1 Rearing Mozambique tilapia in tidally-changing salinities: effects on growth and the growth
2 hormone/insulin-like growth factor I axis.

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Abstract

The growth hormone (GH)/insulin-like growth factor (IGF) axis plays a central role in the regulation of growth in teleosts and has been shown to be affected by acclimation salinity. This study was aimed at characterizing the effects of rearing tilapia, *Oreochromis mossambicus*, in a tidally-changing salinity on the GH/IGF axis and growth. Tilapia were raised in fresh water (FW), seawater (SW), or in a tidally-changing environment, in which salinity is switched between FW (TF) and SW (TS) every 6 h, for 4 months. Growth was measured over all time points recorded and fish reared in a tidally-changing environment grew significantly faster than other groups. The levels of circulating growth hormone (GH), insulin-like growth factor I (IGF-I), pituitary GH mRNA, gene expression of IGF-I, IGF-II, and growth hormone receptor 2 (GHR) in the muscle and liver were also determined. Plasma IGF-I was higher in FW and TS than in SW and TF tilapia. Pituitary GH mRNA was higher in TF and TS than in FW and SW tilapia. Gene expression of IGF-I in the liver and of GHR in both the muscle and liver changed between TF and TS fish. Fish growth was positively correlated with GH mRNA expression in the pituitary, and GHR mRNA expression in muscle and liver tissues. Our study indicates that rearing fish under tidally-changing salinities elicits a distinct pattern of endocrine regulation from that observed in fish reared in steady-state conditions, and may provide a new approach to increase tilapia growth rate and study the regulation of growth in euryhaline fish.

1. Introduction

Growth in vertebrates is largely controlled through the coordinated activities of the growth hormone (GH)/insulin-like growth factor (IGF) axis. GH is an adenohypophysial hormone secreted from the anterior pituitary. In teleosts, GH is involved in the regulation of most major physiological processes such as growth, osmoregulation, metabolism, reproduction, development, and feeding behavior (Madsen and Bern, 1993; McLean et al., 1993; Duan, 1997; Shepherd et al., 1997; Kawauchi and Sower, 2006). GH and its mediator, insulin-like growth factor I (IGF-I), have been demonstrated to facilitate SW adaptation in several salmonids (McCormick et al., 1991; Sakamoto et al., 1991) and in the euryhaline Mozambique tilapia, *Oreochromis mossambicus* (Yada et al., 1994; Sakamoto et al., 1997; Fruchtmann et al., 2000). In the liver and muscle, GH directly stimulates mitosis and differentiation among other cellular functions, and indirectly initiates the production and release of IGF-I and IGF-II, which have been linked to a variety of growth-promoting actions in most tissues (Butler and Le Roith, 2001; Wood et al., 2005; Fan et al., 2009; Duan et al., 2010). GH has been shown to stimulate hepatic gene expression and plasma levels of IGF-II both *in vivo* and *in vitro* in teleosts (Reindl and Sheridan, 2012). In tilapia, IGF-II has been shown to stimulate growth in juvenile fish (Chen et al., 2000).

GH and IGFs act by binding to membrane receptors (Kajimura et al., 2004; Pierce et al., 2007; Duan et al., 2010). In Mozambique tilapia, two GHRs have been discovered and denoted GHR1 and GHR2 (Kajimura et al., 2004; Pierce et al., 2007). Phylogenetic analyses, tissue distribution of mRNAs, and regulation by GH of GHR1 and GHR2 suggest that GHR2 is the primary GH receptor in tilapia (Kajimura et al., 2004; Pierce et al., 2007; Pierce et al., 2012). Therefore, we will focus on GHR2, henceforth referred to as GHR.

The Mozambique tilapia is a euryhaline teleost with a worldwide tropical distribution. Its native habitat includes estuaries and near-shore rivers from the lower Zambezi River to the southeast coast of South Africa not generally more than a mile from the tidal ebb and flow (Trewevas, 1983). Hence, the examination of growth in tilapia that have been reared in tidally-changing salinities may better reflect their natural environmental conditions.

