

# Somatotropic actions of the homologous growth hormone and prolactins in the euryhaline teleost, the tilapia, *Oreochromis mossambicus*

(insulin-like growth factor/growth promotion)

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**ABSTRACT** It is increasingly clear that growth hormone (GH) has growth-promoting effects in fishes, which are mediated in part by the insulin-like growth factor (IGF)-I. Growth-promoting actions of prolactin (PRL) have been reported in higher vertebrates, but are less well established in teleosts. We examined the effects of injecting homologous GH or the two homologous tilapia PRLs (tPRL<sub>177</sub> and tPRL<sub>188</sub>) on the *in vitro* incorporation of [<sup>35</sup>S]sulfate (extracellular matrix synthesis) and [<sup>3</sup>H]thymidine (DNA synthesis) by ceratobranchial cartilage explants and on IGF-I mRNA levels in tilapia liver. Tilapia GH (tGH) and tPRL<sub>177</sub> stimulated sulfate uptake at the highest doses examined. Thymidine incorporation was stimulated by tPRL<sub>177</sub>. tPRL<sub>188</sub> was without these effects. Consistent with its somatotropic actions, tGH elevated IGF-I mRNA levels in the liver. tPRL<sub>177</sub> also elevated liver IGF-I levels. Consistent with the previously described osmoregulatory actions of GH and PRL in teleosts, we observed that tGH elevated and tPRL<sub>177</sub> and tPRL<sub>188</sub> lowered levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. High-affinity, low-capacity binding sites for tGH in the tilapia liver were identified. tPRL<sub>177</sub> binds with lower affinity than tGH to these sites but can displace <sup>125</sup>I-labeled tGH from its receptor. The ability of tPRL<sub>177</sub> to displace tGH was similar to that of ovine GH. tPRL<sub>188</sub> did not displace <sup>125</sup>I-labeled tGH binding. Collectively, this work suggests that tPRL<sub>177</sub> may possess somatotropic actions similar to tGH, but only in freshwater tilapia where tPRL<sub>177</sub> levels are sufficiently high for it to act as a competitive ligand for GH receptors.

Growth hormone (GH), prolactin (PRL), and somatolactin belong to a family of hormones that share similarities in structure and function. Based on the similarities in structure, function, and gene sequences, it has been proposed that these hormones evolved from a common ancestral gene through duplication and subsequent divergence (1, 2, 53).

GH regulates growth in all vertebrates, including fish (2, 3). Recently, GH has been implicated in seawater osmoregulation of salmonid and cichlid teleosts (4–6). The actions of GH are mediated to an important degree through insulin-like growth factor (IGF)-I (7, 8), and the primary source of circulating IGF-I is the liver (8).

PRL is the most versatile of the pituitary hormones. It shows lactogenic, luteotropic, mitogenic, somatotropic, metamorphic, antimetamorphic, and osmoregulatory activities (9–14).

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Among teleosts, the most prominent action of PRL is its osmoregulatory role in freshwater adaptation (10). Since it may be argued that the somatotropic activities ascribed to PRL result from the binding of heterologous PRL to GH receptors (15, 16), more interest has been directed toward examining the growth-promoting and osmoregulatory actions of homologous hormones in teleosts.

Two forms of PRL have been identified in the tilapia, one 177 aa (tPRL<sub>177</sub>) and the other 188 aa (tPRL<sub>188</sub>; refs. 17 and 18). Each is encoded by a separate gene, suggesting unique roles for these two PRLs in tilapia (19). Studies aimed at identifying unique actions of each tPRL have not revealed consistent differences, but it is clear that both hormones facilitate osmoregulatory adaptation to freshwater (17, 20, 21).

Until now, evidence for the somatotropic actions of homologous PRL in teleosts is largely lacking. We examined the effects of homologous tGH, tPRL<sub>177</sub>, and tPRL<sub>188</sub> on growth in hypophysectomized (Hx) tilapia, as determined by ceratobranchial cartilage [<sup>35</sup>S]sulfate and [<sup>3</sup>H]thymidine incorporation *in vitro* and measurements of liver IGF-I mRNA levels. Since the osmoregulatory and growth-promoting actions of GH and PRL are specific and depend upon circulating levels and the availability and specificity of tissue receptors, we evaluated the affinity of hepatic GH receptors for <sup>125</sup>I-labeled tGH. We also evaluated the actions of tGH and tPRLs on gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity.

