Tilapia somatotropin polypeptides: potent enhancers of fish growth and innate immunity

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ABSTRACT

Growth hormone (GH) is a single chain polypeptide of approximately 22 kDa, produced by the pituitary gland and with pleiotropic functions among vertebrates. It mainly regulates body growth, being also involved in reproduction, immunity and osmoregulation in teleost fish. In order to obtain the tilapia (*Oreochromis hornorum*) growth hormone (tiGH) in *Pichia pastoris* cells, its gene was cloned into expression vectors in both with and without a heterologous secretion signal. The tiGH, obtained either intracellularly or extracellularly in *P. pastoris* cells, was characterized showing its production as associated to the cellular rupture precipitate with an approximate molecular weight of 22 kDa; or being secreted with an approximate molecular weight of 18 kDa, respectively. The mass spectrometry analysis of the recombinant protein obtained in the culture supernatant corroborated the identity of the protein as tiGH but lacking 46 aminoacids of its carboxyl terminal sequence. The tiGH biological activity of *P. pastoris* intact cells producing this protein was carried out in tilapia larvae (*Oreochromis* sp.), showing, for the first time, that it is possible to stimulate fish growth by immersion baths with recombinant tiGH-producing yeast. On the contrary what was previously postulated for mammals, the evaluation of *P. pastoris* cells expressing the truncated variant of tiGH demonstrated that this protein is also able to stimulate growth and immune system in fish. This is the first report of a biologically active, truncated GH variant in fish.

Introduction

The development of Aquatic Biotechnology has supported the application of experimental techniques to manipulate fish growth, as diets enriched in specific proteic nutrients and administrating hormones like: prolactin, insulin and growth hormone (GH) [1].

GH is a single chain polypeptide of approximately 22 kDa, produced by the pituitary gland and with pleiotropic functions among vertebrates. It mainly regulates body growth, being also involved in reproduction, immunity and osmosis regulation in teleost fish, and in metabolism regulation through its lipolytic activity and protein anabolism in vertebrates [2].

The structure of fish GH molecule remains unknown. Otherwise, it has been suggested based on aminoacid sequence alignments that it bears functional epitopes similar to the human GH [3] which comprises four anti-parallel ahelixes separated by connecting loops as well as four Cys residues located at positions 53, 165, 182 and 189 loops and with four cystein residues [4]. These residues are highly conserved between GH molecules from fish to mammals [5, 6], suggesting that they may be involved in structural integrity and/or biological activity of the hormone [7]. The information about the interaction of GH with its receptor(s) in fish is scarce, and there are discrepancies on receptors identity. Only one study has proposed that a one-hormone-two-receptors model applies in fish and possibly to all the vertebrates [8]. That study has also postulated the presence of receptor binding site 1 in the goldfish GH-II molecule, comprising three discontinuous regions located in helix 1, together with residues of the middle portion of the molecule and helix 4, and that receptor binding site 2 is located in the middle part of the molecule [8].

GH has also been recognized as relevant for the aquatic industry due to its role on growth and as immune stimulator [9-12]. Hence, the genes coding for GH in different fish species have been cloned and expressed in yeasts and *Escherichia coli* cells [13-15].

Several studies have been conducted to show the growth-stimulating effect of recombinant GH administered by several routes including injection [14-16], oral incubation [17], immersion baths [18-20] and dietary delivery [21-23]. Among these methods, oral incubation and immersion bath are the most feasible for aquaculture avoiding individual manipulation of fish [10, 20, 24]. There are several works demonstrating growth stimulation by feeding fish with diets enriched of GH-producing yeasts [25-27]. However, none of them evaluated the possible effects of GH by supplying GH-producing cells without any other additive and by using immersion procedures. The previous purification GH is the main factor making its use more expensive and prohibitive to be used in aquaculture. The aim of this work was to evaluate for the first time the effect of administering by immersion baths tilapia GH (tiGH)-producing yeast cells or their culture supernatants containing secreted tiGH, to stimulate growth and immune functions in larval and juvenile fish.

