Research Article

The biological role of pituitary adenylate cyclase-activating polypeptide (PACAP) in growth and feeding behavior in juvenile fish

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To date, many technologies have been developed to increase efficiency in aquaculture, but very few successful biotechnology molecules have arrived on the market. In this context, marine biotechnology has an opportunity to develop products to improve the output of fish in aquaculture. Published *in vivo* studies on the action of the pituitary adenylate cyclase-activating polypeptide (PACAP) in fish are scarce. Recently, our group, for the first time, demonstrated the biological role of this neuropeptide administrated by immersion baths in the growth and development of larval fish. In this work, we have evaluated the effects of recombinant *Clarias gariepinus* PACAP administration by intraperitoneal injection on growth performance and feeding behavior in juvenile fish. Our results showed the physiological role of this peptide for growth control in fish, including the juvenile stage, and confirm that its biological functions are well conserved in fish, since *C. gariepinus* PACAP stimulated growth in juvenile tilapia *Oreochromis niloticus*. In addition, we have observed that the growth-promoting effect of PACAP in juvenile tilapia was correlated with higher GH concentration in serum. With regard to the neuroendocrine regulation of growth control by PACAP, it was demonstrated that PACAP stimulates food intake in juvenile tilapia. In general, PACAP appears to act in the regulation of the growth control in juvenile fish. These findings propose that PACAP is a prominent target with the potential to stimulate fish growth in aquaculture. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: PACAP; growth; food intake; PACAP antiserum; juvenile fish

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily, is a multifunctional neuropeptide that plays many roles such as hypophysiotropic hormone, neurotransmitter, neuromodulator, vasodilator, and neurotropic factor in mammals [1]. It was first identified in ovine hypothalamus due to its ability to stimulate cAMP formation in rat anterior pituitary cells [2]. This peptide exists in two molecular forms, with 38 (PACAP38) and 27 (PACAP27) amino acids [2,3]. The sequence of PACAP has been remarkably well preserved from tunicate to human. The high conservation of the PACAP sequence indicates that PACAP fulfills important biological functions in a broad spectrum of organisms, including fish [4].

Studies examining the effect of PACAP on GH secretion in mammals have shown contradictory results [5]. *In vivo* approaches have demonstrated that PACAP increases plasma GH levels in rats and cattle [6,7], but not in humans and ewes [8,9], suggesting that the GH-releasing effect of PACAP may be species specific in mammals. To date, little is known about PACAP function related to GH release in fish *in vivo*. It has been shown that intraperitoneal (IP) injection of goldfish PACAP38 forms (PACAP38a and PACAP38b) elevated GH levels in goldfish at different reproductive stages [10]. In agreement with these findings, more recently it was demonstrated that increases in PACAP gene expression during ovulation correlated with the GH secretion profile in the same species [11]. Additionally, *in vitro* studies have shown that PACAP

provokes a robust stimulation of GH release from coho salmon [12], goldfish [13], eel [14,15], grass carp [16–18], and tilapia [19] pituitary cells, which suggests that this peptide may act as a potent GH secretagog in lower vertebrates.

Until now, studies of PACAP in nonmammalian vertebrates, including fish, have focused primarily on the localization, cloning, and structural evolution of this peptide and very little is yet known about its biological functions *in vivo*. Very recently, our research group demonstrated the biological role of this neuropeptide administered by immersion baths in larval fish growth and development [19].

This work aims at evaluating the effects of the recombinant *Clarias gariepinus* PACAP on growth performance and feeding behavior in juveniles of African catfish (*C. gariepinus*) and Nile tilapia (*Oreochromis niloticus*) after its administration by IP injection. We also investigated, for the first time, *in vivo* serum GH stimulation by PACAP correlated with its effects to increase the growth performance in juvenile tilapia.

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Figure 1. Amino acids sequences of the recombinant and synthetic peptides. (A) Recombinant PACAP38. The metheonine at *N*-terminus was generated from the *E. coli* expression vector. (B) Synthetic PACAP-C11, which corresponds with the last 11 amino acids of the *C*-terminus of the PACAP38 form. A cysteine residue was added at the *C*-terminus for coupling with KLH. (C) Synthetic PACAP38. (D) Synthetic PACAP27.

Materials and Methods

Fish

Juveniles of African catfish *C. gariepinus* and Nile tilapia *O. niloticus* were provided by the Mamposton Aquaculture Research Station, Havana, Cuba, and were kept alive in aerated freshwater under natural photoperiod. The temperature of water was maintained between 28 and 30 °C. All experiments with animals were previously approved by the Ethics Committee of the Center for Genetic Engineering and Biotechnology, Havana, Cuba.

Production of an Antiserum against African Catfish (*C. gariepinus*) PACAP

Synthesis of peptides

Peptides were synthesized at the Center for Genetic Engineering and Biotechnology (Havana, Cuba) at analytical scale. Three amidated forms of the *C. gariepinus* PACAP, with 38 amino acids (PACAP38), with the first 27 amino acids from the *N*-terminus (PACAP27), and with the last 11 amino acids of the *C*-terminus (PACAP-C11) of the PACAP38, were prepared. An additional cysteine residue at the *C*-terminus for coupling with keyhole limpet hemocyanin (KLH) was included in PACAP-C11 (Figure 1).

Peptides were synthesized using manual parallel Fmoc/tBu solid phase chemistry [20] on Fmoc-AM-MBHA resin (400 mg, 0.54 mmol/g). The Fmoc group was removed with 20% of piperidine in *N*,*N*-dimethylformamide. DIC/HOBt activation was used at least fourfold molar excess of Fmoc-amino acids in DMF for each coupling cycle. Washings between coupling and deprotection steps were carried out with DMF using 10 ml/g resin for each wash. In general, no more than 2 h were needed for the completion of the coupling reaction, which was indicated by a negative Kaiser test. All coupling and washing steps were performed under vigorous shaking. After final deprotection, each peptide resin was washed with DMF, methanol, and ethyl ether and dried under vacuum overnight.

Cleavage from the resin and deprotection of side-chain protecting groups were performed by a treatment with TFA/TIS/water (95/2.5/2.5) for 2 h. Cleavage solutions were filtered into 50-ml centrifuge tubes containing cold ether. After centrifugation and decanting, the precipitates were washed four times by the addition of cold, fresh ether. The peptides were finally dissolved in 10 ml of 40% acetonitrile in water and lyophilized.

In all cases, the peptides were purified by RP-HPLC and the collected fractions were analyzed again by analytical RP-HPLC (Figure 2 and Table 1). In short, analytical separation was achieved in a RP C18 column (Vydac, 4.6×150 mm², 5 µm). A linear gradient from% 5 to 60% of solvent B for 35 min at 0.8 ml/min flow rate, with UV detection at 226 nm, was used. Solvent A was 0.1% (v/v)

TFA in water. Solvent B was 0.05% (v/v) TFA in acetonitrile. The software UNICORN 4.11 (GE Healthcare, USA) was used for data processing the RP-HPLC chromatograms. The mass of each peptide was verified by ESI-MS (Table 1).

Molecular mass measurement of peptides

The mass spectra were acquired with a hybrid quadrupole-time-of-flight (Q-TOF2) instrument (Waters, USA) fitted with a nanospray ion source. The capillary and cone voltage were set to 900 and 35 V, respectively. Data acquisition and processing were performed with the MassLynx (version 3.5) package.