## MATERIALS AND METHODS

**Animals and Hypophysectomy.** Animal husbandry is described elsewhere (4, 22). Mature tilapia (*Oreochromis mossambicus*) of both sexes (35–90 g) were Hx as described elsewhere (23). Postoperative procedures are also described elsewhere (4).

**Hormones.** Tilapia pituitary tPRL<sub>177</sub>, tPRL<sub>188</sub>, and tGH were administered by intraperitoneal injection every 2 days for 8 days as described elsewhere (4). In this study, we employed hormone doses of 15 (low), 150 (medium), and 500 (high) ng per g of body weight.

**Experimental Procedure.** Experimental procedures for the injection studies are described elsewhere (4), with the follow-

Abbreviations: GH, growth hormone; tGH, tilapia GH; PRL, prolactin; tPRL<sub>177</sub> and tPRL<sub>188</sub>, 177-aa and 188-aa tilapia PRL, respectively; IGF, insulin-like growth factor; Hx, hypophysectomized.

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ing modifications. Twenty-four hours after the last injection, gill arches were removed and ceratobranchial cartilages were prepared for incubation. Liver tissue and gill filaments were removed, flash-frozen, and stored at  $-80^{\circ}\text{C}$  for subsequent IGF-I mRNA analysis and  $\text{Na}^+, \text{K}^+$ -ATPase analysis, respectively.

**Ceratobranchial Cartilage Bioassay.** The methods employed are those of Duan (24) as modified by Shepherd *et al.* (25) with the following exceptions: (i) no tilapia plasma was used in the incubation medium, and (ii)  $2.5 \mu\text{Ci/ml}$  ( $1 \text{ Ci} = 37 \text{ GBq}$ ) of  $[^3\text{H}]$ thymidine and  $5.0 \mu\text{Ci/ml}$   $[^{35}\text{S}]$ sulfate (DuPont/NEN) were used in the incubation medium.

**Hepatic GH Radioreceptor Assay.** Our methods were identical to those of Hirano (26), which have been used successfully to study liver hormone receptors in different teleost species. Using these procedures, we found that tilapia liver membranes demonstrated high specific binding of  $^{125}\text{I}$ -labeled tGH, which was linear up to 100 mg protein and saturable with increasing amounts of membrane protein. The optimal pH for specific binding was 7.4, and equilibrium was achieved after 15 hr at  $15^{\circ}\text{C}$ .

**RNase Protection Assay.** Total RNA was isolated from frozen tissues by the method of Chomczynski and Sacchi (27) and subsequently quantified by UV spectrophotometry. cDNAs encoding mature IGF-I (B-D domains) derived from teleost species are highly conserved; however, the identity between rainbow trout IGF-I and IGF-II is only 46.1% (2, 28). Total IGF-I mRNA transcripts were measured using a riboprobe complementary to a 213-bp region coding for all rainbow trout IGF-I mRNA forms (29). Crossreactivity of the antisense IGF-I riboprobe with all IGF-I mRNA forms, but not of IGF-II sense cRNA, has been described elsewhere (29). Sense IGF-I cRNA for use in standard curves was generated by transcription of an *Xho*I-linearized construct with T3 polymerase. The antisense IGF-I cRNA probe was generated using a *Not*I-linearized construct with T7 polymerase. The IGF-I riboprobe protects an  $\approx 100$ -bp region of tilapia IGF-I. The protected base component represents all transcripts coding for tilapia IGF-I forms (29).