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*R*esults and discussion

Obtaining the tilapia growth hormone intracellularly in *Pichia pastoris* cells

The DNA fragment coding for tiGH was PCR-amplified from the pTTI plasmid, lacking its signal peptide for intracellular expression [14], and was inserted into the pNAO vector to obtain the pP-tiGH plasmid with the aim of obtaining a *P. pastoris* strain producing tiGH intracellularly. It bears an integration unit composed of homologous regions to the AOX1 locus of *P. pastoris* genome (5'AOX1 and 3'AOX1) for its integration by homologous recombination. The pP-tiGH was digested with the *Cla* I endonuclease and used for transforming of *P. pastoris* cells strain MP36 by electroporation. Southern-blot analyses of three genomic DNA, isolated from three transformants showed a 2.8 kb band in two of them, indicative of double crossovers between the expression cassette and the endogenous AOX1 gene in the host genomes (gene replacement integration)(See Figure 2 in reference [28]). This event generates clones of slow methanol consumption (Mut^S) [29]. The genomic DNA of the untransformed *P. pastoris* strain MP36 was used as negative control of the assay, being detected the 5.5 kb-band corresponding to the native AOX1 gene. Trans-formant C9 was denominated MP36/pP-tiGH and selected for further expression studies.

The expression of tiGH from the MP36/pP-tiGH strain was analyzed in the intracellular fraction under reducing conditions by 15% SDS-PAGE, showing a protein band of an approximate molecular weight between 21 and 31 kDa (Figure 3 in reference [28]). Its molecular weight was similar to the 22 kDa of the tiGH molecule previously obtained in *E. coli* cells. Protein identity was corroborated by Western blot analyses with the aid of an anti-tiGH-HRP monoclonal antibody (Figure 2B in reference [28]). This recombinant protein was absent in analyzed samples of the native MP36 strain.

The intracellular tiGH obtained in *P. pastoris* cells reached approximately 4.3% of the total protein content. This value was determined by optical densitometry from 15% SDS-PAGE gels (Figure 2A in reference [28]). Moreover, the cellular density at the end of culture was approximately of 300 g/L as determined from the wet biomass weight. Recombinant tiGH expression levels were estimated by protein Dot blot, with the aid of a tiGH standard obtained in *E. coli* of known concentration [14], reaching approximately 1.5 g/L of culture (data not shown).

Effect of tiGH from the MP36/pP-tiGH strain on growth of red tilapia (*Oreochromis sp.*) larvae

An experiment was carried out to evaluate the biological activity of tiGH produced by the MP36/pP-tiGH *P. pastoris* strain on stimulating growth of 3-days posthatching tilapia larvae. Intact yeast cells containing tiGH were administered by immersion baths, thrice a week for 6 weeks. Larvae body weight increased significantly four weeks after starting treatment with *P. pastoris* tiGH-producing cells, compared to the ne-

gative control group treated with untransformed *P. pastoris* cells (p < 0.0001) (Figure 4 in reference [28]). Although the stimulatory effect of recombinant GH being administered by immersion baths has been previously demonstrated in fish [18, 20], this is the first report using *P. pastoris* cells expressing a fish GH for that purpose. Previous demonstrations have only used diets enriched with GH-expressing yeast [25-27].

Obtaining an extracellular variant of tiGH in *P. pastoris* cells

The PCR-amplified 0.8 kb DNA fragment from the pTTI plasmid [14] that codes for tiGH was cloned into the pPS7 vector in frame between the *Saccharomyces cerevisiae* suc signal peptide sequence and the GAP terminator, generating the construct pPS7-tiGH. This vector bears an AOX1 homologous integration unit similar to that described above for the pP-tiGH construct.

The pPS7-tiGH construct was digested with the *Sph* I restriction endonuclease and used for transforming of *P. pastoris* cells strain MP36 by electroporation. Southern blot analysis of genomic DNA showed four out of five transformants with a DNA band of 28.7 kb indicating the homologous integration by double crossover of the expression cassette (integration by AOX1 gene replacement) (Figure 1 in reference [30]). These clones showed the slow methanol consumption (Mut^S) phenotype [29]. The 5.5 kb band corresponding to the native host AOX1 gene was observed in the genomic DNA of the control, untransformed *P. pastoris* MP36 strain. The transformant corresponding to lane 1 in figure 1 [30] was selected for expression studies, being denominated MP36/pPS7-tiGH.

