UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Biotecnologia

DISSERTAÇÃO

Clonagem e avaliação da expressão gênica do neuropeptídeo Y no linguado *Paralichthys orbignyanus*

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Pelotas, 2009.

VINICIUS FARIAS CAMPOS

Clonagem e avaliação da expressão gênica do neuropeptídeo Y no linguado *Paralichthys orbignyanus*

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Dedico esta dissertação aos meus pais.

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I get by with a little help from my friends
I get high with a little help from my friends
Gonna try with a little help from my friends

John Lennon e Paul McCartney

RESUMO

CAMPOS, Vinicius Farias. **Clonagem e avaliação da expressão gênica do neuropeptídeo Y no linguado *Paralichthys orbignyanus***. 2009. 48f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Pelotas, Pelotas.

O apetite e a ingestão de alimentos em vertebrados é um processo complexo que envolve várias rotas neurais e endócrinas. Dentre os fatores orexigênicos, destacam-se os neuropeptídeos, como o neuropeptídeo Y (NPY), o qual é constituído de 36 aminoácidos e desempenha papel chave na regulação do apetite. Estudos avaliando a expressão do NPY em peixes demonstraram que este peptídeo está envolvido no estímulo da ingestão. Entretanto, apesar dos avanços recentes, o atual conhecimento sobre a regulação fisiológica do apetite é limitado e baseado em poucas espécies de peixes com evidencias de diferenças interespecíficas. O linguado *Paralichthys orbignyanus* tem sido considerado para a aqüicultura e com isso, torna-se importante compreender os mecanismos de regulação do apetite para incrementar o seu desempenho em cativeiro. Os objetivos deste trabalho compreenderam clonar o gene do NPY, avaliar os níveis de RNA mensageiro nos diferentes tecidos do linguado e também avaliar a expressão do NPY no cérebro durante 24 h por RT-PCR em tempo real. Um fragmento de 597 pb do cDNA do NPY foi clonado a partir do RNAm do cérebro do linguado através do método RACE 3'. A expressão do NPY foi detectada em todos os tecidos analisados (cérebro, baço, coração, intestino, estômago, brânquia, fígado, rim, músculo e testículo), mas com predominante expressão no cérebro. A expressão do NPY não cérebro demonstrou variações durante 24 horas de avaliação. Nenhuma correlação foi observada entre glicose, proteínas totais, colesterol e triglicerídeos plasmáticos. Estes resultados indicam que o NPY pode desenvolver funções em outros tecidos além do cérebro e também pode não estar associado na regulação da alimentação em curto prazo em baixas temperaturas.

Palavras-chave: Alimentação. Clonagem. NPY. *Paralichthys orbignyanus*. PCR em tempo real.

ABSTRACT

CAMPOS, Vinicius Farias. **Clonagem e avaliação da expressão gênica do neuropeptídeo Y no linguado *Paralichthys orbignyanus***. 2009. 48f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Food intake in vertebrates is a complex process involving several endocrine and neural pathways. Among the orexigenic factors, the most notably are the neuropetides as the neuropeptide Y (NPY) that is a 36 amino acid peptide which plays a key role in food intake. Studies evaluating fish NPY expression showed that this peptide is involved in appetite stimulation. However, despite the recent advances, our present knowledge of the regulation of feeding behavior in fish is limited and based in a few fish species, and there is increasing evidence of species-specific differences. 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 during 24 h by real-time RT-PCR. A 597 bp NPY cDNA was cloned from Brazilian flounder brain by 3´RACE method. NPY expression was detected in all peripheral tissues analyzed, but with predominant expression in the brain. No significant differences were observed in brain NPY gene expression over a 24 h evaluation period. No correlation was observed among plasma glucose, total protein, cholesterol, triglycerides and NPY expression levels during 24 hours. These results indicated that NPY may play roles in flounder peripheral tissues and may be not involved short-term regulation of food intake at low-temperatures in Brazilian flounder.

Keywords: cloning, feeding behavior, NPY, *Paralichthys orbignyanus*, real-time RT-PCR.

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LISTA DE ABREVIATURAS E SIGLAS

aa - Aminoácido
ANOVA – Análise de Variância
AP – *primer* adaptador
AUAP – *primer* adaptador universal abreviado
BAC – beta-actina
CART – Transcrito Regulado pela Cocaína e Anfetamina
CCK – Colecistoquinina
cDNA – Ácido Desoxirribonucléico Complementar
CRF – Corticotropina
GRP – Peptídeo Liberador da Gastrina
NPY – Neuropeptídeo Y
PY – Peptídeo Pancreático Y
PYY – Peptídeo YY
qRT-PCR – Transcrição Reversa e Reação em Cadeia da Polimerase quantitativa
RACE – Amplificação Rápida das Extremidades do cDNA
RNAm, mRNA – Ácido Ribonucléico mensageiro

SUMÁRIO

1. INTRODUÇÃO GERAL

Nos vertebrados, o cérebro é o principal centro de controle do apetite, da ingestão de alimentos e do balanço energético. Estudos iniciais usando tanto estimulação elétrica quanto danificações em áreas específicas do cérebro demonstraram que o hipotálamo está envolvido na regulação do apetite (DEMSK; NORTHCUTT, 1983). Informações sobre reservas nutricionais, saciedade, fome, palatabilidade do alimento, entre outras, são comunicados pelo cérebro através de vias neurais ou endócrinas para os órgãos periféricos (STRADER et al., 1998).

