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Effects of hypoxia and elevated ammonia concentration on the viability of red snapper embryos and early larvae

Bardon-Albaret Agnes [1], Saillant Eric [1, *]

[1] University of Southern Mississippi, Department of Coastal Sciences, Gulf Coast Research Laboratory, 103 McIlwain Dr., Ocean Springs, MS 30564, United States

* Corresponding author : Eric Saillant, email address : eric.saillant@usm.edu

Abstract :

The effects of hypoxic conditions and elevated ammonia concentrations on the viability of embryos and newly hatched larvae of the red snapper (*Lutjanus campechanus*) were investigated. In all experiments, tested levels of hypoxia or ammonia concentrations were applied to embryos and unfed newly hatched larvae from three different spawns. Exposures began at 1 h post fertilization (pf) and lasted until all individuals in a group had expired. Survival rates were monitored daily in duplicates for each spawn in each treatment. Fertilized eggs exposed to 2 mg L− 1 dissolved oxygen (29% saturation) showed complete mortality before hatch while 81% of embryos in control groups (> 85% saturation) hatched and subsequently maintained high survival until 5 days pf. Exposure to a moderate hypoxia (target 3 mg L− 1, 43% saturation) reduced significantly the hatch rate and subsequent survival rates; the magnitude of the difference in survival rate between control and exposed groups increased from 10% at hatch to 45% at 5 days pf. When oxygen concentration was maintained high (83% saturation) until 36 h pf and then progressively reduced to reach 3 mg L− 1 at 2 days pf, the survival of exposed embryos and larvae did not differ significantly from those recorded in control groups, although potential delayed or cumulative effects of the treatment after 4 days pf could not be evaluated in this experiment.

Embryos exposed to 10 mg L− 1 total ammonia (TA-N), which corresponded to unionized ammonia (UIA-N) concentrations ranging between 0.307 and 0.468 mg L− 1 in the conditions of the experiment, exhibited significantly reduced hatch rates and complete mortality between 3 and 4 days pf; the latter period corresponds to the onset of exogenous feeding of red snapper. In contrast, control groups (TA-N < 0.26 mg L− 1, UIA-N < 0.006 mg L− 1) maintained high survival rates beyond 5 days pf indicating potential to successfully initiate exogenous feeding. Exposure to 1 mg L− 1 TA-N (0.020 mg L− 1 < UIA-N < 0.054 mg L− 1) did not alter significantly survival with respect to control groups. Significant interactions between the spawn and the tolerance to hypoxia or elevated ammonia were detected in both experiments, indicating that variations among spawns need to be accounted for when determining safe levels for hatchery production.

Statement of relevance

Achieving a reliable supply of high quality eggs and larvae is one of the main challenges of the developing marine aquaculture industry.

Most studies to date have focused on maternal determinants of egg quality but the viability of embryos and newly hatched larvae can be impacted after fertilization if environmental conditions become unfavorable due to intensive hatchery conditions; this topic is poorly documented in marine fishes to date.

This study provides data on the effects of two major stressors acting under high density culture (hypoxia and elevated ammonia concentration) on embryos and newly hatched larvae of the red snapper; the results highlight the importance to consider variations among spawns/parents when determining safe levels for hatchery production and also the high sensitivity of red snapper to these stresses, suggesting that this topic should be investigated in other marine offshore species.

Relevance of the research to commercial aquaculture.

The research contributes to control egg quality.

Highlights

► In aquaculture, incubation of eggs under high density leads to hypoxia and elevated ammonia concentration. ► Knowledge of the effects of these stressors on the viability of marine species is very limited. ► Red snapper embryos and newly hatched larvae were exposed to different levels of hypoxia and elevated ammonia concentrations. ► The minimum dissolved oxygen level and maximum ammonia concentration compatible with short term survival indicate high sensitivity to these stressors and warrant further study. ► The tolerance to hypoxia and elevated ammonia concentration is variable among spawns highlighting the possible occurrence of parental effects and the need to characterize multiple spawns when determining safe levels for hatchery production.

Keywords : *Lutjanus campechanus*, Hypoxia, Ammonia, Egg quality, Stress, Water quality

1. Introduction

Achieving a reliable supply of viable fry for hatchery production is one of the main current challenges faced by the developing marine aquaculture industry (Mylonas et al., 2010). The viability of embryos and pre-feeding larvae is particularly difficult to control because it is determined by several factors (Brooks et al., 1997). To date, most studies have focused on the maternal determinants of egg characteristics, the survival potential of embryos, and the modulation of these maternal effects by parameters such as the age, the size, or the nutritional and stress status of the female parent prior to spawning (Bobe and Labbé, 2010). However, environmental factors can induce stress and affect the viability of eggs and larvae post fertilization (Brooks et al., 1997). In the wild, marine species generally release their gametes into an optimal buffered environment that ensures maximal survival and effective development of their offspring during the early larval period (Rijnsdorp et al., 2009). On another hand, intensive hatchery rearing conditions usually differ from the natural environment (Ashley, 2007), due to the incubation of embryos and newly hatched larvae at high density which can lead to rapid deterioration of the water quality. Depletion of dissolved oxygen (DO) and increase of ammonia concentration result from the metabolic activity (respiration and excretion) of embryos and hatched larvae. Aerobic processes involved in the degradation of dead and unfertilized eggs also contribute to oxygen demand and ammonia production. These changes in water quality have been linked to a reduction in hatch and early larval survival rates, along with other deleterious effects on offspring (Holt and Arnold, 1983; Shang and Wu, 2004). In such a situation, egg quality could be high at fertilization, but if embryos and larvae are cultured under deteriorated conditions, the viability and fitness of fry may be reduced. To date, information on the effects of such stressors on egg and larval quality is still limited in marine species (Yúfera and Darias, 2007). This topic is

of central importance for hatchery production considering the limited respiratory and avoidance capacity at these early stages.

