A novel GH secretagogue, A233, exhibits enhanced growth activity and innate immune system stimulation in teleosts fish

Rebeca Martinez, Kenia Ubieta¹, Fidel Herrera, Alina Forellat¹, Reynold Morales, Ania de la Nuez², Rolando Rodriguez^{2,*}, Osvaldo Reyes², Ayme Oliva and Mario P Estrada

Biotechnology Animal Division, Aquatic Biotechnology Department, CIGB, PO Box 6162, Havana 10600, Cuba

¹Biochemistry Department, Faculty of Biology, Havana University, Havana, Cuba

²Physico-Chemistry Division, Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana 10600, Cuba

(Correspondence should be addressed to M P Estrada; Email: mario.pablo@cigb.edu.cu)

*(R Rodriguez is currently at Computational Medicine, National Institute of Nephrology, Havana, Cuba)

Abstract

In teleosts fish, secretion of GH is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. There is an interaction between immune and endocrine systems through hormones and cytokines. GH in fish is involved in many physiological processes that are not overtly growth related, such as saltwater osmoregulation, antifreeze synthesis, and the regulation of sexual maturation and immune functions. This study was conducted to characterize a decapeptide compound A233 (GKFDLSPEHQ) designed by molecular modeling to evaluate its function as a GH secretagogue (GHS). In pituitary cell culture, the peptide A233 induces GH secretion and it is also able to increase superoxide production in tilapia head-kidney leukocyte cultures. This effect is blocked by

preincubation with the GHS receptor antagonist [D-Lys³]-GHRP6. Immunoneutralization of GH by addition of anti-tilapia GH monoclonal antibody blocked the stimulatory effect of A233 on superoxide production. These experiments propose a GH-mediated mechanism for the action of A233. The *in vivo* biological action of the decapeptide was also demonstrated for growth stimulation in goldfish and tilapia larvae ($P < 0.001$). Superoxide dismutase levels, antiprotease activity, and lectin titer were enhanced in tilapia larvae treated with this novel molecule. The decapeptide A233 designed by molecular modeling is able to function as a GHS in teleosts and enhance parameters of the innate immune system in the fish larvae.

Journal of Endocrinology (2012) **214**, 409–419

Introduction

Growth hormone (GH) has pleiotropic functions in all vertebrates. In addition to its essential role in the regulation of body growth and development, it can also influence reproduction, immunity, osmoregulation, and behavior (Rise *et al.* 2006, Acosta *et al.* 2008, Devlin *et al.* 2009). In teleosts, secretion of GH is regulated by several hypothalamic factors that are influenced by the physiological state of the animal. In fish, GH-releasing hormone, gonadotrophin-releasing hormone (GNRH), neuropeptide Y, and thyrotrophin-releasing hormone (TRH) stimulate the release of GH, effects that have been shown to be influenced by gonadal sex steroids and by the nutritional state of the animal (Sherwood *et al.* 2000). Intrapituitary regulators, including GH, gonadotropin, and inhibin/activin, provide autocrine/paracrine control over GH synthesis and secretion (Chang & Wong 2009). Besides the pituitary gland, the GH gene is also expressed in other tissues of fish, especially in lymphoid organs and cells (Yada 2007).

The synthetic GH secretagogues (GHSs) consist of a family of ligands, first described by Momany *et al.* (1981). They were

initially termed GH-releasing peptides (GHRPs). Multiple efforts have been directed toward finding the active molecules with an increased oral bioavailability and longer clearance times, yielding the discovery of new GHSs (Moulin *et al.* 2007).

GHS bind to a receptor inducing calcium mobilization, as identified by Howard *et al.* (1996) in pigs and humans. Currently, GHS receptor (GHSR) has been identified in teleosts and birds (Palyha *et al.* 2000, Kaiya *et al.* 2009a,b,c, 2010). More than one receptor has been proposed for zebrafish, catfish, and goldfish (Kaiya *et al.* 2008, 2010, Small *et al.* 2009).

The endogenous ligand to this receptor was identified by Kojima *et al.* (1999) as ghrelin (GRLN), which is a 28-amino acid peptide synthesized mainly in the oxyntic mucosal cells of the stomach and which has diverse actions such as potent GH-releasing and orexigenic activities, insulinonistasis, cardiovascular effects, stimulation of gastric motility and acid secretion, adipogenesis, and cell proliferation (Muccioli *et al.* 2007, Veldhuis & Bowers 2010).

Neuroendocrine and immune systems interact in a directional fashion. In this way, the status of pathogen recognition is communicated to the brain and the immune

response is influenced by physiological changes. This explicit communication consequently needs a common language of signaling molecules and receptors. Growing evidence suggests that hormones not only act on neuroendocrine cells but also on immune cells and that cytokines act on both immune and neuroendocrine cells via shared receptors in both systems and modulate their functions. GHSR is expressed predominantly in the brain and pituitary, but it is also expressed in many peripheral organs including immune system cells (Hattori *et al.* 2001). GRLN modulates the immune system (Dixit *et al.* 2004) and it has been shown to stimulate phagocytosis in fish leukocytes, and this effect is mediated in part by GH secreted by leukocytes (Yada *et al.* 2006).

The molecule A233 was described by the exhaustive molecular modeling of the human GRLN receptor using combined techniques of homology modeling, molecular dynamics, and exhaustive conformational search techniques, after which a virtual library was built with several thousands of structures having such characteristics, to perform a conformational analysis, and a massive docking experiment was performed against the receptor model (Rodríguez *et al.* 2007). The aim of this study was to assess the biological activity of synthetic peptide A233 as a stimulator of growth and the innate immune system of teleosts fish, through studies performed *in vitro* and *in vivo*.

In this study, we have characterized a chemical decapeptide compound, A233, designed by molecular modeling, which is able to perform the function of a GH peptide secretagogue. Innate immune system stimulation is also obtained *in vitro* in tilapia head–kidney leukocyte (HKL) cultures using this molecule through a GHSR-dependent pathway. By means of immune neutralization experiments, we have proved the mechanism of action of A233 to be GH-mediated. We also show the biological action *in vivo* of the molecule, for growth stimulation in goldfish and tilapia larvae and enhancing the level of some innate immune parameters and the superoxide dismutase (SOD) in tilapia larvae treated with this novel secretagogue.

Materials and Methods

Fish

Goldfish (*Carassius auratus*) were provided by Nacari Company, Havana, Cuba. Tilapia (*Oreochromis sp.*) juvenile and larvae were obtained from the Center for Aquaculture of Mampostón (CPAM). Fish were kept alive in aerated freshwater under a 12 h light:12 h darkness photoperiod. They were fed commercial dry diet for fish (CENPALAB, Habana, Cuba). Water temperature was maintained at 26 and 28 °C. All animal experiments were previously approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

GH secretagogues

The decapeptide A233 (GKFDLSPEHQ) with a lactam bond between side chains of Lys and Asp (underlined amino acids)

was manually synthesized on a solid-phase support. Crude peptide was purified by reverse-phase, high-performance, liquid chromatography up to 95% on a C-18 preparative column with an acetonitrile/water linear gradient. Trifluoroacetic acid was used in both solvents for counter-ion pair formation. The correct sequence of the purified peptide was confirmed by electrospray mass spectrometry (Micromass, Manchester, UK). The positive control used was GHRP6 (Lipotec, Barcelona, Spain) and the antagonist [D-Lys³]-GHRP6 (Sigma) lyophilized peptides were reconstituted in PBS for use *in vitro* and *in vivo*.