No hipotálamo são produzidos sinalizadores moleculares que estimulam (orexigênicos) e que inibem (anorexigênicos) o apetite. Estes compreendem várias classes de neurotransmissores e agentes endócrinos, que incluem aminoácidos, aminas, citocinas, canabinóides e neuropeptideos (CHEE; COLMERS, 2008). Entretanto, estes sinalizadores não são originados apenas no sistema nervoso central, mas também em órgãos periféricos como intestino, pâncreas, fígado e tecido adiposo. Peptídeos centrais incluem orexigênicos como o neuropeptídeo Y (NPY), as orexinas, a galanina e anorexigênicos como o transcrito regulado pela cocaína e anfetamina (CART) e o fator liberador da corticotropina (CRF) (VOLKOFF et al., 2008). Sinais periféricos incluem estimulantes da ingestão como a Grelina e inibidores, como a colecistoquinina (CCK), o peptídeo liberador da gastrina (GRP), a amilina e a leptina (VOLKOFF et al., 2005). Estas moléculas atuam em redes neurais e endócrinas que controlam a ingestão e o gasto da energia, ou seja, a homeostase energética (VOLKOFF, 2006). Todavia, as funções específicas de cada molécula e as interações com outras na regulação da alimentação e as aplicações biotecnológicas que poderão ser obtidas com este conhecimento ainda estão em

estudos iniciais, ainda que em mamíferos, uma grande quantidade de conhecimento tenha sido produzida recentemente (CHEE; COLMERS, 2008).

1.1. Regulação do apetite em peixes

Nos últimos anos, um número crescente de peptídeos reguladores do apetite, homólogos aos encontrados em mamíferos, vem sendo caracterizados em peixes (GORISSEN et al., 2006), sugerindo que a regulação da alimentação é relativamente conservada entre os vertebrados (VOLKOFF et al., 2008). No entanto, quando comparados aos mamíferos ou outros grupos, os peixes representam um grupo filogeneticamente vasto, o qual demonstra uma significante diversidade no tocante à morfologia, ecologia, comportamento e diferenças genômicas (VOLFF, 2004). Isto sugere que o controle endócrino da alimentação em peixes pode também ser diverso e envolver moléculas e mecanismos espécies-específicas (MACDONALD; VOLKOFF, 2009). Neste sentido, para a caracterização de um peptídeo regulador do apetite em uma espécie de peixe deve-se levar em consideração, não apenas as ações deste hormônio ou neurotransmissor e suas interações com outros fatores reguladores, mas também a filogenia desta espécie, o seu estado fisiológico, bem como o ambiente que a espécie habita (VOLKOFF et al., 2008). A função de peptídeos na ingestão de alimentos pode ser avaliada tanto pela administração destes peptídeos por diversas vias (ALDEGUNDE; MANCEBO, 2006), quanto pela quantificação de seus níveis sanguíneos e teciduais (CARPIO et al., 2007), ou pela avaliação da expressão gênica destas moléculas (MIURA et al., 2006).

Apesar dos recentes avanços, o conhecimento do comportamento alimentar em peixes ainda é muito inicial e, partindo da diversidade dos peixes e do crescente número de moléculas reguladoras do apetite descobertas, estudos em diferentes espécies de peixes, deverão ser realizados para uma melhor compreensão dos mecanismos de regulação da alimentação em peixes.

1.2. Neuropeptídeo Y (NPY)

O NPY é o fator orexigênico mais abundante e potente encontrado no cérebro (CHEE; COLMERS, 2008). É um peptídeo de 36 aminoácidos que está associado a vários processos metabólicos em mamíferos incluindo relações com a reprodução, ritmo circadiano, metabolismo e processos cardiovasculares (DUMONT et al., 1992; LEONARD et al., 2001). Em mamíferos, várias evidências sugerem que o NPY desempenha um papel chave no controle do apetite e do peso corporal. A sua redução endógena via técnicas de RNAm anti-senso levam à diminuição do apetite (STANLEY et al. 1992). Deste modo, estudos na linha da saúde também têm sido desenvolvidos utilizando antagonistas de receptores do NPY com o objetivo de desenvolver medicamentos para o tratamento da obesidade (MACNEIL, 2007).

