Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the Brazilian flounder *Paralichthys orbignyanus*

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Neuropeptide Y (NPY) is one of the most potent stimulants of food intake in vertebrates, mammals and fish. However, the present knowledge about feeding behaviour in fish is still limited and based on studies in a few species. The Brazilian flounder *Paralichthys orbignyanus* is being considered for aquaculture, and it is important to understand the mechanisms regulating feeding in order to improve its performance in captivity. The objectives of this study were to clone NPY cDNA, evaluate the mRNA levels in different tissues of flounder, and also evaluate brain NPY expression to associate food intake with NPY expression levels. A 597 bp NPY cDNA was cloned from Brazilian flounder brain. NPY expression was detected in all the peripheral tissues analysed. No significant differences were observed in brain NPY gene expression over 24 h after food intake at a temperature of 15 ± 3°C. No correlation was observed among plasma glucose, total protein, cholesterol, triglycerides and NPY expression levels during this 24 h period. On the other hand, mRNA levels were increased after two weeks of fasting at elevated temperatures. Our results suggest that NPY mRNA levels in Brazilian flounder are affected by temperature.

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1. Introduction

Food intake in vertebrates is a complex process involving several neural pathways, in which neuropeptide Y (NPY) plays a key role. The stimulatory effects on food intake caused by central injections of NPY have been demonstrated in goldfish (Lopez-Patino *et al.* 1999) rainbow trout (Aldegunde and Mancebo 2006) and tilapia (Kiris *et al.* 2007). Immersion of African catfish fry into water containing NPY also stimulated feeding (Carpio *et al.* 2007), although this treatment has a stimulatory effect only for a short period of time.

NPY is a highly potent stimulant of growth hormone release in fish (Peng and Peter 1997), but growth enhancement can only be achieved when there is sufficient food available (Volkoff *et al.* 2005). Despite recent advances, our present knowledge of the regulation of feeding behaviour in fish is limited and based on a few species, and there is increasing evidence of species-specific differences (Volkoff *et al.* 2009). In goldfish, it was demonstrated that

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NPY mediates the action of ghrelin on feeding (Miura *et al.* 2006) and brain mRNA expression is significantly increased around mealtime in Atlantic cod (Kehoe and Volkoff 2007) and goldfish (Narnaware *et al.* 2000). An increase in brain NPY expression was observed after 72 h of food deprivation in goldfish, but immediately after feeding its expression was normalized (Narnaware and Peter 2001). Two weeks of food restriction significantly increased brain NPY mRNA levels in Winter skate (MacDonald and Volkoff 2009b), while in catfish, three weeks of fasting were needed to alter NPY expression in the brain (Silverstein and Plisetskaya 2000). On the other hand, in Atlantic salmon brain, NPY expression was not increased after six days of food deprivation (Murashita *et al.* 2009) and, as reported in Atlantic cod, seven days of fasting did not increase NPY gene expression (Kehoe and Volkoff 2007). NPY expression has also been detected in the intestine, liver, spleen, muscle and adipose tissue of fish (Liang *et al.* 2007) but little information about its function on these tissues is available.

According to Volkoff *et al.* (2009), the characterization of an appetite-regulating peptide must take into account the phylogeny of the fish, its physiological state as well as the environment it inhabits. The Brazilian flounder *Paralichthys orbignyanus* inhabits the estuarine and coastal waters from Rio de Janeiro (Brazil) to Mar del Plata (Argentina). Tolerance to a wide range of temperatures (Wasielesky *et al*. 1998), salinities (Sampaio and Bianchini 2002), as well as high concentrations of nitrogenous compounds (Bianchini *et al.* 1996) are characteristics that supported the initial studies related to its culture. More recently, studies related to reproduction (Radonic *et al.* 2007; Lanes *et al.* 2008; Sampaio *et al.* 2008), larviculture (Sampaio *et al.* 2007), expression of genes related to growth (Meier *et al.* 2009) and evaluation of transgenic potential (Lanes *et al.* 2009) showed the culture feasibility of this species. However, feeding regulation in this species has never been examined. The role of NPY in the regulation of feeding behaviour of the Brazilian flounder is not known and this information may be helpful to improve its culture.