Coupling of peptide to a carrier protein

To elicit a sustained immune response against the PACAP-C11 form in rabbit, we chose KLH (Sigma, USA) as the protein carrier. KLH has proven to be effective in numerous vaccine applications because of its many foreign epitopes, large molecular mass, and poor solubility [27]. Using the unique thiol on peptide PACAP-C11 added at the *C*-terminus of the sequence as a convenient handle for conjugation to a maleimide-functionalized KLH, we prepared the appropriate KLH carrier complex [21].

For conjugate preparation, KLH protein was dissolved in phosphate buffer solution pH 6 and N-succinimidyl-3-(maleinimido)propionate (MPS) solution was added. The mixture was stirred for 30 min at room temperature. The free MPS was separated from the MPS-activated KLH by gel filtration on a PD10 column. Then, PACAP-C11 dissolved in phosphate buffer solution pH 6 was added to the activated protein and the mixture was stirred for 3 h at room temperature. The free PACAP-C11 peptide was separated from the peptide-KLH conjugate by dialysis against PBS solution pH 7.2 at 4 °C.

Rabbit immunization

New Zealand white rabbits (three females, 6–7 weeks old, without placebo) were immunized with 500 µg of the PACAP-C11 conjugated to KLH. Rabbits were immunized using complete Freund's adjuvant (1 : 1 v/v) (Sigma, USA) on day 0 and incomplete Freund's adjuvant (1 : 1 v/v) (Sigma, USA) on day 15, 36, 51, 72, and 87. They were bled on day 108. In all cases, blood samples were obtained at day 0 (to acquire the preimmune serum) and prior to each immunization.

Animal response was screened using a dot blot assay as follows: 5 µl of synthetic PACAP38, PACAP27, and PACAP-C11, as well as KLH in order to test the antiserum specificity (all at 1 mg/ml in PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 7H2O$, pH 7.3), were bound to a Hybond-ECL nitrocellulose membrane (Amersham, UK) for 2 h at room temperature. After blocking in 5% skim milk
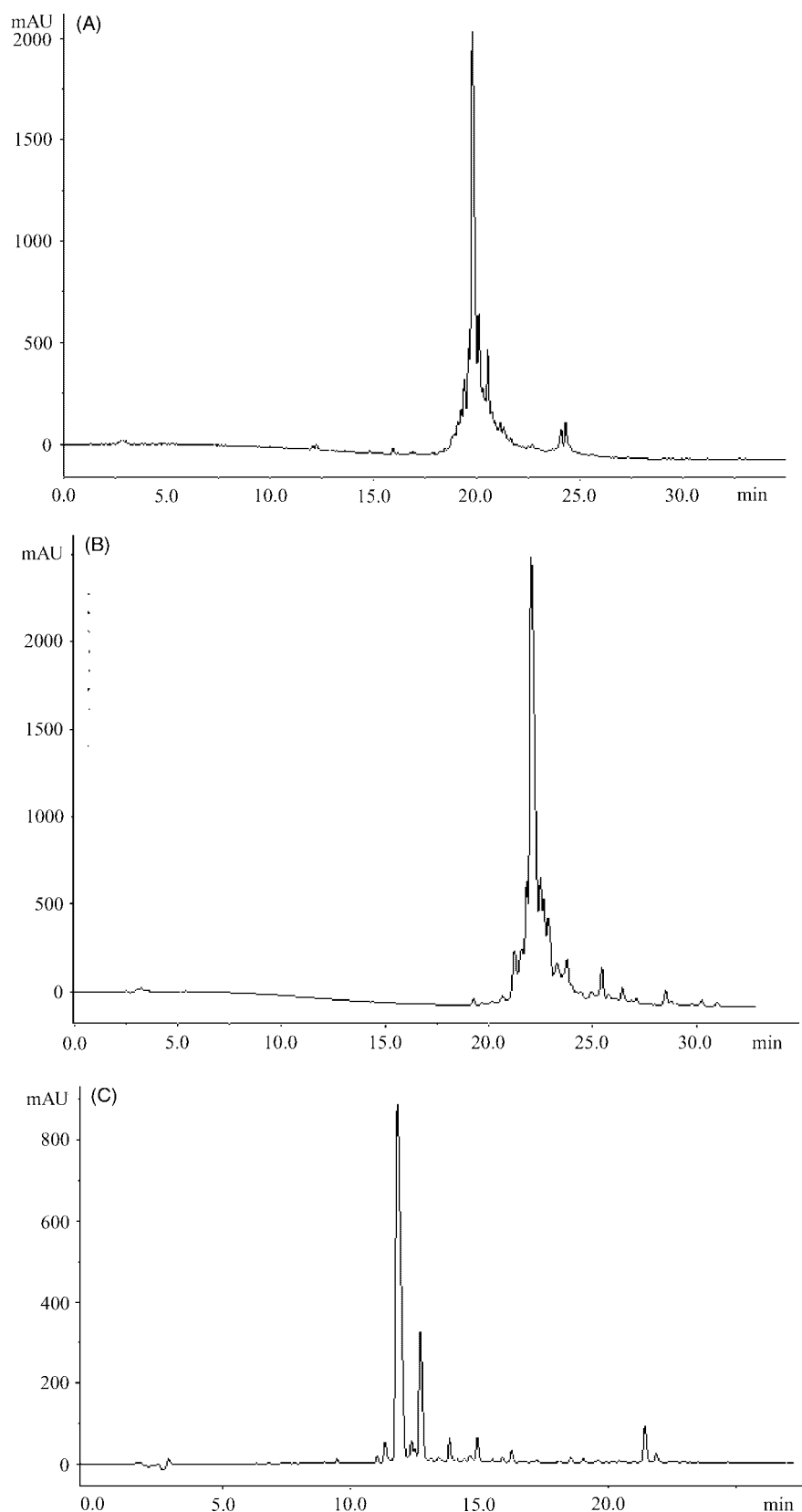
Figure 2. RP-HLPC analysis of the synthetic peptides. Peptides were injected onto RP C18 (Vydac, 4.6×150 mm^2, 5 µm) analytical column. Linear gradient from 5 to 60% of solvent B over 35 min at 0.8 ml/min flow rate, with UV detection at 226 nm. Solvent A was 0.1% (v/v) TFA in water. Solvent B was 0.05% (v/v) TFA in acetonitrile. The arrows denote (A) PACAP38 form; (B) PACAP27 form; and (C) PACAP-C11 (corresponding to the last 11 amino acids of the *C*-terminus of the PACAP38 form).

Table 1.	Physicochemical characterization of the synthetic peptides			
Synthetic peptide	RP-HPLC retention time (min)	Purity (%)	Theoretical mass (daltons)	Experimental mass (Da)
PACAP38	20.01	95.21	4663.50	4663.72
PACAP27	21.63	98.30	3145.65	3145.65
PACAP-C11	11.13	98.20	1637.89	1637.88

(Oxoid, UK) in PBS for 2 h, 2 ml aliquots of serially diluted sera were spotted on antigen-coated membranes, incubated for 2 h, washed 5 min with PBS twice, incubated with horseradish peroxidase-conjugated goat antirabbit antibodies (CIGB, Cuba) diluted 1 : 8000 in PBS for 1 h and washed 5 min with PBS twice. The immunodot was developed using 3,3′-diaminobenzidine (DAB) substrate. In short, 10 µl hydrogen peroxide (30%) was added to 10 ml of 0.05% DAB in PBS and mixed well immediately. Afterward, DAB substrate was transferred to the membranes and then incubated at room temperature with gentle shaking in the dark. The progress of the reaction was monitored carefully until the desired intensity (2–5 min) was obtained.

