, on the musculature and spinal column were responsible for lordotic European sea bass (*Dicentrarchus labrax*), which would be more severe in fish lacking an inflated swimbladder. This was confirmed in the present study when the angle of deformity, an indicator of severity of lordosis, was markedly higher in fish lacking a swimbladder (**Fig. 7b**). Although anecdotal, grouper juveniles were observed to display active caudal fin movement in order to maintain their position in the water column, which may have exacerbated their skeletal deformity.

Kyphosis was found in the mid region and associated with smaller swimbladders or with irregularities in the inflation of the swimbladder (**Fig.6d**). Similarly, Boglione et al. (2009) reported in grouper (*Epinephelus marginatus*) and Grotmol et al. (2005) in cod (*Gadus morahua*) that fish exhibiting an unusually large swimbladder frequently had an abnormal

dorsal curvature of the notochord in the region just behind the cranium. Grotmol et al. (2005) argued that the increased pressure on the notochord might also result in the expansion of abdominal organs although this was not observed in the present study. Caudal deformities, were characterized by dorsal or ventral curvatures, which were associated with uninflated or under inflated swimbladders, producing lordotic or kyphotic fish (**Fig. 6a, b&d**). Little is known about the exact factors involved in vertebral deformities in this species. The fact that fish with an uninflated swimbladder presented higher incidence of vertebral deformities than those with a normal swimbladder, suggests that the mechanical pressure on the vertebral column, during swimming activity, may be involved.

In conclusion, our study on the white grouper (*Epinephelus aeneus*) clearly demonstrated that there was a clear effect of high dissolved $CO_2$ and environmental temperature on growth and skeletal deformity which, in turn, may affect juvenile quality. This may have resulted from reduced or absent swimbladder inflation possibly coupled with increased swimming activity that led to undue mechanical forces on the vertebral column. Furthermore, based on our study, in order to achieve normal skeletal development, dissolved $CO_2$ concentrations in marine rearing systems should be maintained below the medium concentration of $5.64 \pm 0.64$ mg $l^{-1}$.

**5.0 References**

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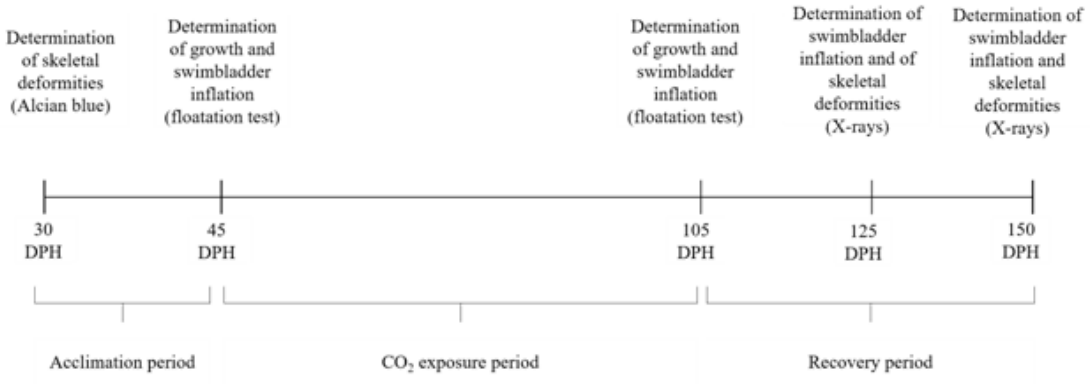
Figure 1. Fish sampling scheme during the experiment.



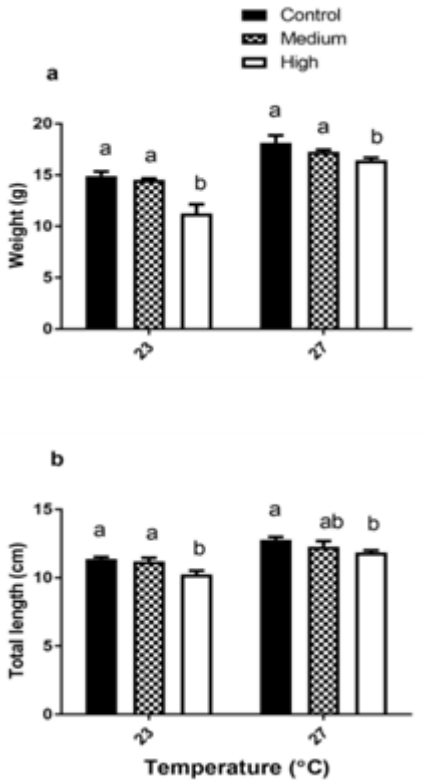



Figure 2. The effect of dissolved $CO_2$ concentration on 105 dph white grouper (*Epinephelus aeneus*) (a) weight and (b) length at 23 and 27 °C. Bars having different letters were significantly different ($P<0.05$, $n=15$, per treatment).