The aims of this study were to clone a cDNA encoding Brazilian flounder NPY, examine NPY expression levels in different tissues and then assess its role on feeding behaviour by evaluating brain NPY mRNA expression over a 24 h period and after fasting by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1 *Animals*

Fish used in this study were obtained by artificial spawning at the Laboratory of Marine Fish Culture at FURG (Brazil). Two male flounder (25.5 ± 2 cm, 200 ± 40 g) were used to clone NPY cDNA and to evaluate tissue distribution. To evaluate 24 h brain NPY expression, animals were allocated to seven tanks with five animals in each tank, and were acclimated for two weeks in seawater. They were fed with commercial pellets (Supra Salmonídeos™/Alisul/Brazil) containing 46% crude protein and 6% lipid once a day at the same time (5 pm) under natural winter temperature (15 ± 3°C) and photoperiod (11L: 13D). Gene expression was measured at seven different times (0–10 min before food intake, 1, 2, 4, 6, 12 and 24 h after food intake). To evaluate the effect of fasting, fed and fasted groups of Brazilian flounder (28.2 ± 4 cm, 250 ± 45 g) were sampled. Five fish (fed control) were sampled from a feeding tank with continuous feeding once a day. Subsequently, the feeding was stopped for two weeks, and five fish were sampled as a fasted group. Fish used in this experiment were acclimated and maintained at the same feeding regimen and photoperiod, and at a temperature of 23 ± 2°C.

2.2 *Cloning of NPY cDNA*

Fish were anaesthetized in benzocain (50 $mg.l^{-1}$) and euthanatized by severing the spinal cord. Whole brains were immediately dissected out, frozen and stored in liquid nitrogen until use. Total RNA was isolated with TRIzol® Reagent (Invitrogen™, Carlsbad, USA). DNase treatment of RNA samples was conducted with a DNA-free® Kit (Ambion™, USA) following the manufacturer's protocol. First-strand cDNA was performed with 2 μg of RNA using SuperScript™ III Reverse Transcriptase (Invitrogen™, Carlsbad, USA) according to the manufacturer's protocol. Two degenerate primer sets NPY 01, 02, 03, 04 (*see* table 1) were designed to clone partial brain NPY cDNA sequences of Brazilian flounder based on the alignment of NPY sequences of other fish species. The PCR parameters were 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. PCR products were sequenced using a MegaBACE 500 automatic sequencer (Amersham Biosciences, USA). To perform 3′rapid amplification of cDNA end (RACE), total brain RNA was reverse transcribed to cDNA in the presence of oligo(dT) adaptor primer (AP) (table 1) also using SuperScript™ III RT (Invitrogen™, Carlsbad, USA) according to the manufacturer's protocol. For PCR, a forward gene-specific primer NPY 01F and reverse AUAP primer were used with the following parameters: 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. The 3′-RACE-PCR products were purified on an agarose gel with GFX™ PCR DNA and Gel Band Purification Kit and sequenced as described above.

Table 1. Primers sequences used in this study

Primers	Sequence 5′→3′	Use
NPY primers		
NPY 01F	TGCATMCTARCTTRGTSAGCT	NPY cloning
NPY 01R	GTGTCCAGAATCYCAGGACTG	NPY cloning
NPY 03F	GACYCTGGGGYTCCTGCTGT	NPY cloning
NPY 04R	ATGGGTYRTAWCTYGACTGTG	NPY cloning
NPY real-time F	CACGTCATTTTCCTCCTGCAT	qRT-PCR and RT-PCR
NPY real-time R	GCATAGCGGCTCGTAGAGGTA	qRT-PCR and RT-PCR
3'RACE primers		
oligo (dT) AP	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT	RACE
AUAP	GGCCACGCGTCGACTAGTAC	RACE
β-actin primers		
BAC real-time F	GACCCAGATCATGTTTGAGACCTT	qRT-PCR
BAC real-time R	AGGGACAGCACAGCTTGGAT	qRT-PCR
BAC semi F	AAGATCTGGCATCACACCTTCTA	RT-PCR
BAC semi R	GGAGTCCATGACGATACCAGTG	RT-PCR

2.3 *Phylogenetic analysis*

Several nucleotide NPY coding sequences, including *P. orbignyanus*, were aligned using CLUSTAL X (Thompson *et al.* 1997) and a phylogenetic analysis was performed using the Phylogeny Inference Package PHYLYP 3.6 (Felsenstein 1997) and the maximum parsimony method (DNAPARS, for details *see* PHYLYP 3.6 manual). *Xenopus laevis* was used as an outgroup. A bootstrapping analysis using 1000 iterations was performed using SEQBOOT.