Primary culture of pituitary cells

The *in vitro* effects of A233 GHS were examined using cells dispersed from whole pituitary. Mature tilapias of both sexes weighing 300–500 g were used after anesthesia in tricaine methanesulfonate (MS-222, Sigma). Pituitaries were collected aseptically in isotonic medium (Krebs bicarbonate–Ringer solution, 330 m Osmolal, pH 7.4) supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and nystatin (250 IU/ml, all from Sigma). The pituitaries were diced with a sterile razor blade and treated with collagenase for 1 h at room temperature in 2.5 ml trypsin–EDTA solution (0.25% trypsin + 0.02% EDTA in PBS, pH 7.4). Tissues were aspirated repeatedly through a pipette during enzymatic treatment to promote dissociation of cells. The process was terminated by the addition of 0.5 ml (20%) fetal bovine serum (Sigma). Cells were counted on a hemocytometer under a light microscope and viability determined by trypan blue exclusion. Viability of the cells was always >95%. Cells were then plated at a density of 4.0×10^5 cells/well into a 24-well plate (Falcon, Primaria 24, Becton Dickinson, Franklin Lakes, NJ, USA) at a volume of 300 µl/well of isotonic medium supplemented with 10% fetal bovine serum. The cells were preincubated for 4 days at 26–28 °C under a humidified atmosphere of 95% O₂ and 5% CO₂, with one change of culture medium at 48 h post-plating. Before each experiment, cells were washed once with serum-free medium. A final 300 µl serum-free medium was added containing A233, GHRP6 (Lipotec), or control medium without hormones. The medium was replaced at 4 h. Incubations were terminated at 8 h, and hormone release was quantified for the 0–4 and 4–8 h intervals. GH release was expressed as secretion per unit volume of medium (ng/ml).

GH assays

The GH secreted *in vitro* was measured using a noncompetitive ELISA as described by Lugo *et al.* (2008). The 96-well MaxiSorp plates (Nalge Nunc International, Roskilde, Denmark) were coated (3 h at 37 °C) with anti-tilapia GH monoclonal antibody 1 (tiGH1 mAb; CIGB, Santi Spiritus, Cuba) at 10 µg/ml in 0.05 M carbonate buffer (pH 9.6, 100 µl/well). The plates were washed two times with PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O,

and 0.05% Tween 20, pH 7.3) and blocked with 3% skim milk (Oxoid, Cambridge, UK) in PBS 1× (200 µl/well) for 1 h at 37 °C. A standard tiGH curve in the range of 35–0.136 ng/ml was obtained by twofold dilutions in 0.5% skim milk in PBS 1×, dispensed by duplicate in the same plate. Test samples were diluted at a ratio of 1:2 as described earlier and were incubated on the plates overnight at 4 °C. After washing the plates four times with PBS-T, 100 µl HRP – tiGH2 mAb conjugate (CIGB), diluted at a ratio of 1:15 000 in PBS 1× containing 0.5% skim milk, were added to each well. The plates were incubated for 1 h at 37 °C and then washed eight times with PBS-T. Then, the substrate buffer (0.2 M Na₂HPO₄, 0.1 M citric acid (pH 5.0) containing 0.5 mg/ml ortho-phenylenediamine and 5 µl 30% H₂O₂) was added (100 µl/well). The reaction was stopped 15 min later by adding 50 µl of 2.5 M sulfuric acid per well. The absorbance was measured at 492 nm using the Titertek Multiskan Plus spectrophotometer. The accepted upper limit of the assay background was 0.094. The lower detection limit of the assay was 0.1 ng/ml. The degree of variation and intra- and interassay coefficients of variation are 3.90 and 13.45% respectively.

Isolation of HKLs

HKLs were isolated as described by Yada *et al.* (2006) with slight modifications. Fish were killed by decapitation and the head–kidney was placed in RPMI-1640 medium (Sigma). The cells were layered on a 34/51% Percoll (Sigma–Aldrich) gradient and centrifuged at 10 000 g for 45 min. The leukocyte band was harvested, washed with Hanks' solution (pH 7.4; Sigma), and suspended in RPMI-1640 medium (Sigma). The leukocytes were counted using a Neubauer Haemocytometer from an aliquot containing 900 µl of 0.1% trypan blue and 100 µl of cell suspension.

Superoxide production in HKLs

To analyze the effect of GHSs on the immune system, the superoxide anion was measured in HKLs as the reduction of NBT (Sigma) as described by Sakai *et al.* (1996) and Yada *et al.* (2006). To examine whether A233 acts through the GHSR, leukocytes were exposed to the GHSR antagonist [D-Lys³]-GHRP6 (10 µM). On the day of treatment, cells were preincubated for 1 h with either control medium or medium containing [D-Lys³]-GHRP6. Preincubation medium was then aspirated and replaced with fresh medium containing either A233 or GHRP6 10 nM.

GH immunoneutralization assay

To assay immunoneutralization against GH, the cells were incubated with GHRP6 or A233 (10 nM) in the presence of anti-tiGH mAb (CIGB) at a concentration of 0.1, 1, and 10 µg/ml for 4 h at 28 °C in 5% CO₂. Superoxide production was measured as described earlier.

Growth performance experiment

The growth promoting activity of A233 was evaluated in tilapia larvae (*Oreochromis sp.*) of 0.007 ± 0.001 g and goldfish (*C. auratus*) larvae of 0.003 ± 0.0008 g mean weight and 0.610 ± 0.09 cm length (*n* = 200). The effect was compared with GHRP6 as positive control. The fish were acclimated at 28 °C in 120 l tanks with running fresh water for 1 week before the experiment. They were fed with a basal diet to satiation twice a day. Before treatment, the tanks were cleaned. The fish were immersed into each treatment for 60 min without water recirculation. The immersion procedure was performed three times a week, for a period of 1 month. Three experimental groups were handled with the following treatments: groups 1 and 2 received the peptides A233 and GHRP6 (positive control) at a dose of 0.1 mg/l, respectively, and a nontreated group in the third one. Growth-promoting effects were evaluated by measuring the body weight and increase in length.

Homogenates of larvae

Larvae were washed three times with sterile PBS 1×: 137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na₂HPO₄·7H₂O at pH 7.3. The larvae were homogenized using 10 ml PBS 1×/g of tissue and centrifuged at 5000 g for 15 min at 4 °C. The supernatants were collected and stored at –70 °C. The protein concentration of larval homogenates was measured with BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions.

Hemagglutination assay for lectins

Serial twofold dilutions of 100 µl larval extracts were performed using PBS (pH 7.2) in U-bottom shaped (96 wells, Greiner Bio-One GmbH, Frickenhausen, Germany, Microtiter) microtiter wells to which an equal volume of freshly prepared 2% erythrocyte suspension (rabbit in PBS) was added (Jung *et al.* 2003). Wells were incubated for 1 h at room temperature and the titer was read visually and was equal to the dilution in the last well to show agglutination (as manifested by an evenly distributed layer of cells over the whole well bottom). The hemagglutinin activity of samples was examined and for each a titer value was obtained. The activity was expressed as titer, i.e. the reciprocal of the highest dilution showing complete agglutination.

Antiprotease activity

Antiprotease activity was performed as described by Magnadottir *et al.* (1999). Briefly, 20 µl larval homogenates were incubated with the same volume of standard trypsin solution (Sigma, 5 mg/ml) for 10 min at 22 °C. To this, 200 µl of 0.1 M phosphate buffer (pH 7.0) and 250 µl 2% azocasein (Sigma) were added and incubated for 1 h at 22 °C. Then, 500 µl of 10% trichloroacetic acid was added and

Figure 1 (A) 2D representation of the peptide amide A233 (GKFDLSPEHQ). The lactam bond between Lys and Asp was formed during the synthesis procedure on solid-phase support and is essential for the biological activity of the molecule. The lactam bond is indicated by shadow rectangle. (B) Dose-related effects of A233 on superoxide anion production by tilapia (*Oreochromis sp.*) HKLs. Cells were incubated with A233 for 4 h at 28 °C in 5% CO₂. Cells with Hanks' solution (pH 7.4) were the control. Data are expressed as the absorbance at 620 nm and bars represent S.D. of the mean (S.E.M., n=5). Statistical differences between groups for Newman-Keuls's test.

incubated for 30 min at 22 °C. The mixture was centrifuged at 6000 g for 5 min. The supernatant (100 µl) was transferred to a 96-well microtray containing 100 µl/well of 1 M NaOH. The OD was read at 450 nm. For a 100% control, buffer replaced the serum and for a negative control, buffer replaced both serum and trypsin. The percentage of inhibition of trypsin activity by each sample was calculated by comparing it to the 100% control sample. All the samples collected were analyzed in triplicates.