In marine environments, hypoxia has been defined as the condition whereby dissolved oxygen levels in the water fall below 2.8 mg DO L^{-1} (Wu, 2002). However, because the availability of oxygen strongly depends on other parameters, particularly water temperature and salinity, hypoxia is better characterized by the oxygen saturation level (Chabot and Claireaux, 2008). European sea bass *Dicentrarchus labrax* and Atlantic cod *Gadus moruha* juveniles avoid waters where oxygen saturation is less than 45-50% (Claireaux et al., 2000; Schurmann et al., 1998) which is considered to be a level of moderate hypoxia (Chabot and Claireaux, 2008). Severe hypoxia (between 15-20% saturation) results in rapid mortality of Atlantic cod juveniles (Claireaux et al., 2000). The effects of hypoxia also depend on the type of exposure (e.g., chronic or acute) and the physiological status of the affected organisms (e.g., active swimming, digestion, stress) which determines their oxygen demand. Fish embryos depend exclusively on cutaneous respiration and their surface area for gas exchange is limited (Elshout et al., 2013). Their sensitivity to hypoxia is therefore expected to be higher than that of adults and juveniles, a hypothesis that is supported by empirical data across several freshwater species (Elshout et al. 2013). To date, information on the tolerance to hypoxia of embryos and larvae of marine fish species is still very limited. Incubation of eggs and larvae under intensive conditions is expected to result in increased levels of dissolved ammonia due to two main processes: (1) ammonia is the main nitrogenous metabolic waste product excreted by teleost fish, and thus is naturally produced in a healthy aquaculture tank, and (2) the degradation of unfertilized eggs and dead embryos also generates ammonia during proteolysis. Sub-lethal or lethal levels can occur as a result from accumulation over time, unless water is renewed at high rates or dissolved ammonia is actively removed by use of biological filters in recirculating aquaculture systems. Total ammonia nitrogen

(TA-N) consists of the highly toxic un-ionized form of ammonia (UIA-N, or NH_3) in equilibrium with the relatively non-toxic form (NH_4^+, Aubrey et al., 2014). The concentration of the two forms of ammonia is regulated primarily by water pH and temperature. Maximum levels of UIA-N between 50 and 200 μg L^{-1} were recommended for marine finfish species by Person-Le Ruyet et al. (1997) and Lemarié et al. (2004), but these recommendations were based on juvenile and adult fish while information on embryos and larvae is still very limited. Available data suggest that the development of embryos can proceed to hatch even in the presence of elevated concentrations of UIA-N in the water, but newly hatched larvae seem much less tolerant to ammonia than eggs (Chen et al., 2012) and can experience mortality with UIA-N levels as low as 0.31 mg L^{-1} to 0.55 mg L^{-1} (Holt and Arnold, 1983). To date, knowledge of the tolerance of embryos and larvae of other marine fishes to elevated levels of ammonia remains very limited.

The red snapper *Lutjanus campechanus* is a candidate for marine aquaculture in the southeastern United States. The current hatchery protocol for this species involves stocking embryos for incubation at a density of 1 egg mL^{-1} under gentle aeration and moderate water turnover (<20% h^{-1}). Larvae are typically transferred 24 hours post hatch to larval tanks where rearing density is lowered to 0.1 larva mL^{-1} or less. Incubation of red snapper eggs at a density of 2 eggs mL^{-1} or higher was shown detrimental to larval survival estimated 36 hours post hatch (Bourque and Phelps, 2007), but the actual changes of water quality parameters such as levels of dissolved oxygen and un-ionized ammonia induced by elevated density were not reported in this experiment. In addition, this study did not document the kinetics of mortality during development, which prevented evaluating the viability of larvae surviving the early phases of exposures and determining the cumulative effects of prolonged exposures.

The objective of this study was to evaluate the tolerance of the red snapper eggs and larvae to individual environmental stressors resulting from high stocking density in intensive

aquaculture. This work focused on the two main water quality parameters discussed above (concentration of dissolved oxygen and total ammonia) and studied the kinetics of mortality during exposures to provide data for the management of water quality during incubation and early larval rearing.

2. Materials and Methods

Each experiment was performed using embryos from three different wild-caught females serving as biological replicates. Females were induced for gamete maturation with human Chorionic Gonadotropin (hCG) following a protocol based on the method developed by Minton et al. (1983). Eggs were collected from each female by manual stripping at ovulation, and immediately fertilized *in vitro* with the sperm of one or two wild-caught males. Conditions at fertilization were: temperature 26±0.5°C, salinity 30±1 psu, dissolved oxygen at 85% saturation or greater (>6 mg L^{-1}), and total ammonia (TA-N) 0 mg L^{-1}. At 1 h post-fertilization (pf), random subsamples of each spawn were transferred to experimental 1-L beakers for challenges. Artificial seawater (BIOSEA® Marinemix, Aqua Craft, Hayward, CA) was prepared using deionized water (Mako RO system, Aquatic Ecosystems) for all experiments.

2.1 Hypoxic Challenges

Exposures of embryos to hypoxic conditions were performed in an oxygen chamber I-Glove incubator glovebox (Biospherix, Lacona, NY), which allowed uninterrupted control of dissolved oxygen (DO) levels in the water. A gas control module PROOX model 360 was used to lower dissolved oxygen concentration in the water to the desired level by injecting nitrogen gas into the oxygen chamber. Infusion of gas exactly matched chamber leakage to hold oxygen level constant. Accordingly, only one reduced (hypoxic) oxygen concentration could be tested per trial.

Three hypoxic challenges were performed, each exposing embryos and larvae to a different hypoxic treatment that was contrasted to control groups unexposed. For each challenge, the eggs from three females were transported in separate closed container at a density of 1 egg mL^{-1} to the location of the oxygen chamber where they were immediately stocked in experimental beakers (approximately 100 eggs estimated volumetrically were stocked in each 1-L beaker). Experimental beakers were maintained under test conditions (control or hypoxic) until the end of the trial.

