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Characterization of Orexin Gene in Tilapia (*Oreochromis niloticus*): Regulator Feeding Appetite, and Correlation with Reproductive Factors

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Abstract. *The mechanism of energy allocation related to increased appetite and feed intake in fish involves several neuropeptides encoded by specific genes. Orexin (OX) is a peptide secreted in the pars tuberalis of the hypophysis that acts as a stimulator in appetite increase (orexigenic factor). However, the expression of different orexigenic factors varies among vertebrate species, reflecting their unique types and lineages. Therefore, this study aimed to identify genes encoding appetite in Tilapia. RNA isolation, complementary Deoxyribose Nucleic Acid (cDNA) cloning, and DNA amplification were performed from brain samples of gonadally mature Tilapia. The PCR products were subsequently sent to Macrogen.Inc for sequencing. The amplification results of Orexin with β-Actin (positive control) observed using agarose gel electrophoresis showed that the size of the nucleotide base product of each gene was 196 bp and 197 bp. Confirmation of sequencing results carried out using the Basic Local Alignment Search Tool (BLAST) method - National Center for Biotechnology Information (NCBI) for Orexin and β-Actin were 97% and 100%. Based on these results, it can be concluded that each target gene isolated from the tilapia brain showed homology/similarity with the sequence available in the NCBI database.*

Keywords: *cloning, feeding appetite, reproduction, tilapia*

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INTRODUCTION

Tilapia is a freshwater species with high economic value and its appetite control is related to reproductive factors (Simanjuntak et al, 2017). The control of feed intake and energy allocation is a complex process in organisms, involving both peripheral nervous system and central nervous system mechanisms in vertebrates, including fish. Energy allocation mechanisms related to increased appetite and feed intake in fish involve several neuropeptides encoded by genes, one of which is Orexin (also known as hypocretin). Orexin is a neuropeptide that has specific G-Protein Coupled Receptors (GPRs) (Kukkonen & Leonard, 2014) and is derived from the precursor derivative prepro-orexin which is secreted in the hypothalamus (Volkoff et al. 2005; Spinazzi et al. 2006). Orexin, along with Neuropeptide Y (NPY), plays an important role in regulating orexigenic factors, which stimulate and increase appetite and feed intake (Sakurai et al. 1998; Matsuda et al. 2012) with other orexigenic peptides.

Feeding and fasting conditions affect Orexin expression and synthesis in the brain, specifically in the hypothalamus in several fish species (Matsuda et al., 2012). One of the influences on increasing Orexin expression is mediated by Leptin in the peripheral nervous system. In addition to being the primary regulator in increasing feed intake and feeding appetite, Orexin also regulates energy homeostasis and balance (Volkoff et al., 2005). Several important roles of Orexin are known as appetite enhancers in mammals and other vertebrates, but the role in various fish species, specifically in tilapia is not yet known.

According to Matsuda et al (2012), Orexin as an orexigenic factor, is believed to influence the reproduction processes in teleost fish. Tilapia, a notable teleost species, is thought to exhibit unique mechanisms for regulating feed intake and reproduction, which are closely interconnected. In the context of increasing appetite, Orexin is posited to have a significant correlation with reproductive factors in fish Sjahdan et al. (2014). One of the reproductive factors involved with Orexin as feeding condition in fish is the Kisspeptin gene (Simanjuntak, 2017). Therefore, this study aimed to investigate the biology of Orexin in Tilapia, suggesting its role in regulating reproductive factors through the Hypothalamic Pituitary Gonad (HPG) axis (Silveyra et al., 2010).

A fundamental investigation of appetite control mediated by Orexin of tilapia is important. This study will be the baseline to improve tilapia growth performance and its correlation to reproductive factors. To date, there have been no studies addressing the biological aspects of appetite regulation by Orexin and its relationship with reproductive factors in Tilapia. Therefore, amplifying and sequencing Orexin is critical for basic biological study to understand its physiological functions in this species, particularly concerning weight gain and physiological processes. Detection of Orexin and examining its correlation with reproductive factors are expected to provide new valuable understanding in the field of fisheries biology in the process of increasing tilapia body weight in accordance with market prices. The role of Orexin amplification and sequencing in this study aims to confirm the coding gene in tilapia and provide updated knowledge about its profile as a precursor for controlling feed intake and energy allocation of fish groups and its relation to reproductive factors. Understanding the correlation between appetite and reproductive factors is needed to improve optimal tilapia growth out and how they play a role in fish physiology.