Determination of SOD in tilapia larval homogenates

SOD (superoxide dismutase) activity was determined based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol as described by Ecobichon (1984).

Statistical analysis

Results were evaluated using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA. All results are expressed as the mean ± S.D. Statistical analysis was performed using one-way ANOVA by Newman-Keuls's or Dunnett's method for data with normal distribution and equal variances: GH assays, superoxide production, GH immuno-neutralization assay, growth performance experiment of

tilapia (*Oreochromis sp.*) larvae, and hemagglutination assay for lectins, antiprotease, and SOD activity. Data with unequal variances were analyzed by the Kruskal-Wallis test followed by Dunn's multiple comparisons post-test: growth performance experiment of goldfish (*C. auratus*) larvae. Treatments were considered to be significantly different if $P < 0.05$.

Results

The molecule A233 (Fig. 1A) was selected from the virtual libraries described by Rodriguez *et al.* (2007), and, in order to determine *in vitro* and *in vivo* whether this novel mimetic molecule of natural or synthetic GHSs maintains some of its biological functions, the following assays were conducted on the growth and immune system.

GH in vitro assays using pituitary cell culture

To evaluate the effect of A233 peptide on GH secretion, we performed an *in vitro* culture of cells in the pituitary gland of tilapia (*Oreochromis sp.*). GH secretion did not increase in culture of cells after 4 h of treatment with A233. At 8 h, the supernatant of cells that received 1 nM A233 peptide did not show a significant increase in GH concentration present; however, the A233 peptide at a concentration of 10 nM stimulated GH secretion by cells in the anterior pituitary gland (Table 1). The stimulatory effect of GRLN and GHSs on the release of GH *in vitro* by the pituitary gland has been reported in mammals, birds, and different species of fish (Kaiya *et al.* 2003a,b, 2008, Unniappan & Peter 2004, Boaz 2005, Fox *et al.* 2007, Picha *et al.* 2009).

Determination and characterization of superoxide anion induction in HKLs

Production of superoxide anion by cultured tilapia (*Oreochromis sp.*) in HKLs was measured by the addition of A233. This peptide induced significant increases in superoxide production. The peptide A233 at concentrations from 0.1 to

Table 1 Effects of the A233 on GH secretion by cultured tilapia pituitary cells. Experiments were tested in triplicate with pituitaries from six animals. Data represent arithmetic mean of the tilapia GH ± S.D.

	GH concentration (ng/ml)	
	4 h	8 h
Treatment		
Control	0.169 ± 0.05	0.13 ± 0.03
A233 (1 nM)	0.16 ± 0.05	0.15 ± 0.05
A233 (10 nM)	0.18 ± 0.07	0.30 ± 0.08 ^a
GHRP6 (1 nM)	0.05 ± 0.04	0.16 ± 0.09
GHRP6 (10 nM)	0.04 ± 0.02	0.42 ± 0.06 ^a

^aSignificantly different from control in the same column ($P < 0.05$).

Figure 2 Effects of the GHSR-specific antagonist, [D-Lys³]-GHRP6, on superoxide production in phagocytic HKLs. Cells were preincubated with [D-Lys³]-GHRP6 (10 µM) for 1 h and incubated with A233 (10 nM) for 4 h. Data are expressed as the absorbance at 620 nm and bars represent s.d. of the mean (s.e.m., $n=5$). *Significantly different from respective control; †significant differences between columns for Student's *t*-test.

10 nM increased superoxide production in the phagocytic HKL in a dose-dependent manner (Fig. 1B). Preincubation of HKLs with [D-Lys³]-GHRP6 (10 µM) for 1 h significantly suppressed the stimulatory effects of A233 (10 nM) on superoxide anion production after 4 h of incubation (Fig. 2).

Owing to the importance of phagocytic cells in the immune response in fish, we evaluated the effect of peptide on A233 *in vitro* superoxide anion production in phagocytic leukocytes isolated from tilapia (*Oreochromis sp.*). The increase of superoxide anion production was statistically higher in cells stimulated with the highest dose of peptide tested (10 nM). Similarly, the stimulatory effect of the peptide, the increase in reactive oxygen species (ROS), was also evident in isolated tilapia peripheral blood leukocytes (data not shown). These results are consistent with those observed in leukocytes from the anterior kidney of rainbow trout (*Oncorhynchus mykiss*) stimulated *in vitro* with different doses of des-GRLN or GRLN VRQ – the same kind (Yada *et al.* 2006). Additionally, this effect is similar to that reported by Acosta *et al.* (2010), who stimulated phagocytic peripheral blood cells of tilapia (*Oreochromis sp.*) with recombinant tiGH (*Oreochromis hornorum*). Addition of anti-tiGH mAb to the cultured HKLs mixed with A233 resulted in significant inhibition of superoxide production, thereby showing a GH-mediated mechanism (Fig. 3).

In vivo biological activity assays

Experiments were performed to evaluate the biological effect of the peptide on the growth rate of goldfish and tilapia larvae. The effect of these peptides on fish growth was determined by measuring the increase in body weight and length. The goldfish larvae treated with A233 showed a highly significant increase in body weight and length compared with the controls just 7 days after treatment and significant differences also compared with the positive control group treated with

GHRP6 (positive control). However, there were no statistically significant differences in larval length between the A233 and GHRP6 treatment groups (Fig. 4).

In the assay with tilapia larvae, the wet body weight and size were recorded at 30 days from the beginning of the experiment. Statistically significant differences in weight were found in tilapia larvae treated with the A233 peptide compared with the nontreated group, as well as larvae treated with GHRP6. No statistically significant difference in length was observed between the GHRP6 and A233 groups during the experiment (Fig. 4).

No statistical differences in protein concentration, anti-protease activity, and lectin titer were found at 20 days from the beginning of the immersion experiment (Table 2). At day 30, increased antiprotease activity and lectins were found in larval homogenates treated with A233 and GHRP6 compared with control group (Table 2).

Determination of SOD induction in tilapia larval homogenates

SOD enzyme activity was measured in larval groups at 20 and 30 days after the beginning of the treatment. There were statistically significant differences between negative controls and the secretagogue-treated groups at 20 days. At 30 days, there were statistically significant differences between the A233 and the GHRP6-treated groups (Table 3).

Discussion

In this study, we demonstrate for the first time the effect of peptidic molecules having internal cycles and composed solely of L-amino acids that are capable of exerting, due to their chemical structure, similar functions to those attributed to GRLN, des-acyl GRLN, and other peptidic GHS. They are a group of peptide compounds and peptides capable of stimulating production and GH secretion *in vitro* and *in vivo*

Figure 3 Effect of GH immunoneutralization on superoxide anion production in HKLs. Cells were incubated with A233 (10 nM) in the presence of anti-tilapia GH mAb (0.1, 1, and 10 µg/ml) for 4 h at 28 °C in 5% CO₂. Data are expressed as the absorbance at 620 nm and bars represent s.d. of the mean (s.e.m., $n=5$). Different letters indicate differences between treatments for Newman-Keuls's test. *Significantly different from respective control for the Student's *t*-test.