Expression and Purification of the Recombinant *C. gariepinus* PACAP

The pTYB vectors are commonly used for cloning and expression of recombinant proteins in *Escherichia coli*. The pTYB1 is a *C*-terminus fusion vector in which the *C*-terminus of the target proteins is fused to the intein tag. This vector is part of the Intein-mediated Purification with an Affinity Chitin-binding Tag (IMPACT™) system (New England Biolabs, Beverly, MA, USA). In this work, the pTYB1 recombinant plasmid containing PACAP cDNA (PTYB1-PACAP), already obtained in the laboratory [19], was transformed into electrocompetent *E. coli* strain BL21 harboring the lambda DE3 lysogen that carries the T7 RNA polymerase under the control of the lac UV5 promoter. One colony was used to inoculate 5 ml of Luria Bertani (LB) medium containing 50 µg/ml of ampicillin for overnight growth at 37 °C with vigorous shaking. This culture was used to inoculate 1 l of LB medium containing 50 µg/ml of ampicillin. The expression of the recombinant PACAP as a fusion protein was induced at an optical density at 600 nm (OD_{600}) of 0.5 by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to 0.5 mM and then the culture was grown for 5 h at 28 °C. After induction, cells were harvested by centrifugation at 5000 *g* for 20 min and stored frozen for later use. The frozen cell pellet was thawed and resuspended in 50 ml of lysis buffer (20 mM Tris–HCl pH 8, 500 mM NaCl, 1 mM EDTA, 20 mM phenylmethylsulphonyl fluoride). The expression of the fusion protein was tested on 10% SDS-PAGE after lysing the cells using a French press. Once the target protein expression had been determined, the clarified lysate containing the fusion precursor was loaded onto the chitin (New England Biolabs, Beverly, MA, USA) affinity gel column (20 ml bed volume) previously equilibrated with 10 volumes of column buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM EDTA). The column chromatography was performed at 1 ml/min. The column was washed with 10 bed volumes of column buffer to remove unbound proteins, then with three bed volumes of cleavage buffer (column buffer plus 50 mM dithiothreitol (DTT)). The flow was stopped and the column was left at 4 °C for 16 h. The protein was eluted by continuing flow with column buffer. Each fraction was checked by both tricine-sodium dodecyl sulfate-polyacrilamide gel (16.5%

T/3% C) electrophoresis (Tricine-SDS-PAGE) [22] and Western blot analysis.

The standard methodologies for staining with Coomassie blue or silver were used. The gels intended for the electrophoretic separation of proteins to be later analyzed by mass spectrometry were stained following the procedure for silver staining without glutaraldehyde, which is downstream compatible with this technique [23].

PACAP fractions purified by affinity chromatography were dialyzed overnight against PBS to remove DTT. The result was checked by Tricine-SDS-PAGE (16.5% T/3% C), Western blot, and mass spectrometry as reported previously by us [19].

Western Blotting Analysis

To verify PACAP expression as an intein-tagged fusion protein, Western blot analyses were carried out. The fused PACAP protein was electrophoretically fractioned by 10% SDS-PAGE under reducing conditions (β-mercaptoethanol to a final concentration of 2 mM to the sample buffer), according to the standard procedure [24] and transferred to a Hybond-ECL nitrocellulose membrane (Amersham, UK) in duplicate. After blocking with 5% skim milk (Oxoid, UK) in PBS, the membrane was cut into two pieces: one was incubated with rabbit serum raised against a peptide derived from the *Bacillus circulans* chitin-binding domain at 1 : 10 000 dilution (New England Biolabs, Beverly, MA, USA) and the other one was incubated with the antiserum generated against catfish PACAP-C11 at 1 : 20 dilution, both for 2 h at room temperature. After washing with PBS-T (PBS 1×, 0.05% Tween 20) once and with PBS twice, the membranes were incubated with a 1 : 5000 dilution of antirabbit polyclonal antibody horseradish peroxidase conjugate (Amersham Biosciences, UK), as secondary antibody with gentle shaking for 1 h at room temperature.

To check the identity of the peptide eluted from the chitin column, the samples were electrophoretically fractioned by 17.5% SDS-PAGE, transferred to a nitrocellulose membrane, and immunodetected as describe above, using the antiserum generated against catfish PACAP-C11 as primary antibody at 1 : 20 dilution for 2 h at room temperature. To confirm the specificity of PACAP antiserum, 5 µg of synthetic PACAP38 and PACAP-C11 were used as positive control. In addition, 5 µg of synthetic PACAP27 and 5 µg of the recombinant crustacean hyperglycemic hormone from *Litopenaeus schmitti* fused to intein protein (rCHH-intein) were used as negative controls. The detections were carried out using DAB substrate as described in Rabbit Immunization section.

Assays for Protein Concentration and Purity

Protein concentration was determined using the BCA Protein Assay Kit (Pierce, USA) according to the manufacturer's instructions. The purity level of the PACAP cleavage from intein was estimated as the percent of peptide versus total proteins in the lane of the tris-tricine gel stained with Coomassie blue (Bio-Rad, USA). The digital gel images used in these analyses were obtained using a *Hewlett Packard Scanjet Plus* scanner. Data were processed with Molecular Analyst software version 1.4.1 (Bio-Rad).

Growth-promoting Effects of the Recombinant *C. gariepinus* PACAP in Juvenile Fish

The growth experiments were done using *C. gariepinus* juveniles (40.4 ± 5.6 g; *n* = 18 per group) and in *O. niloticus* juveniles

Table 2. Hepatosomatic index, muscle dry weight, and serum GH levels in control and recombinant PACAP-treated groups

Species	Experimental groups	Parameters analyzed		
		Hepatosomatic index[a]	Muscle dry weight[b] (g)	Serum GH levels (ng/ml)
C. gariepinus (N = 10)	Control[c]	1.09 ± 0.07	0.50 ± 0.03	ND
	PACAP	1.06 ± 0.05	0.54 ± 0.03	ND
O. niloticus (N = 15)	Control[c]	1.02 ± 0.03	0.53 ± 0.03	5.19 ± 1.93
	PACAP	1.00 ± 0.02	0.56 ± 0.02	13.37 ± 2.84[d]

Data represent arithmetic mean of the tilapia GH ± SE; ND, not determined.
[a] Liver weight/body weight × 100.
[b] Determined using 1 g of muscle from each sample.
[c] Received *E. coli* proteins in an amount equivalent to contaminants present in PACAP.
[d] Statistical difference with respect to its control group ($p < 0.05$).

(32.2 ± 2.7 g; $n = 25$ per group). Each experiment took place independently. In each assay, the groups were acclimated in 500 l tanks with running fresh water, at 28 °C under a 12 : 12 photoperiod regime for one week prior to the experiment. Animals were fed with commercial pelleted feed (Cenpalab, Cuba) to satiation twice a day.