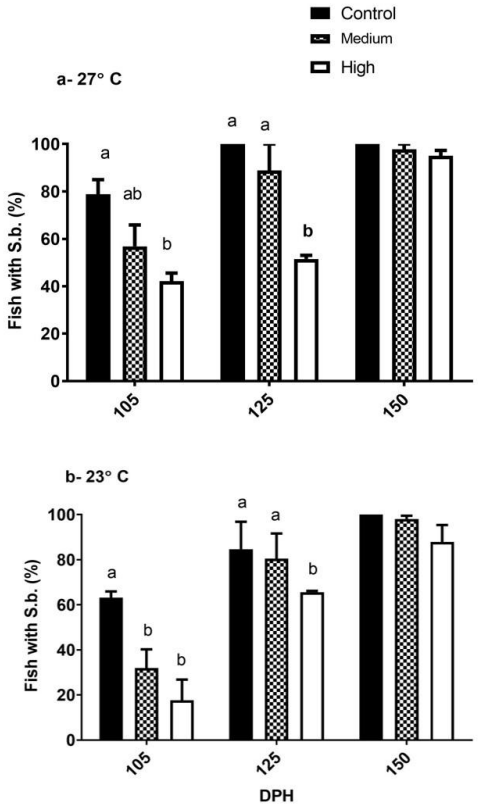



Figure 3. The effect of dissolved $CO_2$ concentration on swimbladder (s.b.) inflation in 105, 125 and 150 dph white grouper (*Epinephelus aeneus*) at (a) 27 $^{o}$C and (b) 23 $^{o}$C. Bars having different letters were significantly different (P<0.05, *n*=15 per tretment).

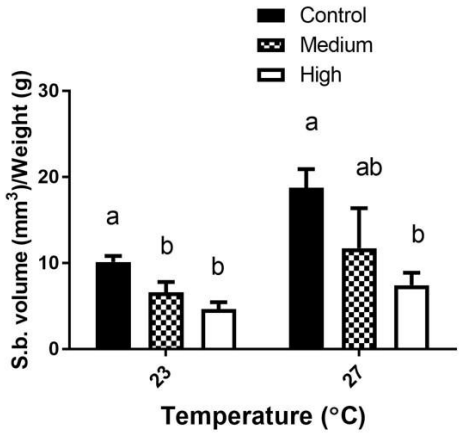



Figure 4. The effect of dissolved $CO_2$ concentration on swimbladder (s.b.) volume per gr ($mm^3\ g^{-1}$) of white grouper at 125 dph. Bars having different letters were significantly different ($P<0.05$, $n$=15 per treatment).

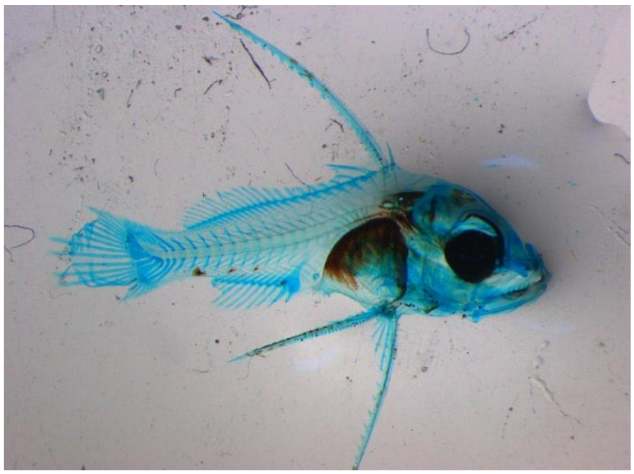


Figure 5. 30 dph white grouper larva with normal vertebral column stained with Alcian blue (*n*=15, per treatment).