Recentemente, os efeitos estimulatórios no apetite, causados por injeções centrais de NPY, têm sido demonstrados em peixes (ALDEGUNDE; MANCEBO, 2006; CARPIO et al., 2007; KIRIS et al., 2007), contudo, este tratamento age por um curto período de tempo. Além disso, o NPY é um potente liberador do hormônio do crescimento em peixes (PENG; PETER, 1997), mas, o aumento do crescimento só acontece quando a quantidade de alimento suficiente for fornecida (VOLKOFF et al., 2005).

Foi demonstrado no goldfish (*Carassius auratus*) que o NPY media, em nível hipotalâmico, a ação orexígena da grelina (MIURA et al., 2006). Também no goldfish (NARNAWARE et al. 2000) e no bacalhau (*Gadus morhua*) (KEHOE; VOLKOFF, 2007) a expressão gênica deste peptídeo é elevada em torno da hora da alimentação.

Estudos no mesmo goldfish demonstraram que após 72 horas de jejum há elevação nos níveis de RNA mensageiro do NPY e rápida normalização (1 h após a realimentação) (NARNAWARE; PETER, 2001) e, duas semanas de jejum, aumentam significantemente a expressão do NPY na *Raja occelatta* (MACDONALD; VOLKOFF, 2009). Porém, sete dias de jejum não são suficientes para a alteração destes níveis no *Gadus morhua* (KEHOE; VOLKOFF, 2007), enquanto que em bagres três semanas são necessárias para alterar estes níveis no cérebro (SILVERSTEIN; PLISETSKAYA, 2000).

A expressão do NPY tem sido detectada em vários tecidos periféricos de peixes como o intestino, fígado, tecido adiposo, baço, músculo, brânquias e ovários (LEONARD et al., 2001; KEHOE; VOLKOFF, 2007; LIANG et al., 2007). Entretanto, pouco se sabe sobre a função deste peptídeo nestes órgãos, além disso, poucos estudos têm empregado métodos mais sofisticados e sensitivos, como a PCR em tempo real, para avaliar a expressão gênica do NPY no cérebro e nos demais tecidos.

A ação do NPY na alimentação é de grande interesse para pesquisadores trabalhando com o crescimento de peixes em cativeiro, pois em estudos como no goldfish, o NPY demonstra interagir como os eixos somatotrófico e metabólico através do sistema do hormônio do crescimento (PENG et al., 1990, 1993). Assim, esta molécula torna-se um importante alvo no estudo do aumento do crescimento de peixes usados na indústria da aqüicultura.

1.3. O Linguado

O linguado *Paralichthys orbignyanus* (Valenciennes) habita regiões estuarinas e litorâneas desde o Rio de Janeiro (Brasil) até Mar del Plata (Argentina), sendo um importante recurso para a pesca nestas localidades (FIGUEREDO; MENEZES, 2000). Vários estudos vêm demonstrando o potencial desta espécie para utilização na aqüicultura, devido ao alto valor comercial e também por possuir ampla tolerância a fatores ambientais (SAMPAIO; BIANCHINI, 2002).

A tolerância a uma larga faixa de temperaturas (WASIELESKY et al., 1998), vários níveis de salinidade (WASIELESKY et al., 1995), bem como à altas concentrações de compostos nitrogenados (BIANCHINI et al., 1996), são características que deram suporte inicial para a sua produção em cativeiro. Recentemente, estudos relacionados à reprodução (RADONIC et al., 2007; LANES et al., 2008; SAMPAIO et al., 2008), larvicultura (SAMPAIO et al., 2007), clonagem e avaliação da expressão de genes relacionados ao crescimento (MEIER et al., 2008) e avaliação do potencial para a transgênese (LANES et al., 2009), demonstraram a viabilidade para a indústria da aqüicultura.

Entretanto, a regulação fisiológica da alimentação desta espécie ainda não foi examinada, não tendo sido demonstrado se o NPY está envolvido na regulação

do comportamento alimentar no linguado, conhecimento que poderá ajudar a incrementar o crescimento em cativeiro.

Baseado no exposto acima, este estudo teve por objetivos, clonar o cDNA do NPY do linguado, avaliar a sua expressão nos diferentes tecidos periféricos e avaliar os níveis de RNAm no cérebro num período de 24 horas. Os resultados e a discussão dos dados obtidos durante a execução deste estudo, bem como a metodologia empregada, são apresentados na forma de artigo científico.

2. ARTIGO

Cloning and evaluation of NPY gene expression over a 24 hours period in Brazilian flounder (*Paralichthys orbignyanus*)

(Artigo científico formatado nas normas do periódico *Aquaculture*)

Cloning and evaluation of brain NPY gene expression over a 24 hours period in Brazilian flounder (*Paralichthys orbignyanus*)

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Abstract

Neuropeptide Y (NPY) is the most potent stimulant of food intake for vertebrates, mammals and fish. However, the present knowledge about feeding behavior in fish is still limited and based in 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 by real-time RT-PCR to associate food intake with NPY expression levels. A 597 bp NPY cDNA was cloned from Brazilian flounder brain by the 3´RACE method. NPY expression was detected in all peripheral tissues analyzed, but with predominant expression in the brain. No significant differences were observed in brain NPY gene expression over a 24 h evaluation period. No correlation was observed among plasma glucose, total protein, cholesterol, triglycerides and NPY expression levels during 24 hours. These results indicate that NPY may be not involved in short-term regulation of food intake at low-temperatures in Brazilian flounder.