MATERIALS AND METHODS

The Tilapia used were male with matured gonads (± 4 months after hatching) and an initial body weight of 30 ± 0.2 g. The fish were obtained from the Ciparay Freshwater Fish Seed Institute (BBI), Bandung Regency. The samples used were first acclimatized for two weeks, then maintained in a closed recirculation system, and fed twice daily. Abiotic parameters such as temperature, pH, and dissolved oxygen were controlled during maintenance.

Tilapia Brain Isolation

Tilapia will be anesthetized using the cold shock method. Isolation of the brain was carried out by dissecting the inner side of the chepal using a dissecting set. The isolated tilapia brain was then soaked in PBS (Phosphate Buffer Saline) solution for 1 minute before being stored in liquid nitrogen until the brain samples were ready for RNA isolation. (Simanjuntak, 2017)

Primer Designing

Primer design will use primer3 plus (https://www.primer3plus.com/index.html). Where the basic material in the primer design process is Orexin CDS sequence (template) will be the target of primer attachment during the PCR process. Then, primer3plus will show the primer results (Table 1)

Table 1. Primer design results of OrexinOrexin and β-Actin using Primer3 Plus

Orexin	196 bp	F	5'-AATGCGCCTCTGTCGACATTG-3'
Orexin		R	5'-ACTGTCCACATCCTGTGGTACCG-3'
β-actin	197 bp	F	5'-AGCATCCCGTCCTGCTCACA-3'
β-actin		R	5'-AGCACAGCCTGGATGGCAAC-3'

Total RNA Isolation

Isolation of total RNA from the tilapia brain was carried out on Simanjuntak (2017). Pure Link RNA Mini Kit (Ambion) and continued with the measurement of quantity and quality of total Ribose Nucleic Acid (RNA). The total RNA quantity was measured by measuring the absorbance of RNA at wavelengths of 260 nm and 280 nm using spectroscopy (Ultraspec 2000, Pharmacia, Biotech). RNA quality was tested by 1.5% agarose gel electrophoresis containing Gel Red in TAE electrophoresis buffer solution. Electrophoresis results showed two gene arrays, including 18s and 28s RNA total contamination-free, undegraded, and ready for cDNA synthesis (Reverse Transcriptase).

cDNA Cloning - Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Total RNA isolated and has a known concentration is then synthesized into cDNA (complementary DNA) through the Reverse Transcription (RT) PCR process using the RevertAidtm First Strand cDNA Synthesis Kit (Thermo Scientific). Based on kit protocols, mix 11 µl RNA template, 1µl Oligo dT primer, reaction buffer 4 µl, riboloc RNAse Inhibitor µl, dNTP Mix, 2 µl and1µl Reverse Transcriptase enzyme will be spindown for ten second. The Sample, after spindown was incubated in the PCR cycle at 42°C for 60 minutes. The reaction was terminated by heating the PCR cycle at 70°C for 5 minutes. The result of cDNA synthesis is ready to be used as a template for PCR amplification.

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PCR Amplification

A total of 1µl cDNA template will be amplified using Gotaq Green Master Mix Kit (Promega) 5 µl, forward and reverse primer 0.5 µl, template 1 µl, nuclease-free water 3 µl with Thermalcycler T100 (Bio-Rad). Orexin target gene is picked using specific primers designed. The PCR process involves several stages, (1) pre-denaturation of template DNA; (2) denaturation of template DNA 95°C during 30 seconds; (3) primer attachment to the template (based optimization of annealing temperature, 57°C, 59°C, 61°C); (4) primer elongation 72°C(extension) and (5) stabilization (post- extension).