2.4 *Evaluation of NPY expression on flounder tissues by semi-quantitative RT-PCR*

Brain and peripheral tissues (liver, spleen, muscle, gill, intestine, heart, kidney, stomach and testis) were examined for NPY expression. Tissue collection, total RNA extraction, DNase treatment and cDNA synthesis were conducted as described in section 2.2. The same NPY primers (NPY 05 and 06, *see* table 1) used for evaluation of brain NPY expression over a 24 h period were also used for semi-quantitative RT-PCR. For endogenous reference, *β*-actin (GenBank accession no. EU542580) primers BAC 03 and 04 were used (*see* table 1). PCR conditions for NPY were: 35 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. For *β*-actin, the conditions were: 35 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, with an additional initial 1 min denaturation at 94°C and a 5 min final extension at 72°C. PCR products for NPY were electrophoresed on a 2.5% agarose gel and for *β*-actin on a 1% agarose gel, both containing 0.5 μg ml^{-1} ethidium bromide.

2.5 *Biochemical parameters*

All fish of each group were anaesthetized at the same time with benzocain (50 mg.l^{-1}). Blood was collected from the first gill-arc artery, centrifuged (15 min at 1000 x *g*), and plasma was frozen and stored in liquid nitrogen until analyses. The blood was treated with EDTA and potassium fluoride as an anticoagulant and anti-glycolytic, respectively. Plasma concentrations of glucose, total protein, triglycerides and cholesterol were measured through analytical enzymatic colorimetric kits (Doles Reagents©, Brazil), following the manufacturer's instructions.

2.6 *Evaluation of 24 h NPY expression and NPY expression after fasting by qRT-PCR*

After sampling blood to evaluate biochemical parameters for the 24 h experiment, brain collection, total RNA extraction, DNase treatment and cDNA synthesis were conducted as described in section 2.2. Real-time PCR was run on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems™, USA) using SYBR® Green PCR Master Mix (Applied Biosystems™, UK). Primers (table 1) for NPY

and the endogenous reference *β*-actin were designed with the Primer Express v. 3.0 software (Applied Biosystems™, USA). Initial validation experiments were conducted to ensure that all primer pairs had equivalent PCR efficiencies. Amplification was carried out at the standard cycling conditions of 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s, 60ºC for 60 s followed by conditions to calculate the melting curve. All PCR runs for each cDNA sample were performed in triplicate. The real-time PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001) and the handbook of *Chemistry guide of applied biosystems*.

2.7 *Data analyses*

Gene expression and biochemical parameters over 24 h following a meal were compared using one-way ANOVA followed by the test of Tukey for multiple comparisons. Evaluation of gene expression in the fasting experiment was compared using the *t* test. Significance was considered at $P<0.05$. All data are expressed as mean ± SEM.

3. Results

3.1 *NPY cloning*

Using RT-PCR coupled to 3′RACE-PCR, a 597 bp fragment of NPY cDNA was cloned from the brain of Brazilian flounder. This was deposited under GenBank accession no. FJ705358 (figure 1). The cloned fragment contained 291 bp of the final part of the open reading frame (ORF) of flounder NPY containing 25 amino acids that formed part of the signal peptide followed by 36 amino acids of mature peptide. The proteolytic processing site Gly-Lys-Arg was followed by 32 amino acids constituting a c-terminal peptide followed by a 3′untranslated region (UTR) of 306 bp. The consensus phylogenetic tree (figure 2) revealed that the NPY of Brazilian flounder and that of other acanthomorph fish (Japanese flounder, grouper, European sea bass, Chinese perch and Atlantic cod) were grouped in the same cluster, whereas the NPY of channel catfish and the cyprinid fishes (goldfish and zebrafish) were grouped in another cluster. Mammalian and chicken NPY were grouped in different clusters and a frog was used as an outgroup.