Key words: cloning, feeding behavior, NPY, *Paralichthys orbignyanus*, real-time RT-PCR.

1. Introduction

Food intake in vertebrates is a complex process involving several neural pathways, where neuropeptide Y (NPY) it plays a key role. This 36 amino acids peptide has also been linked to a variety of other physiological processes in mammals, including cardiovascular, metabolic, circadian rhythm, and reproduction (Dumont et al., 1992; Leonard et al., 2001). This neurotransmitter is abundantly expressed in the nervous system and is the most potent stimulant of food intake in mammals (Chee and Colmers, 2008). The stimulatory effects on food intake caused by central injections of NPY were demonstrated in goldfish (Lopez-Patino et al., 1999) rainbow trout (Aldegunde and Mancebo, 2006), tilapia (Kiris et al., 2007) and African catfish fry (Carpio et al., 2007), although this treatment acts for a short period of time.

Since the discovery of the stimulant action of NPY on food intake, researchers working to enhance growth of fishes for aquaculture production have demonstrated a particular interest in this molecule (Leonard et al., 2001). Moreover, NPY is a highly potent stimulant of growth hormone release in fish (Peng and Peter, 1997), but the growth enhancement can only be achieved when there is sufficient food available (Volkoff et al., 2005). Despite the recent advances, our present knowledge of the regulation of feeding behavior in fish is limited and based in a few fish species, and there is increasing evidence of species-specific differences (Volkoff et al., 2008). In the Goldfish, it was demonstrated that 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 of brain NPY expression was observed after 72 hours of food deprivation in goldfish, but immediately after feeding its expression was normalized (Narnaware and Peter, 2001), and two weeks increased significantly brain NPY mRNA levels in Winter skate (MacDonald and Volkoff, 2008). On the other hand, NPY expression was not increased after 7 days of food deprivation in Atlantic cod (Kehoe and Volkoff, 2007), while in catfish 3 weeks of fasting are needed to alter NPY expression in the brain (Silverstein and Plisetskaya, 2000). Also, the NPY expression has been detected in the intestine, liver, spleen, muscle, and adipose tissue of fish (Liang et al., 2007) but few evidences about the functions on these

tissues have been described. According to Volkoff et al. (2008) to characterize an appetite-regulating peptide, it must be taken into account the phylogeny of the fish, its physiological state as well as the environment of the fish.

The Brazilian flounder *Paralichthys orbignyanus* inhabits estuarine and coastal waters from Rio de Janeiro (Brazil) to Mar del Plata (Argentina) (Figueiredo and Menezes, 2000). 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., 2008) and evaluation of transgenesis potential (Lanes et al., 2009) showed the feasibility of production. However, feeding regulation in this species has never been examined. Its not know whether the NPY are involved in regulation of feeding behavior of the Brazilian flounder and these knowledge may be helpful to improve its culture.

The aims of this study were to clone the NPY cDNA, examine their expression levels in different tissues of Brazilian flounder and after, asses their role on feeding behavior, evaluating brain NPY mRNA expression over a 24 h period by qRT-PCR.

2. Materials and Methods

2.1. Animals

Fish used in this study were from 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. To evaluate 24h brain NPY expression animals were allocated into 7 tanks with 5 animals in each group and were acclimated during 2 weeks in seawater, fed with commercial pellet diet (Supra Salmonídeos TM / Alisul / Brasil) containing 46% crude protein and 6% lipid once a day at the same time (17:00) under natural temperature (15 ± 3°C) and photoperiod (11L: 13D). Gene expression was measured in seven different times (0 – 10 min before food intake, 1, 2, 4, 6, 12 and 24 h after food intake).

2.2. Cloning of NPY cDNA

Fish were anesthetized in benzocain (50mg.L^{-1}) and euthanatized by severing the spinal cord, and then, whole brains were immediately dissected out, frozen and stored in liquid nitrogen until use. Total RNA was isolated with TRIzol® Reagent (Invitrogen™, Carlsbad, USA) following the recommended protocol. Final RNA concentrations were determined by the QuBit® fluorometer (Invitrogen™, Carlsbad, USA) and 2 µg was used for the synthesis of first strand cDNA performed with SuperScript™ III Reverse Transcriptase (Invitrogen™, Carlsbad, USA) according to the manufacturer's protocol. Two degenerate primer sets (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 pa rameters were 40 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min, with an additional initial 1min 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'RACE total brain RNA was reverse transcribed to cDNA in the presence of oligo(dT) adaptor primer (AP) (table 1) using also 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 of 94 °C for 30 sec, 55 °C for 30 sec 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 from agarose gel with GFX™ PCR DNA and Gel Band Purification Kit and sequenced as described above.