Amplicon Sequencing

DNA amplification results are then stored in a -20°C freezer before being sent for the sequencing process. Only one annealing temperature with a clear band will be sequenced. The sequencing process from start to finish requires 14 days of work. The PCR amplification results were subsequently sequenced to determine the nucleotide base sequence of the target gene using specific primers. The sequencing process was conducted by Macrogen (South Korea). The sequencing results were then analyzed using BLAST to compare the sequence of nucleotide sequences of the samples with database nucleotide sequences. BLAST analysis was conducted using BLASTN (https://blast.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

In general, the profile of Orexin target gene amplicons that amplified in annealing temperatures isolated from tilapia gonads are shown in Figure 1. The amplification results, observed qualitatively through agarose gel electrophoresis, showed successful amplification of the amplicons presumed to be strong Orexin genes at each tested temperature. The successfully amplified Orexin and had 196 bp and 197 bp sizes. The size orexin and β-Actin base pairs known at the time primer compiling and Coding Sequence (CDS) of orexin and β-Actin from gene bank. β-Actin is one of the housekeeping gene. The use of housekeeping gene in the amplification process is important. this is because the gene will continue to be expressed in all animal cells and is conserve. Housekeeping gene is a marker that the amplification process is going well. Some examples of housekeeping gen other than β-Actin are GADPH, 18s RNA, β-tubulin.

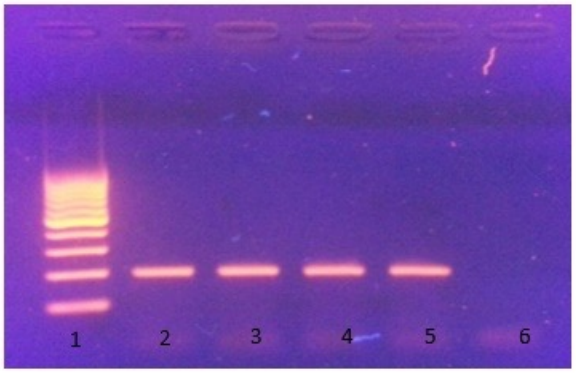

Figure 1. (1) 100 bp ladder; (2) Orexin 57°C; (3) Orexin 59°C; (4) Orexin 61°C; (5) β-Actin; (6) NTC (Negative Control)

The amplification of Orexin and β-Actin target genes was then analyzed for nucleotide sequences using the sequencing method. Subsequently, sequencing is performed to determine the sequence or nucleotide base of the amplification results of specific target genes and confirm the expression gene. The sequence of nucleotide sequence results can be seen in Figure 2. The nucleotide sequence results of Orexin and β-Actin target genes were then BLASTed. Subsequently, BLAST was conducted to

confirm the homology/similarity of the sample sequences with the available database (Gene Bank) sequences (Simanjuntak, 2017). The BLAST analysis results of the suspected target genes can be seen in Figure 3, showing that the Identified Orexin gene shares 97% homology/similarity with the Tilapia Orexin gene derived from the database (gene bank).

Orexin expression is found in all development stages of living things, and its expression will increase if the presence of several energy reserves in adipose tissue, fat reserves, and glucose in the blood begins to deplete or in fasting conditions (Volkoff et al., 2005; Spinazzi et al., 2006). Orexin is a neuropeptide that regulates feed intake (Zohar et al., 2005; Matsuda. 2009; Matsuda et al., 2012). It is a prepro-orexin precursor secreted in the hypothalamus (Volkoff et al., 2005; Spinazzi et al., 2006). Based on its evolution, Orexin has two peptide forms, namely, Orexin A (OXA) and Orexin B (OXB), which are encoded by the same precursor gene and conserved at the same locus (Sakurai et al., 1998). OXA will bind to OX1R (Orexin 1 Receptor), while OXB will bind to OX2R (Orexin 2 Receptor). Both types of receptors are part of the GPCR (Wong et al., 2011). The binding of Orexin with its cognate receptor (OXR) will activate the ERK/CREB (cAMP Response Elements Binding) signaling pathway, whose downstream pathway will activate peptides in stimulating appetite.

>150616-35_A01_orexin_orexin_F.ab1 520
CGACGGGGTGAGTCTCACGGATGATGCGGCCGCTGGGATCCTCACTCTGG
GCAAACGAAAAGAGGACGAGTATCGCTTTCAGAGCCGACTCCAACAGCTC
CTACAGGCTCCAGGAACCAGCAGCAGGGATTCTGACAATGGGGAAGAGAA
CCAGAGAGAGAGCAAAATTAGGAGGTCTGGTTCCTTTATCATTTTTTTTC
TTCTTTTCTCTCCCTCTTTTTCTCCTGTTCTTTTCTTTCTCCTTCTTTTT
TTCTTTTCTCTTTCCCTTCCTCTTTTTTCTTCGCTTTTTTCTTTGTCCCT
TCTTTCTTTTTGTCTTGTTTGTTTTTTTTTTTTTCTTTTTTCTTTTTCTT
CTTTCTTCTTTTTTTTTTTTTTTCCCTTCTCTCTTTTTTTTCTTTTCCTT
CTTTTTTTTTTTCTTTTCTTTTTTTTTATTTTTTTTTTTTTTTTTTTTTT
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Figure 2. Orexin gene sequencing analysis results