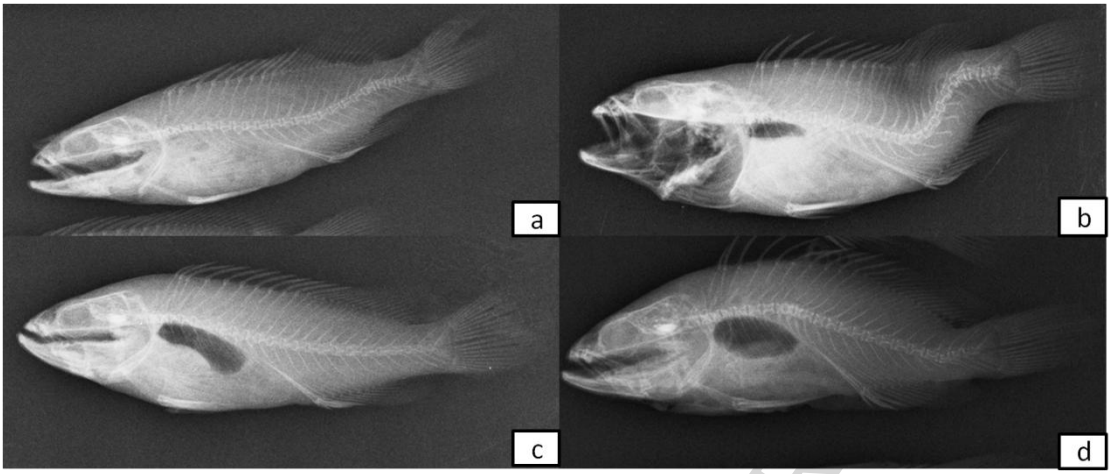



Figure 6. X-rays of 125 dph juvenile white groupers: (a) Absence of swimbladder and caudal deformity, (b) Lordosis and caudal deformity (c) Normal swimbladder and vertebral column, (d), Kyphosis, caudal deformity and an asymmetrical swimbladder.

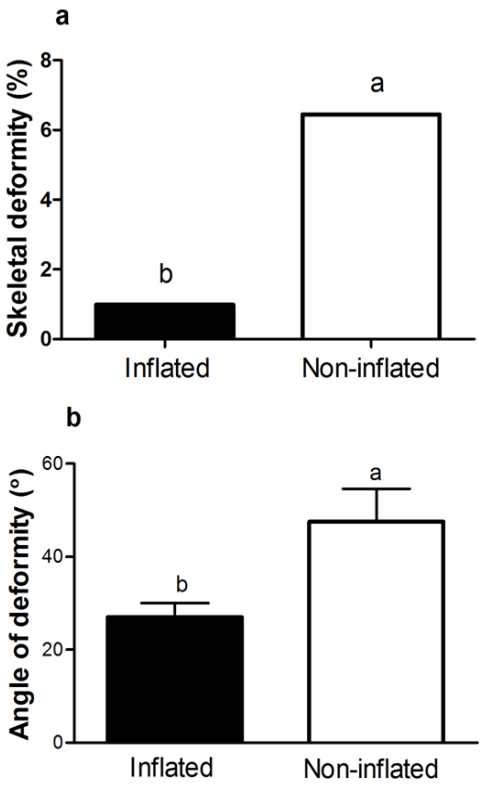



Figure 7. (a) The effect of the presence or absence of swimbladder inflation on the presence (%) of skeletal deformities and (b) on the angle of deformity of the vertebral column in groupers at 125 and 150 dph. The data in the graph include fish from all the six treatments which were divided into two groups with or without inflated swimbladder. Percent and angle values with different letters were significantly different ($P<0.05$, $n=15$ per treatment).


Figure 8. Measurment of β-angle of skeletal deformity (Chatain, 1994).