During the first two challenges, embryos were exposed to a target hypoxic concentration beginning at 1 hour pf. To achieve this treatment, a preliminary trial determined the rate of oxygen depletion in the water contained in experimental beakers and the time needed to reach the desired hypoxic concentration (2 or 3 mg DO L^{-1}); the target hypoxic conditions were reached within 24 hours in the chamber. Based on these results, beakers were placed in the chamber 24 hours prior to introducing eggs to ensure that the desired oxygen concentration was reached at the time of stocking (i.e., eggs were transferred directly from water at >85% saturation to the tested hypoxic conditions). The first trial compared the survival of embryos and larvae exposed to a severe hypoxia (2 mg DO L^{-1}, 29% saturation) to that of controls maintained under normoxic conditions by exposing them to ambient air (oxygen saturation >85%). The second trial compared the survival of embryos exposed to moderate hypoxia (3 mg DO L^{-1}, 43% saturation) to controls. The last hypoxic challenge aimed to expose newly hatched larvae from eggs that had been incubated under normoxic conditions, to a moderate and progressive hypoxia (final DO: 3 mg DO L^{-1}, 43% saturation). To achieve this objective, eggs were stocked in experimental beakers at 1 hour pf as for the previous two challenges, but the oxygen decrease was only initiated at 36 hours pf and reached the target hypoxic concentration (3 mg L^{-1}) at 2 days pf. Therefore, oxygen depletion was progressive and ultimately exposed larvae to a moderate hypoxia beginning at 2

days pf. In all three experiments, control beakers were set beside the chamber where they were exposed to ambient air (oxygen saturation >85%). The water temperature was maintained at 25±1°C by heated water baths for all groups.

Larval survival was determined at three different developmental stages in all experiments: hatching (36 hours pf), 3 days pf (84 hours pf), and 5 days pf (132 hours pf). At each of these three time-points, the number of live and dead embryos/larvae was determined in two replicate beakers for each female in each treatment (hypoxic and control), i.e., 3 females x 2 treatments x 2 replicates beakers were assessed for survival rate per time point. Because of the potential stress inflicted on the larvae by the process of counting, replicate beakers were counted once and then discarded. Subsequent survival counts were performed on other duplicate beakers treated identically throughout the experiment. The level of DO and the temperature were monitored in three additional beakers in each treatment at each sampling date using an optical DO meter (YSI®). Distinct beakers were used for these measurements in order to avoid disturbing embryos and larvae in experimental beakers by immersing an oxygen and temperature probe.

2.2 Ammonia Challenge

The three concentrations of TA-N tested were generated by adding ammonium chloride (NH_4Cl) to artificial salt water (30 psu) maintained at 26±1°C. Target concentrations tested were 1, 10, and 50 mg TA-N L^{-1} and control groups with no ammonium chloride added to the water. Stock solutions for each concentration were prepared in 20-L containers at least 12 hours prior to experiments to ensure TA-N levels were stable and to adjust them prior to stocking of eggs when necessary. The control stock solution was handled identically, except that no ammonium chloride was added to the water. After the determination of the fertilization rate at 1 hour pf, eggs were introduced into 1-L beakers filled with stock solutions that had been equilibrated at the desired TA-N concentration beforehand. The number of live and dead embryos/larvae was determined in

two replicate beakers per ammonia concentration for each female every 24±2 h until total mortality was recorded in all groups. As in the previous challenge, replicate beakers for a given experimental group were counted only once and subsequent survival counts were performed on other duplicate beakers treated identically.

Temperature, TA-N concentration, and pH were measured daily from one replicate beaker per ammonia dose for each female once survival in that beaker had been determined. TA-N concentrations in seawater was determined with a precision of 0.01 mg L^{-1} using a spectrophotometer (Hach DR2800) and the salicylate method (Hach, Method 8155 powder pillows) according to protocols provided by the manufacturer. UIA-N concentrations were determined from the TA-N concentrations accounting for the pH and the dissociation constant pKa. The pKa at the temperature and salinity of the experiment was calculated using equation 3 in Bell et al. (2008). UIA-N and TA-N concentrations were expressed in mg L^{-1}, and pH was read with a pH pen-meter (YSI®, pH-100).

2.3 Statistical Analyses

The percentage of surviving eggs/larvae at each time point in each experiment was arcsine square root-transformed before analyses. Three way ANOVAs were performed in PROC GLM of SAS® 9.3(SAS Institute, Raley, NC) accounting for the fixed factors time and treatment, the random factor female, and their respective interactions. Post-hoc comparisons were performed in PROC GLM using the Tukey procedure.

3. Results

3.1 Hypoxic Challenges

In a first experiment, red snapper embryos were exposed to 2 mg DO L^{-1} (29% saturation) continuously beginning 1 hour pf. Total embryonic mortality was observed at 1 day pf (none of the embryos hatched), while survival rates in controls averaged 81, 73, and 69% at 1, 3, and 5 days pf respectively.

The next two challenges exposed red snapper embryos or newly hatched larvae to a more moderate hypoxia (3 mg DO L^{-1}, 43% saturation). The results of these challenges are presented in Tables 1 and 2.

The first challenge exposed embryos to 3.02±0.33 mg DO L^{-1} (43% saturation on average) beginning 1 hour pf. Oxygen concentration in control groups kept outside the chamber under ambient air averaged 6.01±0.10 mg DO L^{-1} (94% saturation, Table 1, Figure 1 A). The survival of exposed embryos was significantly lower than that of controls, and decreased over time (P<0.0001, Table 3). The interaction between treatment and time was also significant (P=0.0207, Table 3): No significant difference in survival was observed between the two groups at hatching (1 day pf) but survival in the hypoxic treatment was lower than in controls at the two subsequent sampling dates, and the magnitude of the difference between the two treatments increased over time from 10% at 1 day pf to 45% at 5 days pf (Figure 1 A). The survival of embryos and larvae also differed significantly among spawns (Table 1) and, interestingly, the three spawns responded differently to hypoxic conditions as revealed by the significant interaction Oxygen x Spawn (P=0.0468, Table 3).