Recombinant *C. gariepinus* PACAP was injected intraperitoneally twice a week for 6 weeks at the dose of 0.1 µg/g of body weight (0.1 µg/g BW). The dosage was selected based on previously published studies in which different GH secretagogs were used to stimulate growth in fish [10,25]. The negative control received *E. coli* proteins in an amount equivalent to contaminants present in purified PACAP. These *E. coli* proteins were obtained from the chitin column wash prior to PACAP elution. Body weight was measured once a week to adjust PACAP amounts to be injected. At the end of the test, fish were anesthetized with methanesulfonate salt of 3-aminobenzoic ethyl ester (Sigma, USA) dissolved in water and each fish was decapitated and dissected. Livers were collected and weighed to determine the hepatosomatic index calculated as the percent liver weight relative to total body weight. A 1 g fillet from each sample was employed to determine muscle dry weight using the Thermo Control YTC. 01 L (Sartorios, Germany). Growth-promoting effects were evaluated by body weight increase during 42 days. Data were expressed as mean ± standard error (SE).

Plasma Collection

Plasma was collected from the 0.5 ml of heparinized blood following centrifugation at 3000 rpm for 5 min and stored at −80 °C until the GH ELISA was conducted.

GH Assays

To measure the serum GH levels in control and PACAP-treated fish, a noncompetitive ELISA was done as described previously by us [19], with minor modifications. A 96-well MaxiSorp plate (Nalge Nunc International, Denmark) was coated for 3 h at 37 °C with antitilapia GH monoclonal antibody 1 (tiGH1 mAb) (CIGB) at 10 µg/ml in 0.05 M carbonate buffer, pH 9.6 (100 µl per well). Plates were washed twice with PBS-T and blocked with 3% skim milk (Oxoid, UK) in PBS (200 µl per well) for 1 h at 37 °C. After blocking, the plates were washed twice with PBS-T. A standard tilapia GH curve in the range between 32 and 0.5 ng/ml was made by twofold dilutions in 0.5% skim milk in PBS and it was dispensed in duplicate in the same plate. Test samples were prepared by twofold dilutions as describe above, until their optical density fell

into the linear range of the standard curve. Subsequently, the test samples were incubated on the plates overnight at 4 °C. After washing the plates four times with PBS-T, 100 µl of horseradish peroxidase (HRP)-tiGH2 mAb conjugate (CIGB), diluted 1 : 15 000 in 0.5% skim milk in PBS, was added to each well. The plates were incubated for 1 h at 37 °C and then washed eight times with PBS-T. The substrate buffer (0.2 M Na$_2$HPO$_4$, 0.1 M citric acid, pH 5.0) containing 0.5 mg/ml of *ortho*-phenylenediamine and 5 µl of 30% hydrogen peroxide was then added (100 µl per well). The reaction was stopped 15 min later by adding of 50 µl per well of 2.5 M sulphuric acid. The absorbance was measured at 492 nm using a Titertek Multiskan Plus (Flow Laboratories, Irvine, Scotland) plate reader.

Evaluation of Food Intake

Tilapia (*O. niloticus*) with an average body weight of 35 ± 2 g was kept in 80 l tanks with running fresh water, at 28 °C under a 12 : 12 photoperiod regime. Groups of three animals (with three replicates of each group) were assigned into three different treatments: recombinant purified PACAP from *E. coli* (0.5 µg/g BW), GHRP-6 (Lipotec, Spain) at a dose of 0.5 µg/g BW, and *E. coli* proteins (obtained by chitin column wash prior to recombinant PACAP elution, in an amount equivalent to contaminants present in purified PACAP). The group treated with GHRP-6 (a synthetic GH secretagog and agonist of the ghrelin receptor) was used as positive control [26].

Sixteen hours before the experiments began at 10.00 am, each fish was fed at a rate of at least 3% of total fish body weight in each tank. All treatments were administrated by IP injection. Each fish received only one injection at the onset of the experiment, which continued for 16 h. Animals were fed *at libitum*, two times in 16 h, with commercially prepared pellets (Cenpalab). Uneaten feed and feces were siphoned out of each container 6 h after the first feeding operation and 10 h after the last one. Later, they were incubated at 50 °C until constant weight, as it was described previously [27,28]. This amount was deducted from the amount offered to determine food intake.

Statistical Analysis

The statistical analyses were done using GraphPad Prism Statistical Software version 4.00.255 (GraphPad Software Inc., San Diego, California, USA). The normality of the data was evaluated using the Barlett test (χ^2). Data with normal distribution and equal variances were analyzed using Student's *t*-test. The Mann–Whitney *U*-test
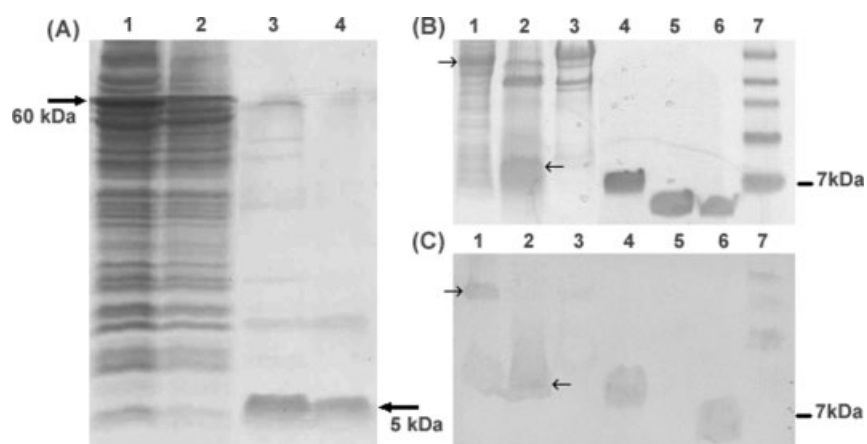
Figure 3. (A) Tricine-SDS-PAGE (16.5% T/3% C) analysis of *C. gariepinus* PACAP purification by affinity chromatography. Lanes: (1) lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP expression vector induced with 0.5 mM IPTG at 28 °C for 5 h (arrow indicates the fused protein PACAP-intein), (2) chitin column flow-through, (3 and 4) fractions of eluted recombinant *C. gariepinus* PACAP (rPACAP) after self-cleavage reaction at 4 °C overnight (arrow indicates the rPACAP). Trichloroacetic-acid-precipitated proteins from 1 ml of fractions corresponding to eluted rPACAP were loaded in lanes 3 and 4. (B and C) Glicine-SDS-PAGE (at 17.5%) and Western blotting, respectively, for both, to confirm the expression in *E. coli* and purification of the *C. gariepinus* PACAP by affinity chromatography and to test an antiserum raised against the last 11 amino acids of the *C*-terminus of the *C. gariepinus* PACAP (PACAP-C11). Lanes (1) lysis supernatant from *E. coli* BL21 (DE3) transformed with pTYB1-PACAP expression vector; (2) recombinant *C. gariepinus* PACAP purified by affinity chromatography; (3) inclusion bodies of the recombinant crustacean hyperglycemic hormone expressed in the *E. coli* BL21 (DE3) as intein fusion protein, (4–6) synthetics PACAP38, PACAP27, and PACAP-C11, respectively; and (7) prestained SDS-PAGE Standards Broad Range (Bio-Rad). Arrow in lanes 1 and 2 indicates the rPACAP.

was used to evaluate differences between groups when data had unequal variances. In the case of the food intake experiment, differences between groups were evaluated by Newman Keuls Multiple Comparison test. Treatments were considered to be significantly different if $p < 0.05$.