2.4 Phylogenetic analysis

Several 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, 1993) and the maximum parsimony method (DNAPARS, for details see PHYLYP 3.6 manual). *Xenopus laevis* was used as outgroup. A bootstrapping analysis using 1000 iterations was performed using SEQBOOT.

2.3. Evaluation of NPY expression on flounder tissues by semi-quantitative RT-PCR

In order to evaluate NPY expression in flounder tissues, brain and other tissues (liver, spleen, muscle, gill, intestine, heart, kidney, stomach and testis) of three animals were examined for NPY expression. Tissue collection and total RNA was prepared as described in 2.1. DNase treatment of RNA samples was conducted with DNA-free® Kit (Ambion™, USA) following recommended protocol. The QuBit® fluorometer (Invitrogen™, Carlsbad, USA) was used to determine RNA concentrations in order to normalize all samples. The first-strand cDNA was synthesized with High Capacity cDNA Reverse Transcription® Kit (Applied Biosystems™, USA), according to the manufacturer's protocol. The same NPY primers used for real-time PCR evaluation were used in RT-PCR (table 1) and for endogenous reference β-actin (GenBank accession no. **EU542580**) was used (BAC semi F and R – see table 1). PCR conditions for NPY were: 35 cycles of 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec, 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: 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec 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 2.5 % agarose gel and for β-actin on 1% agarose gel both containing 0.5 μg ml^{-1} ethidium bromide. Samples of three animals were analyzed. For each gene analyzed, all samples were run in the same gel for comparison. Digitalized images of the gels were analyzed employing the software 1DScan (Scanalytics, USA).

2.4. Evaluation of 24h of NPY expression by real-time RT-PCR (qRT-PCR)

After sample collection to evaluate biochemical parameters, brain collection, total RNA, DNase treatment and cDNA synthesis were carried out as described before. Real-time PCR were 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). Amplification was carried out at the standard cycling conditions of 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 sec, 60ºC for 60 sec following by conditions to

calculate de melting curve. All PCR runs for each cDNA sample were performed in triplicate. The real time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen (2001) and the handbook of Chemistry Guide of Applied Biosystems.

2.5. Biochemical parameters

Plasma concentrations of glucose, total protein, triglycerides and cholesterol were measured through analytical enzymatic-colorimetric kits (Doles Reagents©, Brazil). Blood samples were obtained from anesthetized fish by first gill-arc artery puncture, centrifuged (15 min -1000 x *g*) and serum frozen and stored in liquid nitrogen until analyses. The blood was treated with ETDA and potassium fluoride as anticoagulant and anti-glycolitic, respectively. The absorbance measurements were conducted in triplicate in a plate reader (Biotek, Canada). The manufacturer methods and the wavelength used were: Glucose – glucose oxidase, 510 nm; Total protein – Biuret, 550 nm; Triglycerides – glycerol kinase/Phosfate-peroxidase/4 aminoantipirin, 510 nm; Cholesterol – 4 aminoantipirin, 510 nm.

2.6 Data analyses

In the 24 h evaluation of NPY gene expression experiment different groups were compared using one-way ANOVA and Tukey's test for multiple comparisons. To evaluate biochemical parameters during 24 h the groups were compared using one-way ANOVA Kruskal-Wallis multiple comparisons method. Pearson's correlation analysis was used to detect association among NPY expression and biochemical parameters. In the evaluation of expression on different tissues experiment the groups were also compared using one-way ANOVA Kruskal-Wallis multiple comparisons method. 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 brain of Brazilian flounder that was deposited under GenBank accession no. **FJ705358** (Fig. 1). The cloned fragment contains a 291 bp of final part of the open reading frame (ORF) of flounder NPY containing a 25 amino acid forming part of signal peptide followed by 36 amino acid of mature peptide. The proteolytic processing site Gly-Lys-Arg is followed by 32 amino acid constituting c-terminal peptide followed by a 3'UTR of 306 bp. To reveal the molecular phylogenetic position of NPY, a consensus phylogenetic tree was obtained by maximum parsimony method (Fig. 2). Brazilian flounder NPY and the NPY of other acanthomorph fishes (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) was grouped in another cluster. Mammalian and chicken NPY were grouped in a different clusters and a frog was used as outgroup.

3. 2. Evaluation of NPY expression on flounder tissues

NPY expression was detected in all analyzed tissues with the highest levels in brain followed by heart, gill, spleen, stomach, kidney and liver and the lower levels in muscle and intestine (Fig. 3).

3. 3. Evaluation of brain NPY expression over a 24 h period

No significant differences were observed in the NPY mRNA levels in the 24 h evaluation period (Fig. 4).

3.4. Measurement of biochemical parameters

The values of serum glucose, cholesterol, total protein and triglycerides are showed in table 2. No one significantly association among these parameters and NPY expression level was detected.