In the second hypoxic challenge, embryos were transferred to the hypoxic chamber at 1 hour pf but were not immediately exposed to hypoxia. Beginning after hatch (at 1 day pf), oxygen concentration in the chamber was progressively lowered to reach the target level of

hypoxia at 2 days pf (mean measured DO in the treated group 2.77±0.45 mg DO L^{-1}, 40% saturation, Table 2). The proportion of live embryos and larvae did not differ between the control and treated groups throughout this experiment (*P*=0.4609, Table 3). A rapid decrease in the proportion of live larvae was observed between 3 and 5 days pf in both control and exposed larvae (Figure 1 B). A significant interaction between spawn and time was detected indicating that mortality kinetics differed between spawns (interaction Spawn x Time *P*=0.0002, Table 3) but the mortality pattern was unrelated to the treatment (non-significant interaction Spawn x Oxygen *P*=0.8172, Table 3).

3.2 Elevated Ammonia Challenges

Ammonia concentration significantly affected embryonic and larval survival (*P*<0.0001, Table 4). Survival of embryos and larvae did not differ significantly between the control group (no ammonia added, UIA-N < 0.006±0.002 mg $L^{-}1$) and the group exposed to 1 mg TA-N L^{-1} (average 0.037 mg UIA-N L^{-1}) throughout the entire duration of the experiment (Table 5, Figure 2). In these two groups, survival remained above 75 % on average until 5 days pf. Significant mortality was only recorded at 6 days pf, reflecting starvation and exhaustion of vitellin reserves and was followed by total mortality at 7 dpf. In the group exposed to 10 mg TA-N L^{-1} (average 0.365 mg UIA-N L^{-1}), survival was lower at hatch (Figure 2) and the magnitude of the difference between this treatment and the control amplified rapidly with no remaining survivors in the 10 mg TA-N L^{-1} group at 4 days pf *versus* 81.2% in controls (Table 5), reflecting a significant interaction between time and treatment levels (*P*<0.0001, Table 4). Embryos exposed to 50 mg TA-N L^{-1} (1.498±0.107 mg UIA-N L^{-1}, Table 5) did not hatch.

Hatch rates and subsequent survival rates of larvae differed significantly among the three spawns (*P*<0.0001, Table 4) and ranged from 59 to 92% on average in the control treatment (Table 5).

The magnitude of the effect of the ammonia treatment varied among the three spawns (interaction TA-N x Spawn, $P<0.0001$, Table 4). Differences were noticeable at the first measurement (hatch time) when the larvae from one of the females (spawn 1) were less affected by the 10 mg L^{-1} treatment than those from the other two females (Table 5). However, subsequently, the larvae from another female (spawn 2) retained a higher survival rate, reflecting a significant interaction Spawn x Time x TA-N ($P=0.0004$, Tables 5 and 5).

4. Discussion

In this work, the tolerance of red snapper embryos and newly hatched larvae to two potential stressors resulting from intensive culture was investigated. The transfer of embryos to a DO level of 2 mg L^{-1} (29% saturation) at one hour pf induced complete mortality in all three tested spawns before hatching, indicating that this DO concentration is below the minimum tolerated by red snapper embryos. Groups transferred to moderately hypoxic conditions (3 mg L^{-1}, 45% saturation) showed slightly lower hatch rates than controls (10% difference), and the magnitude of the difference in survival rate between control and exposed groups increased over time. Survival of exposed groups was 50% that of controls at 3 days pf and only 30% at 5 days pf. The increase of the magnitude of the difference between control and treated groups over time could reflect either that there is a cumulative effect of the prolonged exposure to hypoxic conditions, or that hatched larvae are more sensitive to hypoxia than embryos. Interestingly, when the decrease in DO was progressive and reached the same hypoxic condition (44% saturation) at 2 dpf in the second moderate hypoxia challenge, no significant difference in survival rate was detected between control and treated groups. However, in that experiment, almost complete mortality was observed in both the control and the exposed group 3 days after the exposure level was reached (5 days pf), likely reflecting a lower quality of spawns used in

this trial and preventing evaluation of treatment effects at that date. After 1 day of exposure (1 day pf for the first challenge, 3 days pf for the second challenge), the magnitude of the difference between control and exposed groups was similar in the two experiments (10% and 8.3%, respectively). Mortality of exposed larvae became significant and substantial only after 3 days of exposure in the challenge initiated at 1 hour pf and, accordingly, would have been expected to reach significant levels between 3 and 5 days pf in the second challenge. Unfortunately, the two treatments could not be contrasted at 5 days pf in the second experiment because of heavy mortality in all groups as discussed above. Therefore, additional data need to be collected using spawns showing higher viability potential beyond 3 days pf to further determine if mortality patterns differ when hypoxia is induced on larvae *versus* embryos. The question is of importance considering the presumed higher sensitivity of fish embryos to hypoxia (Elshout et al., 2013): in hatched embryos and small larvae, oxygen uptake still occurs primarily through the skin, while the predominant function of gill anlages is ion exchange (Fu et al., 2010). However, small fish larvae may be more tolerant to low oxygen concentration than eggs and embryos thanks to their advantageous surface-to-volume ratio and also due to the absence of the chorion (egg envelope), which has been hypothesized to act as a barrier for gas exchange (Elshout et al., 2013). Future challenges exposing embryos but restoring normoxia shortly after hatch would also be helpful to determine the potential impacts of production protocols in the hatchery where eggs are typically maintained at high density only during the incubation period and then transferred to low density tanks shortly after hatch, where they are unlikely to experience hypoxia.