3.2 *Evaluation of NPY gene expression in flounder tissues and brain*

The fragment corresponding to the NPY cDNA fragment was amplified in all analysed tissues (figure 3). Primers sets for NPY and *β*-actin had efficiencies of 104% and 107%, respectively. No significant differences were observed in the NPY mRNA levels in the 24 h evaluation period (figure 4). Two weeks of food restriction were sufficient to increase the NPY mRNA levels in Brazilian flounder (figure. 5).

3.4 *Measurement of biochemical parameters*

The values of plasma glucose, cholesterol, total protein and triglycerides during the 24 h experiment are shown in

```
1    agcttggtgagctggctggggactctggggctcctgctgtgggcgctgctctgcctgagc 60
1     S  L  V  S  W  L  G  T  L  G  L  L  L  W  A  L  L  C  L  S  20

61   gccctgaccgagggatacccggtgaaaccggagaaccccggggatgacgccccggcggag 120
21    A  L  T  E  G  Y  P  V  K  P  E  N  P  G  D  D  A  P  A  E  40

121  gtactggccaaatactactcagccctgagacactacatcaacctcatcacaagacagagg 180
41    V  L  A  K  Y  Y  S  A  L  R  H  Y  I  N  L  I  T  R  Q  R  60

181  tatgggaagaggtccagtcctgagattctggacacactggtctcagagctgctgctgaag 240
61    Y  G  K  R  S  S  P  E  I  L  D  T  L  V  S  E  L  L  L  K  80

241  gaaagcacagacacgcttccacagtcaagatatgacccatcattgtggtgaatgctgcca 300
81    E  S  T  D  T  L  P  Q  S  R  Y  D  P  S  L  W  *           96

301  tcaacgttgaatccacatcactgccgccgccccgccgctgctgacattctgacctctaaa 360
361  cctctgtcacgtcattttcctcctgcatgccaggagaccgcctgtcttacctctacgagc 420
421  cgctatgcgtaatcaattcctcgtccttaaccatatggacattaggagtccaaactgctt 480
481  gctagtatgtgcgtacaacaattgtaaatagtttactcagttatcatctgtgatacataa 540
541  agctggatgtgagggagggccatgttgtttgtattgtttaaatgtgcaataaagaat    597
```

Figure 1. Partial NPY cDNA sequence cloned from Brazilian flounder brain (GenBank accession no. FJ705358) and deduced amino acid sequence. Deduced amino acid sequence of mature NPY is underlined. The asterisk indicates the stop codon. The 3′ untranslated region is in italics. The nucleotides corresponding to the polyadenylation signal (AATAAA) are in bold.