Results

Expression in *E. coli* of the Recombinant *C. gariepinus* PACAP and Its Purification by Affinity Chromatography

A 114 bp cDNA from *C. gariepinus* PACAP, already cloned in our laboratory [19] into the pTYB1 expression vector, was expressed as an intein fusion protein in *E. coli*.

After IPTG induction, a band corresponding to an approximately 60 kDa intein-tagged PACAP was observed, by SDS-PAGE under reducing conditions, in the lysate supernatant of the *E. coli* BL21(DE3) transformed with pTYB1-PACAP (Figure 3(A)). This band was absent in the lysate supernatant of the *E. coli* BL21(DE3) transformed with pTYB1 vector (data not shown). As reported previously by us [19], 5 h incubation was enough to induce maximum expression of the target protein after IPTG addition at 28 °C. To identify the expressed intein-tagged PACAP, we used rabbit serum raised against the chitin-binding domain that recognizes the intein tag (data not shown). Additionally, we used a rabbit serum raised against the last 11 amino acids of the *C*-terminus of the *C. gariepinus* PACAP38 (PACAP-C11). The Western blotting analysis using this antiserum showed a band of the expected 60 kDa size in the lysate supernatant of the *E. coli* BL21(DE3) transformed with pTYB1-PACAP (Figure 3(C)).

The expression vector pTYB1 allows the target gene to be cloned adjacent to the cleavage site of the intein tag, which results in the purification of a target protein without any extra nonnative residues attached to its terminus. In addition, the chitin-binding domain in the intein tag allows affinity purification of the fusion protein. Affinity chromatography was used to purify

PACAP since this peptide contains a *C*-terminal intein tag. Samples from each purification step were analyzed by Tricine-SDS-PAGE (Figure 3(A)). The binding efficiency of the fusion precursor on the chitin column was almost 100% as seen in Figure 3(A). The quick flush was done using 50 mM DTT and no significant cleavage was observed during this step.

The eluted fractions are shown in Figure 3(A). The molecular weight of the cleavage protein (approximately 5 kDa) coincides with the predicted molecular weight from translation of the PACAP cDNA sequence [19]. Mass spectrometry analysis of the cleavage protein demonstrated that this correspond to PACAP from catfish (data not shown). The identity of the cleavage protein was also corroborated by Western blotting analysis using the antiserum generated against PACAP-C11 (Figure 3(B) and (C)). This antiserum was also able to discriminate between synthetic PACAP38 and PACAP27 (Figure 3(B) and (C)) and specifically detected PACAP38 and PACAP-C11, and did not react with rCHH-intein (an unrelated protein fused to an intein tag) (Figure 3(C)). PACAP was obtained at about 5 mg/l of culture medium and approximately 90% pure after dialysis.

Growth-Promoting Activity of the Recombinant *C. gariepinus* PACAP in Juvenile Fish

Growth-promoting activity in juvenile C. gariepinus

The PACAP-treated group showed a significant increase in body weight, as compared to the control group, 30 days after treatment ($p < 0.05$) (Figure 4). The weight advantage in the group injected with PACAP (0.1 μg/g BW) at this time was 27% relative to the control group. Statistically significant differences ($p < 0.05$) in body weight increment in the PACAP-treated group, as compared to control group, were also observed at days 36 and 42 (43% and 46% greater than in the control group, respectively) (Figure 4).

The possible detrimental effects of high *C. gariepinus* PACAP levels were studied through evaluation of the hepatosomatic index at day 42 from the beginning of the experiment. No significant
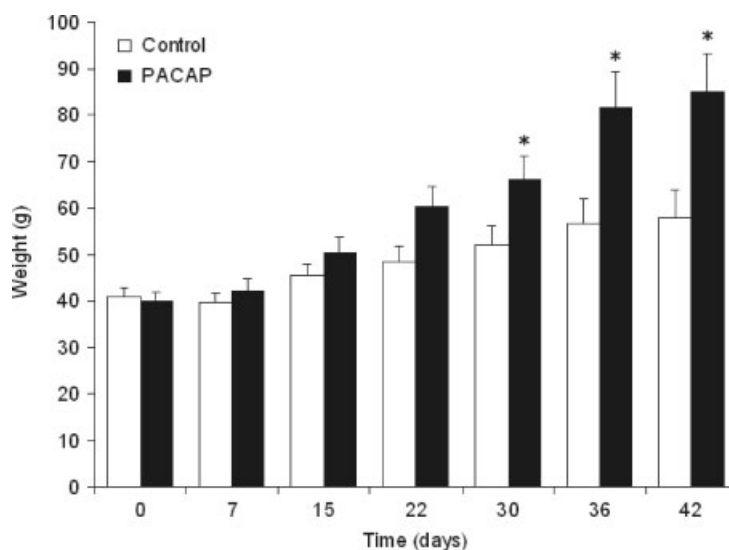
Figure 4. Effect on body weight of recombinant *C. gariepinus* PACAP injection over a 42-day period. Groups of 18 juvenile catfish (*C. gariepinus*) were each intraperitoneally injected twice a week with rPACAP at 0.1 µg/g BW or with a placebo (*E. coli* proteins obtained by chitin column wash prior to recombinant PACAP elution, in an amount equivalent to contaminants present in purified rPACAP). Each animal was weighed weekly. The Mann–Whitney *U*-test was used to evaluate differences between groups. *Significantly different from the control group ($p < 0.05$). Bars indicate media $\pm$ SE.
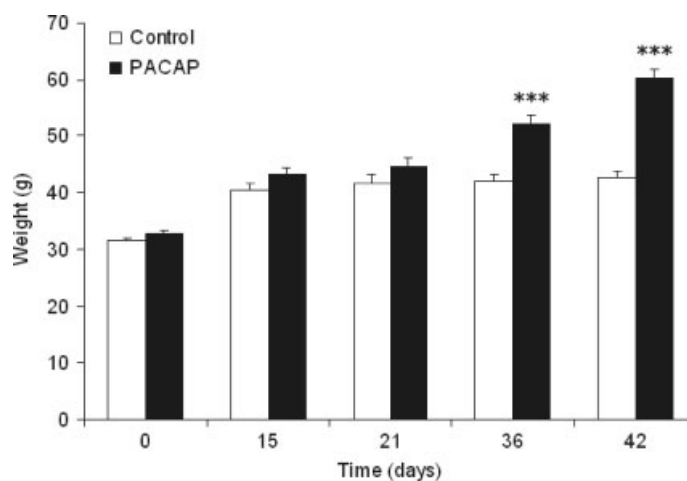

Figure 5. Effect on body weight of recombinant *C. gariepinus* PACAP injection over 42-day period. Groups of 25 juvenile tilapia (*O. niloticus*) were each intraperitoneally injected twice a week with rPACAP at 0.1 µg/g BW dosage or with placebo (*E. coli* proteins obtained by chitin column wash prior to recombinant PACAP elution, in an amount equivalent to contaminants present in purified rPACAP). Each animal was weighed weekly. An unpaired *t*-test was used to evaluate differences between groups. ***Significantly different from the control group ($p < 0.001$). Bars indicate media $\pm$ SE.

difference was observed between fish treated with recombinant PACAP and control group (Table 2). In addition, no significant difference was observed in the muscle dry weight between fish treated with recombinant PACAP and the control group at this time (Table 2).

It was not possible to measure serum GH levels in juvenile *C. gariepinus* due to antibody availability.