4. Discussion

The NPY cDNA fragment with 597 bp of Brazilian flounder was cloned by 3'RACE-PCR method and contains a part of coding region with 291 bp lacking 9 initial bp of the ORF and the 5' UTR. However, deduced amino acid of mature peptide shares high identity with NPY of other fish (table 3). In addition, the amino acid residue present at fourteen positions of the mature peptides NPY and PYY or PY can be used to differentiate their sequences. In peptide YY (PYY) or pancreatic peptide Y (PY) sequences an invariable proline residue is present, while in 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 (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 have high identity with NPY of acanthomorph fishes (composed of 99 aa). Mammalian, avian and amphibian were grouped in different clusters.

4.1. Distribution of NPY mRNA expression

The major NPY expression level was detected in brain of Brazilian flounder as in Chinese perch and other acanthomorph and non-acantomorph fishes (Liang et al., 2007). In this study, considerable mRNA levels of NPY were detected in heart, spleen and gills. In Chinese perch substantial expression of NPY in heart was also described (Liang et al., 2007). In contrast, in Atlantic cod (Kehoe and Volkoff, 2007) and in Grouper (Chen et al., 2005) NPY mRNA was detected in low levels in heart and was not detected in gill and spleen. In mammals, NPY and its receptor Y1 were detected in cardiovascular system by indirect immunofluorescence and was

demonstrated its action in regulation of intracellular calcium (Jacques and Abdel-Samad, 2007). Also NPY mRNA was detected in mammalian spleen with stimulant effects on angiogenesis (Kitlinska et al., 2002). In fish, the effects of NPY expression in heart, spleen and gills have not been demonstrated yet.

Lower NPY mRNA levels were found in liver, kidney and stomach than those found in heart, spleen and gill. In Atlantic cod, NPY expression was not detected in liver while in kidney high expression levels were found (Kehoe and Volkoff, 2007) differently from grouper which demonstrates expression in stomach and liver (Chen et al., 2005) and Chinese perch which also expresses NPY in liver (Liang et al., 2007). NPY has been detected in kidney of pufferfish (Sundstrom et al., 2005) but not in kidney of catfish according to Leonard et al. (2001). These authors as well as Kehoe and Volkoff (2007), described the NPY mRNA expression in fish ovarian tissue, besides NPY affects the reproduction in goldfish (Peng et al., 1994) and the release of GnRH in catfish (Gaikwad et al., 2005). According with these previous studies, we showed that NPY mRNA was found in the Brazilian flounder testis. We detected mRNA also in intestine and muscle in very low levels, in contrast to Kurokawa and Suzuki (2002) that not found NPY expression in intestine of Japanese flounder. The presence of NPY in intestine might be due a connection to ghrelin action as demonstrated in goldfish (Miura et al., 2006).

Differences in NPY expression level among peripheral tissues in Brazilian flounder might be the result of a differential NPY expression regulation. Rats in chronic food restriction differentially affect NPY gene expression in hypothalamus and in liver suggesting that NPY expression may be also differentially regulated in other tissues (Sucajtys-Szulc et al. 2008), while in fish it has not been demonstrated yet. Therefore, it may be risky to draw functional conclusions from anatomical studies in a single species (Söderberg et al., 2000).

4.2. Evaluation of brain NPY expression over a 24 h period

In the present study, NPY gene expression in Brazilian flounder brain was examined by real-time RT-PCR. To out knowledge, is the first time that NPY expression is evaluated over a 24 h period. NPY expression not changed in a 24 h period. These results suggest that NPY might have no action as a shot-term food

intake regulation in *P.orbignyanus*. These results are in contrast with previous studies in goldfish which NPY expression levels were increased 1-3 h before food intake and decrease 1-3 hours after food intake in hypothalamus. However, in the optic tectum-thalamus these levels were not increased and a weak increase was detected 1 h after 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 gene expression evaluation, describe an increasing in NPY mRNA levels around the mealtime and a decrease 2 h after in Atlantic cod. Nevertheless, in our study we used whole forebrain and we might have masked changes in NPY expression within discrete brain regions. However, by using qRT-PCR, which is a high sensible method, small changes could be detected in the expression levels.

Factors as the temperature can reduce food intake and modify the digestive physiology in several fish species. The Brazilian flounder is a eurythermal fish that undergo natural temperature fluctuations and can survive in a wide range of temperatures, from 11 to 30°C (Wasielesky et al., 1997). However the adequate temperature to captivity growth for this species is around to 23°C. In our study the low temperature conditions (15 ± 3°C) may be reduced the required time to total food digestion and this fact might be influenced the NPY mRNA levels. This is supported by the serum glucose levels that were significantly increased 24 h after food intake and the animals sampled were still with the full stomach. The pleuronectiform winter flounder (*Pleuronectes americanus*) stop feeding entirely and do not grow during the winter months (Stoner et al., 1999) and the NPY might be involved in long-term feeding regulation (MacDonald, 2008 apud Volkoff et al., 2008) as also demonstrated in winter skate (MacDonald and Volkoff, 2009) . In the contrast, in the Atlantic cod, that is species which inhabits low temperature environments, the low temperature can reduce food intake in captivity, but brain NPY mRNA levels appear to be not influenced by this condition (Kehoe and Volkoff, 2008).