Previous studies on the intersections between environmental salinity, growth, and the GH/IGF axis in Mozambique tilapia have focused on the effects of fasting and refeeding within various steady-state salinities; these studies varied the amount and protein content of feed,

hormonal supplementation, and acclimation salinity (Ron et al., 1995; Shepherd et al., 1997; Riley et al., 2003; Sparks et al., 2003; Uchida et al., 2003; Fox et al., 2006; Magdeldin et al., 2007; Pierce et al., 2007; Fox et al., 2010). Mozambique tilapia reared in seawater (SW) grow faster than fish reared in fresh water (FW) or brackish water (BW) (Kuwaye et al., 1993; Ron et al., 1995; Shepherd et al., 1997; Riley et al., 2002; Riley et al., 2003; Sparks et al., 2003). The faster growth of tilapia in SW seems, at least in part, to be attributed to activation of the GH/IGF axis. Circulating and pituitary GH have been shown to increase in response to a transfer from FW to SW, and pituitary GH release has been found to increase in response to elevation of extracellular osmolality *in vitro* (Borski et al., 1994; Seale et al., 2002; Seale et al., 2006; Pierce et al., 2007; Breves et al., 2010b; Breves et al., 2010c). Several studies suggest that pituitary GH mRNA, however, may be a better indicator of growth than circulating GH and IGF-I levels (Riley et al., 2002; Riley et al., 2003; Magdeldin et al., 2007). Other studies point to conflicting effects of salinity on the GH/IGF system. While some authors have shown greater hepatic IGF-1 expression in SW-acclimated tilapia (Magdeldin et al., 2007), others have reported the opposite trend during a transfer from FW to SW (Link et al., 2010). While interesting, results from one-way salinity transfers are generally limited to a time course that may neither fully reflect long-term rearing salinities nor dynamically-changing salinities.

In recent studies, we examined how osmoregulatory mechanisms are affected by rearing Mozambique tilapia in tidally-changing salinities that are characterized by changes between FW and SW every 6 hours (Moorman et al., 2014; Moorman et al., 2015). While we found that tilapia reared in tidally-changing salinities are able to maintain a narrow range of osmoregulatory parameters that are similar to those of SW-acclimated fish (Moorman et al., 2015), the effects of this dynamic rearing paradigm on growth and the endocrine control of growth have remained unexplored. Hence, the main goal of this study was to determine how rearing Mozambique tilapia in a tidally-changing salinity affects growth and the GH/IGF axis, and how these parameters differ from fish reared in steady-state FW and SW. To this end, we measured plasma levels of GH and IGF-I, pituitary GH mRNA expression, and gene expression of IGF-I, IGF-II, and GHR in muscle and liver.

2. Materials and Methods

2.1 Fish Maintenance and treatments

Mozambique tilapia (*O. mossambicus*) yolk-sac fry were collected from broodstock tanks maintained in FW at the Hawai'i Institute of Marine Biology (Kāne'ohe, HI). In each of the replicate experiments fish were transferred into tanks using the following method. The collected fry were kept in 75 L glass aquaria supplied with circulating FW until yolk-sac absorption was complete. Fry were then combined into one 75 L aquarium containing FW. Two days after yolk-sac absorption they were distributed into ten 75 L glass aquaria supplied with FW (3 L/min) and stocked at a density of 100 fry per tank (mean weight, 12 ± 1 mg). Water temperature was maintained at $25 \pm 1^\circ\text{C}$ in all tanks. The fish were exposed to a 12L:12D cycle. After two days in FW, eight of the ten tanks were transitioned from FW to BW (10 ‰) over the course of 3 h; BW was composed of sea water (SW; 35 ‰, Kāne'ohe, HI) diluted with FW. After an additional two days in BW, two of the BW tanks were transitioned to SW and the other six tanks were maintained under a tidally-changing salinity. As a result, the salinity of the ten tanks was set as follows: two tanks with FW, two tanks with SW, and six tanks maintained in a tidally-changing salinity.