Growth-promoting activity in juvenile O. niloticus

Thirty-six days after the beginning of the experiment, the PACAP-treated group showed a significant increase in body weight compared with the control group ($p < 0.001$). The additional increment in the body weight in the PACAP-treated group with respect to the control group was 24% at this time (Figure 5). The weight increase advantage in the group injected with PACAP

(1 µg/g BW) was 40% ($p < 0.001$) relative to the control group at day 42 from the beginning of the experiment (Figure 5).

No significant difference was observed in the hepatosomatic index and muscle dry weight between fish treated with recombinant PACAP and the control group at the end of the experiment (at day 42) (Table 2).

A statistically significant increase in the serum GH levels ($p < 0.05$) was observed in the group treated with PACAP at day 42 (Table 2).

Effect of the Recombinant *C. gariepinus* PACAP in the Tilapia Food Intake

PACAP administration (0.5 µg/g BW) by IP injection increased average food intake of juvenile tilapia within 6 and 16 h compared to the control group ($p < 0.05$) (Figure 6). GHRP-6 (0.5 µg/g BW)
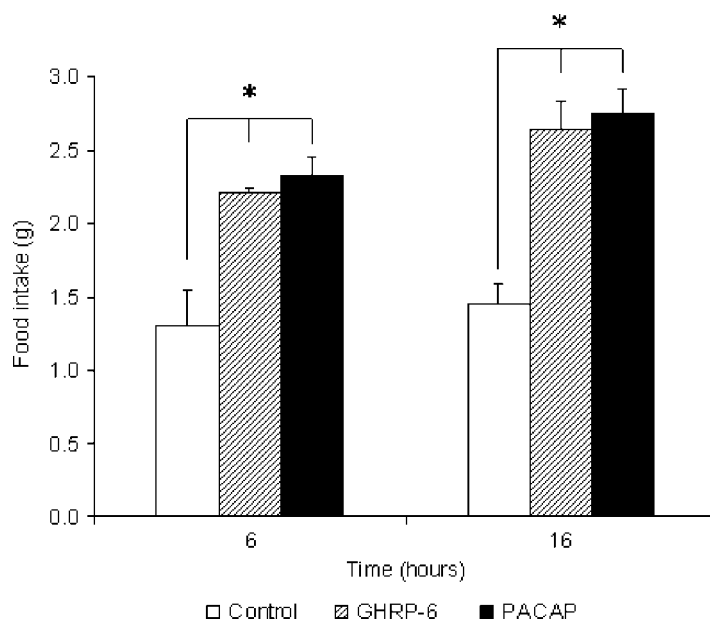
Figure 6. Effect of intraperitoneal injection (0.5 µg/g BW) of rPACAP on food intake of juvenile *O. niloticus* at 6 and 16 h post treatment. A group was treated with GHRP-6 at 0.5 µg/g BW dosage as positive control. The negative control group was injected with placebo (*E. coli* proteins obtained by chitin column wash prior to recombinant PACAP elution, in an amount equivalent to contaminants present in purified rPACAP). Each value is the mean of three replicates ($n = 3$ per replicate). The Newman–Keuls multiple comparison test was used to evaluate differences between groups. *Significantly different from the control group ($p < 0.05$). Bars indicate media ± SE.

used as positive control also showed an increase in food intake within 6 and 16 h compared to the control group ($p < 0.05$). No statistical differences were observed between PACAP and GHRP-6 groups (Figure 6).

Discussion

Recently, we reported an efficient recombinant expression of the mature fish *C. gariepinus* PACAP and PACAP-related peptide (PRP) in *E. coli*, showing that the growth rate of African catfish (*C. gariepinus*), Nile tilapia (*O. niloticus*), and Common carp (*Cyprinus carpio*) larvae are enhanced by the unpurified *C. gariepinus* PACAP or PRP in the *E. coli* lysate supernatant, when it is administered by immersion baths [19]. In this study, we have purified PACAP using the IMPACTsystem, in order to examine its biological function in juveniles of African catfish (*C. gariepinus*) and Nile tilapia (*O. niloticus*) when administered directly by IP injection. In addition, we have obtained a PACAP antiserum, which specifically recognizes the PACAP38 form. This antiserum was useful to identify the recombinant peptide expressed in *E. coli*, in both forms, fused to intein and cleaved from intein. To date, physiological studies of PACAP in various tissues of nonmammalian vertebrates have been based principally on a radioimmunoassay developed for mammalian PACAP [15,29–31]. Until this study, there have been only one previous published data described an antiserum against a teleost (the stargazer) fish PACAP, specifically against PACAP27 [32].

The expression of PACAP as an intein fusion protein allowed a rapid purification by affinity chromatography, thereby avoiding extra purification steps and protecting the target protein from degradation by proteases. When the column was incubated at 4 °C for 16 h, the cleavage was almost complete and approximately 5 mg of recombinant *C. gariepinus* PACAP was obtained from 1 l induced culture. This result is in agreement with previous findings

that showed the production of a comparable amount (4.8 mg/l) of maxadilan, a potent vasodilatory peptide present in the salivary glands of the sand fly that is also agonist specific for the PAC1 receptor (a PACAP receptor), using the same purification system [33]. In addition, because the intact fusion precursor protein and the intein–chitin-binding domain tag remained bound to the chitin resin during the intein-mediated cleavage and the target peptide elution, the recombinant PACAP was obtained with high purity (approximately 90%).

In this work, growth experiments were performed to evaluate the biological effect of the recombinant *C. gariepinus* PACAP administered by IP injection on the growth performance of juvenile African catfish (*C. gariepinus*) and Nile tilapia (*O. niloticus*). To date, there has been only one previous *in vivo* study showing the PACAP functions related to growth regulation in fish [19]. As mentioned above, in this study our research group has demonstrated that the growth rate of fish larvae is enhanced by PACAP when administered by immersion baths [19]. Herein, we have shown similar results in juvenile fish, confirming the physiological role of PACAP on growth control in teleost fish. The results also confirmed that PACAP biological functions are well conserved in fish, since *C. gariepinus* PACAP stimulates growth in juvenile tilapia *O. niloticus* as well as in juvenile catfish *C. gariepinus*.

No significant difference was observed in the hepatosomatic index and muscle dry weight between fish treated with recombinant PACAP and control groups at the end of the experiments. This result suggests that no detrimental effects were produced by IP injection of the recombinant catfish PACAP.

GH plays an important role as metabolic regulator, especially in stimulating lipid mobilization and protein increase, both of which encourage growth in fish [34]. Recently, using hemipituitary gland culture from tilapia (*O. niloticus*), we established that *C. gariepinus* PRP and PACAP are able to stimulate GH secretion *in vitro* [19]. In addition, it has been demonstrated that PACAP provokes a robust stimulation of GH release from salmon [12], goldfish [13],

eel [14,15], and grass carp [16–18] pituitary cells. *In vivo* there are a few studies about the effect of PACAP on GH secretion in mammals. It is known that this peptide increases the GH levels in rat and bovine plasma *in vivo* [6,7], whereas in ewes and humans it does not produce this effect [8,9]. To date, there have also been a few previous *in vivo* studies that show an effect of PACAP on GH release in fish. Wong *et al*. in 2000 observed that IP injection of goldfish PACAP38a and 38b (two isoforms of PACAP38) were effective in elevating plasma GH levels in goldfish at different reproductive stages [10]. More recently, it was demonstrated that PACAP, instead of gonadotropin-releasing hormone, is involved in the control of GH secretion during ovulation in goldfish [11]. In this study, a statistically significant increase in serum GH levels was observed in the PACAP-treated group as compared to control group at the end of the growth-promoting experiment in tilapia. Taking into account our results and the well-known effects of GH in vertebrate growth, the action of PACAP in juvenile fish growth enhancement might be mediated by GH. However, further studies are necessary to elucidate whether the action of this neuropeptide on growth performance is achieved solely through serum GH stimulation or in conjunction with another, more direct mechanism, affecting cell growth.