The RNase protection assay was performed as described elsewhere (29, 30), with the following modifications. Briefly, RNA ( $50 \mu\text{g}$ ) was hybridized with probe overnight at  $50^{\circ}\text{C}$  in  $30 \mu\text{l}$  of hybridization buffer. Digestion was carried out under stringent conditions with the addition of  $250 \mu\text{l}$  of RNase-T2 (GIBCO/BRL) solution ( $30 \text{ units/ml}$ ) for 1 hr at  $37^{\circ}\text{C}$ . For validation, protected fragments were isolated, precipitated, and separated on 6% polyacrylamide/8 M urea gels and exposed to x-ray film. For quantification, the digested RNA was precipitated by the addition of  $300 \mu\text{g}$  of yeast tRNA and  $300 \mu\text{l}$  of ice-cold 10% trichloroacetic acid and kept on ice for 10 min. Precipitates were collected on GF/C glass fiber filters (Whatman) using a vacuum-manifold harvester (Brandel, Bethesda, Maryland). Filters were washed three times with ice-cold 20 mM sodium pyrophosphate in 5% trichloroacetic acid, washed once with 70% ethanol, air-dried, and processed for scintillation counting on a Beckman LS-3801 counter. The amount of IGF-I mRNA in a sample was calculated from standard curves. Results are expressed as pg of mRNA per  $\mu\text{g}$  of RNA. To determine whether equal amounts of total RNA were used in our assays, levels of 18S rRNA were assessed by Northern blot procedures as described elsewhere (30).

**Gill  $\text{Na}^+, \text{K}^+$ -ATPase Activity.** Gill  $\text{Na}^+, \text{K}^+$ -ATPase activity was analyzed at  $25^{\circ}\text{C}$  according to the method of McCormick and Bern (31), and values were expressed as  $\mu\text{mol}$  of ADP per mg of protein per hr.

**Statistical Analyses.** Significant differences among group means were evaluated using one-way analysis of variance (MINITAB, State College, PA) coupled with Fisher's protected least significant difference (FPLSD) test for predetermined pairwise comparisons (32).

## RESULTS

### Cartilage $[^{35}\text{S}]$ Sulfate and $[^3\text{H}]$ Thymidine Incorporation.

Hypophysectomy lowered levels of sulfate incorporation into cartilage tissues compared with sham-operated control values (Fig. 1). tGH increased sulfate uptake in tissues in a dose-related manner. tPRL<sub>177</sub> treatment increased sulfate uptake in tissues at the two highest doses. Tissues from Hx controls showed lower levels of thymidine incorporation than sham-operated animals (Fig. 2). Thymidine incorporation in tissues from tPRL<sub>177</sub>-injected animals was stimulated at the two highest doses. tPRL<sub>188</sub> had no effect on sulfate or thymidine incorporation. This study was repeated and we observed some differences: (i) hypophysectomy did not measurably alter thymidine incorporation in cartilage tissues (data not shown); (ii) tPRL<sub>177</sub> injection at the lowest dose significantly ( $P < 0.01$ ) stimulated sulfate uptake; and (iii) tGH at the highest dose significantly ( $P < 0.001$ ) stimulated thymidine incorporation.

**Presence of Hepatic tGH- and tPRL<sub>177</sub>-Dependent IGF-I mRNA Expression.** The effects of tGH and tPRL(s) replacement on IGF-I mRNA levels in liver tissue were examined by RNase protection assay. Compared with sham-operated values, hypophysectomy had no effect on IGF-I mRNA levels (Table 1). tGH treatment significantly increased IGF-I mRNA levels at the highest dose. tPRL<sub>177</sub> stimulated IGF-I mRNA levels at the two highest doses. tPRL<sub>188</sub> had no effect on IGF-I mRNA levels (Table 1). Northern blot procedures for 18S rRNA revealed that equal amounts of RNA were used in our RNase protection assay; therefore, our IGF-I mRNA values were normalized to total RNA and values are expressed as pg of IGF-I mRNA per  $\mu\text{g}$  of total RNA.

**Hepatic Radioreceptor Assay: Conditions and Scatchard Analysis.** Scatchard analysis indicated a single class of binding sites with a capacity of  $241 \pm 99 \text{ fmol per mg}$  of membrane protein and a binding affinity of  $0.75 \pm 0.11 \text{ nM}^{-1}$ . Significant specific binding was also found in membrane preparations from gill, heart, kidney, muscle, and testis, but levels were  $< 30\%$  of that observed from liver (data not shown). tGH competed equally well as eel GH with eel liver membrane preparations (data not shown). Eel GH competed as well as tGH with tilapia liver membrane preparations (data not shown). In tilapia liver membrane preparations, ovine GH was almost 20-fold less potent than tGH (Fig. 3 and Table 2). We