Salinity in the tanks subjected to a tidally-changing regime alternated between FW and SW every 6 h, yielding a complete salinity transfer within 1.5 h. The flow of FW and SW was controlled by submersible pumps connected to electric timer outlets that were set out of phase by 6 h, as previously described (Moorman et al., 2014). The fish were maintained in these conditions until sampling. After two months, half of the fish were removed from each tank to reduce crowding. Forty fish from each tank were sampled after four months. Of the six tidal tanks, three tanks of fish were sampled at the end of the FW phase (tidal FW; TF) and the other three tanks of fish were sampled at the end of the SW phase (tidal SW; TS). The experiment was replicated using 10 different tanks, which were arranged in a different order than the first experiment to control for tank effects. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

2.2 Diet and Feeding

Fish were fed crushed Silver Cup Flake feed (Silver Cup, Harrietta, MI; consisting of not less than 41% protein and 6% fat and not more than 3% fiber and 12% ash) *ad libitum* twice daily, once in the morning between 8AM and 10AM and once in the late afternoon between 2PM and 4PM. Feed was crushed into a powder for feeding fry, placed into individual vials for each tank, and weighed at the beginning of each day. In feeding, first a portion of the feed in each vial was added to each tank. After 30 min the tanks were visually inspected, and if no feed remained in the tank, additional feed was added; this cycle of feeding and inspection at every 30 min was repeated until there was feed remaining in the tank. At the end of the day, the weight of the feed vial with any remaining feed was recorded and subtracted from the weight of the vial and feed at the beginning of the day to determine feed consumption in each tank. After two months fish were provided 2 mm Trout Chow pellets (Skretting, Tooele, UT) instead of flake food. The method of feeding remained the same as with the flake food.

2.3 Sampling

To determine the differences in growth rates between the treatments throughout the experiment, fish were weighed at 25 days following the start of the experiment and every 21 days thereafter, until termination. All fish were removed from the tanks and individually weighed to avoid any biases associated with sampling a subset of the tank. Sample size for weight data was based on the number of fish in each treatment; 400 in FW, 400 in SW, and 1200 in tidal tanks.

After four months, fish were netted and anesthetized with 2-phenoxyethanol (0.3 ml/L). Blood was collected with a needle and syringe coated with sodium heparin (200 U/ml, Sigma-Aldrich, St. Louis, MO). Plasma was separated by centrifugation (10 min at 12,000 G) and stored at -80°C until later analyses. Following blood collection, fish were euthanized by rapid decapitation. Muscle, liver, and pituitary samples were harvested and then immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. Plasma and tissues were sampled from 10 fish taken from 4 to 6 tanks for each treatment (4 tanks in FW and SW, and 6 tanks in TF and TS), totaling 35 to 60 individual samples for each salinity regime.

2.4 Feed Conversion Ratio and Specific Growth Rate

Feed conversion ratio (FCR) was calculated for all fish in each experimental group at each time point using the following formula:

$$FCR = \text{Total amount of dry feed consumed (g)} / (W_f - W_i)$$

The denominator stands for the wet weight gain of fish, that was calculated by subtracting the weight of all fish within a tank at the beginning of the sampling period (W_i) from that of the end of the sampling period (W_f).

Specific growth rate (SGR) was calculated for each treatment for the entire experiment using the following formula:

$$SGR = (\ln(W_f) - \ln(W_i)) / (\text{time in days}) * 100$$

The sample size for FCR and SGR is based upon the number of tanks of a given treatment combined from two replicate experiments; 4 FW tanks, 4 SW tanks, and 12 tidal tanks.