Moreover, Kehoe and Volkoff (2007) have proposed that NPY can be a hunger signal prior to meal and differences in NPY expression pattern among fishes can be attributed to differences in the diet and digestive physiology as well as susceptibility to stress. The Japanese flounder *Paralichthys olivaceus* 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 fish were netted rapidly during sampling to minimize stress, it is possible that NPY levels might have been affected. Correlations among biochemical parameters and brain NPY expression were not detected in the present study. However, the values are in agreement with indicated to Brazilian flounder (Robaldo, 2003) and congeneric species *Paralichthys olivaceus* (Cho et al., 2007).

4.3 Summary and conclusion

We demonstrate for the first time cDNA cloning of NPY from Brazilian flounder. NPY mRNA is mainly expressed in brain and also expressed at different levels in several peripheral tissues suggesting that NPY may be involved in other functions in these tissues. No significant differences were observed in brain NPY mRNA levels over a 24 h period suggesting that NPY may be not involved in short-term regulation of food intake at low temperatures in *P. orbignyanus*.

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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

Table 2. Blood chemistry evaluation of Brazilian flounder *Paralichthys orbignyanus* during 24 hours.

	Time (hours)						
Parameters	0	1	2	4	6	12	24
Glucose (mg/dL)	33.1 ± 5.4 a	43.5 ± 1.1 a	81.4 ± 10.5 ab	87.6 ± 11.5 ab	67.2 ± 11.7 ab	93.6 ± 16.0 ab	113.7 ± 14.7 b
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 ab	96.5 ± 8.2 ab	79.1 ± 17.6 ab	77.7 ± 7.4 a	52.6 ± 6.4 a	86.9 ± 16.1 ab	162.9 ± 18.9 b
Triglycerides (mg/dL)	525.7 ± 77.3 ab	530.4 ± 80.1 ab	493.6 ± 24.3 ab	487.1 ± 56.7 ab	371.6 ± 39.4 a	466.5 ± 62.8 ab	615.6 ± 28.5 b

*Data are expressed as means ± SEM (n=5). Different letters indicate differences between means. (Kruskal-Wallis ANOVA; p<0,05).

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

Species	Amino acid identity (%)
Epinephelus coioides	97
P. olivaceus	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 % identical amino acids compared to *P. orbignyanus* NPY.

