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Effect of salinity on three tilapia (*Oreochromis* sp.) strains: hatching rate, length and yolk sac size

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Abstract: Salinity is an important factor for fish embryonic development. In tilapias, tolerance to salinity at any age is influenced by physiological responses of each species and can be transferred into hybrids. We evaluated the differences with respect to hatching rate (HR), total length at hatching (TL) and yolk sac area (YS) in eggs obtained from breeders kept at 0‰ and directly transferred to salinities of 0, 5, 15, 25, 35, 45, 55 and 65 ‰, after egg collection of the following varieties of tilapia: red tilapia (*Oreochromis* sp) (RT), a hybrid 1 (H1) obtained from the cross of Nile tilapia (male) X red tilapia (female) and a new red synthetic tilapia as hybrid 2 (H2): Pargo-UNAM. The results showed that salinity above 35-45‰ significantly reduced hatching rates in H1 and H2 until reaching 0% HR at 55 and 65‰, while RT eggs hatched at all salinities. TL decreased significantly >35‰, with an interval with no differences between 0-25‰. YS also decreased >25‰ for H1 and H2, with an overall smaller size for RT. These results provide an insight of morphological differences related to salinity tolerance at early stages, as smaller hatching size and increased yolk sac utilization at salinity >35‰.

Key Words: hatching, salinity, tilapia, yolk sack

Introduction

Tilapia farming under high salinity conditions has been the subject of many studies and is practiced in commercial operations all over the world. This kind of farming is viable given the particular physiological adaptations of this fish, which, in all case reports, shows better growth rates and lower oxygen consumption under these conditions (Ron *et al*., 1995). Tilapia hybrids, in particular, have more tolerance for salinity variations, and their tolerance increase as they develop from early to more advances stages, as reported for Florida red tilapia,

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Oreochromis urolepis hornorum (female) X *O. mossambicus* (male), given its tolerance to direct transfer to sea water starting from 10 days after hatching (dph), which ranged from a few hours (190 min) to 3910 min at 70 dph (Watanabe *et al*., 1990); Thailand red tilapia also showed this kind of tolerance (*O. niloticus* X *O. mossabicus*) (Villegas, 1990).

In general, it is well established that salinity conditions during incubation and rearing are highly relevant for embryonic development, affecting variables such as hatching rate, and even later causing a lower survival rate and deformities in larvae (Okamoto *et al*., 2009), or affecting larval size, particularly when salinity is above the species tolerance, producing smaller fish when reared at higher salt concentrations (Steward Fielder *et al*., 2005). Optimal salinity conditions can vary greatly between fish species; therefore, proper determinations of such conditions need to be outlined for each particular fish. In tilapia, salinity tolerance has a major genetic component, as described by El-Zaeem *et al*. (2012) when crossing female Red tilapia X male Nile Tilapia, a cross that showed significantly better productive performance when compared with the reciprocal cross. At this moment, a new synthetic strain of red tilapia (Pargo-UNAM) produced by a cross of Florida Red tilapia, Rocky Mountain Tilapia and red *O. niloticus* is being evaluated for productive performance in rearing conditions of high salinity, with noteworthy results (Ramirez-Paredes *et al*., 2012).

There is information available describing specific physiological responses of salinity tolerance in Red and Nile tilapia, both at the cellular level (including cell proliferation and enzyme expression in several tissues such as yolk sac membrane, gills and intestine) and activated responses involving the secretion and production of specific hormones (growth hormone) and other molecules such as prolactin, and cortisol, among others; this information was clearly presented in a comprehensive review by Yan *et al*. (2013), where the authors amply describe many of the physiological responses mentioned above and also mention some desirable characteristics of salt tolerant tilapias, which include a wide tolerance interval, good growth rate to market size (500 g), uniformity in growth performance and morphology, possibility of large-scale reproduction and wide genetic diversity to reduce the potential for inbreeding. In general, specific adaptations involve mostly to intestinal and gill adaptations, including the regulation of drinking rates and chloride cell proliferation, both for efficiency in intestinal and gill ionic transport and as adaptive mechanisms in hypersaline-tolerant fish species (Laverty and Skadhauge, 2012). This information is quite relevant; however, it does not provide an insight of how to assess the salinity tolerance of a specific tilapia strain using the morphological responses of tilapia hatched after direct transfer

of embryos to different salinities.

