E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(1): 485-489

www.fisheriesjournal.com
Received: 06-11-2016
Accepted: 07-12-2016

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Changes in NPY gene expression during exogenus starvation in *H. fossilis*

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Abstract
Starvation condition is experienced in many fish species every year, due to various environmental conditions. Also, it is evident that starvation results in mortality. Metabolic enzymes, RNA/DNA ratio and protein also get affected during fasting. We aimed to investigate the distribution of endocrine cell immunoreactivity for the neuropeptide-Y (NP-Y) in stomach and intestinal region of digestive tract of teleost cat fish *Heteropneustes fossilis*, by using immunocytochemistry. The present study also focused to access the effect of starvation on mRNA gene expression in the intestine of the fish. The results revealed the NP-Y immunoreactive endocrine cells from the intestine of *Heteropneustes fossilis* were quite varied as compared to other teleost fish species. Stomach showed complete absence of immunoreactivity. mRNA concentration increased significantly in middle region due to increased in starvation period. Above findings confirmed the role of NPY in regulation of feeding and digestion.

Keywords: Immunocytochemistry, mRNA, Endocrine cell, Neuropeptide Y, Gene Expression

1. Introduction
Neuropeptides in vertebrates and in invertebrates have been suggested to play key roles as signalling molecules in the regulation of physiology, behaviour and development, (Tensen *et al*., 1998) [27]. These peptides also shows several effects on the digestive and feeding behaviour (Toni 2004 and Jensen 2001) [28, 11]. In fish digestive tract more than 45 gastro-intestinal peptides have been restored (Kiliaan *et al.,* 1997; Girolamo *et al*., 1999; Lucini *et al.,* 1999; Domeneghini *et al.,* 2000; Youson *et al.,* 2001; Burrin *et al.,* 2003) [13, 9, 16, 8, 32, 2]. The key regulators to control appetite in fish includes neuropeptide Y (NP-Y), ghrelin (GRLN), cocaine and amphetamine regulated transcript (CART) and various others (Volkoff *et al*., 2005) [30].
Heteropneustes fossilis (*Bloch*, 1794), the stinging catfish of India is an important commercial fish due to high market value. It is highly appreciated due to low fat content and high amount of iron and calcium. *H. fossilis* is also reported for high value of genetic variability within and between the population.
Literature review performed in past decades described the distribution and relative frequency of endocrine cells present in the gastrointestinal tract of various species of fish.
However, investigations into the neural regulations of appetite in different fish species has only recently began.
Several peptides in the gut of fish, which play a key role in gastrointestinal physiology. Gastrin (GAS), CCK-8, Calcitonin Gene Related Peptide (CGRP) and Neuropeptide Y (NPY) are regarded as few important peptides which regulate digestive processes as well as feeding behaviour (Lopez-Patino *et al*., 1999; Jensen 2001; Olssen and Holmgren, 2001; Martinez-Alvarez *et al*., 2009) [15, 11, 23, 19].
Neuropeptide -Y, a highly conserved peptide is expressed both in the brain and gastrointestinal tract. It is known to be involved in regulation of feeding mechanism. In addition, NPY mRNA expression levels are seen to be increased in brain of *Pacific salmon* (*oncorhynchus* sp.) (Silverstein *et al*., 1998) [26] and goldfish (Narnaware and Peter, 2002) [22] in restricted food conditions. In mammals NP-Y is one of the most potent orexigenic factor (Chee and Colmers, 2008) [5]. Neuropeptide Y (NPY) is a powerful stimulant of eating behavior in many species, including goldfish (Lopez-Patino *et al.,* 1999; Narnaware *et al.,* 2000) [15, 20].
The first aim of the present investigations was to study the distribution of endocrine cells that

are NP-Y immunoreactive from the stomach and intestine of *H. fossilis*. Secondly, in order to provide new information on peptide regulation of *H.fossilis*, we focussed to access the effect of starvation on the mRNA gene expression in the intestine.

2. Materials and Methods

2.1 Animals and sample preparation

H. fossilis fish of either sex (weigh 180-200 gram) were procured from local market. Approximately 45 fish were acclimatized in the aquaria at temperature 21 ^{0}C. for one week. They were divided into three groups, a control group which were regularly fed for 900 hr. while the other two groups fish did not receive food i.e. they were starved for 24hrs and 48hrs respectively. Fish from each group were anesthetized by 2-phenoxyethanol (sigma cat = P1126 1I) and perfused transcardially with ice-cold PBS at pH 7.45. Intestine tissue was taken for RNA extraction and immunocytochemistry. The tissue was stored at -20 ^{0}C in RNA later (Invitrogen) and further processed for total RNA extraction.