2.5 Quantitative real-time PCR (qPCR)

Total RNA was extracted from frozen muscle and liver samples with TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Using the High Capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), 500 ng of total RNA was reverse transcribed into first-strand cDNA. The quantitative real-time PCR (qPCR) was set up as previously described (Pierce et al., 2007), using primer pairs listed in Table 1. To generate a standard curve for quantification, cDNAs transcribed from total RNAs of muscle or liver of FW fish were serially diluted and used as the standard templates. The PCR mixture (15 μ L) contained Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), 200 nM of forward and reverse primers, and 2 μ L of cDNA with a concentration of 250ng/ μ L. The following cycling parameters were employed using the StepOnePlus real-time PCR system (Applied Biosystems): 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The R^2 values and amplification efficiencies for standard curves were as follows: 0.993 and 98.2% (elongation factor 1 α , EF1 α), 0.996 and 93.7% (GH), 0.981 and 88.3% (IGF-I), 0.997 and 86.3% (IGF-II), and 0.995 and 83.4% (GHR). After verification

that expression did not vary across treatments (muscle, $P = 0.14$; liver, $P = 0.39$; pituitary, $P = 0.75$), EF1 α was used as a reference to normalize target genes. Data are expressed as fold-change from FW values.

2.6 Plasma Parameters

Plasma levels of GH were measured by homologous radioimmunoassay (Ayson et al., 1993). For plasma IGF-I measurement, total IGF-I was extracted from 25 μ l of plasma with 100 μ l of acid-ethanol, as previously described (Shimizu et al., 1999; Riley et al., 2003; Fox et al., 2010). Total IGF-I extracted was then measured by a radioimmunoassay employing recombinant salmon IGF-I as a standard and anti-barramundi IGF-I antiserum (GroPep, Adelaide, Australia) (Shimizu et al., 2000; Riley et al., 2003; Fox et al., 2010).

2.7 Statistical Analysis

Data for each parameter were analyzed by two-way analysis of variance (ANOVA), with tank replicate and salinity regime as main factors. After the absence of tank effects were verified, the data from replicate tanks within each salinity regime were combined and statistical analyses were conducted using a one-way (ANOVA), with salinity regime as the independent variable. Significant main effects of salinity regime ($P < 0.05$) were followed up by the Bonferroni multiple comparison test. Data are expressed as means $\pm$ S.E.M. Data were tested and met assumptions of normality and equal variance. Statistical calculations were performed using a statistical software program, Prism 5.0 (GraphPad, La Jolla, CA).

3. Results

3.1 Growth and feed conversion ratio

The effects of salinity on the growth of the tilapia are shown in Fig. 1. Tilapia reared in a tidally-changing environment grew significantly faster than fish reared in FW or SW ($P < 0.001$).

Differences in growth between the treatments were seen at the first 25 days of the experiment and persisted through the end of the experiment.

There was a significant difference in FCR at the 46-day time point ($P < 0.01$); SW fish showed the highest FCR followed by FW fish, and tidal fish had the lowest. The difference between FCR of SW and that of tidal fish was statistically significant ($P < 0.01$) (Table 2). Specific Growth Rate was significantly higher for fish reared in a tidally-changing salinity than fish reared in FW or SW ($P < 0.05$) (Table 2).

3.2 Plasma GH, IGF-I, and pituitary GH expression

No effect of tank replicate was observed on plasma GH and IGF-1 levels ($P = 0.19$ and 0.15 , respectively), nor in pituitary GH mRNA expression ($P = 0.13$). Plasma GH was significantly higher in TF fish than in FW and SW fish ($P < 0.05$) (Fig. 2A). There was no significant difference in plasma IGF-I between FW fish and TS fish. Plasma IGF-I was significantly lower in SW and TF fish than in FW and TS fish ($P < 0.05$) (Fig. 2B). Pituitary GH mRNA expression was the lowest in FW fish, intermediate in SW fish, and highest in TF and TS fish, with significant differences among levels in FW, SW and tidal fish ($P < 0.001$) (Fig. 2C).