Figure 4 Growth promotion experiment in *Carasius auratus* and *Oreochromis* sp. larvae immersed in GHRP6 and A233 peptide at a dose of 0.1 mg/l. (A) Effect on length and weight over 7 days of treatment in goldfish larvae; samples of 50 animals were sized and weighted at 7 days. The control group did not receive any treatment. Kruskal–Wallis test followed by Dunn’s multiple comparison post-test was used for length and body weight comparisons among groups. Different letters represent statistical significance. Data are expressed as mean $\pm$ s.d. ($n=50$). (B) Effect on length and weight over 30 days of treatment in tilapia larvae; samples of 50 animals were sized and weighted at 30 days. The control group did not receive any treatment. Newman–Keuls multiple comparison tests were used for length and body weight comparison among groups. Different letters represent statistical significance. Data are expressed as mean $\pm$ s.d. ($n=50$).

(Tannenbaum & Bowers 2001, Moulin *et al.* 2007), and subsequent investigations have revealed different receptors that can recognize these molecules and the diverse actions they can promote (Veldhuis & Bowers 2010).

Our results are similar to those obtained in tilapia (*Oreochromis mossambicus*), where the effect of GRLN on GH secretion *in vitro* was dependent on the concentration of the endogenous secretagogue used (Kaiya *et al.* 2003b). Meanwhile, Boaz (2005) observed the stimulating effect of CP-477 335 secretagogue on the secretion of GH by the pituitary gland of

Mozambique tilapia (*O. mossambicus*) by incubating the body for 8 h with the synthetic secretagogue. Other researchers have reported the stimulatory effect of the synthetic peptides GHRP6, PACAP, and PACAP-related peptide from *Clarias gariepinus* on the release of GH *in vitro* by the pituitary gland of tilapia (*Oreochromis niloticus*; Lugo *et al.* 2008).

There are reports of increased levels of GH *in vivo* after treatment with synthetic peptide secretagogues. These results have been observed in tilapia (*O. mossambicus*) and rainbow trout (*O. mykiss*) following i.p. injection of the peptide GHRP2 (Shepherd *et al.* 2000, 2007). However, other researchers did not observe the same effects in the carp (*Ctenopharyngodon idellus*) treated with GHRP6 or hexarelin (Xiao *et al.* 2002). The results obtained in the present investigation related to the secretion of GH *in vitro* show that A233 is a peptide GHS. Despite the *in vitro* biological studies that are not enough, it is necessary to carry out *in vivo* tests to corroborate the stimulating effect of A233 peptide on GH release.

In teleosts, the morphology of the anterior kidney is similar to the bone marrow of higher vertebrates and is a major hematopoietic organ where phagocytic cells are formed (Whyte 2007). Phagocytic cells produce ROS such as superoxide anion, which help to eliminate many of the pathogens and parasites that infect these animals (Magnadottir 2006, Secombes 2009). The superoxide anion is a ROS produced by the NAPH oxidase complex and are well-known central components in the antimicrobial arsenal of activated phagocytes (Brown *et al.* 2009). In our case, the stimulation of ROS by A233 could be achieved by the aforementioned NAPH pathway or through the increase in production of mitochondrial-derived ROS. Further experiments should be performed to clarify this mechanism. The phagocytic cells capable of producing these reactive oxygen ions in teleost fish are the monocytes, macrophages, and granulocytes (Zapata & Amemiya 2000).

The presence of GHSR has been reported in head–kidney of tilapia (*O. mossambicus*; Fox *et al.* 2007, Kaiya *et al.* 2009a,b,c), peripheral blood leukocytes, and leukocytes from the anterior kidney of rainbow trout (*O. mykiss*), among other tissues (Yada *et al.* 2006). It has recently been described in two variants of GHSR1a in zebrafish (*Danio rerio*; Kaiya *et al.* 2008, Olsson *et al.* 2008), catfish, and rainbow trout (*O. mykiss*; Kaiya *et al.* 2009a,b,c). Moreover, it is reported that the peptide [D-Lys³]-GHRP6 inhibits the mechanism of signal transduction of synthetic GHSs (Chan *et al.* 2004, Moulin *et al.* 2007). Leukocytes isolated from tilapia anterior kidney were treated with the specific antagonist secretagogue receptor [D-Lys³]-GHRP6 and subsequently stimulated with peptide A233. The stimulatory effect of peptide A233 on the increased production of superoxide anion was inhibited by pretreatment with the antagonist (Fig. 2). Similar results were observed in the phagocytic leukocytes of rainbow trout (*O. mykiss*), where the effect of GRLN was eliminated by the presence of GHSR1a antagonist (Yada *et al.* 2006). The results related to the specific antagonist secretagogue receptor

Table 2 Protein concentration and innate immunity parameters (anti-trypsin activity and lectins) in control, GHRP6-, and A233-treated groups at 20 and 30 days from the beginning of the immersion experiment. Data represent arithmetic mean $\pm$ s.d. at 20 days ($n=5$ pools of four larvae) and 30 days ($n=10$ pools of four larvae)

Groups	20 days			30 days		
	Protein concentration (mg/g of tissue)	Percentage of antiprotease activity (anti-trypsin)	Lectins (titer)	Protein concentration (mg/g of tissue)	Percentage of anti-protease activity (anti-trypsin)	Lectins (titer)
Control	10.34 $\pm$ 2.19	22.00 $\pm$ 2.84	3.00 $\pm$ 1.10	14.47 $\pm$ 5.92	13.19 $\pm$ 2.31	6.00 $\pm$ 2.19
GHRP6	10.61 $\pm$ 2.54	21.10 $\pm$ 3.41	4.67 $\pm$ 2.73	9.53 $\pm$ 1.75	23.11 $\pm$ 3.98 ^a	14.67 $\pm$ 9.35 ^a
A233	9.51 $\pm$ 0.95	19.85 $\pm$ 2.72	5.33 $\pm$ 2.07	13.97 $\pm$ 5.47	28.69 $\pm$ 4.43 ^a	28.00 $\pm$ 9.79 ^a

^aSignificantly different from control in the same column ($P<0.05$).

suggest that the A233 peptide action on tilapia leukocytes (*Oreochromis sp.*) is GHSR1a secretagogue receptor mediated.

The expression of GH and GH receptor has been found in tissues and cells of rat, cattle, and human immune systems, in addition to the distribution of GH mRNA in tissues of the fish immune system (Yada 2007, Hattori 2009). This suggests that GH acts in an autocrine/paracrine manner on the immune system (Yada *et al.* 2006, Yada 2007). Other studies show that the effects of GHSs on immune cells may be mediated by the action of GH produced by these cells (Poppi *et al.* 2002, Yada *et al.* 2006, Yada 2007, Hattori 2009).

In this study a mixture of monoclonal anti-tiGH peptide with the A233 was administered. The effect of the peptide A233 fell after being administered with the mixture of antibodies in a dose-dependent manner, being observed in the cells treated with 10 μ g/ml mix, a response similar to that found in the negative control. Yada *et al.* (2006) obtained similar results by administering GRLN and a polyclonal anti-GH of salmon to the white blood cells of rainbow trout (*O. mykiss*). Moreover, these authors reported the increase in the expression levels of GH and SOD in leukocytes stimulated with the endogenous secretagogue. There are several studies that demonstrate the effect of GH on phagocytic cells. In fish, the increase has been observed in the ingestion of particles by phagocytic leukocytes after *in vitro* administration of GH, suggesting activation of phagocytosis in these cells (Sakai *et al.* 1996, Calduch-Giner *et al.* 1997). Other researchers reported that incubation of peripheral blood leukocytes of rainbow trout (*O. mykiss*) with isolated GH salmon pituitary significantly stimulated the proliferation of cells (Yada *et al.* 2004).

The stimulatory effect of A233 on superoxide production was abolished by immunoneutralization with an anti-tiGH mAb mixture, suggesting the importance of GH secreted by leukocytes, as described by Yada *et al.* (2006), indicating that the effect of these peptides is mediated through local production of GH.

In vivo studies on the biological function of GHS in fish are scarce and focus mainly on the action of GRLN (Kaiya *et al.* 2003b, Unniappan & Peter 2004, Riley *et al.* 2005, Matsuda *et al.* 2006, Fox *et al.* 2007, Jönsson *et al.* 2007, Picha *et al.* 2009).