In the present study, three tilapia strains, including red tilapia (*Oreochromis* sp.) (RT), a hybrid 1 (H1) obtained from the cross of Nile tilapia (male) X red tilapia (female) and Pargo-UNAM (H2) were evaluated for salinity tolerance from early developmental stages, assessing their hatching rate, total length and yolk size area at hatching within the range of 0, 5, 15, 25, 35, 45, 55 and 65 ‰ salinity. This work provides an interesting insight on using morphological traits as salinity tolerance indicators in tilapia fry production, potentially eliminating the necessity of an acclimation period prior to culturing tilapia under high salinity conditions. On this subject there are very few references available in terms of comparative studies, in particular for Pargo-UNAM (H2) given its potential as a new alternative for brackish and marine tilapia production in México (Ramirez-Paredes *et al*., 2012) and to elucidate its early development performance at different salinities when compared with other tilapias.

Materials and Methods

For the present work, the following strains of tilapia broodstock were used: Male *Oreochromis niloticus* provided by a government-owned aquaculture center located in Chametla, Sinaloa, Mexico; males and females of red tilapia (*Orechromis* sp.) from a group of organisms held at FACIMAR-UAS, Mazatlán, Sinaloa Mexico; Tilapias Pargo-UNAM provided by CEIEGT-UNAM, located at Tlapacoyan, Veracruz, Mexico. After the fish were fully identified as male or female by means of staining the genital papilla with a colorant solution, they were placed in several 450 L tanks with 0‰ salinity, divided into the following groups: Group 1 (RT), red tilapia, both males and females; group 2 (H1), Nile tilapia (male) X red tilapia (female); and group 3 (H2), tilapia Pargo-UNAM, both males and females, with a minimal weight of 100-150 g for all fish with a male:female ratio of 1:3-1:5 (two tanks per group). Seven to ten days afterwards, the breeders were stocked; the fish were checked daily for a week to collect fertilized eggs after removing them from the oral cavity of the females. The collected fertilized eggs (1 spawn per experimental group) were directly transferred to 1 L polyethylene cylindrical containers with water with following salinity levels: 0, 5, 15, 25, 35, 45, 55, 65‰. To obtain a salinity level below 35‰, sea water was diluted with fresh water, both filtered to 1 µm and UV-sterilized prior to being mixed as water treatment; for salinities >35‰, 1 g/L of unprocessed sea salt purchased at a local producer, was dissolved in treated seawater until salinity values of 45, 55 and 65‰ were achieved; a temperature compensated refracto-meter was used in all cases (Vital sine®). Each container was stocked with 25-30 fertilized eggs with three replicates for each salinity level,

using eggs from each group. All experimental units were placed in plastic shelves in a facility at 27±2°C, with controlled photoperiod 14:10 and constant aeration; incubation was carried out for 36-48 hours until hatching.

The hatching rate (HR) was quantified as the number of larvae/total eggs per replicate at each salinity level. We quantified total length (TL) in millimeters (mm) and yolk sac area (YS) in square millimeters (mm^2) of each larvae at hatching after digital analysis of pictures taken using a dissecting microscope at 7x magnifications equipped with a microscope digital camera (5 megapixels; model 2500 Moticam®) and Motic Images Plus 2.0 ML software for morphometric measurements after calibration.