3.3 Muscle and Liver gene expression of IGF-I, IGF-II, and GHR

There was no effect of tank replicate on muscle mRNA expression of IGF-I, IGF-II, and GHR ($P = 0.10$, 0.15 , and 0.06 , respectively), nor on hepatic mRNA expression of IGF-I, IGF-II, and GHR ($P = 0.10$, 0.17 , and 0.09 , respectively). IGF-I gene expression in muscle was significantly higher in SW fish than in FW fish ($P < 0.05$). Nevertheless, there was no significant difference in muscle IGF-I gene expression between the two phases of the tidal cycle, which was intermediate to FW and SW fish (Fig. 3A). There were no significant differences in the gene expression of IGF-II in the muscle of tilapia in any of the sampled conditions (Fig. 3B). GHR gene expression in the muscle of FW, SW and TS fish did not differ significantly, while GHR expression in TF fish was 2.5-fold greater compared with TS fish ($P < 0.001$) (Fig. 3C).

Hepatic expression of IGF-I of TF fish was 2-fold greater than that in TS fish ($P < 0.001$). Conversely, hepatic IGF-I expression did not differ significantly among FW, SW and TF

fish (Fig. 4A). Hepatic IGF-II expression was 2-fold greater in SW fish than in FW fish ($P < 0.01$). In tidal groups, on the other hand, hepatic IGF-II expression was 2-fold greater in TF fish than in TS fish ($P < 0.01$) (Fig. 4B). Hepatic GHR gene expression was significantly greater in TF fish than in TS fish ($P < 0.01$). There was no significant difference in the hepatic GHR gene expression between FW and SW fish (Fig. 4C).

3.4 Correlations

To determine if there were correlations between growth and the other parameters of the GH/IGF axis measured in this study, we calculated the linear regression of each plasma parameter and expression of each target gene against the total weight of the corresponding fish. The strongest positive correlation was found between pituitary GH mRNA expression and growth ($R^2 = 0.13$; $P < 0.001$). Additionally, there were significant positive correlations between muscle GHR mRNA expression and growth ($R^2 = 0.05$; $P < 0.01$), and between hepatic GHR mRNA expression and growth ($R^2 = 0.05$; $P < 0.01$). There was no correlation, however, between growth and plasma IGF-I ($R^2 = 0$; $P = 0.60$).

4. Discussion

The purpose of this study was to characterize patterns of growth and differences in responses of the GH/IGF axis in Mozambique tilapia reared in a tidally-changing environment in comparison with fish raised in FW and SW. Our major findings are: 1) fish reared in a tidal environment grew faster than those reared in steady-state environments (Fig. 1 and Table 2); 2) fish reared in a tidal environment showed oscillation patterns between the two phases of the tidal cycle with respect to plasma IGF-I, muscle GHR gene expression, and hepatic IGF-I, IGF-II, and GHR gene expression, while pituitary GH gene expression was consistently higher in both phases of the tidal cycle when compared with fish reared in steady-state environments (Figs. 2, 3 and 4).

While different growth rates between fish reared in different steady-state salinities have been observed in several euryhaline teleosts, including a variety of species of tilapia, blenny, and European seabass (Ron et al., 1995; Plaut, 1999; Boeuf and Payan, 2001; Sparks et al., 2003), the