Growth in fish is regulated by the brain neuroendocrine GH–insulin-like growth factor axis [35]. Thus, growth can be stimulated by manipulation of selected neuroendocrine regulators of GH; however, it cannot occur without adequate food intake [36]. The control of food intake is highly complex and involves numerous external and internal factors. Recently, some reports have indicated that PACAP may play a role in the regulation of feeding behavior in vertebrates, including rodents [37–39], chick [40–42], and goldfish [43–46]. In general, the information obtained from the literature with respect to the actions of PACAP on feeding behavior in fish, as well as in mammals, showed contradictory results. In goldfish, the studies to investigate the effect of PACAP on food intake described PACAP as an anorexigenic neuropeptide in the goldfish brain [43–46]. However, although in channel catfish, *I. puntatus*, both central (intracerebroventricular) and peripheral (IP) injection of glucagon-like peptide (GLP-1), another member of the secretin/glucagon/VIP superfamily, induces anorexia, peripheral treatment has a weaker effect, suggesting that the major effects of GLP-1 on food intake are centrally mediated [47]. In addition, IP injection of VIP, a closed PACAP-related peptide, at relatively high dose (88 pmol/g BW) did not inhibit food intake and this result suggests that IP injection of VIP at high dose may induce the downregulation or desensitization of the food intake mechanism, but this cause is unknown [43]. In this work, we observed an increase in food intake when PACAP was administrated at a dose of 0.5 µg/g BW by IP injection in juvenile tilapia, within 6 and 16 h compared to the control group. In accord with our result, it was demonstrated that PACAP-knockout mice decreased high carbohydrate food intake. In addition, PACAP stimulated neuropeptide Y, the most potent orexigenic factor known to date in mammals, in the arcuate nucleus [38]. Orexigenic action of PACAP is also supported by decreased food intake in VPAC2-R (a PACAP receptor)-deficient mice [48]. In summary, although the reason for this apparent paradox is unclear, PACAP may exert a dual action on the appetite-regulating system in the brain, as PACAP shows physiological and functional variation in its effects, as described above. Further studies are needed to fully determine the mechanisms involved in the feeding regulation by PACAP. The use of transgenic or gene knockout fish could be of great interest

for determining the precise mechanisms through which PACAP interacts to regulate food intake in fish.

In conclusion, the results we obtained through IP injection of PACAP showed the biological role of this peptide in growth and feeding behavior in juvenile of African catfish (*C. gariepinus*) and Nile tilapia (*O. niloticus*). These findings identify PACAP as a prominent target with the potential to stimulate teleost fish growth in aquaculture. Certainly, further molecular research is needed to evaluate the practicality of developing PACAP into a novel fish biotechnology product.

Conflict of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

1 Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. *Jpn J. Physiol*. 1998; **48**: 301–331.
2 Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD, Coy DH. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun*. 1989; **164**: 567–574.
3 Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N, Arimura A. Isolation of a neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP38). *Biochem. Biophys. Res. Commun*. 1990; **170**: 643–648.
4 Gonzalez BJ, Basille M, Vaudry D, Fournier A, Vaudry H. Pituitary adenylate cyclase-activating polypeptide. *Ann. Endocrinol. (Paris)* 1998; **59**: 364–405.
5 Montero M, Yon L, Kikuyama S, Dufour S, Vaudry H. Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. *J. Mol. Endocrinol*. 2000; **25**: 157–168.
6 Jarry H, Leonhardt S, Schmidt WE, Creutzfeldt W, Wuttke W. Contrasting effects of pituitary adenylate cyclase activating polypeptide (PACAP) on in vivo and in vitro prolactin and growth hormone release in male rats. *Life Sci*. 1992; **51**: 823–830.
7 Radcliff RP, Lookingland KJ, Chapin LT, Tucker HA. Pituitary adenylate cyclase-activating polypeptide induces secretion of growth hormone in cattle. *Domest. Anim. Endocrinol*. 2001; **21**: 187–196.
8 Chiodera P, Volpi R, Capretti L, Caffarri G, Magotti MG, Coiro V. Effects of intravenously infused pituitary adenylate cyclase-activating polypeptide on adenohypophyseal hormone secretion in normal men. *Neuroendocrinology* 1996; **64**: 242–246.
9 Sawangjaroen K, Curlewis JD. Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) on prolactin, luteinizing hormone and growth hormone secretion in the ewe. *J. Neuroendocrinol*. 1994; **6**: 549–555.
10 Wong AO, Li WS, Lee EK, Leung MY, Tse LY, Chow BK, Lin HR, Chang JP. Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochem. Cell Biol*. 2000; **78**: 329–343.
11 Canosa LF, Stacey N, Peter RE. Changes in brain mRNA levels of gonadotropin-releasing hormone, pituitary adenylate cyclase activating polypeptide, and somatostatin during ovulatory

luteinizing hormone and growth hormone surges in goldfish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2008; **295**: 1815–1821.

12 Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM. Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* 1997; **138**: 414–423.

13 Wong AO, Leung MY, Shea WL, Tse LY, Chang JP, Chow BK. Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. *Endocrinology* 1998; **139**: 3465–3479.

14 Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S, Vaudry H. Localization of pituitary adenylate cyclase-activating polypeptide in the central nervous system of the European eel *Anguilla anguilla*: stimulatory effect of PACAP on GH secretion. *Ann. NY Acad. Sci.* 1998; **865**: 475–477.

15 Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S, Vaudry H. Distribution, characterization, and growth hormone-releasing activity of pituitary adenylate cyclase-activating polypeptide in the European eel, *Anguilla anguilla*. *Endocrinology* 1998; **139**: 4300–4310.

16 Sze KH, Zhou H, Yang Y, He M, Jiang Y, Wong AO. Pituitary adenylate cyclase-activating polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp: II. Solution structure of a brain-specific PACAP by nuclear magnetic resonance spectroscopy and functional studies on GH release and gene expression. *Endocrinology* 2007; **148**: 5042–5059.

17 Xiao D, Chu MM, Lee EK, Lin HR, Wong AO. 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* 2002; **76**: 325–338.

18 Wong AO, Li W, Leung CY, Huo L, Zhou H. Pituitary adenylate cyclase-activating polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp. I. Functional coupling of cyclic adenosine 3′,5′-monophosphate and Ca2+/calmodulin-dependent signaling pathways in PACAP-induced GH secretion and GH gene expression in grass carp pituitary cells. *Endocrinology* 2005; **146**: 5407–5424.

19 Lugo JM, Rodriguez A, Helguera Y, Morales R, Gonzalez O, Acosta J, Besada V, Sanchez A, Estrada MP. Recombinant novel pituitary adenylate cyclase-activating polypeptide from African catfish (*Clarias gariepinus*) authenticates its biological function as a growth-promoting factor in low vertebrates. *J. Endocrinol.* 2008; **197**: 583–597.