The data obtained for all variables (HR, TL and YS) were tested for normality and homoscedasticity. HR data were arcsine transformed. Each variable was tested using one-way Anova for each experiment (breeders group) and Fisher's test was used to establish differences between treatment means. All statistical analyses were performed using SAS 9.1 software at a significance level of 95% (SAS Institute, Inc., Cary, N.C. EUA).

Results

Hatching rate: Tilapia larvae from the RT group showed the highest tolerance to salinity, with a hatching rate of approximately 63.2% at all tested salinities; the H1 hybrid hatched at salinities of up to 45‰, with a mean value of 68%; the H2 hybrid (Pargo-UNAM) only hatched within a salinity range of 0-25‰, showing significant lower hatching rate values above 15‰ (39.5%) (Tab. 1).

Total length: In all three experiments, total length values showed a tendency to decrease as salinity increased. These changes varied in magnitude as follows: a decrease of 0.5 mm, or 12%, in RT for 0-65‰; a decrease of 1 mm, or 18%, in H1; and a decrease of 1.2 mm, or 23%, in H2, (Tab. 1).

Yolk sack area: This variable showed different tendencies in each tilapia group. In RT, there was a reduction from 4.6 to 3.9 mm^2, equivalent to almost 15%, for 65‰ salinity. In H1, the tendency was an upsurge of YS as salinity increased, equivalent to a 17% increase at 45‰ salinity (Tab. 1). In H2, a decrease of 5% in YS was noticeable; despite the low magnitude of the change, there was still a significant difference between 0 and 25‰ salinity (Tab. 1).

Discussion

This research work provides information on the feasibility of a direct transfer of tilapia offspring to water of different salinities at early developmental stages of embryonic growth,

Tab. 1: Observed values for hatching rate (HR), total length (TL) and yolk sack area for each salinity per tilapia experiment (mean± standard deviation)

Experiment	Salinity	Hatching (%)	Total length (mm)	Yolk sack area (mm^2)
Red Tilapia (RT)	0	66.7±5.70	5.08±0.32^{acd}	4.60±0.42^{a}
	5	71.8±14.3	4.99±0.16^{cd}	4.07±0.19^{be}
	15	65.8±31.6	5.13±0.34^{c}	4.06±0.46^{bc}
	25	46.2±32.8	4.90±0.35^{cd}	4.24±0.33^{de}
	35	59.9±29.1	4.77±0.44^{d}	4.22±0.30^{cde}
	45	65.4±14.4	4.78±0.36^{d}	4.13±0.33^{cde}
	55	66.1±18.3	4.69±0.25^{db}	4.23±0.44^{e}
	65	64.2±24.2	4.51±0.50^{b}	3.91±0.28^{f}
Nile tilapia (male) X red tilapia (female) (H1)	0	68.7±10.8	5.27±0.33^{a}	2.44±0.28^{a}
	5	77.1±9.60	5.25±0.28^{a}	2.49±0.31^{a}
	15	66.7±7.20	5.06±0.50^{ac}	2.55±0.29^{a}
	25	70.8±19.1	4.93±0.55^{bc}	2.57±0.29^{a}
	35	75.0±6.30	4.61±0.31^{d}	2.89±0.22^{b}
	45	50.0±16.5	4.28±0.44^{e}	2.86±0.34^{b}
	55	n.d.	n.d.	n.d.
	65	n.d.	n.d.	n.d.
Pargo-UNAM (H2)	0	88.8±4.11^{a}	5.53±0.48^{a}	2.87±0.29^{a}
	5	86.3±3.82^{a}	5.73±0.45^{b}	2.78±0.23^{bc}
	15	92.1±2.40 ^{a}	5.38±0.40^{c}	2.80±0.23^{ac}
	25	39.5±9.64 ^{b}	4.27±0.46^{a}	2.75±0.18^{bc}
	35	n.d.	n.d.	n.d.
	45	n.d.	n.d.	n.d.
	55	n.d.	n.d.	n.d.
	65	n.d.	n.d.	n.d.