Orexin significantly regulates orexigenic factors or stimulates appetite and feed intake with NPY (Sakurai et al., 1998; Matsuda et al., 2012) and other orexigenic peptides, and its expression can be affected by feeding and fasting conditions. This regulation can affect Orexin synthesis in the hypothalamus in several fish species (Matsuda et al., 2012). One of the influences on increasing Orexin expression is mediated by Leptin in the peripheral nervous system.

Besides being the primary regulator in increasing feed intake and feeding appetite, Orexin retrieved also regulates energy homeostasis, energy balance, and reproduction. Control of metabolism and appetite will affect reproductive factors, and vice versa. In the case of mammals that have been castrated, Orexin affects increasing body weight. The increase is influenced by energy metabolism.

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According to Sjahdan et al. (2014), orexigenic regulation can stimulate the reproductive axis. This stimulation is related to inhibition of the reproductive pathway, where increased expression of Orexin can inhibit spawning and sex steroids through GnRH in fish. In contrast, increased expression of GnRH can reduce Orexin expression (Hoskins et al., 2008). This process suggests the involvement of Orexin in regulating reproductive factors through the HPG axis (Silveyra et al., 2010).

Several studies have shown a close relationship between orexigenic and reproductive factors mediated by orexigenic peptides in vertebrates. The role of neuropeptides in the central nervous system and metabolic signals in the peripheral system in regulating the reproductive system shows a correlation between energy balance and reproduction, as observed in some fish species. Stimulation in the form of inhibition of sex steroid formation in the reproductive pathway shows that energy flow is compensated for growth (growth out).

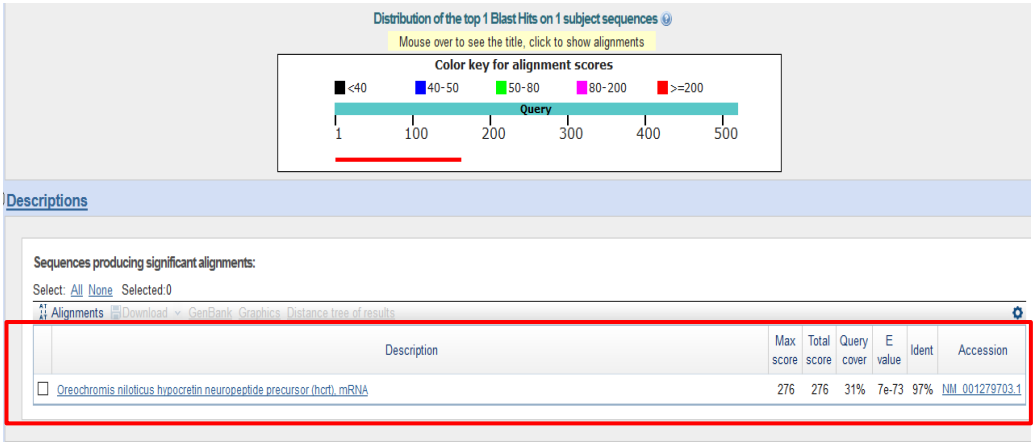

Figure 3. Blast Result

CONCLUSION

In conclusion, the amplification results showed DNA bands for both target genes, with Orexin showing a nucleotide length of 196 bp with 97% similarity/homology. Orexin played a significant role regulating feeding appetite and highly correlated with reproduction factors in tilapia.

AUTHOR CONTRIBUTION

A.B; **I.W** designed and supervised all the studies, **R.F.S.** collected samples from wet and dry laboratories and drafted the hypothesis

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CONFLICT OF INTEREST

We do not have any conflict of interest. By that, we as an author have disclosed those interests fully to the journal, and I have in place an approved plan for managing any potential conflicts arising from (that involvement).

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