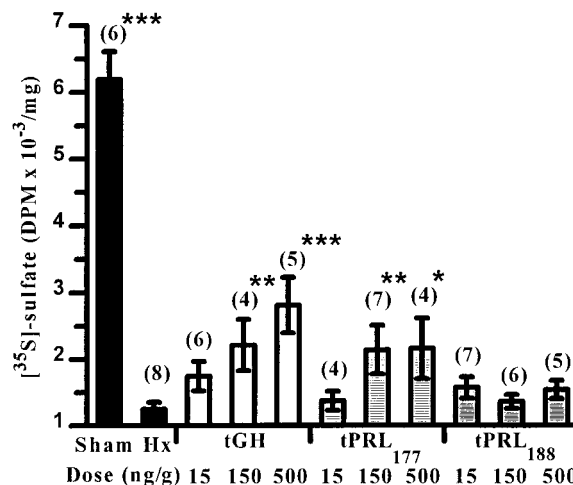


FIG. 1. Effects of hypophysectomy and homologous tGH, tPRL<sub>177</sub>, and tPRL<sub>188</sub> replacement at different doses on the *in vitro* uptake of  $[^{35}\text{S}]$ sulfate by ceratobranchial cartilage explants. Sham-operated and Hx controls were injected with vehicle only. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with Hx controls (FPLSD). Values represent the mean  $\pm$  SEM and are expressed as decays per minute (DPM) per mg of dry weight of cartilage. Numbers in parentheses denote sample size.

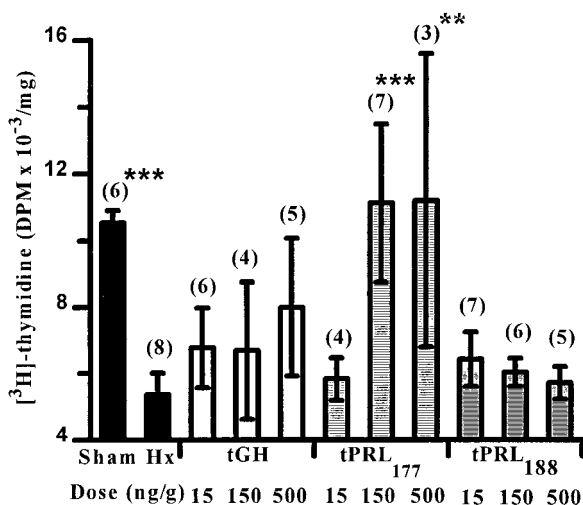


FIG. 2. Effects of hypophysectomy and homologous tGH, tPRL<sub>177</sub>, and tPRL<sub>188</sub> replacement at different doses on the *in vitro* uptake of [<sup>3</sup>H]thymidine by ceratobranchial cartilage explants. Sham-operated and Hx controls were injected with vehicle only. \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with Hx controls (FPLSD). Values represent the mean  $\pm$  SEM and are expressed as decays per minute (DPM) per mg of dry weight of cartilage. Numbers in parentheses denote sample size.

also observed that <sup>125</sup>I-labeled tGH can be displaced equally well by tPRL<sub>177</sub> and ovine GH, but not to any appreciable degree by tPRL<sub>188</sub> (Fig. 3 and Table 2) or ovine PRL (data not shown). tPRL<sub>177</sub> was 50-fold less potent than tGH at displacing <sup>125</sup>I-tGH from tilapia liver binding sites (Table 2). In eel liver membrane preparations, tPRL<sub>177</sub> did not compete for eel GH binding sites (data not shown).

**Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity.** As seen in Table 3, hypophysectomy lowered levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase compared with sham-operated values. tGH elevated Na<sup>+</sup>,K<sup>+</sup>-ATPase at all doses compared with Hx control values. tPRL<sub>177</sub> at the low and high doses had no effect on gill Na<sup>+</sup>,K<sup>+</sup>-ATPase, but at the middle dose it was effective at depressing Na<sup>+</sup>,K<sup>+</sup>-ATPase levels below Hx values. tPRL<sub>188</sub> at the two lowest doses had no effect, but the high dose showed a mean Na<sup>+</sup>,K<sup>+</sup>-ATPase level that was lower than levels observed at the other two doses and in the Hx group.