20 Atherton E, Fox H, Harkiss D, Logan CJ, Sheppard RC, Williams BJ. A mild procedure for solid phase peptide synthesis: use of fluorenylmethoxycarbonylamino-acids. *J. Chem. Soc. Chem. Commun.* 1978; 537–539.

21 Carter MJ. Techniques for conjugation of synthetic peptides to carrier molecules. In *Peptide Analysis Protocols*, Pennington MW, Dunn BM (eds). Humana Press: Totowa, NJ, 1994; 155–191.

22 Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 1987; **166**: 368–379.

23 Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 1996; **68**: 850–858.

24 Laemmli UK, Beguin F, Gujer-Kellenberger G. A factor preventing the major head protein of bacteriophage T4 from random aggregation. *J. Mol. Biol.* 1970; **47**: 69–85.

25 Leedom TA, Uchida K, Yada T, Richman III NH, Byatt JC, Collier RC, Hirano T, Grau EG. Recombinant bovine growth hormone treatment of tilapia: growth response, metabolic clearance, receptor binding and immunoglobulin production. *Aquaculture* 2002; **207**: 359–380.

26 Miura T, Maruyama K, Shimakura S, Kaiya H, Uchiyama M, Kangawa K, Shioda S, Matsuda K. Regulation of food intake in the goldfish by interaction between ghrelin and orexin. *Peptides* 2007; **28**: 1207–1213.

27 Nunes AJP, Parsons GJ. Size-related feeding and gastric evacuation measurements for Southern brown shrimp *Penaeus subtilis*. *Aquaculture* 2001; **187**: 133–151.

28 Carpio Y, Acosta J, Morales A, Herrera F, Gonzalez LJ, Estrada MP. Cloning, expression and growth promoting action of Red tilapia (*Oreochromis sp.*) neuropeptide Y. *Peptides* 2006; **27**: 710–718.

29 Reglodi D, Somogyvari-Vigh A, Vigh J, Li M, Lengvari I, Arimura A. Pituitary adenylate cyclase activating polypeptide is highly abundant in the nervous system of anoxia-tolerant turtle, Pseudemys scripta elegans. *Peptides* 2001; **22**: 873–878.

30 Yon L, Feuilloley M, Chartrel N, Arimura A, Fournier A, Vaudry H. Localization, characterization and activity of pituitary adenylate cyclase-activating polypeptide in the frog adrenal gland. *J. Endocrinol.* 1993; **139**: 183–194.

31 Yon L, Jeandel L, Chartrel N, Feuilloley M, Conlon JM, Arimura A, Fournier A, Vaudry H. Neuroanatomical and physiological evidence for the involvement of pituitary adenylate cyclase activating polypeptide in the regulation of the distal lobe of the frog pituitary. *J. Neuroendocrinol.* 1993; **5**: 289–296.

32 Matsuda K, Onoue S, Kashimoto K, Hamakawa A, Kikuchi M, Uchiyama M, Mochizuki T, Arimura A. A newly developed enzyme-immunoassay for measuring the tissue contents of PACAP in fish. *Peptides* 2002; **23**: 1741–1750.

33 Yu R, Yi T, Zhang L, Hong A, Dai Y, Zhou T. Intein-mediated rapid purification of recombinant maxadilan and M65 and their acute effects on plasma glucose. *Acta Biochim. Biophys. Sin. (Shanghai)* 2008; **40**: 1015–1022.

34 Hernandez Llorente MD, Dato Gomez MJ, de Costa Ruiz J, Mendiola Lopez P, Zamora Navarro S. Effect of recombinant human GH and GHRH on plasma metabolite levels in rainbow trout (*Oncorhynchus mykiss*). *J. Physiol. Biochem.* 2004; **60**: 211–218.

35 Peter RE, Marchant TA. The endocrinology of growth in carp and related species. *Aquaculture* 1995; **129**: 299–321.

36 Silverstein JT, Wolters WR, Shimizu M, Dickhoff WM. Bovine growth hormone treatment of channel catfish: strain and temperature effects on growth, plasma IGF-I levels, feed intake and efficiency and body composition. *Aquaculture* 2000; **190**: 77–80.

37 Adams BA, Gray SL, Isaac ER, Bianco AC, Vidal-Puig AJ, Sherwood NM. Feeding and metabolism in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology* 2008; **149**: 1571–1580.

38 Nakata M, Kohno D, Shintani N, Nemoto Y, Hashimoto H, Baba A, Yada T. PACAP deficient mice display reduced carbohydrate intake and PACAP activates NPY-containing neurons in the rat hypothalamic arcuate nucleus. *Neurosci. Lett.* 2004; **370**: 252–256.

39 Mizuno Y, Kondo K, Terashima Y, Arima H, Murase T, Oiso Y. Anorectic effect of pituitary adenylate cyclase activating polypeptide (PACAP) in rats: lack of evidence for involvement of hypothalamic neuropeptide gene expression. *J. Neuroendocrinol.* 1998; **10**: 611–616.

40 Tachibana T, Saito ES, Takahashi H, Saito S, Tomonaga S, Boswell T, Furuse M. Anorexigenic effects of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal peptide in the chick brain are mediated by corticotrophin-releasing factor. *Regul. Pept.* 2004; **120**: 99–105.

41 Tachibana T, Saito S, Tomonaga S, Takagi T, Saito ES, Boswell T, Furuse M. Intracerebroventricular injection of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibits feeding in chicks. *Neurosci. Lett.* 2003; **339**: 203–206.

42 Tachibana T, Tomonaga S, Oikawa D, Saito S, Takagi T, Saito ES, Boswell T, Furuse M. Pituitary adenylate cyclase activating polypeptide and vasoactive intestinal peptide inhibit feeding in the chick brain by different mechanisms. *Neurosci. Lett.* 2003; **348**: 25–28.

43 Matsuda K, Maruyama K, Nakamachi T, Miura T, Uchiyama M, Shioda S. Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, *Carassius auratus*. *Peptides* 2005; **26**: 1611–1616.

44 Matsuda K, Maruyama K, Miura T, Uchiyama M, Shioda S. Anorexigenic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: feeding-induced changes in the expression of mRNAs for PACAP and its receptors in the brain, and locomotor response to central injection. *Neurosci. Lett.* 2005; **386**: 9–13.

45 Maruyama K, Miura T, Uchiyama M, Shioda S, Matsuda K. Relationship between anorexigenic action of pituitary adenylate cyclase-activating polypeptide (PACAP) and that of corticotropin-

releasing hormone (CRH) in the goldfish, *Carassius auratus. Peptides* 2006; **27**: 1820–1826.

46 Matsuda K, Maruyama K, Nakamachi T, Miura T, Shioda S. Effects of pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal polypeptide on food intake and locomotor activity in the goldfish, *Carassius auratus. Ann. NY Acad. Sci.* 2006; **1070**: 417–21.

47 Silverstein JT, Bondareva VM, Leonard JB, Plisetskaya EM. Neuropeptide regulation of feeding in catfish, *Ictalurus punctatus*: a role for glucagon-like peptide-1 (GLP-1)? *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 2001; **129**: 623–631.

48 Asnicar MA, Koster A, Heiman ML, Tinsley F, Smith DP, Galbreath E, Fox N, Ma YL, Blum WF, Hsiung HM. Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate. *Endocrinology* 2002; **143**: 3994–4006.

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