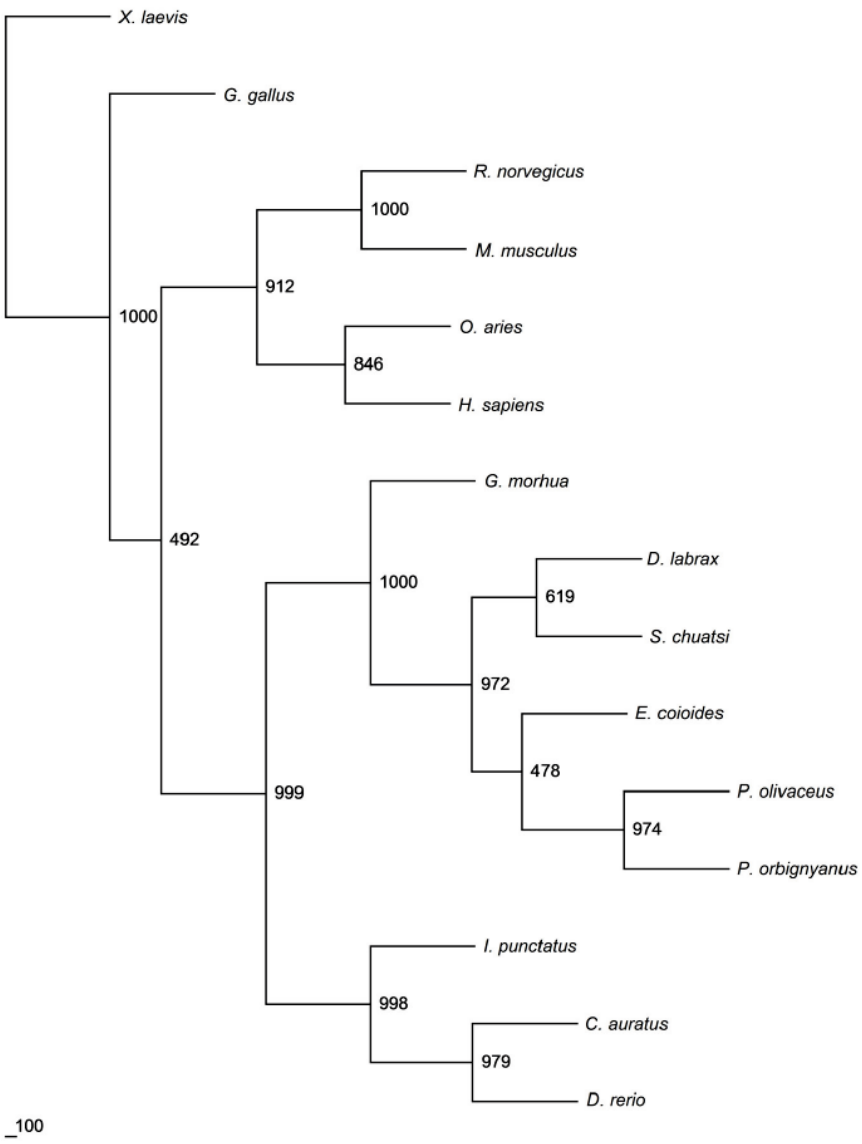

Figure 2. Phylogenetic tree of NPY coding sequences of Brazilian flounder (*Paralichthys orbignyanus* FJ705358), Japanese flounder (*Paralichthys olivaceus* AB055211), Atlantic cod (*Gadus morhua* AY822596), goldfish (*Carassius auratus* M87297), zebrafish (*Danio rerio* NM_131074), Chinese perch (*Siniperca chuatsi* EF554594), Channel catfish (*Ictalurus punctatus* AF267164), European sea bass (*Dicentrarchus labrax* AJ005378), grouper (*Epinephelus coioides* AY626561), frog (*Xenopus laevis* BC080115), chicken (*Gallus gallus* NM_205473), rat (*Rattus norvegicus* M20373), mouse (*Mus musculus* NM_023456), sheep (*Ovis aries* NM_001009452), human (*Homo sapiens* NM_000905).

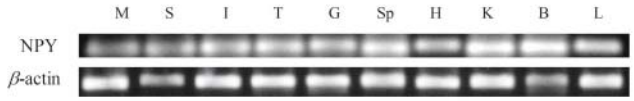

Figure 3. Semi-quantitative NPY mRNA expression in tissues of Brazilian flounder *Paralichthys orbignyanus*. M, muscle; S, stomach; I, intestine; T, testis; G, gill; Sp, spleen; H, heart; K, kidney; B, brain; L, liver.

table 2. No significant association among these parameters and NPY expression level was detected (NPY × glucose, $P = 0.6$; NPY × total protein, $P = 0.52$; NPY × cholesterol, $P = 0.22$; NPY × triglycerides, $P = 0.71$).

4. Discussion

The NPY cDNA fragment of 597 bp from Brazilian flounder was cloned by the 3′RACE-PCR method and contains a part

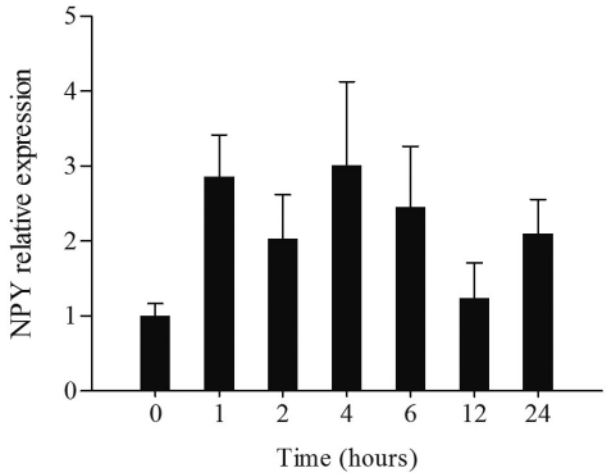

Figure 4. Evaluation of *Paralichthys orbignyanus* brain NPY expression over a 24 h period. Data are expressed as means ± SEM (*N*=5).