examination of growth under long-term dynamically-changing salinities has not been previously explored. By experimentally simulating a tidal cycle, we have found for the first time that a dynamically-changing salinity regime enhances growth in a euryhaline teleost when compared with fish kept in steady-state FW or SW. Previous studies with Mozambique tilapia have demonstrated differences in growth between fish reared in FW versus SW. By the end of six to seven month long experiments, researchers found that SW fish grew 2-times faster than FW fish, but significant differences were not seen during the first 4 months (Kuwaye et al., 1993; Ron et al., 1995). In other studies, growth rate was reduced when fish were transferred from SW to FW, for at least 21 days (Riley et al., 2003; Magdeldin et al., 2007). It is worth mentioning, that a major difference with the earlier experiments in Mozambique tilapia is that in the current study, fish were fed *ad libitum* and the amount of feed eaten quantified, while in previous studies fish were fed different percentages of their body weight based on their age, and the calculation of feed amount was based on the weight of the fish at the start of a 30 day weigh period (Kuwaye et al., 1993; Ron et al., 1995; Sparks et al., 2003). This makes it unclear whether the fish were being overfed or underfed and does not account for the weight of uneaten feed. When comparing the effects of different treatments on growth in the studies above, it is not possible to determine whether the different growth rates of experimental groups derive from differences in the feed consumption or from the efficiency by which feed is converted into body mass, since FCR was not examined (Kuwaye et al., 1993; Ron et al., 1995; Sparks et al., 2003). In the current study, we found that the difference in growth among experimental groups was not explained by differences in feed consumption as fish reared in tidally-changing salinities had the same or lower FCR (i.e. by 46 days) than other groups. Previous work on Nile tilapia has shown that FCR is dependent on fish size, with smaller fish having lower FCRs than larger fish (Teshima et al., 1987). Nonetheless, we found that that even though fish in a tidally-changing salinity were larger, they had a lower FCR than the smaller fish reared in FW or SW. In addition, fish reared in a tidally-changing salinity had a higher SGR than FW and SW fish. Together, these results indicate that fish reared in tidally-changing salinities not only grow at a faster rate, but also possess greater efficiency of converting fish feed into body mass than fish reared in steady-state salinities.

The GH/IGF axis has been shown to be the main regulator of growth in vertebrates (McCormick et al., 1992; Duan and Plisetskaya, 1993; Negatu and Meier, 1995; Moriyama et al.,

2000; Uchida et al., 2003; Norbeck et al., 2007) and, in many fish species, such as Coho salmon, Gulf killifish, Rainbow trout, and Mozambique tilapia, the endocrine mediator of the effects of environmental salinity on growth (McCormick et al., 1991; Borski et al., 1994; Sakamoto et al., 1997; Sakamoto and McCormick, 2006; Pierce et al., 2007). In tilapia, while the effects of environmental salinity on plasma GH levels have been somewhat inconsistent (Riley et al., 2003; Pierce et al., 2007; Breves et al., 2011), pituitary GH mRNA expression has been demonstrated to be a better indicator of growth (Riley et al., 2003; Magdeldin et al., 2007; Breves et al., 2010b). In the current study, we found no significant difference in plasma GH levels between FW and SW fish, while plasma GH was significantly higher in TF fish than either of the steady-state salinities. Furthermore, there was no correlation between growth and plasma GH in any of the experimental groups, supporting the previous findings that plasma GH is a poor indicator of growth (Riley et al., 2003). By contrast, of all endpoints measured, the best indicator of growth was pituitary GH mRNA, which was highest in fish sampled at TF and TS relative to those in steady-state salinities. Higher gene expression of GH was also observed in the pituitary of SW fish relative to FW fish. This is consistent with the finding that total pituitary GH content was shown to be higher in fish reared in SW than in FW, suggesting a role for GH in SW osmoregulation (Borski et al., 1994). While pituitary GH expression was consistently high in both TF and TS fish, GHR expression in muscle and liver was higher in TF fish than in TS fish. The combination of higher pituitary GH expression in fish in a tidally changing salinity and higher GHR expression in the muscle and liver of TF fish may underlie the elevated growth in fish reared under a tidal regimen, despite the differences in the latter between TF and TS fish. We are uncertain of the reason for the high GHR gene expression in TF fish; it may be due to a lag in upregulation of GHR mRNA from the last exposure to SW, or due to anticipation of the upcoming SW phase. A future time course study will be needed to better understand the cause of this response.

