REPORT

Growth-stimulating neuropeptides and the innate immune system in aquatic organisms

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ABSTRACT

The manipulation of the growth characteristics of aquatic organisms constitutes a high priority for the biotechnological industry, given the expected worldwide increase in food demand and the current food shortages, especially in animal protein, for significant segments of the population. The stimulation of growth, aimed at reducing the extension of harvest cycles for aquaculture and at decreasing the high mortality typical of larval stages in fish; as well as the use of immunostimulants to allow aquacultural populations to cope with the conditions of intensive culture, are two of the main targets of modern aquacultural biotechnology. Recent data about the immune-endocrine connection in fish have shown that growth hormone has a stimulatory effect on several parameters of the innate immune system. Therefore, the use of biotechnological means for the stimulation of growth hormone and, consequently, the innate immune system, is a promising mean for fulfilling this goal. The present work summarizes our study of the influence of the pituitary adenylate cyclase-activating peptide (PACAP) and the PACAP-related peptide (PRP) from *Clarias gariepinus*, as well as neuropeptide Y from *Oreochromis* sp. on the growth characteristics and innate immune system of fish. The results show that these neuropeptides not only have growth-promoting and development-related effects, but also stimulate several elements of the innate immune response such as lysozyme, lectins, nitric oxide and anti-oxidative defenses (catalase, superoxide dismutase and reduced glutathione levels).

Keywords: PACAP, PRP, NPY, growth, immunity, fish

*I*ntroduction

The pituitary adenylate cyclase-activating peptide (PACAP) was first isolated in 1989 from bovine hypothalamus by Miyata *et al.*[1]. The same authors showed that PACAP stimulated the secretion of growth hormone (GH) by the activation of adenylate cyclase [1].

PACAP, as well as the PACAP-related peptide (PRP) belong to the glucagon/secretin peptide superfamily, which also includes the vasoactive intestinal peptide, glucagon-like peptides 1 and 2, histidine/methionine and histidine/Isoleucine dipeptides and the glucose-dependent insulinotropic peptide [2]. PRP is the only peptide from the glucagon family whose biological role has not been completely elucidated [3]. Although this peptide might perform an important function in lower vertebrates (such as fish), perhaps it has lost this function in mammals, where the gene coding for its receptor has been deleted from the genome [4].

PACAP-coding genes have been isolated from several vertebrate species as well as a proto-chordate (a tunicate). Recently, a cDNA coding for the PRP/PACAP polypeptide was isolated from C*larias gariepinus* [5]. PACAP stimulates the release of melanocyte-stimulating hormone (MSH) by the melanotrophic cells of the anterior pituitary [6]. The first evidences supporting a role of PACAP in skin pigmentation in fish were reported by Lugo *et al.*, 2008 [5].

The role played by PACAP in the immune system of mammals is well documented, and several patents claim its use in humans as an immunomodulator. The modulation of the immune response in fish by PACAP was first demonstrated in 2008 by Lugo *et al.*, 2008 [5].

The Recombinant bacterial expression of PACAP and PRP was first reported recently in Cuba and it was demonstrated that both PACAP and PRP can increase growth rates in fish [5]. The results obtained *in vivo* for catfish (*Clarias gariepinus*), tilapia (*Oreochromis niloticus*) and carp (C*yprinus carpio*) showed that PRP, and specially PACAP, play a major role in growth control in this vertebrate group [5]. From a theoretical standpoint, these results further support the existence of the neuroendocrine axis proposed by the current literature in order to explain the hypothalamic regulation of growth in lower vertebrates. On the other hand, they evidence the potential of recombinant PACAP as a biotechnological product aimed at increasing aquacultural productivities.

