*Brief communication*

# Neuropeptide Y gene expression around meal time in the Brazilian flounder *Paralichthys orbignyanus*

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Neuropeptide Y (NPY) is considered the major stimulant for food intake in mammals and fish. Previous results indicate that NPY is involved in the feeding behaviour of the Brazilian flounder, *Paralichthys orbignyanus*. In this study, we evaluated hypothalamic NPY expression before (−2 h), during (0 h) and after feeding (+2 h) in two independent experiments: (1) during a normal feeding schedule and (2) in fish fasted for 2 weeks. During normal feeding, changes in the levels of NPY mRNA were periprandial, with expression levels being significantly elevated at meal time ($P<0.05$) and significantly reduced 2 h later ($P<0.05$). Comparing the fasting and unfasted groups, NPY mRNA levels were significantly higher ($P<0.05$) at −2 h and +2 h in the fasting group, but there was no difference at 0 h. In addition, the higher NPY mRNA levels that were observed in the fasting group were maintained throughout the sampling period. In summary, our results show that NPY expression was associated with meal time (0 h) in food intake regulation.

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## 1. Introduction

Changes in feeding behaviour and appetite are often associated with changes in gene expression and/or protein concentration levels of appetite-regulating hormones or their receptors in a variety of vertebrates including fish (Aldegunde and Mancebo 2006; Carpio *et al.* 2007; Volkoff *et al.* 2010; Kamijo *et al.* 2011), mammals (Ferretti *et al.* 2011; Sucajtys-Szulc *et al.* 2008) and chicken (Saneyasu *et al.* 2011). Thus, changes in mRNA/protein levels of a given hormone following fasting or feeding likely reflect its physiological role in feeding regulation (Lopez-Patino *et al.* 1999; Valassi *et al.* 2008).

Neuropeptide Y (NPY) is the most important stimulant of food intake and body weight gain in vertebrates, including mammals and fish (Valassi *et al.* 2008; Volkoff *et al.* 2009). In fish, NPY appears to have a role in both daily feeding and long-term energy homeostasis. Meal time changes in NPY mRNA brain levels have been reported for goldfish (Narnaware *et al.* 2000) and Atlantic cod (Kehoe and Volkoff 2007). Food deprivation also increases NPY mRNA in the hypothalamus of goldfish, Coho salmon, Chinook salmon and in winter skate (Narnaware *et al.* 2000; MacDonald and Volkoff 2009), an effect that can be reversed by refeeding (Narnaware and Peter 2001).

**Keywords.** Food intake; mRNA expression; NPY; *Paralichthys orbignyanus*



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Recently, we demonstrated the cloning of the NPY gene from the Brazilian flounder and reported a significant increase in NPY mRNA levels 2 weeks after fasting, but changes in hypothalamic NPY mRNA levels were not observed in fish maintained at low temperature (Campos *et al.* 2010). In addition, we demonstrated that NPY was also involved in gonadal maturation in the Brazilian flounder (Campos *et al.* 2011a, b). Despite recent advances, the current knowledge of how feeding behaviour is regulated in fish is limited, based on only a few species, while there is increasing evidence of species-specific differences (Volkoff *et al.* 2009, 2010).

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 inhabits estuarine and coastal waters ranging from Rio de Janeiro (Brazil) to Mar del Plata (Argentina). Recently, studies focusing on reproduction (Radonic *et al.* 2007; Lanes *et al.* 2008; Sampaio *et al.* 2008; Lanes *et al.* 2010; Campos *et al.* 2011b), larviculture (Sampaio *et al.* 2007), expression of genes related to growth (Meier *et al.* 2009) and reproduction as well as evaluation of transgenic potential (Lanes *et al.* 2009) demonstrated the culture feasibility of this species. However, the role of NPY in food intake regulation of the Brazilian flounder was unknown and this information may be of use in improving its aquaculture.

In this study, two independent experiments measured NPY expression around meal time. NPY gene expression was evaluated in animals with a standard feeding schedule (fed once a day) and in fasting animals, in order to evaluate NPY expression around meal time after a fasting period.