The two exposures conducted at 3 mg L^{-1} led to moderate and non-significant mortality after one day of exposure, and some larvae survived after three days of hypoxia (even 5 days in the first experiment). The DO level tested corresponds to 45% saturation at the temperature of

our experiments and is considered a moderate level of hypoxia for juvenile and adult fish in other species (Claireaux et al., 2000). Few studies have examined hypoxia effects on embryo and newly hatched larvae of marine fishes with pelagic larvae such as the red snapper. Eggs and larvae of these species are less likely to encounter low oxygen concentrations in their natural habitat and have been hypothesized to be more sensitive to hypoxic conditions (Miller et al. 2002). Eggs of Greenland halibut (*Reinhardtius hippoglossoides*), a cold-water species, hatched at DO levels as low as 20% of oxygen saturation (Mejri et al. 2012). In contrast, the estuarine black bream (*Acanthopagrus butcheri*), a relatively warm-water species, showed a delayed embryonic development at 45% saturation (3.1 mg DO L^{-1}), a treatment that also resulted in 100% deformed larvae (Hassell et al., 2008). Newly hatched larvae of the tropical yellowfin tuna (*Thunnus albacares*) died rapidly when exposed to 2.2 mg DO L^{-1} (34% saturation at 28°C) and showed a moderate reduction of survival to first feeding (23% lower than control) when exposed to 2.65 mg DO L^{-1} or 40% saturation (Wexler et al., 2011). Available data on warm-water species are consistent with our results on red snapper, where moderate mortality was observed at or close to 45% saturation and complete mortality before hatch at 29% saturation. Overall, these results suggest that the minimum level of dissolved oxygen compatible with survival of red snapper embryos and larvae is higher than 45% saturation under the conditions of this experiment. At 45% saturation, larval survival was less than 30% that of controls at 5 days pf. Thus, a small fraction of the larvae were still alive during the transitional period when larvae need to initiate exogenous feeding (between 3 and 5 days pf). Considering the high rates of mortality already detected at 3 days pf, the few larvae still alive at that point were likely stressed and had a low likelihood to initiate successfully exogenous feeding. Additional studies targeting higher intermediate levels of hypoxia will be needed to clarify the range of oxygen concentrations suitable for hatchery production. Assessment of effects on growth and deformities would also be

helpful considering observations in other species (Buentello et al., 2000, Petersen and Pihl, 1995, Tran-Duy et al., 2008). The results at 3 mg L^{-1} (minimal effect at hatch but major effects on survival at 3 and 5 days pf) also highlight the need to monitor treatments during incubation and early larval life when refining the minimum DO level tolerable for embryos. Indeed, hypoxic treatments between 3 and 4 mg DO L^{-1} may reveal no significant effect on hatch rate, but could induce significant mortality during the first few days of life if carryover effects occur as previously reported in brown trout *Salmo trutta* (Roussel, 2007). The occurrence of such carryover effects in red snapper remain to be tested.

Finally, significant differences among spawns in sensitivity to hypoxia were detected during the first moderate challenge. The potential mechanisms involved in these differences are multiple and include genetic and maternal factors. The interaction pattern between the viability of spawns under normoxia and their sensitivity to hypoxia seems complex. During this experiment, the spawn with highest viability (under normoxia) showed highest tolerance to hypoxia, but the second best spawn appeared more sensitive to the treatment than the spawn displaying lowest quality. Thus, improving the viability of spawns, as measured under normoxic conditions, would not necessarily improve their tolerance to hypoxia. Minimum threshold DO levels for incubation should therefore be determined during challenges involving several spawns to ensure identification of safe levels for all spawns.

The challenge evaluating the tolerance of embryos and newly hatched larvae to ammonia was based on a similar principle. Three doses of TA-N were tested by immersing embryos directly from water where TA-N was ~0 mg L^{-1} to different concentration levels at 1 hour pf, and maintaining them unfed in the tested static conditions until complete mortality was observed. Survival of embryos and larvae exposed to a TA-N concentration of 1 mg L^{-1} did not differ from those in control groups throughout the monitoring period, and no significant mortality was

detected until 6 days pf in these two groups. Hatch rates and subsequent survival rates were significantly lower in the groups exposed to 10 mg TA-N L^{-1}, where complete mortality was observed at 4 days pf. Complete mortality was observed before hatch in all groups exposed to 50 mg TA-N L^{-1}. Thus, in our experimental conditions, exposure had no detectable effects on survival at 1 mg L^{-1} but significant reduction in survival at the 10 mg L^{-1} dose. In the latter group, survival was 54 % of that in controls at the time when larvae are expected to initiate exogenous feeding (3 days pf), and complete mortality was recorded in this treatment the next day, indicating that larvae would not have initiated feeding successfully. Interpretations of these results in terms of range of tolerance for red snapper embryos and larvae need to account for the actual concentration of unionized ammonia (UIA-N), which is the toxic form of ammonia found in equilibrium with the non-ionized (and non-toxic) form (Bower and Bidwell, 1978). In the conditions of our experiment, the average concentration of UIA-N in the 1 mg L^{-1} treatment was estimated to be 0.04 mg L^{-1}. Concentrations of UIA-N between 0.02 and 0.05 mg L^{-1} have been shown to be harmless to first feeding marine larvae (Brownell, 1980), consistent with the lack of detectable effect at this dosage in this challenge. In contrast, the 10 mg L^{-1} TA-N treatment was expected to yield 0.37 mg L^{-1} UIA-N, a value that exceeds the upper range of toxicity tolerance for most aquatic organisms (Francis-Floyd et al., 2009). However, most exposure results reviewed by Francis-Floyd et al. (2009) were based on the tolerance of juveniles and adults of studied species, and few studies reported the effect of ammonia on embryos and larvae of marine fishes, even though available data suggest that larvae seem to be the most sensitive life history stage to UIA-N (Harcke and Daniels, 1999). At 26±1°C, more than 90% of spotted sea trout and red drum eggs hatched when exposed to 6.0 mg UIA-N L^{-1} or higher, but significant mortality of larvae occurred in both species at concentrations as low as 0.26 mg UIA-N L^{-1} (Daniels et al., 1987; Holt and Arnold, 1983). In red snapper, 0.37 mg UIA-N L^{-1} affected embryonic survival

resulting in lower hatch rate, denoting a higher sensitivity of embryonic development to ammonia exposure than the two estuarine sciaenids discussed above. The subsequent increase in mortality rate at this exposure concentration suggests that hatched larvae are more sensitive to UIA-N than embryos, and/or that effects of the prolonged exposure to ammonia might be cumulative.

The causes for an increased sensitivity of red snapper embryos to ammonia are unclear. While some authors argued that the structure and function of the chorion could provide a high degree of protection to the embryo against ammonia (Bucking et al., 2013), other' studies suggested that ammonia can penetrate the chorion (Steele et al., 2001; Braun et al., 2009). Also, very early in development, urea synthesis has been hypothesized to protect teleost embryos from toxic nitrogenous end-products generated during yolk absorption (Wright and Land, 1998; LeMoine and Walsh, 2013). Thus, increased sensitivity of red snapper embryos to ammonia could be related to a higher permeability of the chorion to ammonia than in other species, or a lower efficiency of mechanisms protecting embryos from ammonia such as urea synthesis.