The effect of recombinant PACAP and PRP administration, by immersion baths, on important parameters of the innate immunity and anti-oxidant defenses of African catfish (*C. gariepinus*) larvae was also evaluated. As a result, it was demonstrated for the first time that recombinant *C. gariepinus* PACAP not only promotes growth, but it also increases the lysozyme activity, the concentration of nitric oxide-derived metabolites and anti-oxidant defenses in fish larvae [5]. These data suggest a new function for PACAP as an important modulator of the immune system in teleostean fish, in addition

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2. Campbell RM and Scanes CG. Evolution of the growth hormone-releasing factor (GRF) family of peptides. Growth Regul 1992;2:175-91.

3. Tam JK, Lee LT, Chow BK. 2007 PACAP-related peptide (PRP)-Molecular evolution and potential functions. Peptides 2007;28:1920-9.

4. Lee LT, Siu FK, Tam JK, Lau IT, Wong AO, Lin MC et al. Discovery of growth hormone-releasing hormones and receptors in non-mammalian vertebrates. Proc Natl Acad Sci USA 2007;104:2133-8.

5. Lugo JM, Rodríguez A, Helguera Y, Morales R, González O, Acosta J, et al. 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. J Endocrinol 2008;197:583-97.

to its well-known physiological function in growth control.

Another peptide involved in the growth regulation cascade of fish is neuropeptide Y (NPY), a 36 amino-acid long molecule belonging to the Y peptide family that has a highly conserved sequence from fish to mammals [7]. This peptide is widely distributed throughout several structures of the vertebrate central nervous system, including that of teleostean fish [8].

The intracerebral-ventricular injection of NPY in goldfish has been shown to influence appetite and, therefore, food intake. Furthermore, diet restrictions and food deprivation result in a significant increase in growth hormone in the serum of these organisms, correlated with an increased expression of the NPY gene [9]. The results imply a dynamic involvement of NPY in daily food intake in fish, which supports the thesis that it constitutes an important orexigenic neuropeptide.

NPY was also obtained as a recombinant molecule, which will contribute to basic knowledge of fish physiology. The effect of the peptide on food intake and growth of tilapia was determined, evaluating its potential application in aquacultural settings.

*P*ACAP and PRP from *Clarias gariepinus*

Isolation of a cDNA coding for PACAP/PRP by reverse transcription/polymerase chain reaction

A complementary DNA (cDNA) coding for PRP/PACAP from *C. gariepinus* was obtained by reverse transcription/polymerase chain reaction (RT-PCR) from total RNA isolated from brain, using specific oligonucleotide primers designed from the catfish (*Ictalarus puntatus*) PRP/PACAP nucleotide sequence. The amplified region included from the signal peptide to the 3' untranslated sequence (figure 2 from reference [5]). The *C. gariepinus* PRP/PACAP cDNA was cloned into a T-vector, obtaining plasmid pPRP-PACAP. The complete nucleotide sequence of the *C. gariepinus* PRP/PACAP cDNA is shown in figure 2 from reference [5].

The pPRP-PACAP plasmid was in turn used as a template for amplifying the cDNA coding for the mature PRP and PACAP peptides. Figure 1, at lane P. shows the amplification of the cDNA for *C. gariepinus* PACAP; a similar procedure was used for the amplification of a cDNA coding for PRP from *C. gariepinus* (Figure 2).

Subcloning of PACAP and PRP cDNA into an *Escherichia coli* expression vector

In order to obtain high quantities of PRP and PACAP peptides in *E. coli*, a strategy was designed for subcloning their corresponding cDNA under control of the T7 promoter in vector pTYB1 of the IMPACT-CN kit (New England Biolabs, cat. # E6900S). This methodology allowed the development of a protocol for the purification of these peptides with a high degree of purity, while at the same time retaining their biological activities for further *in vivo* biological assays.

Based on the nucleotide sequence of the PRP/PACAP neuropeptide from *C. gariepinus*, isolated by RT-PCR and cloned into a commercially available T-vector (plasmid pPRP-PACAP), PCR was used to amplify DNA fragments of 114 and 145 bp coding for PACAP and PRP, respectively (Figures 1 and 2). The oligonucleotides were designed to bear *Nde* I recognition sites and a methionine codon for translational initiation for the case of sense primers, and a *Sap* I recognition site for the case of the antisense primers. This design allows the later cloning of the amplified cDNA into the bacterial expression vector.