The Western blot analysis of culture supernatants from 15% SDS-PAGE under reducing conditions showed a protein band immunoreactive to an anti-tiGH monoclonal antibody. Its molecular weight was between 15 and 25 kDa (Figure 1), lower than both the

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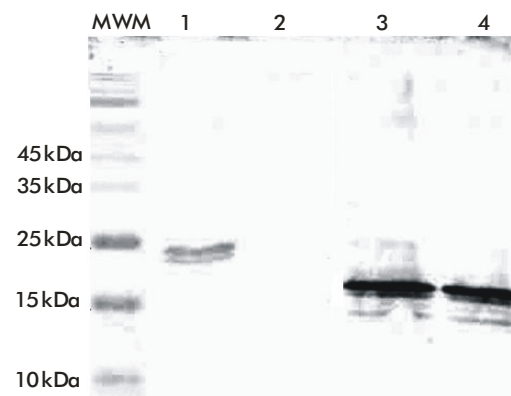
Figure 1. Western blot analysis of tiGH gene expression by the MP36/pPS7-tiGH transformant strain in 15% SDS-PAGE gels under reducing conditions. Lane 1: MP36/pPS7-tiGH intracellular extracts. Lane 2: Supernatants of native MP36 strain. Lanes 3 and 4: MP36/pPS7-tiGH culture supernatants. MWM: Molecular weight marker.

22 kDa native tiGH [14] and the intracellular tiGH produced in *P. pastoris* cells [28]. The recombinant protein was absent in culture supernatants of the native untransformed MP36 strain. Its expression levels were estimated by Dot blot, comparing the previously mentioned tiGH standard obtained in *E. coli* [14], reaching 40 and 80 mg/L (data not shown). Mass spectrometry analyses corroborated the identity of the protein as tiGH but lacking the 46 carboxyl A similar experiment to evaluate the intracellular tiGH effect on growth of tilapia larvae was carried out, but using this time culture supernatants from the MP36/pPS7-tiGH strain containing the truncated tigGH to stimulate growth of 3-days post-hatching tilapia larvae. Treatment was applied by immersion baths thrice a week for 7 weeks. As for the intracellular variant, larvae body weight increased significantly four weeks after starting treatment with culture supernatants from MP36/pPS7-tiGH cells, compared to the negative control group treated with supernatants from untransformed *P. pastoris* cells ($p < 0.0001$) (Figure 2), reaching a 2.2-fold increase after 7 weeks ($p < 0.0001$) (Figure 2). These results demonstrated that the truncated tiGH variant lacking the 46 carboxyl terminal aminoacids from the native tiGH is biologically active.

Comparison of biological activities of the cellular lysate and culture supernatant from the respective intracellular and truncated tiGH variants-expressing cells

The stimulatory biological effects of both tiGH variants (intracellular complete tiGH or secreted truncated tiGH) on the innate immunity and growth of tilapia larvae were evaluated in of 3-days post-hatching larvae. Treatment was applied by immersion baths thrice a week for 45 days. Larvae were analyzed at 21 and 45 days after the start of the experiment for growing parameters and innate immune functions (lysozyme and hemmaglutination activities).

Growth stimulating activity

A significant increase in body weight was observed in day 21 for tilapia larvae treated with culture supernatants containing the truncated tiGH, compared to both the larvae control group ($p < 0.001$) and the group treated with lysates of the untransformed native *P. pastoris* strain ($p < 0.05$) (Figure 4 in reference [30]). Moreover, larvae treated with intracellular tiGH-containing *P. pastoris* cell lysates also significantly increased body weight compared to the untreated control group ($p < 0.001$) (Figure 4 in reference [30]).