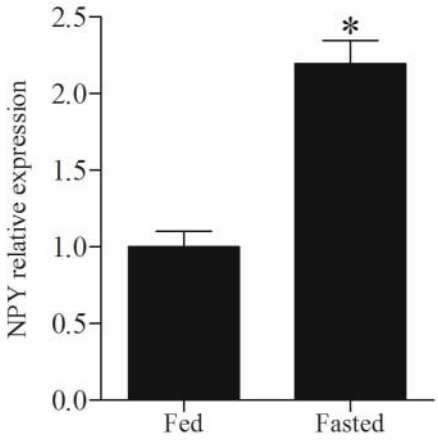

Figure 5. Evaluation of *Paralichthys orbignyanus* brain NPY expression in fed and fish fasted for two weeks. Data are expressed as means ± SEM (*N*=5).

Table 2. Blood chemistry evaluation of Brazilian flounder *Paralichthys orbignyanus* over 24 h following a meal

	Time (h)						
Parameters	0	1	2	4	6	12	24
Glucose (mg/dl)	33.1 ± 5.4^{a}	43.5 ± 1.1^{ab}	81.4 ± 10.5^{bc}	87.6 ± 11.5^{c}	67.2 ± 11.7^{bc}	93.6 ± 16.0^{c}	113.7 ± 14.7^{c}
Total protein (g/dl)	4.1 ± 0.6^{a}	4.7 ± 0.5^{a}	6.2 ± 1.1^{a}	7.1 ± 1.4^{a}	5.3 ± 1.1^{a}	3.3 ± 0.3^{a}	24.9 ± 0.4^{b}
Cholesterol (mg/dl)	116.2 ± 12.1^{bc}	96.5 ± 8.2^{ab}	79.1 ± 17.6^{ab}	77.7 ± 7.4^{ab}	52.6 ± 6.4^{a}	86.9 ± 16.1^{ab}	162.9 ± 18.9^{c}
Triglycerides (mg/dl)	525.7 ± 77.3^{a}	530.4 ± 80.1^{a}	493.6 ± 24.3^{a}	487.1 ± 56.7^{a}	371.6 ± 39.4^{a}	466.5 ± 62.8^{a}	615.6 ± 28.5^{a}

*Data are expressed as means ± SEM (*N*=5). Different letters indicate differences between means. Glucose data were transformed by log (X).

of coding region with 291 bp lacking 9 bp of the initial part of the ORF and the 3′ UTR. However, the deduced amino acid of the mature peptide shares high identity with the NPY of other fish (table 3). In addition, the amino acid residue present at 14 position of the mature peptides NPY and peptide YY (PYY) or pancreatic peptide Y (PY) can be used to differentiate their sequences (Leonard *et al.* 2001). In the PYY or PY sequences, an invariable proline residue is present, while in the NPY sequences an alanine residue is present, except for trout NPY which has a treonine (Larhammar *et al.* 1993), and channel catfish which has a valine residue (Leonard *et al.* 2001). The deduced amino acid sequence for mature Brazilian flounder NPY determined in the present study has an alanine residue. The phylogenetic analysis of nucleotide sequences that encode NPY demonstrate that Brazilian flounder has high identity with the NPY of acanthomorph fish (composed of 99 aa). Mammalian, avian and amphibian were grouped in different clusters.