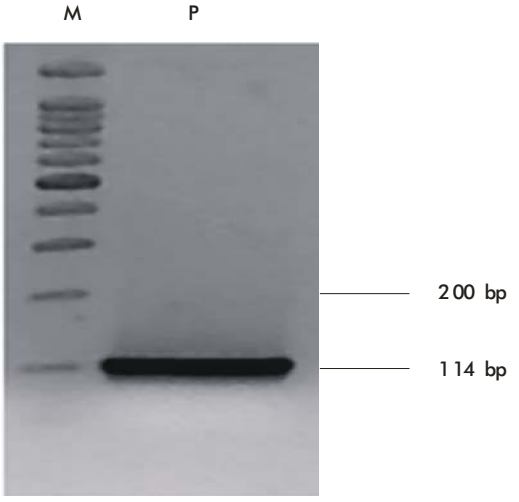

Figure 1. Agarose (2% w/v) gel electrophoresis of DNA showing the results of the RT-PCR reactions for the amplification of a cDNA coding for PACAP from C. *gariepinus*. M, 100 bp molecular weight ladder (Promega, cat. # G6951); P, DNA fragment corresponding to PACAP.

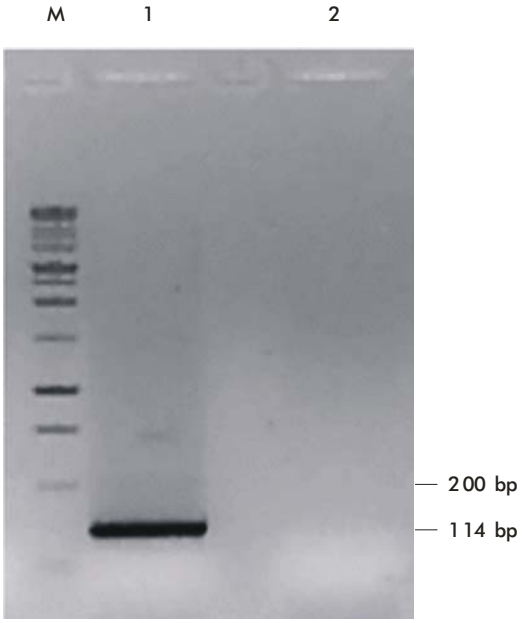

Figure 2. Agarose (2% w/v) gel electrophoresis of DNA showing the results of the RT-PCR reactions for the amplification of a cDNA coding for PRP from C. *gariepinus*. M, 100 bp molecular weight ladder (Promega, cat. # G6951); lane 1, amplification of the cDNA coding for PRP from C. *gariepinus*; lane 2, negative control (no-template PCR).

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7. Larhammar, D. Evolution of neuro-peptide Y, peptide YY and pancreatic polypeptide. Regul Pept Lett 2000;62:1–11.

8. McDonald JK. NPY and related substances. Crit Rev Neurobiol 1988;4:97-135.

The sequences of the recombinant plasmids obtained from each subcloning procedure (named pTYB1-PACAP and pTYB1-PRP) were verified using a universal T7 primer and automatic sequencing. The obtained sequences were analyzed with BLASTX (http://www.ncbi.nlm.nih.gov/blast/blastx), showing high levels of homology with the nucleotide sequences reported for orthologs from different species in literature.

Induction of gene expression in *E. coli*

The *E. coli* strain BL21(DE3) was transformed with plasmids pTYB1-PACAP and pTYB1-PRP in order to induce the expression under the T7 promoter of the genes coding for the PACAP and PRP peptides. No signal peptides were included in the PRP and PACAP coding sequences cloned into pTYB1, and therefore the polypeptides resulting from the induction of the T7 promoter may end up as soluble products in the cytoplasm or as inclusion bodies. In order to determine the specific cytoplasmic location of the recombinant proteins, the *E. coli* cells were lysed mechanically after induction in a French press, separating the soluble (rupture supernatant) from the insoluble (rupture pellet) fractions by centrifugation, followed by analysis through protein electrophoresis and Western blotting (Figure 4 in reference [5]).