n.d.: Non determined, zero hatching observed. Superscripts indicate significant differences per column for each variable per experiment ($p<0.05$).

being more relevant for RT offspring followedby H1 and H2. We describe the morphological variations of tilapia larvae of evaluated strains as potential indicators of osmotic stress and as a tool for the rapid on-hatchery evaluation that could be applied to any particular tilapia strain. Based on our results, we can outline an evident genetic component of the tolerance to salinity of tilapia larvae from early developmental stages after direct transfer from fresh water to water with higher salt concentrations, particularly when red tilapia is involved in the cross; as described by Watanabe and Kuo (1985), who mention that Nile tilapia has a relatively low

salinity tolerance above 25‰; however, a significant effect is also noticeable when breeders are older than 2 years of age. The stock of breeders of all strains used in our work was younger than 1 year of age; it is thus possible to continue our research by reexamining these results using different broodstock year classes. In addition, when red tilapia is used as female component for hybrid production, salinity tolerance can be increased, as described in a study where red tilapia was used as maternal component in crosses of female red tilapia X male Nile tilapia, showing a significant effect on salinity tolerance up to 32 ppt (El-Zaeem *et al*., 2012), which was similar to our results for H1, which hatched in salinity levels up to 45‰. Lutz *et al*., 2010 also indicates that when Red Florida tilapia and *O. mossambicus* are involved as maternal component in reciprocal crosses with pure breed tilapias such as *O. aureus* and three lines of *O*. *niloticus* including two red Nile tilapia strains, mean salinity tolerance values were among 41.1 to 52.5‰; therefore, H1 reported results for hatching performance in our trials at salinity values of 45‰ are due to the maternal component as RT in the produced cross.

In our study, the eggs of all three tilapia strains were collected at similar times, all within a maximum of 36 hours after fertilization, reducing the possibility that the variations observed in hatching rates were due to the timing of egg collection and of the transfer to saline water. Fridman *et al*. (2012) in a relevant study of the tolerance to salinity of Nile tilapia describes that the time of transfer after fertilization can indeed be a key factor in tolerance to salinity after direct transfer to water with salinity levels of 0-25‰ for Nile tilapia, as hatching success can be reduced up to 50% if eggs are transferred 48 h after fertilization. The hatching rate improved greatly when the eggs were transferred within 4 hours post-fertilization. No hatching was observed at 32 ‰. Embryonic development was also delayed at salinities above 15 ‰, at which level hatching took up to 20 hours longer than at 0 and 7.5 ‰. In addition, Hui *et al*. (2014) described that in Nile tilapia, the interaction between temperature, salinity and pH values and their effect on fertilization and hatching efficiency, instead of evaluating salinity as a single factor; their results show that 9.2 ‰ at 27.1 °C and pH 7.4 is the best combination for obtaining higher hatching rates. Furthermore, incubation temperatures were kept constant for all three trials, with no noteworthy pH variations (7.1-7.3 values), allowing us to discard the potential interaction of the above-mentioned factors as a potential source of variation of the results. There is a potential effect on survival of Nile tilapia due to the salinity sources, as seawater, table salt or common salt, especially for swim up fry as is not as significant for yolk sack larvae, at 4-12 ‰ salinity values (Bart *et al*., 2013). Nevertheless, as we used common sea

salt (non-oidated) directly from a local salt producer, we can discard a potential negative effect on hatching rates for all evaluated strains and assume that the Nile tilapia genetic component of both hybrids was the major factor on the reduction of hatching efficiency, both for H1 and H2.