Larvae treated with supernatants containing the truncated tiGH showed the best growing results 45 days after treatment. They had a 3.6-fold increase of body weight compared to the untreated group ($p < 0.001$), a 2.1-fold increase treated with untransformed *P. pastoris* culture supernatants ($p < 0.001$), 2.9-fold in the group treated with untransformed *P. pastoris* cell lysates ($p < 0.001$) and 1.6-fold in the group treated with intracellular tiGH-containing cell lysates ($p < 0.05$) (Figure 4 in reference [30]). Likewise, the group treated with the intracellular tiGH-containing lysates showed a 2.2- and 1.8-fold increase in body weight compared to the untreated control group ($p < 0.001$) and the group treated with untransformed *P. pastoris* cell lysates ($p < 0.001$), respectively (Figure 4 in reference [30]).

Effects of tiGH variants on innate immunity

Lysozyme activity

Lysozyme activity was undetectable in larvae homogenates 21 days after starting treatment. There were statistically significant differences at day 45 only between the group treated with culture supernatants

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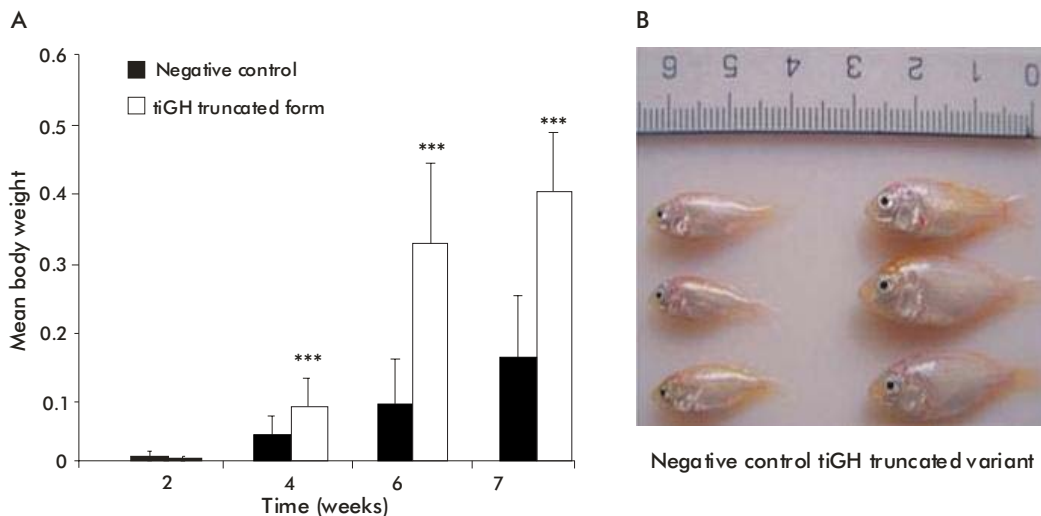Negative control   tiGH truncated variant

Figure 2. Growth stimulating activity of truncated tiGH-containing supernatant of *P. pastoris* cell cultures. A) Effect in body weight of treated and negative control groups of fish after 2, 4, 6 and 7 weeks of starting treatment. Bars represent mean body weight + stardard deviation (n = 30). Data were statistically analyzed by a Student´s t test with Welch´s correction on weeks 2, 4 and 6, and without it on week 7. Asterisks (***) indicate *p* < 0.0001. B) Photograph showing the phenotype of treated and control fish on week 7.

containing truncated tiGH compared to the untreated control group ($p < 0.05$) (Table 1 in reference [30]).