The PRP and PACAP peptides expressed in *E. coli* were also analyzed by mass spectrometry. Figure 3 shows the peptides from recombinant PRP and PACAP that were sequenced.

Effects of PACAP and PRP on the growth of *C. gariepinus* larvae

The effect of neuropeptides PACAP and PRP on growth were analyzed by measuring body weight and length of treated larvae from *C. gariepinus*. Eight days after starting the experiment, the animals treated with rupture supernatants from *E. coli* BL21(DE3) transformed with the respective expression vectors showed a significant increase in body weight compared to those treated with rupture supernatants from *E. coli* BL21(DE3) transformed with the empty vector or not treated (CN) (Figure 6 in reference [5]). These differences were maintained by days 15 and 21 after starting the experiment (Figure 6 in reference [5]).

Figure 4 shows the differences in body weight for catfish larvae per experimental treatment, 21 days after starting the experiment.

Effects of PACAP and PRP on the growth of *Oreochromis niloticus* larvae

PACAP is the most conserved member of the glucagon superfamily (Sherwood *et al.*, 2000) [10]. There is a high degree of homology between the aminoacid sequences reported for PACAP in different species of

PACAP MHSDGIFTDSYRKQMAVKKYLAAVLGRRYRQRFRNK

PRP MHADGLLDRALRDILVQLSARKYLHSLTAVRVGEEEEDEEDSEPLS

Figure 3. Aminoacid sequences of the peptides corresponding to PACAP and PRP from *C. gariepinus* expressed in *E. coli* and determined by mass spectrometry. The sequence coverage is depicted by underlining the zones corresponding to the sequenced peptides. M is the methionine added as the result of recombinant expression in bacteria.

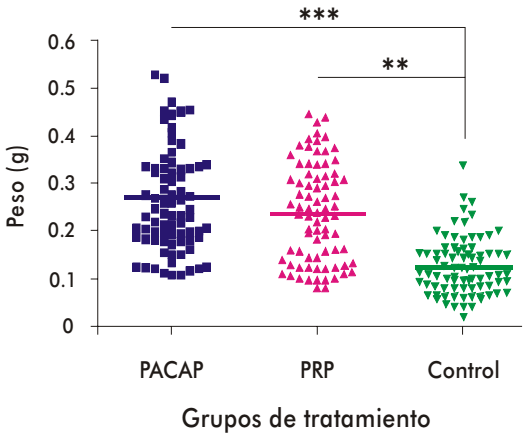

Figure 4. Effect of the PACAP and PRP peptides on the growth of *Clarias gariepinus* larvae at 21 days after the start of the treatment with 200 µg/L doses. Groups (n=80) were analyzed with ANOVA and Newman-Keuls tests (**- P < 0.01, ***-P <0.001).

fish; *e.g.*, there is a 94.7% of identity between the sequences for catfish and herring [11].

Based on the above, several *in vivo* experiments were performed in order to evaluate the effect of the *C. gariepinus* PACAP on the growth of tilapia larvae belonging to *Oreochromis niloticus*, evaluating animal body weight and length.

Eight days after the experiment, the average weight of the larvae treated with PACAP or PRP was higher than that of the CN group, and this difference was statistically significant. The differences were maintained until day 21 after the start of the experiment (Figure 7 in reference [5]). Figure 5 shows the differences in body weight for the tilapia larvae, per experimental treatment, by day 30 after the beginning of the experiment.