In our experiments, a direct response to salinity tolerance was outlined, indicating that, as expected, red tilapias have greater capacity for osmotic regulation; mostly as intestinal and gill adaptations, drinking rate regulation and chloride cell proliferation an adaptive mechanism in hypersaline-tolerant fish species (Laverty and Skadhauge, 2012), we previously mentioned that in particular, red tilapia expresses a significant amount of physiological adaptations to high salinity conditions, including both at the cellular level (including cell proliferation and enzyme expression in several tissues such as yolk sac membrane, gills and intestine) and activated responses involving the secretion and production of specific hormones (growth hormone) and other molecules such as prolactin, and cortisol (Yan *et al*., 2013), such adaptations allow red tilapia *O. mossambicus* to reproduce even at 49 ‰ (Bhujel, 2000) and survive at salinity conditions as high as 120‰ (Whitfield and Blaber, 1979); consequently, observed hatching performance at 65‰ and 45‰ for RT and H1 respectively, provide relevant complimentary salinity adaptive capacity information for red tilapia and its hybrids.

The observed differences with respect to hatching rates as well as observed morphological variations across evaluated salinities have many possible explanations, one of them being the genetic component of red tilapia (*O. mossambicus*) in the tilapia strains assessed. This species has a remarkable capacity for osmotic regulation due to the proliferation of specific cells, similarly to what occurs with most marine fish larvae. Chloride cells are particularly relevant, in terms of osmotic regulation, as the salinity of the water in which the fish are reared increases (Kaneko *et al*., 2002). The proliferation of these cells is a direct physiological response of fish larvae regardless of their developmental stage. Although most larvae possess considerable osmotic tolerance, which can improve as the fish grows (Varsamos *et al*., 2005), still, determining the degree of proliferation of chloride cells in tissues such as yolk sac and gill epithelia is time consuming, as it requires microscopic histological observation; therefore, the hatching rate and the morphometric responses of hatched larvae could provide a fast criteria to assess the tolerance to direct transfer to saline water of both embryos and young tilapia in a follow-up experiment, with a significant histology component, predominantly for H2 as a more comprehensive characterization of salinity adaptation at early stages.

Previous similar experiments evaluated the incubation of eggs from breeders kept at different salinities using a single strain of Nile tilapia, indicating that if eggs are produced at higher salinities, the larvae have a better chance of successfully surviving specific salinity conditions (Watanabe *et al*., 1985). Although our breeders were kept in fresh water, the observed results on hatching rate suggest a preliminary approach, that is, to determine the breakpoint of salinity tolerance for this variable for each tested tilapia variety. These results are particularly relevant for Pargo-UNAM, a synthetic strain of red tilapia produced by a cross of Florida Red tilapia, Rocky Mountain Tilapia and red *O. niloticus* (Ramirez-Paredes *et al.*, 2012), given the genetic component of Florida red tilapia in this particular group and the fact that no previous information is available on this topic. Thus, the special management of breeders of this genetic line could be useful for increasing the salinity tolerance of tilapia offspring, perhaps by increasing the levels of protein and lipids in the diet, as recommended for Nile tilapia (El-Sayed *et al*., 2003).

There are many physiological implications of salinity tolerance on fish larvae, including those of tilapia; a key issue is the capability of mozambique tilapia for chloride cell proliferation in most teguments, particularly the tissue surrounding the yolk sac, similarly to what occurs with several marine fish (Kaneko *et al*., 2002). Thus, we propose further research to establish whether chloride cell proliferation could be used as valid criteria to determine if produced offspring of red tilapia crosses express tolerance to salinity when incubated in salinity conditions, particularly in the case of Pargo-UNAM (H2) and other new strains. Several preliminary trials have been conducted on the growth of H2 in marine water, with relevant results in terms of growth rates and low feed conversion ratios (Jimenez-Salcedo, 2012). Therefore, this particular criterion could indicate a significant phenotypic expression of salinity tolerance for early stages of this particular new strain of tilapia. However, if the physiological adaptation of salinity tolerance appears in later stages, once the fish reach a large size, as happens with most euryhaline fishes (Varsamos *et al*., 2005), then perhaps such an early transfer to seawater might be not advised for Pargo-UNAM given its hatching rate results above 25‰ salinity.