IGF-I mediates many of GH's growth-promoting actions in teleosts (McCormick et al., 1991; Duan, 1997). In Mozambique tilapia, plasma IGF-I has been shown to be affected by environmental salinity and nutritional state. Plasma IGF-I in tilapia is elevated in SW fish compared with FW, and hepatic IGF-I mRNA expression rises as environmental salinity increases (Fiess et al., 2007; Magdeldin et al., 2007). Plasma IGF-I is also elevated in fed fish compared with fasted fish, while plasma GH is higher in fasted fish compared with fed fish

(Uchida et al., 2003; Fox et al., 2006; Fox et al., 2010; Breves et al., 2014). In addition, it has been shown that IGF-I mRNA expression in hepatocytes is stimulated by GH (Pierce et al., 2011). In the present study, we found that IGF-I mRNA expression in the muscle is significantly higher in SW fish than in FW fish, while there was no variation between TF and TS fish. By contrast, hepatic gene expression of IGF-I did not vary between FW and SW fish, but was significantly different between TF and TS fish. Based on our current findings and those of Magdeldin and colleagues cited above, it is possible that the increase of hepatic IGF-I expression in TF fish occurs in anticipation of the SW entry that the fish regularly experience every 6 hours.

Muscle IGF-II expression has been shown to be sensitive to nutritional status (Fox et al., 2010), but had not been previously examined in tilapia with respect to changes in salinity. We observed no effect of salinity on muscle IGF-II mRNA expression, but found that fish reared in SW had higher hepatic IGF-II mRNA expression than those in FW; hepatic IGF-II mRNA expression was also higher in fish sampled in TF relative to TS, indicating dynamic regulation of this transcript within 6 h. As with hepatic IGF-I expression, it is unclear whether the fluctuations of hepatic IGF-II mRNA are driven by the combined changes in circulating GH and GHR availability. Together with other parameters that we found to vary between TF and TS, it is possible that the expression of IGFs in liver is directly sensitive to changes in plasma osmolality experienced by the fish as salinity varies over the tidal cycle, or that fluctuations are driven by a circum-tidal rhythm, entrained over the course of four months of rearing in tidally-changing conditions (Moorman et al., 2014). It would be of value to sample fish at short intervals during each phase of the tidal cycle in combination with a parallel sampling of fish for which the change in salinity is delayed.

In summary, the rearing paradigm for Mozambique tilapia employed in this study provided a means with which to study growth and the GH/IGF axis of euryhaline fish under a simulation of naturally occurring dynamic salinity conditions. Mozambique tilapia reared under tidally-changing salinities grew faster than fish reared in either FW or SW and showed patterns of gene expression of elements of the GH/IGF axis that differed from those observed in fish held in constant FW or SW conditions, such as the increase and decrease of GHR mRNA levels in muscle and liver with each phase of the tidal cycle. Tidally-changing salinity is characteristic of this tilapia's native habitat and we hypothesize that it has undergone selection to perform best under these conditions. Together with our previous studies, these findings suggest that tilapia

reared in tidally-changing salinities exhibit a markedly different endocrine control of osmoregulation and growth compared with that observed in steady-state environments. Our findings further suggest that rearing fish under tidally-changing salinities may provide a new approach to increase tilapia growth rate without increasing the amount of feed and invites further studies into the nature of growth regulation under conditions where salinity changes cyclically.

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Figure Legends:

Figure 1. The effects of rearing condition on the growth of the tilapia during 1-109 days post yolk-sac absorption. Values are expressed as means $\pm$ S.E.M. ($n = 400-1200$ for the first 3 time points and $n = 150-450$ for the last 3 time points). Means not sharing the same letter are significantly different (one-way ANOVA followed by Bonferroni multiple comparison test, $P < 0.05$). Measurements during 1-25 days are also shown as an inset with magnified Y axis.