## 2. Materials and methods

### 2.1 *Animals and experimental design*

Fish were obtained by artificial spawning at the Aquaculture Station at Universidade Federal do Rio Grande (FURG, Rio Grande, RS, Brazil). Experimental fish were acclimatized in seawater for 4 weeks in 500 L tanks (5 fish per tank). During acclimatization, fish were maintained under a scheduled feeding regimen and fed with commercial pellets, containing 46% crude protein and 6% lipid (Supra Salmonídeos, Alisul, Brazil), once a day at the same time (12:00) and at a natural summer temperature (23±2°C) and photoperiod (13 L:11D). This feeding method ensured that all the pellets offered to the fish were consumed and did not sink to the bottom of the tank. After acclimation the fish were sampled.

*2.1.1 Experiment 1 – Effects of meal time on NPY mRNA expression:* Five groups (five animals/group) were used in this experiment. Fish were sampled from the feed tanks at different times with respect to the scheduled feeding time: three groups of fish were sampled 2 h before feeding time (10:00, −2 h), at meal time (12:00, 0 h) or 2 h after feeding time (14:00, +2 h). The two groups that were not fed at meal time were sampled at time 0 h and at time +2 h to evaluate if the lack of feeding would affect NPY mRNA expression levels. Fish sampled at meal time were allowed to feed for 10 min and subsequently euthanized – they were anaesthetized with benzocaine (50 mg $L^{-1}$) followed by severing of the spinal cord. The brain, minus the cerebellum and medulla oblongata, was quickly dissected out and the hypothalamus was frozen in liquid nitrogen until use as described previously (Amano *et al.* 2004).

*2.1.2 Experiment 2 – Effects of fasting on NPY expression around meal time:* Six groups (five animals/group) of acclimatized fish were used in this experiment. Feeding was stopped for 2 weeks (three groups) and the feeding schedule described above was maintained for the three remaining groups. After 2 weeks, the fish were sampled. As described in the previous experiment, fish from the six groups were sampled at three different times (−2, 0, +2 h). Sampling was carried out as described above.

### 2.2 *RNA extraction and cDNA synthesis*

Total RNA extraction and cDNA synthesis were carried out as described previously (Campos *et al.* 2010, 2011b). Briefly, RNA samples were isolated using TRIzol Reagent (Invitrogen, Carlsbad, USA) and samples were DNase-treated with a DNA-free kit (Ambion, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 μg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) as recommended by the manufacturer (Campos *et al.* 2010).

### 2.3 *Real-Time PCR*

Real-Time PCR (qPCR) reactions were run on a Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, UK) using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, USA). NPY mRNA expression levels were measured using previously described primers (Campos *et al.* 2010) (GenBank # FJ705358 – forward 5′CACGTCATTTTCCTCCTGCAT, reverse 5′GCATAGCGGCTCGTAGAGGTA). The Brazilian flounder β-actin gene-specific primers (GenBank # EU542580 – forward 5′ GACCCAGATCATGTTTGAGACCTT, reverse 5′AGGGACAGCACAGCTTGGAT) were used as the

endogenous reference. Initial validation experiments were conducted to ensure that both above primer pairs had equivalent qPCR efficiencies (Campos *et al.* 2010). Amplification was carried out at cycling conditions of 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 60°C for 60 s followed by calculation of the melting curve. The qPCR assays for each cDNA sample were performed in duplicate. Amplification, dissociation curves and gene expression analyses were performed using the MxPro v4.1 software (Stratagene). The relative Ct (ΔΔCt) method (Livak and Schmittgen 2001) was used to quantify NPY gene expression. Briefly, the fold change of each target gene was normalized to the housekeeping gene (β-actin), and expressed relative to a calibrator sample. In the case of experiment one, the average fold expression of sample from the −2 h group (reference group) was set to 1 and the expression levels of all the other groups were expressed as a percentage relative to this group. In the case of experiment two the reference group was the −2 h from the unfasted group.