NPY expression was detected in several peripheral tissues as described by MacDonald and Volkoff (2009a). Possible differences in NPY expression level among the peripheral tissues of Brazilian flounder might be the result of a differential regulation of NPY expression. Rats submitted to chronic food restriction had differentially affected NPY gene expression in the hypothalamus and liver, suggesting that NPY expression may also be differentially regulated in other tissues (Sucajtys-Szulc *et al.* 2008), while in fish it has not yet been demonstrated. Therefore, it may be risky to draw functional conclusions from anatomical studies in a single species (Soderberg *et al.* 2000).

In the present study, NPY gene expression in Brazilian flounder brain was examined. To our knowledge, this is the first time that NPY expression has been evaluated over a 24 h period. We show that NPY expression did not change within a 24 h period following a meal. These results are in contrast with previous studies in goldfish in which NPY expression levels were increased 1–3 h before food intake and decreased 1–3 h after food intake in the hypothalamus. However, in the optic tectum-thalamus, these levels were not increased and a weak increase was detected 1 h after

Table 3. Amino acid identity (%) between mature NPY from Brazilian flounder *Paralichthys orbignyanus* and other species[a]

Species	Amino acid identity (%)
Paralichthys olivaceus	97
Epinephelus coioides	97
Siniperca chuatsi	94
Dicentrarchus labrax	94
Gadus morhua	88
Danio rerio	88
Ictalurus punctatus	88
Ovis aries	88
Carassius auratus	86
Xenopus laevis	86
Rattus	83
Mus musculus	83
Homo sapiens	83
Gallus gallus	80

[a] Calculated from the ClustalW multiple alignment as percentage of identical amino acids compared with *P. orbignyanus* NPY

food intake (Narnaware *et al.* 2000). These results suggest that post-prandial changes in NPY expression may be area-specific. Besides, Kehoe and Volkoff (2007), using whole forebrain to evaluate gene expression, described increasing NPY mRNA levels around mealtime followed by a decrease 2 h later in Atlantic cod.

Indeed, exogenous factors such as temperature may result in a reduction in food intake and modify the digestive physiology in several fish species. In the winter flounder, hypothalamic NPY expression is not affected by fasting of two or even four weeks in the winter months, but these same starvation periods increase NPY levels in the summer (MacDonald and Volkoff 2009a). Here, we demonstrate that in Brazilian flounder, NPY mRNA levels increased after two weeks of fasting in the summer, as demonstrated in the winter skate (MacDonald and Volkoff 2009b). In our study, the low temperature (15 ± 3°C) during the 24 h experiment could increase the time required or food digestion and absorption, and this fact might have influenced the levels of NPY mRNA. This is supported by the increase in plasma glucose levels even 24 h after food intake, and also by the presence of a few pellets in the stomach. Hence, these low temperatures may not be sufficient to increase NPY expression. In contrast, low temperature can reduce food intake of Atlantic cod in captivity, but brain NPY mRNA levels do not appear to be influenced by this condition (Kehoe and Volkoff 2008).

Moreover, Kehoe and Volkoff (2007) have proposed that NPY can be a hunger signal prior to a meal and differences in NPY expression pattern among fish can be attributed to differences in the diet and digestive physiology, as well as susceptibility to stress. The Japanese flounder is very susceptible to transport and manipulation stress (Hur *et al.* 2007) and previous studies in rainbow trout (Doyon *et al.* 2003, 2006) demonstrate the influence of stress in NPY mRNA expression. Although all fish of each group were anaesthetized at the same time and were netted rapidly during sampling to minimize stress, it is possible that NPY levels might have been affected. Although not significant, the biochemical parameters, including glucose, increased at each sampling time, showing that digestion and absorption were in progress. However, correlations between biochemical parameters and brain NPY expression were not detected in the present study, showing that these biochemical variables could not be involved in NPY mRNA expression.

In summary, we demonstrate for the first time cDNA cloning of NPY from Brazilian flounder. NPY mRNA tends to be expressed mainly in the brain and is also expressed at different levels in several peripheral tissues, suggesting that NPY may be involved in other functions in these tissues. No significant correlations were found between NPY and biochemical parameters, and no significant differences were observed in brain NPY mRNA levels over a 24 h period after feeding at low temperatures. On the other hand, mRNA levels were increased after two weeks of fasting at elevated temperatures. Our results suggest that NPY mRNA levels in Brazilian flounder are affected by temperature.

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