2.2 Immunocytochemistry

For immunocytochemistry, the tissue was passed through 10%, 20% and 30% sucrose solution. Streptavidin-biotin peroxidase method was employed to localize NPY in tissue sections at light microscopic level. All the incubations and washing procedure were performed in humid chamber. Briefly, the tissue sections were washed in PBS and treated with 1%Bouin Serum Albumin (BSA). Section were further incubated with primary antibody against (NPY rabbit) (sigma cat # N- 9528) diluted in PBS (1:2000). After wash in PBS biotinylated secondary antibody IgG (GeNei, India, 021031) was applied for 2 hour followed by streptavidin- peroxidase conjugate (GeNei India 091072) for 2 hour. The reaction was visualised through chromogen AEC (3-amino 9 ethyl carbazole). Section were rinsed twice in distilled water and mounted in glycerol gelatin.

2.3 Specificity of the Antibody

Control procedure like (1) omission of the primary antibody from the reaction, (2). Replacing the anticera against NPY with bovine serum albumin, (3).Preabsorption of the antibody with NPY at 10^{-5} M for 24h before incubation resulted in total loss of immunoreactivity.

All the prepared slides were observed under the Olympus light microscope.

2.3 RNA extraction and quantification

Total RNA was isolated using trizol /chloroform- reagent according to the manufacturer's protocol and then quantified by using Qubit®2.0 Flurometer (Life Technologies USA), which provides an accurate and selective method for the quantitation of high abundance RNA samples. The integrity of the RNA was verified by visualization of the Agarose gel Electrophoresis. (Agarose from Invitrogen, USA and electrophoresis apparatus from BioRad Company).

2.4 cDNA Synthesis

One microgram of total RNA from each tissue was reverse – transcribed using the DTT, MLV-RT (Life Technologies, USA) and Nuclease free water, according to the protocol provided by the manufacturer. Quantitative real – time PCR was performed using the SYBR Green PCR mix (TAKARA Biosciences).PCR detection system (BioRad) was employed to quantify NPY and mRNA. Primer and probe sequences for NPY are listed in Table 1.

Table 1: List of primers and their annealing temperature

Primer Name	Primer sequence(F) (Forward)	Primer sequence(R)	Primer annealing Temp. (^{0}C)
NPY-q	CCCGAAGCACT AATGATGAC	CATGGAAGGTTC ATCATACCTAA	52
EF-q	AAGGAAGCTGC TGAGATGGG	CAGCTTCAAACT CACCCACA	52

2.5 RT – PCR

Two primers (NPY_q and EF_q) were designed to clone intestinal NPY. cDNA sequences of *H. fossilis* by PCR parameters were 40 cycles at 94 ^{0}C. These cycles were used for efficient amplification of all genes in real time PCR (Applied Biosystems Stepone Plus)

The genes of interest were normalized to the reference gene (EF_q) and expression levels were compared using ΔΔCt method. Amplification, dissociation curves and gene expression analysis were performed (Applied Biosystems Stepone Plus). The reference gene EFq was tested for starvation affect gene expression level from all the three regions of intestine.

3. Results

Immunocytochemistry results of NP-Y in Intestine of *H. Fossilis* are shown in table 2.

Table 2: Immunocytochemistry

Region of Intestine	NP-Y Reactivity		
	Control	24Hr	48 Hr
Proximal Region	++	++	++
Middle Region	++++	+++	++++
Distal Region	+++	++	++

Relative densities for immunoreactive elements are indicated by the following symbols:

Highest: ++++ Moderate: +++ and Weak: ++

Starvation condition is experienced in many fish species every year, due to various environmental conditions. Also, it is evident that starvation results in mortality. Metabolic enzymes, RNA/DNA ratio and protein also get affected during fasting. Therefore the present study was planned to illustrate the effect of starvation on the appetite regulating peptide NP-Y. Immunoreactivity for NP-Y peptide was studied in different cell types of stomach and intestinal region during control and starve conditions of *H. fossilis* for the first time. The NP-Y Immunoreactivity exhibited moderately in the proximal and distal intestine region in the endocrime cells, while the middle region showed intense NP-Y Immunoreactivity in the control group of fish. Stomach region of the digestive tract both from control as well as experimental group (24 hr. and 48 hr. starve) showed complete absence of NP-Y Immunoreactivity. NP-Y +ve

endocrine cells were observed to show varied morphology from elongated, sac and tadpole shape with cell body and cytoplasmic processes in the epithelial lining of apical region of lamina propria and in middle region of intestinal mucosal fold. In 24 hours starved group, the NP-Y Immunoreactive endocrine cells were sparsely decreased in intensity as compared to the control. Endocrine cells were less frequent in the proximal region than in the middle and distal region of intestine. The reactivity was detected in the apical part of proximal intestine, while in the middle intestine region endocrine cells were sporadically observed in apical, middle and bottom area of epithelial mucosa.