Hemagglutinating activity

There were no statistically significant differences 21 days after beginning treatment. Consistently, after 45 days, the highest hemagglutinating activity was obtained in the group treated with truncated tiGH-containing supernatants (1024 ± 1), statistically significant when compared to the untreated control group (128 ± 1; $p < 0.001$) and groups treated either with untransformed *P. pastoris* culture supernatants (512 ± 1; $p < 0.01$), untransformed *P. pastoris* cell lysates (256 ± 1; $p < 0.001$) or intracellular tiGH-containing cell lysates (323 ± 2.5; $p < 0.001$) (Figure 5 in reference [30]). All the treatments stimulate haemagglutinin activity compared to non-treated group (Figure 5 in reference [30]). No differences among groups were found at day 21 (data not shown). This was the first report linking GH administration to lectin activation. In this sense, we cannot exclude a synergic or individual influence on this parameter of some factors present in culture supernatants activating innate immunity in fish, coming either from *P. pastoris* cells (intracellular and/ or secreted) or even from culture medium components. As far as we know, there are no reports on this matter. Nevertheless, it has been reported that *S. cerevisiae* cell wall components such as B-1,3-D-glucan, B-1,6-Dglucan, mannoproteins and chitin [31] individually increase innate defense mechanisms and/or disease resistance when purified ad administered to vertebrates [32-34]. B-glucans and mannoproteins are the main yeast cell wall components. The former enhance fish humoral (lysozyme and complement activity) and cellular (phagocytic and respiratory burst activities and bacterial killing) immune responses both *in vitro* and *in vivo* [35, 36]. The least abundant yeast cell wall compound, but one that is very important, is chitin. Some studies have shown that chitin and its derivatives modulate the immune system in mammals as well as in fish [33, 34, 37]. Finally, the combination of all the yeast cell compounds, not only cell wall sugars but also vitamins and genetic material, might produce an optimum physiological status in fish due to multiple interactions, particularly with regard to the immune system [38-40]. In addition, the effect of GH by itself can be either direct by interacting with hormone regulators of the immune response or indirect by just accelerating the development of larval immune cell populations. All these require further investigation.

This is the first report of a tiGH polypeptide fragment lacking the last 46 aminoacids of the native tiGH which shows *in vivo* biological activity for stimulating growth and being involved in innate immune stimulation of tilapia larvae. Differences observed between the truncated and the intracellular tiGH variants cannot be attributed to the tiGH molecule itself, since both variants were tested coming from complex samples subjected to different procedures and none of them included tiGH purification. Moreover, they were supplied at different tiGH molar concentrations and without knowing the possible influence of their different tiGH molecular tertiary structures.

Based on these facts, we decided to use the culture supernatant containing the truncated tiGH molecule

for further studies of its industrial application in aquaculture.

Effect of truncated tiGH-containing supernatant of *P. pastoris* cells in ornamental fish

Effect on growth and survival of goldfish larvae (Carassius auratus)

The effect of truncated tiGH-containing supernatant on growth and survival of goldfish larvae was evaluated from 5-days post-hatching larvae. Treatment was applied by immersion baths thrice a week for 75 days. Treated larvae showed a 3.1-fold increase in body weight compared to the untreated group ($p < 0.0001$; Figure 3) after 45 days of started treatment, that remained two-fold after 75 days ($p < 0.0001$; Figure 3), also showing increased coloration (Figure 3B). Besides, truncated tiGH-containing supernatant also enhanced survival 22% after 75 days, compared to the animals of the control group.

Effect on growth of larvae and juvenile carp (Cyprinus carpio)

The truncated tiGH-containing supernatant was also evaluated in different developmental stages of trout (*Cyprinus carpio*). For this purpose, 5 days posthatching larvae and juveniles (1.2 ± 0.2 g) were treated by immersion baths thrice a week. Larvae were sampled from 15 to 30 days after starting the experiment, showing a 3.5-fold body weight increase compared to the control group ($p < 0.0001$) at day 15, which remained 2.2-fold ($p < 0.0001$) after 30 days (Figure 4).

Trout juveniles were sampled 0, 15 and 28 days showing significant increased body weight compared to the control group ($p < 0.05$), which was 1.5-fold after 28 days ($p < 0.0001$) (Figure 5).

Effect on growth and anatomical parameters of angelfish (Pterophyllum scalare) juveniles

The effect of truncated tiGH-containing supernatant on growth and quality-related parameters of