Figure 2. Effects of rearing condition on plasma GH (A), plasma IGF-I (B), and pituitary GH mRNA expression (C) in tilapia sampled from different salinity conditions. Values are expressed as means $\pm$ S.E.M. ($n = 35-60$, from 4 to 6 replicate tanks depending on treatment). Means not sharing the same letter are significantly different (one-way ANOVA followed by Bonferroni multiple comparison test, $P < 0.05$).

Figure 3. Effects of rearing condition on muscle mRNA expression of IGF-I (A), IGF-II (B), and GHR (C) in tilapia sampled from different salinity conditions. Values are expressed as means $\pm$ S.E.M. ($n = 35-60$, from 4 to 6 replicate tanks depending on treatment). Means not sharing the same letter are significantly different (one-way ANOVA followed by Bonferroni multiple comparison test, $P < 0.05$).

Figure 4. Effects of rearing condition on hepatic mRNA expression of IGF-I (A), IGF-II (B), and GHR (C) in tilapia sampled from different salinity conditions. Values are expressed as means $\pm$ S.E.M. ($n = 35-60$, from 4 to 6 replicate tanks depending on treatment). Means not sharing the same letter are significantly different (one-way ANOVA followed by Bonferroni multiple comparison test, $P < 0.05$).

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Table 1. Primers used for qPCR

Gene Name	Primer Sequence (5'-3')	Reference	Accession Number
EF1 α	Forward AGCAAGTACTACGTGACCATCATTG Reverse AGTCAGCCTGGGAGGTACCA	(Breves et al., 2010a)	AB075952
GH	Forward TTACATCATCAGCCCGATCG Reverse AGATCGACAGCAGCTTCAGGA	(Magdeldin et al., 2007)	AF033806
GHR	Forward CACACCTCGATCTGGACATATTACA Reverse CGGTTGGACAATGTCATTAACAA	(Pierce et al., 2007)	EF452496
IGF-I	Forward CTGCTTCCAAAGCTGTGAGCT Reverse GATCGAGAAATCTTGGGAGTCTTG	(Kajimura et al., 2004)	AF033796
IGF-II	Forward GCTTTTATTTTCAGTAGGCCAACCA Reverse CACAGCTACAGAAAAGACACTCCTCTA	(Davis et al., 2008)	AH006117

EF1 α : Elongation Factor 1 α ; GH: Growth Hormone; GHR-2: Growth Hormone Receptor 2; IGF-I: Insulin-like Growth Factor I; IGF-II: Insulin-like Growth Factor II

Table 2. Feed Conversion Ratio (FCR) and Specific Growth Rate (SGR) of Mozambique tilapia reared in FW, SW, and a tidally-changing salinity. Values are expressed as means $\pm$ S.E.M. (n = 4-12). The combined data of two replicate experiments gives a sample size of 4 FW tanks, 4 SW tanks, and 12 tidal tanks. Means not sharing the same letter are significantly different from the other treatments at the same time point (one-way ANOVA followed by Bonferroni multiple comparison test, $P < 0.05$).

Treatment	FCR (Day 1-25)	FCR (Day 26-46)	FCR (Day 47-67)	FCR (Day 68-88)	FCR (Day 89-109)	SGR (% of body mass/day) (Day 1-109)
FW	1.89 $\pm$ 0.21	1.46 $\pm$ 0.23 ^{AB}	1.04 $\pm$ 0.16	0.84 $\pm$ 0.04	0.98 $\pm$ 0.13	6.51 $\pm$ 0.07 ^B
SW	1.57 $\pm$ 0.07	1.54 $\pm$ 0.52 ^B	0.90 $\pm$ 0.00	0.80 $\pm$ 0.02	0.93 $\pm$ 0.07	6.62 $\pm$ 0.06 ^B
Tidal	1.33 $\pm$ 0.17	1.05 $\pm$ 0.05 ^A	0.82 $\pm$ 0.03	0.76 $\pm$ 0.02	0.91 $\pm$ 0.02	6.80 $\pm$ 0.03 ^A

Figure 1

Figure 2

Figure 3

Figure 4