In the three trials conducted, we observed different patterns in the variables of total length and yolk sac utilization; for example, in RT and H2, salinity increments did produce a decrease in size for both strains. Whereas in RT there was a significant decrease of total yolk sack area of 10-15% when salinity ranged from 0 to 65‰, in H2 the decrease was of smaller magnitude, while in H1 we observed the opposite effect, the yolk sac area increasing in size at higher salinity values. For Nile tilapia, Fridman *et al*. (2012) reports that the mean

weight and standard length of the fish at hatching were significantly different between the evaluated salinities; the fish were smaller in length above 20 ‰ but showed higher mean dry weight at all salinities above 7.5 ‰. Several factors influence yolk sac size, such as the pre-reproductive management of broodstock, the protein and lipid composition of the yolk sac (Mccormick, 1999), and other factors such as the genetic contribution of the male, as significant changes in yolk sac size can be observed in haddock *Melanogrammus aeglefinus*, depending on the specific male used for larvae production (Nikolaus *et al*., 2006). In addition, it has been reported that, in fact, salinity changes reduce yolk sac size, while incubation of turbot *Scophthalmus maximus* at different salinity levels resulted in a significant reduction at lower salinities, a phenomenon that the authors attributed to higher energy expenditure for osmoregulation and buoyancy in early hatched larvae (Kamler, 2008). All the factors mentioned provide a potential explanation for the observed differences in yolk sac area at different salinity levels between the tilapia strains used in this study. Further work is needed, including repeated measurements (larger number of spawns) for each tilapia strain, to obtain a more comprehensive understanding of the described morphological dissimilarities between different tilapia strains as a potential criterion for an *a priori* estimation of salinity tolerance at early stages.

Observed differences in yolk sack area within each evaluated tilapia strain showed different tendencies, which could be related to different expression of salinity tolerance for each group. For example, RT and H2 performed in a similar fashion as yolk sack area decreased with each salinity increment; but H1 did not displayer the same trend as yolk sack reduction. On a first approach, is difficult to conclude what was the main factor that influenced such response, either as increment or decrement of yolk sack area for each strain; nevertheless there is a potential effect due to specific differences in water drinking rates correlated with circulating levels of cortisol as compensating osmoregulation mechanism for each strain, that reduces water content and increases dry weight in tilapia larvae, with minimal changes in yolk sack diameter as reported for *O mossambicus* at rearing salinities up to 35‰ (Lin *et al*., 2000) or a 30% reduction of dissolved protein per mg of dry weight in the same tilapia at salinities of 10 and 20‰ (Lee *et al*., 2005) that could account for the reduction in yolk sack area for RT and H2 as less material stored on this structure; however, individual chances and the magnitude of such differences on larvae dry weight at each salinity may represent more accurately increased yolk sack utilization in further studies.

These kind of studies are usually followed by physiological, functional genomics and proteomic studies, given their importance for

outlining the relationship between metabolism and osmoregulatory physiology, which is particular relevant for tilapia aquaculture (Cnaani and Hulata, 2011). Thus, the main differences between Red tilapia and Nile tilapia in terms of adaptive physiological responses are gene expression of prolactin, GH secretion and enzyme activity in the gills, specifically the response time of these mechanisms (Velan *et al*., 2011). There is little information on the subject for tilapia hybrids. Other authors, taking a more drastic approach, have attempted to increase the salinity tolerance of tilapia by incorporating foreign DNA into Nile tilapia gonads by means of microinjection, using sea bream *Sparus aurata* and *Artemia salina* DNA, which show high salinity tolerance but also considerable growth and fry production rates (El-Zaeem *et al*., 2013). In the meanwhile, the differences in hatching rates and the morphological variations produced by different salinity levels could serve as a first-line analysis for salinity acclimation of a particular tilapia strain using a low cost analysis in hatchery conditions.

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