The value of GHSs as useful growth enhancement molecules is clear. These synthetic molecules are effective in stimulating production and release of endogenous hormone as a physiological response, with no side effects on the pituitary or toxicity potential; besides their low molecular weight, it makes a better entrance to the organism.

In our laboratory, we have successfully employed the immersion bath technique to study the effects of nutritional supplements and growth factors on growth control and the immune system in fish (Acosta *et al.* 2008). The immersion bath method used for our studies requires little manipulation and causes minimum stress to fish during treatment. There is evidence suggesting that the gill pillar cells are a possible entry site for some molecules when fish are treated by immersion bath (Palyha *et al.* 2000).

This study evaluated the biological function of synthetic peptide A233 on somatic growth of tilapia (*Oreochromis sp.*) and goldfish larvae (*C. auratus*). Tilapia larvae showed a significant increase in growth at 20 and 30 days of treatment with peptide A233 (0.1 mg/l). All animals received the same commercial diet, so the increase in weight and height is due to the administration of peptide A233. The positive control group represented by the fish treated with the peptide GHRP6 significantly increased their growth only at 30 days.

Table 3 Determination of SOD induction in tilapia larval homogenates at 20 and 30 days after the beginning of the treatment^a. Data represent arithmetic mean ($n=5$ pools) $\pm$ s.d.

Experimental groups	SOD activity (U/ml per min) ^b	
	20 days	30 days
Control	2.04 $\pm$ 0.45	1.06 $\pm$ 0.09
GHRP6	6.09 $\pm$ 2.82 ^c	7.54 $\pm$ 1.36 ^c
A233	9.06 $\pm$ 2.05 ^c	10.3 $\pm$ 2.8 ^c

^aThe treatment was performed three times a week by the immersion bath of larvae with the secretagogues as described in the Materials and Methods section.

^bThe SOD activity was expressed in units per milliliter of larval homogenates per minute (U/ml per min).

^cSignificantly different from control in the same column ($P<0.05$).

In the second fish species used, as early as 1 week after the application of the secretagogue peptide to goldfish larvae, statistically significant increases in body weight and length were obtained in the treated fish larvae compared with the control group. The time difference to show growth enhancement in the species chosen for this work probably reflects species-specific growth characteristics and the important fact that there have been four GHSR1a identified in goldfish derived from four distinct genes. In this species, two uniquely functional GHSRa receptors exist, with differential expression in tissues and distinct ligand selectivity, the GHSR1a acts on energy metabolism (Kaiya *et al.* 2010).

These results are similar to those found in mice where there was an increase in body weight after dosing by s.c. injection of the synthetic peptide GHRP2 (Tschöp *et al.* 2002). In adult rats, GHRP6 also increased body weight (Svensson *et al.* 2000) as well as other GHSR agonists, like SM-130686 administered orally (Nagamine *et al.* 2001) and BIM-28131 administered by s.c. injection (Strassburg *et al.* 2008). It has been recently reported that GHSR1a agonist, a pentapeptide with D-amino acids, promotes weight gain in rats, by i.p. administration during 7 days (Dong *et al.* 2009). Moreover, the administration, by the same method, of GH tilapia (*O. hornorum*) secreted into the culture supernatant of yeast *Pichia pastoris* (Acosta *et al.* 2008) to larvae of tilapia (*Oreochromis sp.*), significantly increased growth of these animals. Administration of tilapia recombinant neuropeptide Y (*Oreochromis sp.*) to larvae of African catfish (*C. gariepinus*) also produced an increase in animal weight (Carpio *et al.* 2007). In addition, treatment with PACAP and PACAP-related peptide from *C. gariepinus* larvae of African catfish (*C. gariepinus*), tilapia (*O. niloticus*), and common carp (*Cyprinus carpio*) increased body weight and length in three fish species (Carpio *et al.* 2008, Lugo *et al.* 2008).

Innate immunity is the first line of defense against pathogens that infect fish (Magnadottir 2006, Whyte 2007, Alvarez-Pellitero 2008). In fish embryos and larvae, innate immunity is very important because they lack acquired immunity (Magnadottir *et al.* 2005). Moreover, several studies report the use of nutritional supplements, hormones, and peptides as nonspecific immunostimulant immunity in cultured fish larvae as a strategy to improve survival of these organisms at this stage (Peddie *et al.* 2002, Bricknell & Dalmo 2005, Martínez *et al.* 2006, Acosta *et al.* 2008, Carpio *et al.* 2008).

In this study, we evaluated some parameters of the innate immune response in larvae treated with the peptide A233. Among the parameters analyzed were the title of lectins and antiprotease activity. Additionally, we assessed the enzymatic activity of SOD as an indicator of antioxidant defense.

Lectins have the function of defense against viruses and bacteria are part of the defense of the fish integument and systemic humoral defenses of innate immunity (Ellis 2001). Carbohydrate-binding proteins are present in pathogens, leading to opsonization, phagocytosis, and activation of the complement system (Magnadottir 2006). Many bacteria produce toxins with proteolytic activity that digest proteins

in the host tissue as a result of these protease inhibitors that have a role in defense against pathogens that infect fish (Zuo & Woo 1997, Woo 2001).

In this study, as the results found in the title of lectins, we identified only a significant increase of antiprotease activity within 30 days of treatment using the peptides. These results suggest that the immunological state enhancing effect of the peptide A233 on some parameters of the immune system of tilapia larvae depends on the number of treatments with the peptide. Moreover, these results are consistent with previous reports showing that immersion baths of nutritional supplement Acuabio 1 increase antiprotease activity and lectin titer in fish larvae (*Oreochromis sp.*; Martínez *et al.* 2006). Additionally, the results are similar to those found in larvae of tilapia (*Oreochromis sp.*) treated with GH tilapia, lacking the 46 last amino acids of the C-terminal, through dips, where the only significant difference was found in antiprotease activity and lectins titer within 30 days of treatment (Acosta *et al.* 2011).

The action of GRLN and GHS on the cells of the immune system is in correspondence with the widespread distribution of GHS receptors in these tissues (Hattori 2009). In humans, the expression of GHSR has been reported in the spleen, lymph nodes, and lymphocytes (Gnanapavan *et al.* 2002). The presence of GHSR has also been found in mouse spleen cells and spleen, gill, kidney, and leukocytes in teleost fish (Xia *et al.* 2004, Yada *et al.* 2006, Kaiya *et al.* 2009a,b,c).

The treated larvae exhibit a better growth rate as well as an enhancement of the some innate immune response parameters, improving the larvae quality. It could potentially give them a higher resistance to pathogens and better efficient adaptive response due to the cross talk between innate and acquired immune response. Next, experiments should be conducted to verify how adaptive response is affected in fish with an enhanced innate immune response due to A233 administration. The different parameters of the innate immune response are highly variable, and the application of immunostimulant allows priming of the innate immune response in the larvae population.

Hormones have been reported to modulate antioxidant enzyme activities in mammals (Hauck & Bartke 2000). In this study, we determined the activity of the antioxidant enzyme SOD in homogenates of larvae treated with peptide A233. In the test performed two times was an increase in the activity of the enzyme in the larvae treated with the peptides. There are reports of fish showing modulation of antioxidant activity due to variations in energy metabolism (Wilhelm Filho *et al.* 1993, Martínez-Alvarez *et al.* 2005). Administration or overexpression of GH produced a significant increase in metabolic rate and oxygen consumption in Atlantic salmon (*Salmo salar*; Seddiki *et al.* 1995, 1996, Stevens *et al.* 1998, Cook *et al.* 2000, Herbert *et al.* 2001) and tilapia (*Oreochromis sp.*; McKenzie *et al.* 2000). In addition, administration by immersion baths of neuropeptide Y recombinant African catfish larvae (*C. gariepinus*) increased the concentration of reduced glutathione and SOD activity, without producing any effect on the activity of catalase (Carpio *et al.* 2007). Also, in larvae

of tilapia treated with recombinant tilapia, GH was increased in the activity of SOD and catalase (Acosta *et al.* 2011).