## DISCUSSION

Results of the ceratobranchial sulfate/thymidine assay and analyses of liver IGF-I mRNA levels from the present study offer evidence, in a teleost fish, that the homologous pituitary hormones tPRL<sub>177</sub> and tGH promote growth and alter levels of liver IGF-I in Hx adult animals.

In teleosts, liver IGF-I and IGF-II production is GH-dependent, and the incorporation of [<sup>35</sup>S]sulfate into cartilage has been shown to be stimulated by IGF-I (24, 29). We have

Table 1. Liver IGF-I mRNA levels

Treatment	Dose		
	15 ng/g	150 ng/g	500 ng/g
tGH	1.8 $\pm$ 0.7	2.5 $\pm$ 0.9	15.0 $\pm$ 5.0*
tPRL <sub>177</sub>	0.2 $\pm$ 0.0	2.6 $\pm$ 0.6*	2.9 $\pm$ 0.5*
tPRL <sub>188</sub>	1.6 $\pm$ 1.2	1.2 $\pm$ 0.8	0.8 $\pm$ 0.3

Effects of hypophysectomy and hormone replacement on liver IGF-I mRNA levels. IGF-I values for vehicle-injected Hx and sham-operated control groups are 0.2  $\pm$  0.0 and 0.8  $\pm$  0.3 pg per mg of total RNA, respectively. Values represent the mean  $\pm$  SEM ( $n = 3$ ) of duplicate determinations and are in pg per mg of total RNA.

\* $P < 0.001$  compared with Hx controls (FPLSD for those groups being compared).

Table 2. Potencies of tGH and tPRLs in radioreceptor assays

Membrane	tGH ED <sub>50</sub> , ng per tube	Potency relative to tGH*		
		tGH	tPRL <sub>177</sub>	tPRL <sub>188</sub>
Tilapia liver	0.68	100	2.0	0.15
Eel liver	0.70	100	<0.0006	<0.0006

\*Potency of the tGH standard was designated as 100%.

found that tGH and tPRL<sub>177</sub> treatment increase sulfate uptake *in vitro* in cartilage. Studies on other vertebrates have also shown a stimulatory effect of both heterologous or homologous PRLs on cartilage sulfation and on liver IGF-I activity (for review see refs. 3 and 11). In contrast, Duan *et al.* (33, 34) found that GH and somatotactin stimulated liver IGF-I mRNA expression in sockeye and coho salmon, but PRL did not. The differences observed between our work and that of Duan *et al.* (33, 34) may derive from the use of PRL in animals in physiological states wherein PRL is known to not be active (as in salmonid smolts) or antagonistic to seawater survival (4, 6, 35, 36). We observed that hypophysectomy did not affect liver IGF-I mRNA levels. This may be due to the extended period of fasting (14–16 days during our experiment), which reduces circulating IGF-I levels and liver IGF-I gene expression in other vertebrates (37, 38).

Some work on tilapia has focused on identifying the possible differences between the two PRLs (17, 20, 39, 40), but results have been inconsistent (41, 42). Recent work, however, suggests that tPRL<sub>188</sub> possesses greater sodium-retaining and calcitropic activity than does tPRL<sub>177</sub> (4, 20, 21).

GH and PRL have osmoregulatory actions in tilapia and other teleosts (4, 5, 10, 43). GH treatment results in increased levels of gill Na<sup>+</sup>,K<sup>+</sup>-ATPase that are consistent with the seawater-adapting role of this hormone in salmonids and cichlids (4, 6). Conversely, PRL treatment reduces gill Na<sup>+</sup>,K<sup>+</sup>-ATPase levels, consistent with its role as the freshwater osmoregulatory hormone (6, 35). More important to this study is that the actions of our GH and PRL preparations are consistent with their osmoregulatory roles in this teleost (4, 22) and strongly suggest that the somatotropic activity of our tPRL<sub>177</sub> preparation is not due to tGH contamination.