Figure 1. 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 italic. The nucleotides corresponding to the polyadenylation signal (AATAAA) are in bold.

```
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 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**).

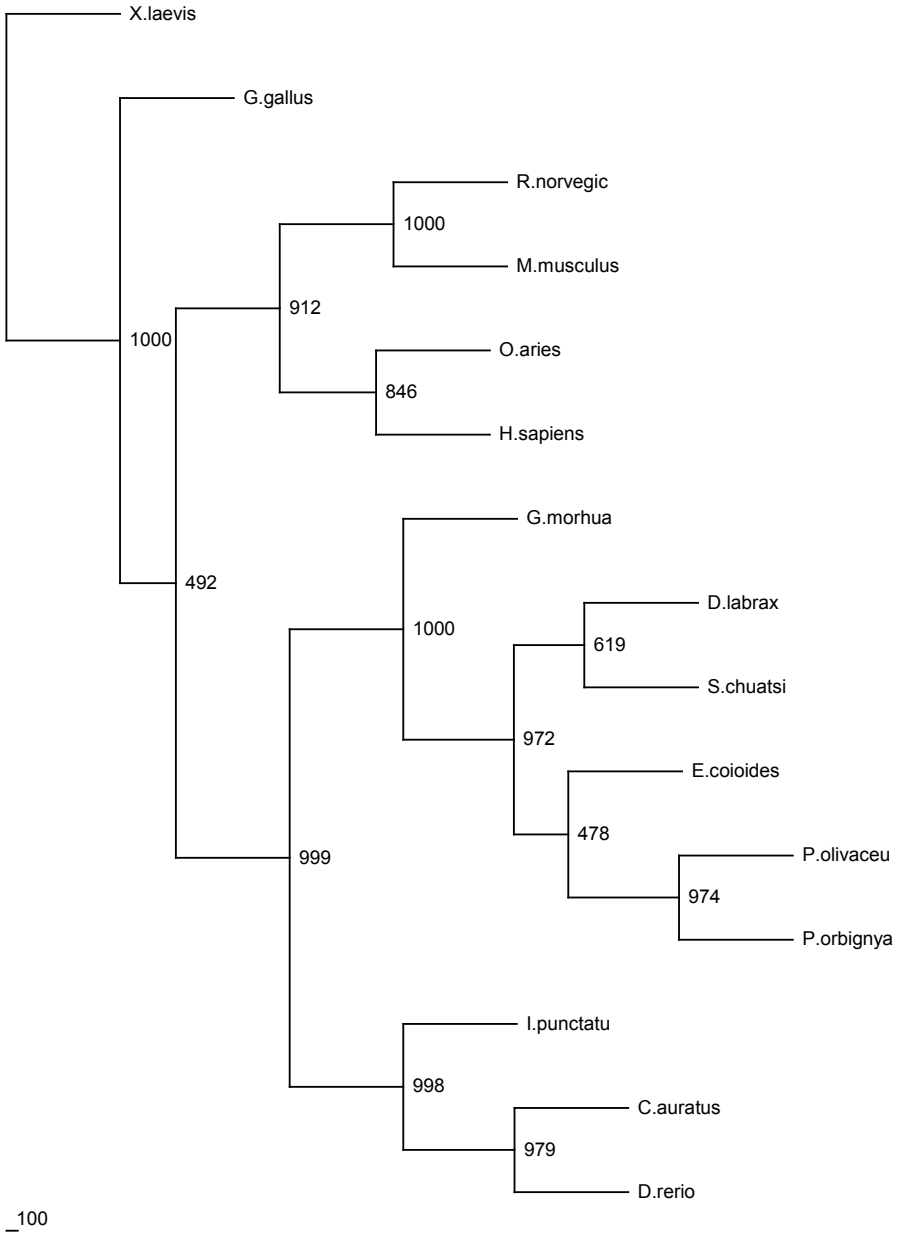


Figure 3. Semi-quantitative NPY mRNA expression in tissues of Brazilian flounder *Paralichthys orbignyanus*. B = brain, S = stomach, I = intestine, Sp = spleen, T = testis, G = gill, L = liver, M = muscle, K = kidney, H = heart. Data are expressed as means ± SEM (n=3). Significant differences (p<0.05) between groups were detected using a one-way ANOVA. Groups that differ significantly are indicated by different letters above bars.

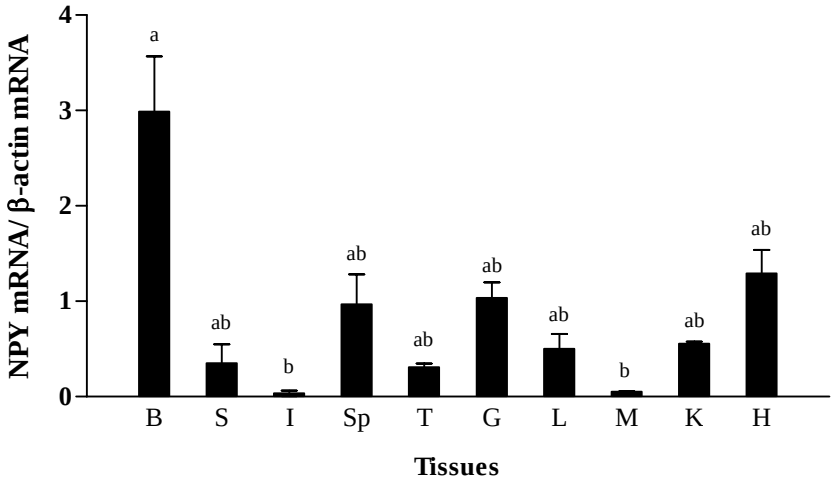
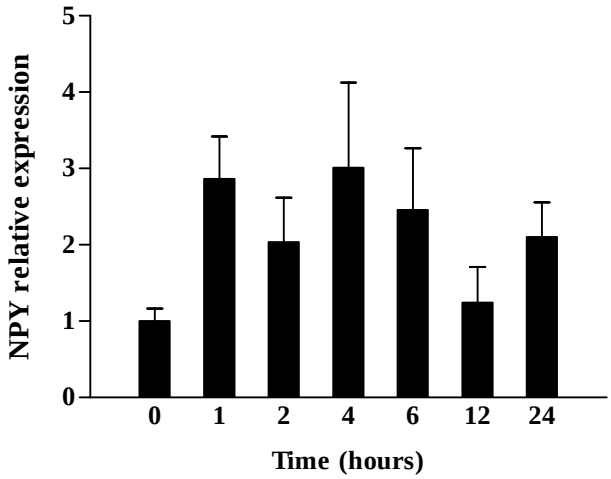


Figure 4. Evaluation of *Paralichthys orbignyanus* brain NPY expression by real-time RT-PCR over a 24 h period. Data are expressed as means ± SEM (n=5).



3. CONCLUSÃO

O estudo realizado demonstrou pela primeira vez a clonagem do gene do NPY no linguado. Demonstrou também que este gene é expresso em vários tecidos periféricos em diferentes níveis e que os níveis de RNAm no cérebro não variam dentro de um período de 24 h sugerindo que o NPY pode não estar associado na regulação da alimentação em curto prazo em baixas temperaturas.

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