The sensitivity of red snapper embryos and larvae to ammonia UIA-N between 0.04 and 0.37 mg UIA-N L^{-1} should be further investigated to refine sub lethal conditions and maximum thresholds tolerable during incubation and larval rearing in aquaculture. As discussed in the case of hypoxia above, long term effects such as deformities could result from an acute exposure during early development (including embryonic development), and/or cumulative effects could lead to worsening mortality under a given dosage if the exposure is prolonged. In consequence, treatments need to be evaluated several days after hatch as was done in this study even if exposure is ended at hatch. Information on the effects of exposure on larval growth and deformities is also needed based on observations in other species (Holt and Arnold, 1983; Lemarié et al., 2004; Schram et al., 2010).

A specific consideration to ammonia challenges is the importance of the pH, as this parameter directly influences the concentration of toxic UIA-N. If the pH had been 8.6 in the present experiment, the proportion of UIA-N would have been expected to reach 0.15 mg L^{-1} in the 1-mg TA-N L^{-1} treatment. This exposure is in the upper range of tolerance for a variety or organisms, and, considering the major effects detected at 0.37 mg UIA-N L^{-1} in this study, could lead to significant mortality before the transitional feeding period. Monitoring and controlling the pH is therefore essential when designing and interpreting future experiments and, most importantly, when characterizing safe levels for incubation and larval rearing of the red snapper.

Significant maternal effects on the sensitivity to ammonia exposure were detected as revealed by a significant female x treatment interaction. The main aspect of this interaction is that the embryos from the female showing lower viability under control conditions seemed more resilient to the 10 mg.l^{-1} TA-N exposure than the embryos from the other females. As discussed above, this indicates that improving the viability under normal conditions will not necessarily increase tolerance to stressful levels of ammonia and the variability among spawns will need to be accounted for by evaluating several spawns when designing protocols to identify a safe range of ammonia concentrations for red snapper embryos and larvae.

In conclusion, the results of challenge experiments revealed that the minimum dissolved oxygen concentration suitable for incubation of red snapper embryos and newly hatched larvae is higher than 3 mg L^{-1}, and the maximum concentration of unionized ammonia is lower than 0.04 mg L^{-1}. Future studies should focus on the effects of exposures restricted to the incubation period until hatch, which represents the rearing phase where density is high and hypoxia and elevated ammonia concentration are more likely to happen. Another aspect that needs to be evaluated is the interaction between hypoxia and elevated ammonia stresses when applied

simultaneously. Indeed, incubation at high density often results in both increase in ammonia and reduction of oxygen levels, and the combination of these two stressors may not be simply additive; thus, identification of safe levels may need to account for both dissolved oxygen and UIA-N concentrations. Characterization of the changes of oxygen demand over time during incubation would be helpful when evaluating density treatments for aquaculture operations as this parameter is expected to increase during the course of embryonic and larval development (Wright and Wood, 2012). Similarly, the kinetics of increase of ammonia in incubators due to embryo metabolic processes and to the degradation of dead and unfertilized eggs would need to be quantified to predict possible levels of UIA-N under different density scenarios. The results of these studies will be useful to determine incubation and early larval rearing conditions that will preserve egg quality after fertilization.

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Table 1. Percentages of live larvae (%Live) recorded at 1, 3 and 5 days pf in experimental groups exposed to moderate hypoxia (target 3 mg L^{-1} DO, 45% saturation) beginning 1 hour post fertilization and in controls. Data from three spawns (S1 to 3) with two replicates (Rep A and B) per time point; DO target: targeted oxygen concentration (3 mg L^{-1}), Ctrl: control treatment (ambient air), DO meas.: measured oxygen concentration (% saturation), n: sample size.

			S1		**S2**		**S3**	
			Rep A	Rep B	Rep A	Rep B	Rep A	Rep B
Day pf	DO target	DO meas.	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)
1	Ctrl	6.59 (92%)	75.0 (72)	57.1 (205)	87.6 (137)	85.2 (155)	96.7 (60)	92.4 (132)
	3	3.19 (46%)	58.3 (72)	51.2 (86)	69.7 (76)	73.8 (149)	93.3 (75)	85.9 (156)
3	Ctrl	6.57 (94%)	67.9 (140)	28.0 (132)	82.4 (108)	84.6 (104)	91.9 (99)	94.3 (70)
	3	2.96 (42%)	27.2 (158)	41.3 (317)	44.0 (75)	9.1 (55)	64.7 (51)	37.5 (64)
5	Ctrl	6.39 (91%)	7.5 (67)	54.7 (159)	84.9 (93)	69.5 (105)	88.0 (75)	86.0 (86)
	3	2.9 (42%)	5.1 (276)	4.4 (183)	15.0 (140)	0.0 (128)	52.5 (99)	45.8 (96)

Table 2. Percentages of live larvae (%Live) recorded at 1, 3 and 5 days pf in experimental groups exposed to moderate hypoxia (target 3 mg L^{-1} DO, 45% saturation) beginning 1 day post fertilization (see text for detailed protocol) and in controls. Data from three spawns (S4 to 6) in two replicates (Rep A and B) per time point; DO target: targeted oxygen concentration (control or 3 mg L^{-1}), Ctrl: control treatment (ambient air), DO meas.: measured oxygen concentration (% saturation), n: sample size.