Effect of PACAP and PRP on the growth of larvae from *Cyprinus carpio*

In order to evaluate the effect of neuropeptides PACAP and PRP on the growth of carp larvae, body weight

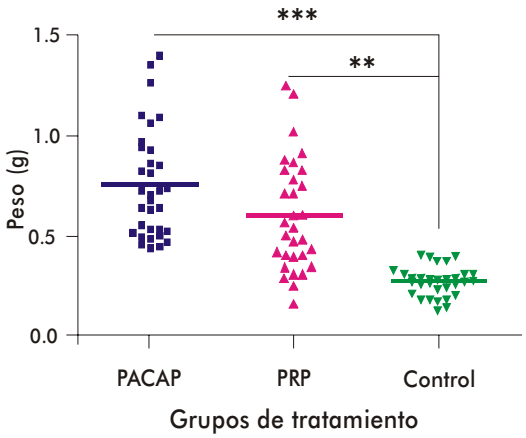

Figure 5. Effect of the PACAP and PRP peptides on the growth of *Oreochromis niloticus* larvae at 30 days after the start of the treatment with 200 µg/L. doses. Groups (n=30) were analyzed with ANOVA and Newman-Keuls tests (**- P < 0.01, ***- P <0.001).

9. Narnaware YK, Peyon PP, Lin X, Peter RE. Regulation of food intake by neuropeptide Y in goldfish. Am J Physiol Regul Integr Comp Physiol 2000;279:1025-34.

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(g) and length (cm) of treated and untreated animals were evaluated. Eight days after the start of the experiment, the animals treated with rupture supernatants from *E. coli* BL21(DE3) expressing the PACAP and PRP peptides showed statistically significant increases in body weight in comparison to those treated with rupture supernatants from the same strain transformed with the empty vector (NC group). This result was repeated at days 15 and 27 of the evaluation period (Figure 8 in reference [5]). Figure 6 shows the differences in body weight of carp larvae, per experimental treatment, at day 27.

The experiment also showed that peptides PACAP and PRP stimulate skin pigmentation (Table 4 in reference [5]), which constitutes a very important parameter in the ornamental fish industry.

Effects on the innate immunity of C. *gariepinus* larvae

There is abundant evidence on the influence of PACAP and PRP over the immune system in higher vertebrates. Therefore, it was decided to investigate the effect of PACAP and PRP treatments on three immunological parameters of fish larvae: lysozyme activity, lectins and nitric oxide (NO). Lysozyme is one of the most important surrogates for the status of innate immunity in fish, as well as the concentration of soluble lectins, which can be easily studied by hemagglutination assays. NO, on the other hand, constitutes a reactive nitrogen species that is generated during the respiratory burst of macrophages, which displays a potent antimicrobial effect. NO degrades quickly into NO_2 and NO_3, which are more stable and less volatile.

After 21 days of treatment with PACAP, a significant increase in lysozyme activity was observed for the larvae under study, in comparison with the negative controls and the group treated with PRP (Figure 2 in reference [5]).

The lectin assays performed in homogenates of *C. gariepinus* larvae used as negative control a 2% suspension of erythrocytes in 1X PBS, which did not yield detectable hemagglutination in these conditions. At day 15 of the observation period, there was a significant increase in lectin titers for the group treated with PACAP, compared to the negative control group (BL21) and the PRP-treated group. At day 21 of the observation period, however, the PRP group had lectin titers which were significantly higher than those of the negative control group (BL21) (Figure 3 in refe-rence [12]).

The estimation of NO concentrations in catfish homogenates was performed by measuring total NO_2 concentrations by means of a $NaNO_2$ standard. At day 21 of the observation period the NO_2 concentrations for the PACAP-treated group were significantly higher than for the remaining groups. There were no statistically significant differences between the latter groups (Figure 4 in reference [12]).

Effects on the anti-oxidant defenses of C. *gariepinus* larvae

The activities of catalase (CAT) and superoxide dismutase (SOD), as well as the concentration of reduced glutathione (GSH), were also measured, since these molecules play key roles in the defense mechanisms developed by living beings in order to avoid cellular damage upon the presence of an oxidative stress.