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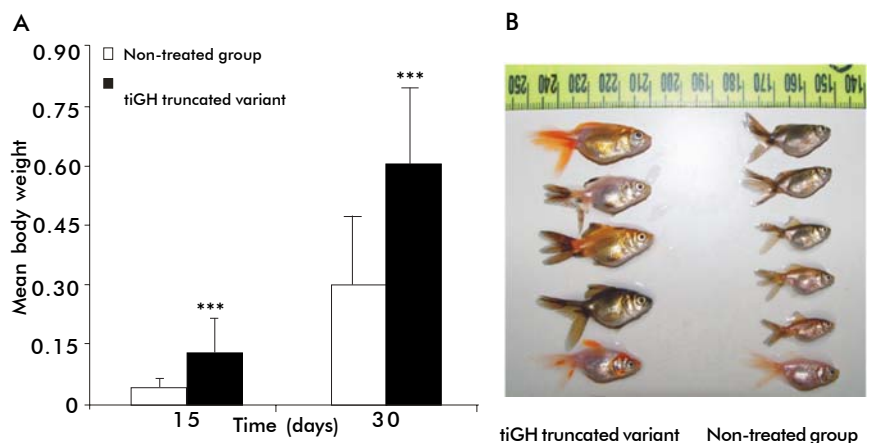
Figure 3. Growth-stimulating activity of the truncated tiGH-containing *P. pastoris* supernatant in goldfish larvae. A) Body weight after 45 and 75 days of treatment. Bars represent mean weight + standard deviation (n = 30). Data were statistically analyzed by a Student´s t test with and without Welch´s correction on days 45 and 75, respectively. Asterisks (***) indicate $p < 0.0001$. B) Photograph showing the phenotype of treated and control fish after 75 days.

A

B

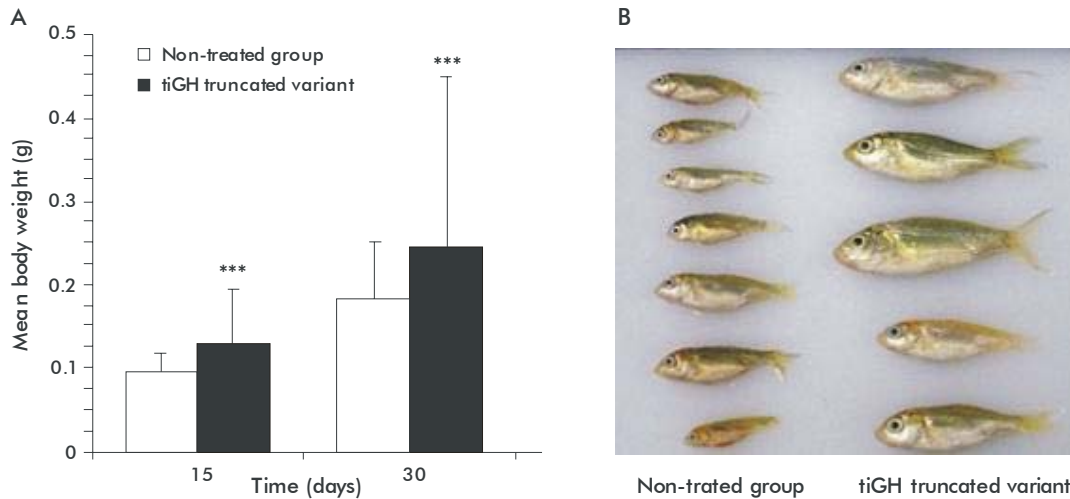Non-trated group　　　tiGH truncated variant

Figure 4. Growth-stimulating activity of the truncated tiGH-containing *P. pastoris* supernatant in trout larvae. A) Body weight after 15 and 30 days of treatment. Bars represent mean weight + standard deviation (n = 60). Data were statistically analyzed by a Student´s t test with Welch´s correction. Asterisks (***) indicate p < 0.0001. B) Photograph showing the phenotype of treated and control fish after 30 days.
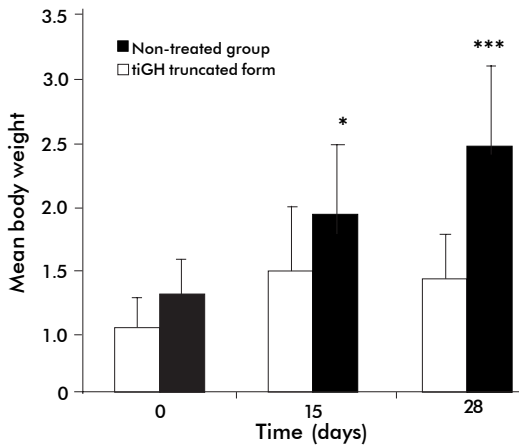

Figure 5. Growth-stimulating activity of the truncated tiGH-containing *P. pastoris* supernatant in trout juveniles. Bars represent mean weight + standard deviation (n = 20). Data were statistically analyzed by a Student´s t test on days 0 and 15, and by a Student´s t test with Welch´s correction after 28 days. Asterisks (*) indicate *p* < 0.05, and (***) indicate *p* < 0.05.

ornamental fish was also evaluated in angelfish juveniles (3.1 ± 0.6 g) receiving immersion baths thrice a week. Fish showed gained weight 1.6-fold after 30 days than those of the untreated group (p < 0.01, Figure 6). Besides, their body length (p < 0.01), width (p < 0.01) and length of the dorsal fin (*p* < 0.001) significantly increased in the treated group (Table 1).