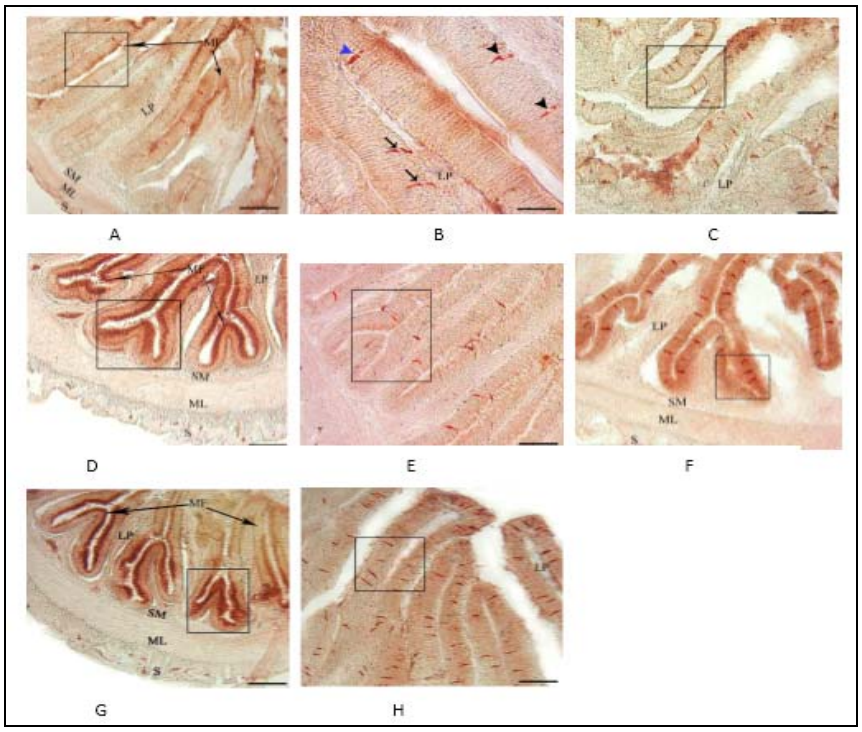

Fig 1: A: Proximal intestine region of control group *H. fossilis* showing NP-Y immunoreactive endocrine cells in the epithelial lining of apical & middle region of lamina propia.(Scale bar 15µm). **B:** Magnified view of sac (→), tadpole (➤) and elongated (➤) open NPY immunoreactive endocrine cells observed in the apical and middle region of proximal intestinal mucosal fold. (Scale bar 20µm). **C, D:** Middle and distal intestine region of control group showing NPY immunoreactive endocrine cells detected in the intestinal mucosal fold and lamina propria (LP) respectively. (Scale bar 15µm). **E, F, G:** Proximal, Middle and Distal intestinal region of 24 hr starvation group showing NPY immunoreactive endocrine cells respectively (Scale bar 15µm**). H:** Middle intestine region of 48 hr starvation group showing NPY immunoreactive endocrine cells detected in the intestinal mucosal fold with lamina propria (LP). (Scale bar 15µm).
In figure: SM: submucosa; ML: muscularis; S: serosa; LP: Lamina propria; MF: mucosal fold.

Distal region focused less NP-Y immunoreactivity in middle and bottom part of intestinal fold. 48 hr. starvation group was confirmed with huge amount of immunoreactivity in the middle intestinal region, moderate in distal while less in proximal.

3.1 Effect of starvation on mRNA gene expression

Although, on the basis of immunocytochemistry, NP-Y immunoreactivity appeared to have got increased in middle intestine of control and starved fish. There were no significant changes in NP-Y gene expression in the distal region (B, D, F) and proximal region (A, C, E) of control and starved fish. Relative quantitation of gene expression for NP-Y gene from middle region (M1,M2 and M3) suggest that M2 region (24 hr.) shows 1.3 fold expression as compared with M1 and there is no significant expression change in M3 sample.

Table 3: Total relative quantification of control, 24hrs starve and 48hrs starve sample.

Sample	Target	Reference	Target Mean Ct	dCt Mean	ddCt	RQ
A	NPY	EF	37.81	-0.16	0.00	1.00
B	NPY	EF	38.38	-0.68	-0.52	1.43
C	NPY	EF	29.24	-0.07	0.10	0.93
D	NPY	EF	29.26	-0.04	0.12	0.92
E	NPY	EF	30.99	0.75	0.91	0.53
F	NPY	EF	29.27	-0.03	0.14	0.91
M1	NPY	EF	19.37	-1.46	-1.30	2.46
M2	NPY	EF	18.04	-0.92	-0.76	1.69
M3	NPY	EF	18.72	-1.58	-1.41	2.66

In table 3:-control fish sample: A: anterior intestine, B: posterior intestine, M1: middle intestine; 24hrs starve fish sample: C: anterior intestine, D: distal intestine, M2: middle intestine; 48hrs starve fish sample: E: anterior intestine, F: distal intestine, M3: middle intestine.