CAT activity in catfish larvae treated with PACAP displayed a significant increase after 21 days of peptide administration by immersion baths (Figure 5a in reference [12]).

SOD activity, on the other hand, showed no statistically significant differences between the different experimental groups by day 21 of the treatment (Figure 5c in reference [12]). The reduced glutathione concentration was significantly higher in the larvae treated with PACAP when compared to the PRP-treated or negative control groups at this time point (Figure 5b in reference [12]).

The results concerning PACAP and PRP have a high potential for applicability in an aquacultural setting, since they can be used for improving growth and survival of fish in larval stages. From a scientific point of view, these results show for the first time the potential role of PACAP as an immunomodulator in aquatic organisms, and provide further data on the biological function of PRP, which has been unknown in the case of aquatic organism.

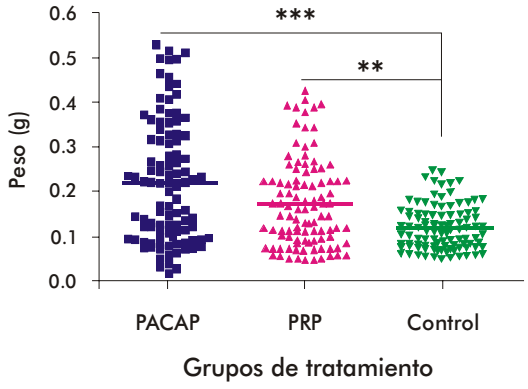

Figure 6. Effect of the PACAP and PRP peptides on the growth of *Cyprinus carpio* larvae at 27 days after the start of the treatment with 200 μg/L. doses. Groups (n=150) were analyzed with ANOVA and Newman-Keuls tests (**- P < 0.01, ***- P <0.001).

*N*europeptide Y from tilapia

Isolation of complementary DNA

The RT-PCR technique was used to amplify a 192 bp DNA fragment from brain RNA of red tilapia (*Oreochromis* sp.), corresponding to the sequence encompassing the signal peptide and the coding sequence of the NPY 36-aminoacid peptide. The primers were designed based on the sequences from two species of the *Percomorpha* genus (European sea bass and bastard halibut), selecting highly conserved stretches for the 5' and 3' ends. The obtained nucleo-tide sequence (GenBank accession Nr. AY779047) was highly homologous to those of previously characterized species, including mammals and birds, figure 1 in reference [13].

Recombinant expression and purification of mature neuropeptide Y

After NPY cDNA isolation from tilapia and the characterization of its sequence, it was used for the amplification and subcloning of a signal peptide-less NPY

12. Carpio Y, Lugo JM, León K, Morales R, Estrada MP. Novel function of recombinant pituitary adenylate cyclase-activating polypeptide as stimulator of innate immunity in African catfish (*Clarias gariepinus*) fry. Fish Shellfish Immunol 2008;25(4) 439-45.

into the commercially available vector pTYB1 for expression in bacteria. After inducing the expression of the heterologous gene with IPTG a reinforced band of approximately 60 kDa was observed during denaturing protein electrophoresis (SDS-PAGE), which corresponded to NPY fused to the intein from the vector. The highest levels of expression were obtained 6 h post-induction with IPTG. The mature peptide was generated at an 80% purity after incubation with DTT for 16 h at 4 °C, which yielded a species with an approximate molecular weight of 4.4 kDa, as predicted from the sequence of its cDNA. The molecular weight of the purified NPY was confirmed by mass spectrometry, resulting in a value of 4395.21 Da, in accordance with the expected mass. Two peptides were sequenced; one corresponding to the N-terminal 20 aminoacids, which confirmed the identity of the isolated polypeptide (figure 4 in reference [13]).

Food intake experiment for measuring appetite and growth-promoting activity in tilapia larvae

The administration of NPY increased food intake in juvenile tilapias (37 ± 3 g) injected intraperitoneally in a twenty-four-hour period, in comparison with the negative controls (See figure 5 in reference [13]).