Binding sites for GH and PRL have been identified in several tissues of the tilapia (15). Few studies, however, have

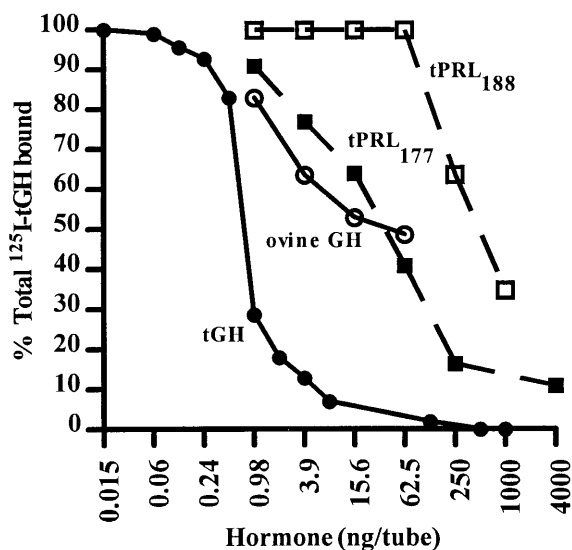


FIG. 3. Displacement of <sup>125</sup>I-labeled tGH from high-affinity, low-capacity hepatic binding sites by tGH (●), ovine GH (○), tPRL<sub>177</sub> (■), and tPRL<sub>188</sub> (□). Specific binding is shown as a percentage of total radioactivity in each tube. Each point represents the mean of duplicate determinations.

Table 3. Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity

Treatment	Dose		
	15 ng/g	150 ng/g	500 ng/g
tGH	1.4 ± 0.2*	1.3 ± 0.1†	1.2 ± 0.1‡
tPRL <sub>177</sub>	1.0 ± 0.1	0.7 ± 0.1‡	0.8 ± 0.1
tPRL <sub>188</sub>	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.0

Effects of hypophysectomy and homologous tGH, tPRL<sub>177</sub>, and tPRL<sub>188</sub> replacement at different doses on gill filament Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Na<sup>+</sup>,K<sup>+</sup>-ATPase values for vehicle-injected Hx and sham-operated control groups are 0.9 ± 0.1 and 1.9 ± 0.1 μmol of ADP per mg of protein per hr, respectively. Values represent the mean ± SEM (*n* = 6-20) and are in μmol of ADP per mg of protein per hr.

\**P* < 0.001; †*P* < 0.005; and ‡*P* < 0.05, compared with Hx controls (FPLSD).

examined the relationships between environmental adaptation and the hormones and receptors involved (44–46). We have undertaken radioreceptor studies to understand how GH and PRL exert their biological activities in the tilapia. We have identified high-affinity, low-capacity binding sites for GH in tilapia liver. tPRL<sub>177</sub> binds to these sites, albeit with lower affinity than tGH, but equally as well as ovine GH, the actions of which on growth in teleosts have been described (12, 47, 48). tPRL<sub>188</sub> does not compete for tGH binding sites. In eel liver membrane preparations, tPRL<sub>177</sub> did not compete with eel GH or tGH for binding sites, which suggests that the ability of tPRL<sub>177</sub> to displace tGH may not be general in teleosts. It is interesting that tPRL<sub>177</sub>, but not tPRL<sub>188</sub>, can displace <sup>125</sup>I-labeled tGH from its receptor given the structural similarities of these hormones and their receptors (49, 50).

tPRL<sub>177</sub> may also have a novel cartilage or liver receptor to which tPRL<sub>188</sub> does not bind. Such a possibility is suggested by the stimulatory effect of tPRL<sub>177</sub> on cartilage [<sup>3</sup>H]thymidine incorporation observed in our first study. Recent work on *Oreochromis niloticus* has demonstrated the presence of a single class of PRL receptors and a recombinant PRL receptor, which have higher affinity for tPRL<sub>188</sub> than for tPRL<sub>177</sub> (45, 50). This suggests that the actions of tPRL<sub>177</sub> may overlap with those of tPRL<sub>188</sub> and tGH through its ability to bind to their receptors. This may be analogous to the phenomenon of primate GH binding to GH and PRL receptors (9, 51).

Our findings suggest that tPRL<sub>177</sub>, in addition to its freshwater osmoregulatory role, may also be somatotrophic in freshwater tilapia, where circulating levels are high (22, 25, 39). Also suggested by our work and that of others is that GH possesses both osmoregulatory and somatotrophic actions in seawater tilapia (4, 25, 52). Collectively, these findings point to the existence of a GH/PRL-IGF-I axis in the tilapia.

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