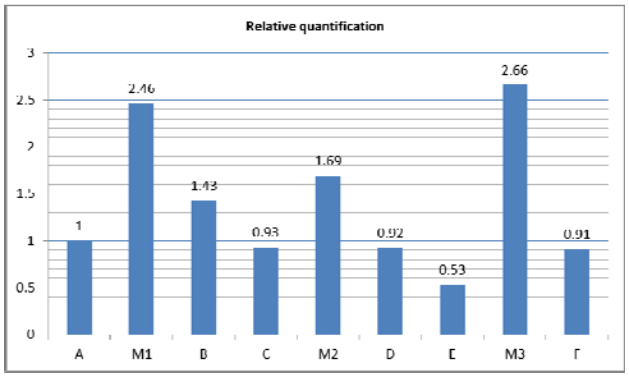

Chart 1: Relative quantification of gene expression in control, 24hrs starve and 48hrs starve fish intestine sample**.** Control fish sample: A: anterior intestine, M1: middle intestine, B: distal intestine; 24hrs starve fish sample: C: anterior intestine, M2: middle intestine, D: distal intestine; 48hrs starve fish sample: E: anterior intestine, M3: middle intestine, F: distal intestine.

4. Discussion

The gastrointestinal mucosa of a fish is known to produce a large number of endocrine secretions. These secretions are called as peptides and are detected in the lamina propria, glands, mucosal nerve ganglions and intermuscular nerve plexus. (Himick and Peter, 1994; Cimini *et al*, 1989; Pan *et al*., 2000) [10, 7, 24]. Literature review on immunohistochemical studies have focused that as in mammals, several neuropeptides may be present in the gastrointestinal tract of fish. Published literature gives information of several gastrointestinal tract peptides in various fish species.

Hence, in the present study we examined NP-Y immunoreactive endocrine cells in the intestine of teleost fish *H. fossils* under feeding and starve conditions. No examination are reported in *H. fossils* fish, to study whether the NP-Y containing endocrine cells *get al*tered during different nutritional conditions, like starvation. Present study reveals that NP-Y immunoreactivity was maximum in the middle region of intestine of all the three experimental groups, i.e. control and two starve groups. The proximal intestine region of control fish showed higher immunoreactivity as compared to that of starve fish group, while, in fish *O. banariencies* showed higher number of neuroendocrine cells immunoreactive to NPY in the proximal intestine, which could indicate a role of this region as a primary source of signals to stimulate food intake in the absence of food (Vigliano *et.al.,* 2011) [29].

In fishes, *p. reticulate and L. idus melanotus* no changes were recorded occur in the density of endocrine cells, nerve fibres or nerve cell bodies of the intestinal region after starvation (Bukhardt-Holm and Holmgren, 1989) [1]. These findings are not consistent with that of our results, where the density of immunoendocrine cells varied with respect to starvation.

In fish and other vertebrates feeding and satiation are controlled through orexigenic and anorexigenic signals, from the central nervous system and peripheral signals that are provide. It has been suggested that NPY is a key orexigenic peptide in the regulation of appetite in teleost fish (Cerda-Reverter and Larhammar, 2000, Larhammar, 1996; Volkoff *et al.,* 2009) [3, 14, 18, 19, 31].

In both goldfish (Narnaware and Peter, 2001a) [21] and winter flounder (McDonald, 2008) [17], the fasting period induces increase in hypothalamic NPY mRNA expression, suggesting that NPY acts as a long- term regulator of feeding in fish.

Within the fish *H. fossilis*, NP-Y mRNA expression was detected in all the three regions of intestine i.e. proximal, middle and distal in both fed and unfed (starved) fish group. However, 48 hr starvation showed an increase in mRNA expression in the middle region of intestine. While proximal and distal region of fed fish group showed more mRNA expression of NP-Y as compared to 24 hr and 48 hr starve group.

Previous studies detected NP-Y mRNA expression in gastrointestinal tract of several other fish including *winter flounder* (MacDonald and Volkoff, 2009) [18, 31], cod (Kehoe and Volkoff, 2007) [12], orange spotted grouper (Chen *et. al*., 2005) [6] and goldfish (Peng *et al*., 1994) [25].

In conclusion, the region wise distribution of NP-Y immunoreactive endocrine cells from the intestine of *H. fossilis* was quite varied as compared to other teleost fish species. Stomach showed complete absence of NP-Y immunoreactiviy. mRNA concentration increased significantly in middle region due to increase in starvation period. Above findings confirmed the role of NPY in regulation of feeding and digestion.

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