Table 1. Water quality parameters (mean ± STDEV) recorded during the experiment, salinity 25‰.

| Temperature | $CO_2$ (mg $l^{-1}$) | pH | Alkalinity (meq $l^{-1}$ as $CaCO_3$) | $NH_4$-N (mg $l^{-1}$) | $O_2$ (sat. %) |
|---|---|---|---|---|---|
| 27 °C | 0.8±0.2 | 7.93±0.1 | 1.78±0.15 | 0.15±0.03 | 97.55±10 |
| | 5.64±0.6 | 7.1±0.1 | 1.42±0.12 | 0.15±0.03 | 96±10 |
| | 28.58±3 | 6.2±0.1 | 0.98±0.18 | 0.11±0.03 | 94.8±10 |
| 23 °C | 0.78±0.1 | 8±0.1 | 1.85±0.1 | 0.15±0.07 | 95.5±10 |
| | 5.45±0.4 | 7.1±0.1 | 1.5±0.15 | 0.2±0.03 | 97±10 |
| | 29.1±2.5 | 6.2±0.1 | 0.95±0.05 | 0.12±0.05 | 96±10 |

Table 2. The effect of dissolved $CO_2$ concentration on 105 dph white grouper (*Epinephelus aeneus*) (a) weight and (b) length at 23 and 27 °C. Bars having different letters were significantly different ($P<0.05$, n=15, per treatment).

| Temperature | $CO_2$ concentration | 45 DPH | | 105 DPH | |
|---|---|---|---|---|---|
| | | weight (g) | length (cm) | weight (g) | length (cm) |
| 27 °C | Control | 0.409±0.014 | 2.15±0.08 | 18.14±0.7 [a] | 12.78±0.2 [a] |
| | Medium | 0.406±0.02 | 2.25±0.1 | 17.26±0.2 [a] | 12.28±0.4 [ab] |
| | High | 0.41±0.012 | 2.17±0.15 | 16.42±0.2 [b] | 11.83±0.2 [b] |
| 23 °C | Control | 0.402±0.015 | 2.25±0.16 | 14.91±0.43 [a] | 11.38±0.15 [a] |
| | Medium | 0.41±0.01 | 2.15±0.12 | 14.5±0.16 [a] | 11.21±0.25 [a] |
| | High | 0.407±0.016 | 2.2±0.15 | 11.25±0.9 [b] | 10.25±0.25 [b] |

Table 3. The effect of dissolved $CO_2$ concentration on swimbladder (s.b.) inflation in 105, 125 and 150 dph white grouper (*Epinephelus aeneus*) at (a) 27 $^{o}$C and (b) 23 $^{o}$C. Bars having different letters were significantly different ($P<0.05$, $n=15$ per treatment).

| Temperature | $CO_2$ concentration | 105 DPH | 125 DPH | 150 DPH |
|---|---|---|---|---|
| 27 °C | Control | 78.89±6.1 $^{a}$ | 100±00 $^{a}$ | 100±00 |
| | Medium | 56.81±9.1 $^{ab}$ | 88.89±11.5 $^{a}$ | 97.78±2.22 |
| | High | 42.2±3.4 $^{b}$ | 51.5±1.5 $^{b}$ | 96.07±2.25 |
| 23 °C | Control | 63.2±2.8 $^{a}$ | 84.68±12.13 $^{a}$ | 100±00 |
| | Medium | 31.93±8.35 $^{b}$ | 80.53±11.1 $^{a}$ | 98.06±1.48 |
| | High | 17.64±9.2 $^{b}$ | 65.63±0.56 $^{b}$ | 87.88±7.48 |

Table 4. The effect of dissolved $CO_2$ concentration on swimbladder (s.b.) volume per gr ($mm^3$ $g^{-1}$) of white grouper at 125 dph ($P<0.05$, $n=15$ per treatment).

| $CO_2$ concentration/ Temperature | 23 °C | 27 °C |
|---|---|---|
| Control | 10.12±0.72 [a] | 18.78±2.15 [a] |
| Medium | 6.61±1.2 [b] | 11.74±4.64 [ab] |
| High | 4.65±0.8 [b] | 7.43±1.45 [b] |

Highlights

1. High aqueous $CO^2$ reduced swimbladder volume and body weight in white grouper juveniles.

2. A reduction in swimbladder volume or failure to inflate the swimbladder increased lordosis and kyphosis in white grouper.

3. White grouper juveniles reared at 27 °C had a significantly higher proportion of inflated swimbladders then fish reared at 23 °C.

4. Kyphosis was associated with irregularities in the inflation of the swimbladder.

5. In order to achieve normal skeletal development in white grouper larvae and juveniles, dissolved $CO_2$ concentrations in marine rearing systems should be maintained below 5 mg $l^{-1}$.