The observed increase in antioxidant defenses may neutralize deleterious byproducts of metabolism and counteract the oxidative stress associated with growth. Similar results were obtained by Brown-Borg & Rakoczy (2003), who observed body weight increase and alteration of multiple components of the antioxidative defense system after GH administration to dwarf mice.

The results obtained in this research associated with the parameters of the innate immune response and antioxidant defense of the larvae of tilapia indicate, for the first time, the role of the A233 peptide as a stimulator of the immune system of tilapia (*Oreochromis sp.*).

It would be interesting to determine the temporal and tissue-specific characteristics for activation of tilapia receptors by the new A233 secretagogue peptide. The decapeptide A233 designed by molecular modeling is able to function as a GHS in teleosts. This biological activity was corroborated by the capability of A233 to accelerate the growth rate in fish larvae and to enhance some parameters of the innate immune response and antioxidant defenses through a GH-mediated mechanism.

One of the biggest problems facing aquaculture is the high rates of mortality during the larval stage of fish because of various factors that include diet, physical and chemical factors, and diseases caused by pathogens (Helvik *et al.* 2009). Therefore, stimulation of growth directed to reduce the time of harvest and high mortality in the larval stages of fish and the use of immunostimulants to prepare them to cope with intensive farming are the key objectives of modern biotechnology. The delivery of A233 by immersion baths to fish larvae stimulates growth, and due to the action of GH, there is also stimulation on various parameters of innate immunity, as evidence of the relationship between the immune and endocrine systems in fish.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

Acosta J, Carpio Y, Besada V, Farnos O, Morales R, Sánchez A, Curbelo Y, Ayala J & Estrada MP 2008 Recombinant truncated tilapia growth hormone enhances growth and innate immunity in tilapia fry (*Oreochromis sp.*). *General and Comparative Endocrinology* **157** 49–57. (doi:10.1016/j.ygcen.2008.03.009)

Acosta J, Carpio Y, Morales R, Aguila JC, Acanda Y, Herrera F & Estrada MP 2010 New insights into the biological activity and secretion properties of a polypeptide derived from tilapia somatotropin. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **156** 264–272. (doi:10.1016/j.cbpb.2010.04.001)

Acosta J, Ruiz O, Carpio Y, Morales R, Aguila JC, Valdés J, Martínez E & Estrada MP 2011 Thermal treatment enhances the stability and biological activity of a truncated tilapia somatotropin contained in *Pichia pastoris* culture supernatant. *Journal of Biotechnology* **151** 175–179. (doi:10.1016/j.jbiotec.2010.11.017)

Alvarez-Pellitero P 2008 Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology* **126** 171–198. (doi:10.1016/j.vetimm.2008.07.013)

Boaz S 2005 Effects of the growth hormone secretagogue, CP-477335, on the GH/IGF-I axis in tilapia, *Oreochromis mossambicus*. *Journal of Young Investigators* **12**.

Bricknell I & Dalmo RA 2005 The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology* **19** 457–472. (doi:10.1016/j.fsi.2005.03.008)

Brown-Borg HM & Rakoczy SG 2003 Growth hormone administration to long-living dwarf mice alters multiple components of the antioxidative defense system. *Mechanisms of Ageing and Development* **124** 1013–1024. (doi:10.1016/j.mad.2003.07.001)

Brown KL, Christenson K, Karlsson A, Dahlgren C & Bylund J 2009 Divergent effects on phagocytosis by macrophage-derived oxygen radicals. *Journal of Innate Immunity* **1** 592–598. (doi:10.1159/000235583)

Calduch-Giner JA, Sitja-Bobadilla A, Alvarez-Pellitero P & Pérez-Sánchez J 1997 Growth hormone as an *in vitro* phagocyte-activating factor in the gilthead sea bream (*Spanus aurata*). *Cell Tissue Research* **287** 535–540. (doi:10.1007/s004410050777)

Carpio Y, Leon K, Acosta J, Morales R & Estrada MP 2007 Recombinant tilapia neuropeptide Y promotes growth and antioxidant defenses in African catfish (*Clarias gariepinus*) fry. *Aquaculture* **272** 649–655. (doi:10.1016/j.aquaculture.2007.08.024)

Carpio Y, Lugo JM, Leon K, Morales R & Estrada MP 2008 Novel function of recombinant pituitary adenylate cyclase-activating polypeptide as stimulator of innate immunity in African catfish (*Clarias gariepinus*) fry. *Fish & Shellfish Immunology* **25** 439–445. (doi:10.1016/j.fsi.2008.06.004)

Chan CB, Leung PK, Wise H & Cheng CH 2004 Signal transduction mechanism of the seabream growth hormone secretagogue receptor. *FEBS Letters* **577** 147–153. (doi:10.1016/j.febslet.2004.08.088)

Chang JP & Wong AOL 2009 Fish neuroendocrinology. Chapter 4 growth hormone regulation in fish: a multifactorial model with hypothalamic, peripheral and local autocrine/paracrine signals. *Fish Physiology* **28** 151–195. (doi:10.1016/S1546-5098(09)28004-6)

Cook JT, McNiven MA & Sutterlin AM 2000 Metabolic rate of pre-smolt growth-enhanced transgenic Atlantic salmon (*Salmo salar*). *Aquaculture* **188** 33–45. (doi:10.1016/S0044-8486(00)00332-X)

Devlin R, Sakhrania D, Tymchuk W, Rise M & Goh B 2009 Domestication and growth hormone transgenesis cause similar changes in gene expression in coho salmon (*Oncorhynchus kisutch*). *PNAS* **106** 3047–3052. (doi:10.1073/pnas.0809798106)

Dixit VD, Schaffer EM, Pyle RS, Collins GD, Sakthivel SK, Palaniappan R, Lillard JW & Taub DD 2004 Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. *Journal of Clinical Investigation* **114** 57–66.

Dong JZ, Zhang J, Taylor JE, Halem H, Datta R, Culler M & Eynon J 2009 GHS-1a agonists that effectively stimulate food intake and body weight gain. *Peptides for Youth: the Proceedings of the 20th American Peptide Symposium, Advances in Experimental Medicine and Biology* **611** 487–488. (doi:10.1007/978-0-387-73657-0_210)

Ecobichon DJ 1984 Glutathione depletion and resynthesis in laboratory animals. *Drug and Chemical Toxicology* **7** 345–355. (doi:10.3109/01480548408998263)

Ellis AE 2001 Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology* **25** 827–839. (doi:10.1016/S0145-305X(01)00038-6)