### 2.4 *Data analyses*

In both experiments, gene expression data were compared using two-way ANOVAs followed by Tukey's tests for multiple comparisons. In experiment one, two factors were considered: feeding status (fed or unfed fish, two levels) and meal time (−2, 0, +2 h, three levels). In experiment two, two factors were considered fasted or unfasted fish (two levels) and time point evaluation (−2, 0, +2 h, three levels). In both experiments two-way ANOVAs were performed to compare the effects of feeding or fasting and time or the interaction between these variables on NPY gene expression. That the data were normally distributed was verified using Kolmogorov-Smirnov's test. Significance was considered significant at $P<0.05$. All data are expressed as mean RNA expression ± SEM.

## 3. Results

### 3.1 *Experiment 1: NPY expression at meal time*

NPY expression at meal time was observed that both meal time and feeding status affects NPY gene expression: $F(2,24)=15.15$, $P<0.0001$. In the fed group, when the NPY mRNA levels at specific times (−2, 0 and +2 h) were compared, the highest significant levels were observed at 0 h and the lowest at +2 h ($P<0.05$) (figure 1). In the unfed group, although the NPY levels varied at −2, 0 and +2 h, the differences were not significant (figure 1). When comparing the fed and unfed group, NPY mRNA levels remained significantly higher in the unfed group: at +2 h in comparison to the fed group at the same time point ($P<0.05$) (figure 1).

### 3.2 *Experiment 2: NPY expression around meal time after fasting*

In this experiment it was observed that both meal time and fasting affects NPY gene expression: $F(2,24)=11.18$, $P=0.0004$. NPY levels in the unfasted group were significantly higher at 0 h than at −2 and +2 h ($P<0.05$, figure 2), similar to that observed in experiment (figure 1). However, in contrast to the first experiment, there was no difference in the NPY levels in the unfasted group at −2 and +2 h. While the NPY levels in the fasting group varied across the −2, 0 and +2 h

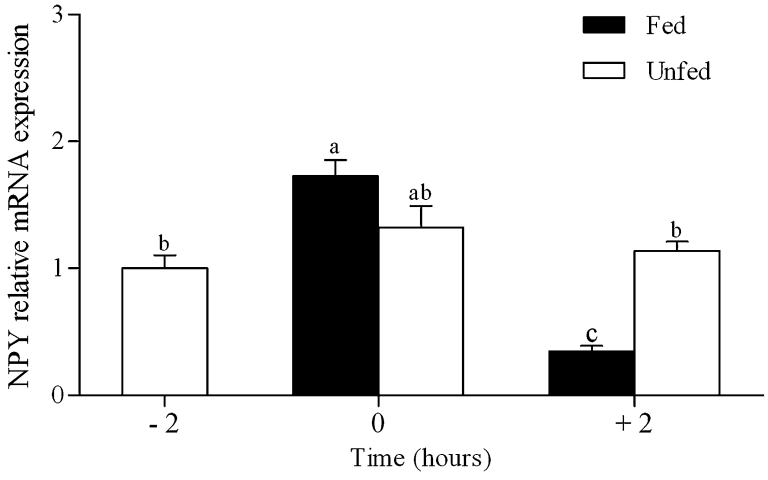


**Figure 1.** NPY gene expression at meal time in the Brazilian flounder. Different letters above the bars indicate significant differences. Data are expressed as means ± SEM ($n=5$).

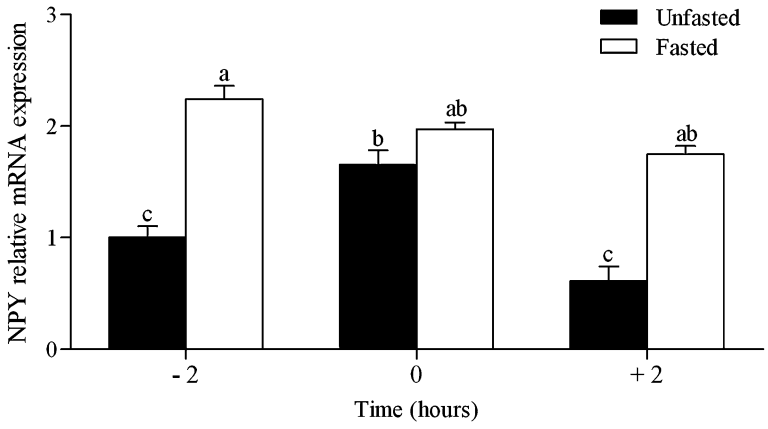


**Figure 2.** NPY gene expression around meal time after fasting in the Brazilian flounder. Different letters above the bars indicate significant differences. Data are expressed as means ± SEM ($n$=5).