The growth experiment used 12 animals with 0.8 ± 0.1 g of weight per group, which were kept in 250 L tanks at 28°C under a 12/12 h light/darkness cycle, fed twice a day with commercial fodder (CENPALAB) *ad libitum*. Recombinant NPY was delivered by intraperitoneal injection twice per week, for a total of 5 weeks. One group received a dose of 1 mg/g body weight, another a dose of 0.1 mg/g body weight, and the control group was inoculated with PBS 1X. At the end of the experiment the livers were collected and weighed in order to calculate the hepatosomatic index (HSI), which is the liver: body weight ratio. Additionally, the protein and water contents of muscle tissue were measured.

The treatment with NPY stimulated the growth of the injected animals at 4 weeks after the delivery of the 1 µg/g dose (figure 6 in reference [13]). There were no differences in the HSI of the treated animals *vs.* the control. The protein contents of muscular tissue were significantly increased ($p<0.05$), in contrast to the water contents which remained constant (figure 6 and table 1 in reference [13]).

In conclusion, the cDNA coding for the signal peptide-mature NPY from red tilapia (*Oreochromis* sp.) was isolated for the first time. High amino-acid sequence conservation was observed when compared to previously known orthologs from fish, amphibians, birds and mammals. The peptide can be obtained in a biologically active form from rupture supernatants of *E. coli* using the IMPACT-CN system. As purified, the recombinant peptide stimulates appetite upon administration to juvenile tilapias. Additionally, it was shown that the intraperitoneal administration of NPY promotes growth and increases the protein concentration of muscle tissue, in dependence to the administered dosage [13].

Effect on growth and the innate immune system of C. *gariepinus* larvae

The influence of the endocrine system on the level of anti-oxidative activities has been previously described in mammals [14]. Therefore, we decided to investigate the effect of NPY on these activities using catfish larvae weighing 0.0072 ± 0.0001 g, with 9.3 ± 0.2 mm length.

The body weight of the animals treated with NPY was larger than that of the negative control group at days 15 and 30 after starting the experiment, with increases of 87 and 64%, respectively. The size of the individuals in the treated group was also larger, with increases (compared to the negative control group) of 27 and 16% at days 15 and 30 of the experiment, respectively (see figure 1 in reference [15].

No statistically significant differences were detected regarding protein concentration, lysozyme activity and lectin titers at day 15 of the experiment. At day 30, however, there was a detectable increase in SOD activity and in the concentration of reduced glutathione on the larvae treated with NPY. No differences in CAT activity were found among the experimental groups; see table 1 in reference [15].

The results summarized above constitute the first demonstration of increase in fish growth rate (*Clarias gariepinus* larvae) upon administration by immersion of a recombinant NPY peptide obtained in *E. coli*. This confirms the high degree of conservation of the biological roles of this molecule and its function as an orexigenic factor in fish. In addition, NPY seems to modulate anti-oxidative defense mechanisms, as underlined by the increases in SOD activity and GSH concentration (figure 2 in reference [15]).

Conclusions

The genes coding for PACAP and PRP from the African catfish (*Clarias gariepinus*), as well as the gene coding for neuropeptide Y from tilapia (*Oreochromis* sp.), were isolated and cloned for the first time. It was shown that the corresponding recombinant gene products promote growth and enhance survival upon administration to different fish species in different developmental stages, under conditions of intensive culture. Furthermore, the study of the relationship between these peptides and the innate immune system in fish yielded the first evidences for an immunomodulatory activity of PACAP. Other findings of this study were the orexigenic activity of NPY and the evidences supporting the role of PACAP as a major hypophisiotrophic factor involved in the regulation of growth hormone secretion.

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15. Carpio Y, León K, Acosta J, Morales R, Estrada MP. Recombinant tilapia Neuropeptide Y promotes growth and antioxidant defenses in African catfish (*Clarias gariepinus*) fry. Aquaculture 2007;272:649-55.