- Fox BK, Riley LG, Dorough C, Kaiya H, Hirano T & Grau EG 2007 Effects of homologous ghrelins on the growth hormone/insulin-like growth factor-I axis in the tilapia, *Oreochromis mossambicus*. *Zoological Science* **24** 391–400. (doi:10.2108/zsj.24.391)
- Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB & Korbonits M 2002 The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *Journal of Clinical Endocrinology and Metabolism* **87** 2988–2991. (doi:10.1210/jc.87.6.2988)
- Hattori N 2009 Expression, regulation and biological actions of growth hormone (GH) and ghrelin in the immune system. *Growth Hormone & IGF Research* **19** 187–197. (doi:10.1016/j.ghir.2008.12.001)
- Hattori N, Saito T, Yagyu T, Jiang BH, Kitagawa K & Inagaki C 2001 GH, GH receptor, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. *Journal of Clinical Endocrinology and Metabolism* **86** 4284. (doi:10.1210/jc.86.9.4284)
- Hauck SJ & Bartke A 2000 Effects of growth hormone on hypothalamic catalase and Cu/Zn superoxide dismutase1. *Free Radical Biology & Medicine* **28** 970–978. (doi:10.1016/S0891-5849(00)00186-6)
- Helvik JV, Hamre K, Hordvik I, van der Meeren T, Ressem H, Scharlt M, Tveiten H & Oie G 2009 The fish larva: a transitional life form, the foundation for aquaculture and fisheries. In *The Research Council of Norway*, pp 5–35. Norway.
- Herbert NA, Armstrong JD & Björnsson BT 2001 Evidence that growth hormone-induced elevation in routine metabolism of juvenile Atlantic salmon is a result of increased spontaneous activity. *Journal of Fish Biology* **59** 754–757. (doi:10.1111/j.1095-8649.2001.tb02379.x)
- Howard AD, Feighner SD, Cully DF, Arena JP, Liberators PA & Rosenblum CI 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273** 974–977. (doi:10.1126/science.273.5277.974)
- Jönsson E, Forsman A, Einarsdóttir IE, Kaiya H, Ruohonen K & Björnsson BT 2007 Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **147** 1116–1124. (doi:10.1016/j.cbpa.2007.03.024)
- Jung WK, Park PJ & Kim SK 2003 Purification and characterization of a new lectin from the hard roe of skipjack tuna, *Katsuwonus pelamis*. *International Journal of Biochemistry & Cell Biology* **35** 255–265. (doi:10.1016/S1357-2725(02)00176-0)
- Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG & Kangawa K 2003a Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. *Journal of Endocrinology* **176** 415–423. (doi:10.1677/joe.0.1760415)
- Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T & Grau EG 2003b Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **135** 421–429. (doi:10.1016/S1096-4959(03)00109-X)
- Kaiya H, Miyazato M, Kangawa K, Peter RE & Unniappan S 2008 Ghrelin: a multifunctional hormone in non-mammalian vertebrates. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **149** 109–128. (doi:10.1016/j.cbpa.2007.12.004)
- Kaiya H, Kodama S, Ishiguro K, Matsuda K, Uchiyama M, Miyazato M, Uchiyama M, Miyazato M & Kangawa K 2009a Ghrelin-like peptide with fatty acid modification and O-glycosylation in the red stingray, *Dasyatis akajei*. *BMC Biochemistry* **10** 30. (doi:10.1186/1471-2091-10-30)
- Kaiya H, Mori T, Miyazato M & Kangawa K 2009b Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **153** 438–450. (doi:10.1016/j.cbpa.2009.04.612)
- Kaiya H, Riley LG, Janzen W, Hirano T, Grau EG & Miyazato M 2009c Identification and genomic sequence of a ghrelin receptor (GHS-R)-like receptor in the Mozambique tilapia, *Oreochromis mossambicus*. *Zoological Science* **26** 330–337. (doi:10.2108/zsj.26.330)
- Kaiya H, Miura T, Matsuda K, Miyazato M & Kangawa K 2010 Two functional growth hormone secretagogue receptor (ghrelin receptor) type 1a and 2A in goldfish *Carassius auratus*. *Molecular and Cellular Endocrinology* **327** 25–39. (doi:10.1016/j.mce.2010.06.004)
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660. (doi:10.1038/45230)
- Lugo JM, Rodríguez A, Helguera Y, Morales R, González O, Acosta J, Besada V, Sánchez A & Estrada MP 2008 Recombinant novel pituitary adenylate cyclase activating polypeptide (PACAP) from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth promoting factor in low vertebrates. *Journal of Endocrinology* **197** 583–597. (doi:10.1677/JOE-07-0555)
- Magnadóttir B 2006 Innate immunity of fish (overview). *Fish & Shellfish Immunology* **20** 137–151. (doi:10.1016/j.fsi.2004.09.006)
- Magnadóttir B, Jónsdóttir H, Helgason S, Björnsson B, Jørgensen TO & Pilstrom L 1999 Humoral immune parameters in Atlantic cod (*Gadus morhua* L.). II. The effects of size and gender under different environmental conditions. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **122** 181–188. (doi:10.1016/S0305-0491(98)10157-8)
- Magnadóttir B, Lange S, Gudmundsdóttir S, Bogwald J & Dalmo RA 2005 Ontogeny of humoral immune parameters in fish. *Fish & Shellfish Immunology* **19** 429–439. (doi:10.1016/j.fsi.2005.03.010)
- Martínez R, Carpio Y, Gómez Y, Raíces M, Morales A, Herrera F, González O, Morales R & Estrada MP 2006 Acuabio 1 estimula el metabolismo anaerobio y el sistema inmune innato de las larvas de goldfish y tilapia. *Biología Aplicada* **23** 287–293.
- Martínez-Alvarez RM, Morales AE & Sanz A 2005 Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and Fisheries* **15** 75–88. (doi:10.1007/s11160-005-7846-4)
- Matsuda K, Miura T, Kaiya H, Maruyama K, Shimakura S, Uchiyama M, Kangawa K & Shioda S 2006 Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. *Peptides* **27** 2321–2325. (doi:10.1016/j.peptides.2006.03.028)
- McKenzie DJ, Martínez R, Morales A, Acosta J, Taylor EW, Steffensen JF & Estrada MP 2000 Metabolic rate, exercise performance and hypoxia tolerance of growth hormone transgenic tilapia (*Oreochromis sp.*). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **126** S66. (doi:10.1016/S0305-0491(00)80130-3)
- Momany FA, Bowers CY, Reynolds GA, Chang D, Hong A & Newlander K 1981 Design, synthesis and biological activity of peptides which release growth hormone, *in vitro*. *Endocrinology* **108** 31–39. (doi:10.1210/endo-108-1-31)
- Moulin A, Ryan J, Martínez J & Fehrentz JA 2007 Recent developments in ghrelin receptor ligands. *ChemMedChem* **2** 1242–1259. (doi:10.1002/cmdc.200700015)
- Muccioli G, Baragli A, Granata R, Papotti M & y Ghigo E 2007 Heterogeneity of Ghrelin/Growth Hormone Secretagogue Receptors. Toward the Understanding of the Molecular Identity of Novel Ghrelin/GHS Receptors. *Neuroendocrinology* **86** 147–164.
- Nagamine J, Nagata R, Seki H, Nomura-Akimaru N, Ueki Y, Kumagai K, Tajiri M & Noguchi H 2001 Pharmacological profile of a new orally active growth hormone secretagogue, SM-130686. *Journal of Endocrinology* **171** 481–489. (doi:10.1677/joe.0.1710481)
- Olsson C, Holbrook JD, Bompadre G, Jönsson E, Hoyle CH, Sanger GJ, Holmgren S & Andrews PL 2008 Identification of genes for the ghrelin and motilin receptors and a novel related gene in fish, and stimulation of intestinal motility in zebrafish (*Danio rerio*) by ghrelin and motilin. *General and Comparative Endocrinology* **155** 217–226. (doi:10.1016/j.ygcen.2007.05.016)
- Palyha OC, Feighner SD, Tan CP, McKee KK, Hreniuk DL & Gao YD 2000 Ligand activation domain of human orphan growth hormone (GH) secretagogue receptor (GHS-R) conserved from Pufferfish to humans. *Molecular Endocrinology* **14** 160–169. (doi:10.1210/me.14.1.160)
- Peddie S, Zou J & Secombes CJ 2002 Immunomodulation in the rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal administration of Ergosan. *Veterinary Immunology and Immunopathology* **86** 101–113. (doi:10.1016/S0165-2427(02)00019-3)