intervals, the differences were not significant. Comparing the fasting and unfasted, NPY expression levels at −2 and +2 h were significantly higher ($P$<0.05), in the fasting group, but there were no differences observed at 0 h between the two groups (figure 2).

## 4. Discussion

In the current study, NPY gene expression was evaluated in two independent experiments in order to associate its expression with food intake and meal time. Under a standard feeding procedure, hypothalamic NPY mRNA levels significantly increased at meal time and then significantly dropped after feeding. The expression levels of orexigenic factors such as NPY usually increase before or during a meal in fish as previously reported for Atlantic cod (Kehoe and Volkoff 2007) and goldfish (Narnaware and Peter 2001). Our results corroborate this, showing that NPY levels are elevated around feeding time in the Brazilian flounder. In addition, in animals that were not fed at meal time, NPY expression was not reduced as observed for those that fed normally, suggesting that the signal for food intake remained high acting as a hunger signal. These results are in agreement with previous studies in goldfish in which hypothalamic NPY expression levels were increased 1–3 h before food intake and decreased 1–3 h after food intake (Narnaware *et al.* 2000). Similarly, in rats, NPY peptide and mRNA levels decreased progressively after a meal (Kalra *et al.* 1991; Sahu *et al.* 1992).

Our previous results (Campos *et al.* 2010) suggested that NPY mRNA levels in the Brazilian flounder were affected by temperature. There were no significant differences in the NPY mRNA levels over the 24 h evaluation period at low temperatures (winter temperatures, approximately 15°C). This low temperature during the 24 h experiment could increase the time required for food digestion and absorption, and this fact might have influenced the expression of NPY. 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, and acute temperature changes might be necessary to induce more pronounced changes in NPY expression in cod (Kehoe and Volkoff 2008). In this study, using summer temperatures (approximately 23°C) we demonstrate that NPY expression was affected by meal time, suggesting that temperature could affect NPY gene expression; however, further studies are needed to clarify this issue.

Food deprivation usually induces an up-regulation in the expression of orexigenic factors such as NPY. Our previous results showed that NPY gene expression increased after fasting (Campos *et al.* 2010); however, we did not know the effects of fasting around meal time on NPY gene expression. Here we evaluate the effects of fasting on NPY gene expression around meal time. We observed that at all sampling points (−2, 0 and +2 h) the NPY mRNA levels in fasted animals were higher than those in fed animals, except at meal time (0 h) where NPY mRNA levels from fed animals were similar to those seen in the fasted group. The data confirmed that fasting increased NPY mRNA levels and that these changes were not influenced by the pre-established periprandial feeding schedule. Our findings indicate that after 2 weeks of fasting, NPY mRNA levels remained high throughout the day.

In goldfish, NPY expression levels increased after 72 h of food deprivation (Narnaware and Peter 2001), while in catfish, 3 weeks of food deprivation were required before there were any detectable changes in NPY expression (Silverstein and Plisetskaya 2000). In Atlantic cod, a 1 week period of food deprivation did not affect NPY mRNA expression

levels, suggesting that short-term starvation might not be sufficient to cause significant changes in NPY levels (Kehoe and Volkoff 2007). Yet, in the tiger puffer, a 1 week period of food deprivation was enough to increase NPY mRNA levels (Kamijo *et al.* 2011).

In summary, our results suggest that NPY expression is associated with meal time in food intake regulation. In addition, we demonstrated for the first time that fasting increased and maintained NPY mRNA levels and this was not associated with a scheduled feeding time. Furthermore, the findings presented in this study may be important for aquaculture of the Brazilian flounder.

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