These results demonstrate that treatment with truncated tiGH-containing supernatants not only increases growth, but also stimulates quality-related body parameters in ornamental fish. It was also demonstrated its action in larvae and juvenile fish developmental stages.

Conclusions

For the first time, these findings provide differences of the hormone receptor interaction and the structure-function relationship between fish and mammals, as well contribute to understand the interactions of GH with its receptors in fish. Moreover, because the truncated tiGH variant being supplied in *P. pastoris* culture supernatants showed to be a potent

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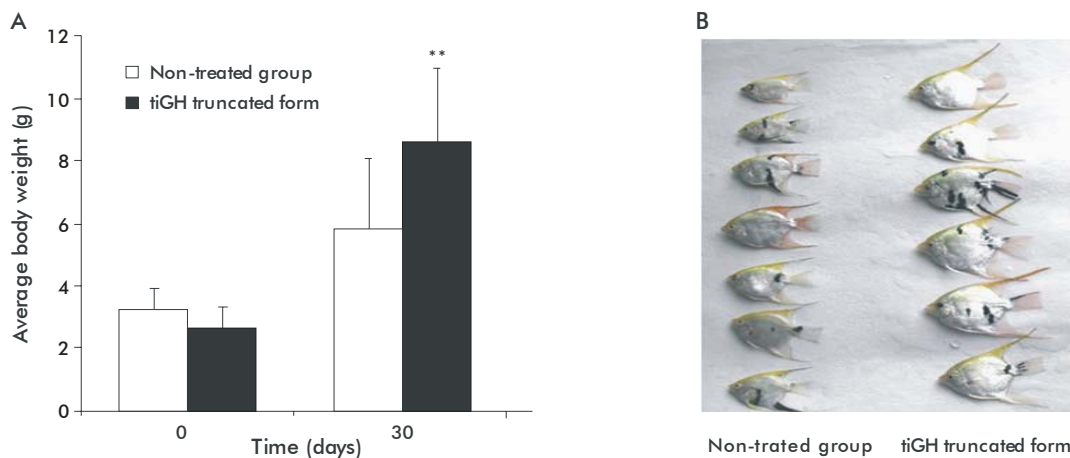
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Non-trated group　　　tiGH truncated form

Figure 6. Growth-stimulating activity of the truncated tiGH-containing *P. pastoris* supernatant in scalar juveniles. Bars represent mean weight + standard deviation (n = 10). Data were statistically analyzed by a Student´s t test. Asterisks (**) indicate *p* < 0.01.

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Table 1. Effect of truncated tiGH-containing *P. pastoris* supernatants on body parameters of scalar juvenile fish

	Body length (mm)	Abdominal fin (mm)	Body width (mm)	Dorsal fin (mm)	Caudal fin width (mm)
Treated group	73.1 ± 4.58 [a,**]	48.8 ± 8.76	40.0 ± 3.78 **	44.4 ± 6.78 ***	29.4 ± 5.63
Untreated group	62.9 ± 8.09	41.4 ± 4.76	33.7 ± 3.45	29.6 ± 3.10	25.0 ± 5.00

[a] Data are expressed as the mean ± standard deviation (n = 10).
** Statistical significance by Student´s t test with Welch´s correction for $p < 0.01$.
*** Statistical significance by Student´s t test with Welch´s correction for $p < 0.001$.

enhancer for growth, survival, quality of larvae and immune parameters, it can be used in aquaculture mainly for ornamental fish production. It allows to have treated fish of increased growing rates and more resistant to stress conditions and infections, and reducing production costs.

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