- Picha ME, Strom CN, Riley LG, Walker AA, Won ET, Jhnstone WM & Borski RJ 2009 Plasma ghrelin and growth hormone regulation in response to metabolic state in hybrid striped bass: effects of feeding, ghrelin and insulin-like growth factor-I on *in vivo* and *in vitro* GH secretion. *General and Comparative Endocrinology* **161** 365–372. (doi:10.1016/j.ygcen.2009.01.026)
- Poppi L, Dixit VD, Baratta M, Giustina A, Tamanini C & Parvizi N 2002 Growth hormone secretagogue (GHS) analogue, hexarelin stimulates GH from peripheral lymphocytes. *Experimental and Clinical Endocrinology & Diabetes* **110** 343–347. (doi:10.1055/s-2002-34991)
- Riley LG, Fox BK, Kaiya H, Hirano T & Grau EG 2005 Long-term treatment of ghrelin stimulates feeding fat deposition, and alters the GH/IGF-I axis in the tilapia (*Oreochromis mossambicus*). *General and Comparative Endocrinology* **142** 234–240. (doi:10.1016/j.ygcen.2005.01.009)
- Rise ML, Douglas S, Sakharani D, Williams J, Ewart KV, Rise M, Davidson WS, Koop BF & Devlin RH 2006 Multiple microarray platforms utilized for hepatic gene expression profiling of GH transgenic coho salmon with and without ration restriction. *Journal of Molecular Endocrinology* **37** 259–282. (doi:10.1677/jme.1.02031)
- Rodriguez R, De la Nuez A, Estrada MP, Martinez R, Chinae G, Reyes O, Fernandez R, Garcia D, Berlanga A & Musacchio A 2007 Compounds analogous to growth hormone peptide secretagogues and preparations containing them. (Patent publication No.: WO 2007/098716 A1).
- Sakai M, Kobayashi M & Kawachi H 1996 Mitogenic effect of growth hormone and prolactin on chum salmon *Oncorhynchus keta* leukocytes *in vitro*. *Veterinary Immunology and Immunopathology* **53** 185–189. (doi:10.1016/0165-2427(95)05507-X)
- Secombes CJ 1997 The NonspecificImmune System: Cellular Defenses. *Fish Physiology* **15** 63–103. (doi:10.1016/S1546-5098(08)60272-1)
- Seddiki H, Maxime V, Boeuf G & Peyraud C 1995 Effects of growth hormone on plasma ionic regulation, respiration and extracellular acid-base status in trout (*Oncorhynchus mykiss*) transferred to seawater. *Fish Physiology and Biochemistry* **14** 279–288. (doi:10.1007/BF00004066)
- Seddiki H, Boeuf G, Maxime V & Peyraud C 1996 Effects of growth hormone treatment on oxygen consumption and sea water adaptability in Atlantic salmon parr and pre-smolts. *Aquaculture* **148** 49–62. (doi:10.1016/S0044-8486(96)01407-X)
- Shepherd BS, Eckert SM, Parhar IS, Vijayan MM, Wakabayashi I & Hirano T 2000 The hexapeptide KP-102 (D-al¹-D-beta²-Nal³-ala⁴-trp⁵-D-phe⁶-lys⁷-NH²) stimulates growth hormone release in a cichlid fish (*Oreochromis mossambicus*). *Journal of Endocrinology* **167** 7–10. (doi:10.1677/joe.0.167R007)
- Shepherd BS, Johnson JK, Silverstein JT, Parhar IS, Vijayan MM, McGuire A & Weber GM 2007 Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **146** 390–399. (doi:10.1016/j.cbpa.2006.11.004)
- Sherwood NM, Krueckl SL & McRory JE 2000 The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocrine Reviews* **21** 619. (doi:10.1210/er.21.6.619)
- Small BC, Quiniou SMA & Kaiya H 2009 Sequence, genomic organization and expression of two channel catfish, *Ictalurus punctatus*, ghrelin receptors. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **154** 451–464. (doi:10.1016/j.cbpa.2009.07.027)
- Strassburg S, Anker SD, Castaneda TD, Burget L, Perez-Tilve D, Pfluger PT, Nogueiras R, Halem H, Dong J, Culler M *et al.* 2008 Long-term effects of ghrelin and ghrelin receptor agonists on energy balance in rats. *American Journal of Physiology. Endocrinology and Metabolism* **295** E78–E84. (doi:10.1152/ajpendo.00040.2008)
- Stevens ED, Sutterlin A & Cook T 1998 Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. *Canadian Journal of Fisheries and Aquaculture Sciences* **55** 2028–2035. (doi:10.1139/F98-078)
- Svensson SJ, Lall S, Dickson L, Bengtsson B-Å, Romer J, Ahnfelt-Ronne I, Ohlsson C & Jansson J-O 2000 The GH secretagogues ipamorelin and GH-releasing peptide-6 increase bone mineral content in adult female rats. *Journal of Endocrinology* **165** 569–577. (doi:10.1677/joe.0.1650569)
- Tannenbaum GS & Bowers CY 2001 Interactions of growth hormone secretagogues and growth hormone-releasing hormone/somatostatin. *Endocrine* **14** 21–27. (doi:10.1385/ENDO:14:1:021)
- Tschöp M, Statnick MA, Suter TM & Heiman ML 2002 GH-releasing peptide-2 increases fat mass in mice lacking NPY: indication for a crucial mediating role of hypothalamic agouti-related protein. *Endocrinology* **143** 558–568. (doi:10.1210/en.143.2.558)
- Unniappan S & Peter RE 2004 *In vitro* and *in vivo* effects of ghrelin on luteinizing hormone and growth hormone release in goldfish. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **286** R1093–R1101. (doi:10.1152/ajpregu.00669.2003)
- Veldhuis JD & Bowers CY 2010 Integrating GHS into the ghrelin system. *International Journal of Peptides* **2010** 1–40. (doi:10.1155/2010/879503)
- Whyte SK 2007 The innate immune response of finfish – a review of current knowledge. *Fish & Shellfish Immunology* **23** 1127–1151. (doi:10.1016/j.fsi.2007.06.005)
- Wilhelm Filho D, Giulivi C & Boveris A 1993 Antioxidant defenses in marine fish. I. Teleosts. *Comparative Biochemistry and Physiology* **106** 409–413.
- Woo PTK 2001 Cryptobiosis and its control in north American fishes. *International Journal for Parasitology* **31** 566–574. (doi:10.1016/S0020-7519(01)00169-2)
- Xia Q, Pang W, Pan H, Zheng Y, Kang JS & Zhu SG 2004 Effects of ghrelin on the proliferation and secretion of splenic T lymphocytes in mice. *Regulatory Peptides* **122** 173–178. (doi:10.1016/j.regpep.2004.06.016)
- Xiao D, Chu MM, Lee EK, Lin HR & Wong AO 2002 Regulation of growth hormone release in common carp pituitary cells by pituitary adenylate cyclase-activating polypeptide: signal transduction involves cAMP- and calcium-dependent mechanisms. *Neuroendocrinology* **76** 325–338. (doi:10.1159/000066627)
- Yada T 2007 Growth hormone and fish immune system. *General and Comparative Endocrinology* **152** 353–358. (doi:10.1016/j.ygcen.2007.01.045)
- Yada T, Muto K, Azuma T & Ikuta K 2004 Effects of prolactin and growth hormone on plasma levels of lysozyme and ceruloplasmin in rainbow trout. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* **139** 57–63. (doi:10.1016/j.cca.2004.09.003)
- Yada T, Kaiya H, Mutoh K, Azuma T, Hyodo S & Kangawa K 2006 Ghrelin stimulates phagocytosis and superoxide production in fish leukocytes. *Journal of Endocrinology* **189** 57–65. (doi:10.1677/joe.1.06187)
- Zapata G & Amemiya T 2000 Phylogeny of lower vertebrates and their immunological structures. In *Origin and Evolution of the Vertebrate Immune System*, ch 8. pp 67–110. Heidelberg: Springer-Verlag.
- Zuo X & Woo PTK 1997 Natural anti-proteases in rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis* and the *in vitro* neutralization of fish [α]₂-macroglobulin by the metalloprotease from the pathogenic haemoflagellate, *Cryptobia salmositica*. *Parasitology* **114** 375–382. (doi:10.1017/S0031182096008578)

Received in final form 10 May 2012

Accepted 15 June 2012

Made available online as an Accepted Preprint
15 June 2012