			S4		**S5**		**S6**	
			Rep A	Rep B	Rep A	Rep B	Rep A	Rep B
Day pf	DO target	DO meas.	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)
1	Ctrl	5.87 (84%)	81.2 (128)	92.4 (198)	72.3 (65)	65.5 (113)	21.2 (113)	20.8 (101)
	Ctrl	5.81 (83%)	73.5 (98)	87.6 (113)	61.0 (82)	52.6 (76)	31.1 (193)	27.0 (115)
3	Ctrl	5.96 (85%)	90.3 (113)	86.5 (141)	53.2 (62)	69.1 (181)	35.1 (168)	33.3 (87)
	3	2.45 (35%)	54.2 (59)	85.9 (78)	68.5 (73)	63.3 (79)	28.2 (110)	15.9 (88)
5	Ctrl	6.22 (89%)	3.0 (132)	5.7 (105)	3.2 (95)	4.4 (91)	4.0 (100)	4.8 (63)
	3	3.09 (43%)	8.8 (114)	0.0 (100)	11.4 (70)	3.9 (77)	3.1 (260)	2.9 (204)

Table 3. Results of three way ANOVAs conducted for two moderate hypoxia challenges (see text for protocols applied in the two challenges).

		3mg.l^{-1} from 1 hour pf		3mg.l^{-1} 2 day pf	
Factor	DF	*F*-value	*P*-value	*F*-value	*P*-value
Oxygen	1	43.17	<0.0001	0.64	0.4350
Time	2	19.11	<0.0001	151.77	<0.0001
Spawn	2	21.88	<0.0001	38.46	<0.0001
Oxygen x Time	2	4.85	0.0207	1.21	0.3219
Oxygen x Spawn	2	3.65	0.0468	0.20	0.8172
Spawn x Time	4	0.64	0.6384	9.80	0.0002
Oxygen x Spawn x Time	4	0.89	0.4895	1.74	0.1844

Table 4. Results of three way ANOVAs accounting for the ammonia treatments (TA-N), duration of exposure (time), spawn and the interactions between these factors.

Effects and interactions	DF	*F*-value	*P*-value
TA-N	2	984.33	<0.0001
Time	6	887.29	<0.0001
Spawn	2	137.06	<0.0001
TA-N * Time	12	110.53	<0.0001
TA-N * Spawn	4	40.10	<0.0001
Spawn * Time	12	7.24	<0.0001
TA-N * Spawn * Time	24	2.92	0.0004

Table 5. Percentages of live larvae (%Live) with sample size (n) recorded daily from 1 to 7 days post fertilization in experimental groups exposed to three total ammonia concentrations (TA-N, 1, 10 and 50 mg L^{-1}) beginning 1 hour post fertilization and in controls (no ammonia added). Data from three spawns (S1 to 3) with two replicates (Rep A and B) per spawn. Target TA-N concentration (TANt), mean ± SD of measured TA-N (TANm), calculated UIA-N in mg L^{-1}, and measured pH.

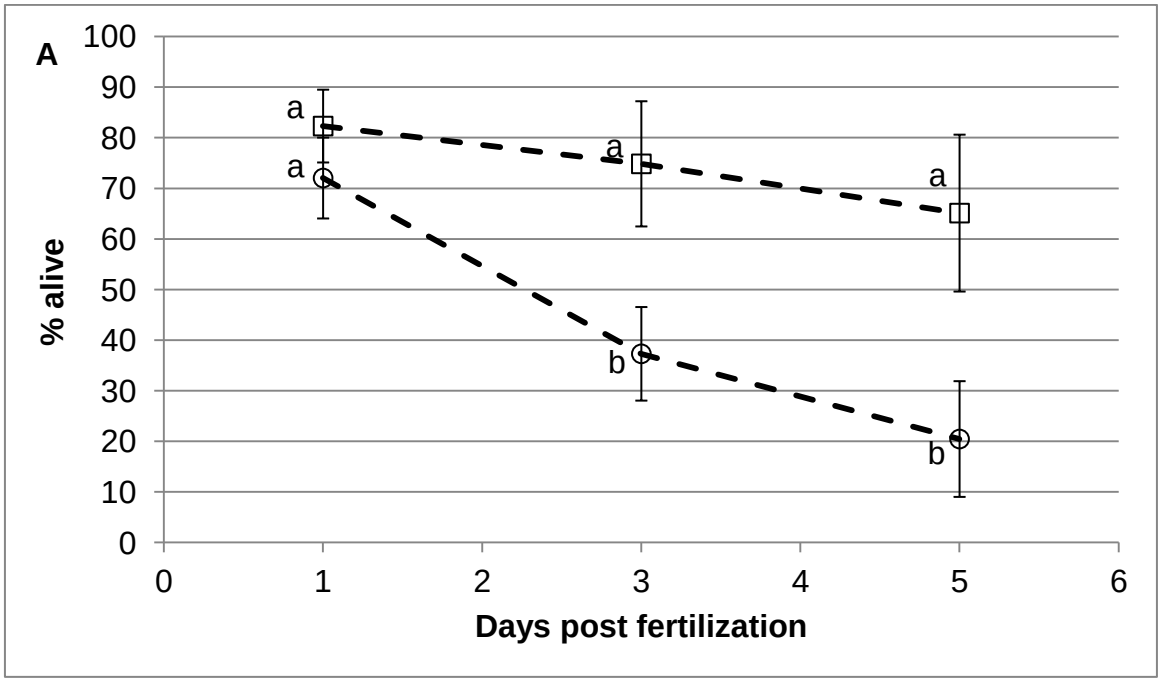
					S1		S2		S3	
					Rep A	Rep B	Rep A	Rep B	Rep A	Rep B
dpf	TANt	TANm	UIA-N	pH	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)	%Live (n)
1	0	0.02 ±0.01	0.000 ± 0.000	7.23 ±0.21	59.4 (180)	58.9 (180)	77.7 (121)	78.5 (121)	91.9 (186)	92.2 (217)
	1	1.17 ±0.25	0.020 ± 0.005	7.60 ±0.04	63.6 (118)	66.4 (122)	75.7 (140)	86.0 (136)	92.8 (138)	92.3 (156)
	10	9.00 ±0.00	0.307 ± 0.017	7.89 ±0.03	53.6 (125)	47.3 (93)	59.2 (98)	61.6 (99)	60.6 (104)	56.8 (125)
	50	46.67 ±2.89	1.498 ± 0.107	7.80 ±0.02	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)
2	0	0.06 ±0.04	0.002 ± 0.002	7.73 ±0.39	61.5 (117)	54.3 (140)	82.7 (75)	75.7 (144)	96.1 (234)	91.1 (124)
	1	0.97 ±0.12	0.034 ± 0.014	7.88 ±0.26	64.6 (175)	51.3 (119)	85.0 (147)	84.8 (138)	90.0 (130)	94.1 (170)
	10	9.33 ±0.58	0.330 ± 0.150	7.87 ±0.23	50.3 (155)	45.7 (70)	51. (81)	61.3 (106)	62.0 (108)	28.0 (50)
3	0	0.12 ±0.03	0.004 ± 0.000	7.82 ±0.07	51.9 (156)	60.3 (121)	81.3 (75)	84.6 (162)	87.1 (93)	95.9 (169)
	1	1.30 ±0.17	0.035 ± 0.009	7.78 ±0.06	55.2 (183)	63.9 (83)	86.2 (94)	80.2 (126)	92.7 (206)	88.1 (109)
	10	12.0 ±1.00	0.468 ± 0.132	7.94 ±0.11	32.1 (78)	44.7 (94)	45.7 (92)	52.2 (69)	15.6 (64)	28.2 (78)
4	0	0.19 ±0.06	0.003 ± 0.001	7.58 ±0.06	60.2 (108)	64.3 (140)	85.7 (91)	87.9 (257)	94.0 (235)	95.2 (63)
	1	1.53 ±0.25	0.03 ± 0.004	7.65 ±0.05	56.3 (48)	64.5 (172)	74.7 (91)	83.3 (96)	93.4 (136)	96.9 (162)
	10	14.67 ±2.31	0.355 ± 0.020	7.74 ±0.05	0.0 (82)	0.0 (103)	0.0 (93)	0.0 (51)	0.0 (71)	0.0 (97)
5	0	0.26 ±0.02	0.006 ± 0.002	7.73 ±0.13	52.3 (128)	56.7 (164)	81.9 (116)	79.1 (139)	84.6 (188)	93.3 (180)
	1	1.40 ±0.10	0.048 ± 0.012	7.89 ±0.14	61.3 (173)	49.2 (128)	82.7 (150)	85.6 (97)	88.6 (140)	89.9 (139)
6	0	0.15 ±0.03	0.003 ± 0.001	7.69 ±0.29	8.5 (118)	6.4 (109)	1.4 (140)	4.3 (188)	6.3 (175)	16.3 (166)
	1	1.10 ±0.10	0.054 ± 0.005	8.06 ±0.02	0.0 (149)	1.6 (124)	2.2 (91)	3.8 (78)	11.1 (153)	16.3 (129)
7	0	0.26 ±0.05	NA	NA	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)
	1	1.60 ±0.28	NA	NA	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)	0.0 (NA)

Figure Legends:

Figure 1. Survival rate of red snapper (±SD) exposed to moderate hypoxia (circles) and controls (squares) beginning at 1 hour post fertilization (A) or 36 hours post fertilization (B). Data are presented as the mean (±SD) of 3 spawns with 2 replicate groups per spawns. Letters (a, b) denote homogeneous groups identified during post-hoc tests.

Figure 2. Survival rate of red snapper exposed to three ammonia concentrations (0, 1, and 10 mg L^{-1}) beginning 1 hour post fertilization. Data are presented as the mean (±SD) of three spawns with 2 replicates per spawn. Letters (a, b, and c) denote homogeneous groups identified during post-hoc tests.

Figure 1



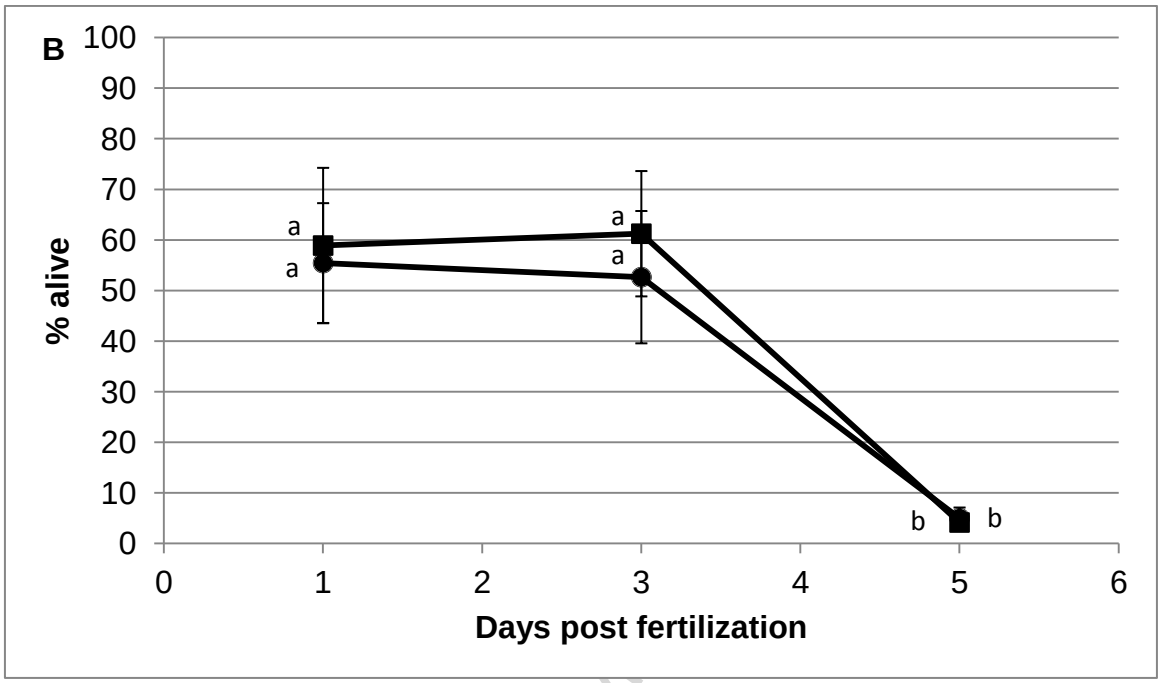


Figure 2