

## Research Article

# Changes in the Neuropeptide Y mRNA Expression in *Oncorhynchus mykiss* at Different Feeding Frequencies

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Received 22 June 2022; Revised 29 July 2022; Accepted 11 August 2022; Published 24 August 2022

Academic Editor: Xiangjun Leng

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In this study, the relationship between feeding time preference and appetite in *Oncorhynchus mykiss* with scheduled feeding frequencies was investigated using neuropeptide Y (NPY). Samples ( $n = 288$ , initial weight:  $38.72 \pm 2.99$  g) were stocked in three treatments of different feeding frequencies with six replications. Treatments included one feeding time at 09:00 (T1), three feeding times at 09:00, 13:00, and 17:00 (T2), and five feeding times at 09:00, 11:00, 13:00, 15:00, and 17:00 (T3) every day based on 5% of body weight for 30 days. On day 31, each experimental treatment was divided into fed and unfed groups with triplicates. To measure NPY expression, fish were sampled from both groups 15 minutes earlier than feeding hours. In a comparison of the NPY expression in T1 of the fed group, the highest level of NPY occurred at 08:45. In T2, the NPY was maximized at 08:45, 12:45, and 16:45. In T3, the NPY increased significantly at 08:45 compared to the other sampling hours. In T1 and T2 of the unfed group, the highest NPY was recorded at 10:45 and 14:45, respectively. In T3, upward and downward changes in the NPY were observed from 08:45 to 12:45 and from 12:45 to 16:45, respectively. Concerning changes in the NPY levels, the fed groups in the three treatments were significantly different at all hours, except for 08:45. In the unfed groups, significant differences were recorded between the treatments at 10:45 and 14:45. According to the fish appetite, it can be concluded that feeding *O. mykiss* three times a day at about 4 h intervals may be appropriate.

## 1. Introduction

As with any other behavior, feeding behavior includes a series of biochemical and physiological reactions. Feed intake is regulated by feeding centers in the brain that receive information from endocrine signals and the surrounding environment [1–5]. Environmental factors (temperature and light), different internal factors (digestive hormones, ghrelin), and metabolic factors (e.g., glucose) transmit feeding condition information to the fish brain either directly or indirectly. The brain, particularly the hypothalamus, plays an important role in receiving and processing appetite-related signals administered by two groups of neurons, one of which stimulates feed intake through the

regulation of neuropeptide Y (NPY) and agouti-related peptide gene expression levels [6]. Feeding regulation is affected by several neuronal pathways, and NPY plays a pivotal role in feed intake control and feed efficiency, affecting the final body weight. This hormone influences the pituitary-hypothalamus axis, metabolism, and appetite. So NPY increases during starvation and decreases after feeding [7]. Several studies have reported feed intake stimulation in fish influenced by NPY expression [8–12].

Studies indicate that the improved understanding of physiological rhythms and neurohormonal mechanisms regulating feeding in farmed fish can modify feeding time and frequency, improve feeding and production efficiency, reduce losses and feces, and minimize the environmental

impacts of aquaculture [13]. Feeding time preference (feeding rhythm) is a topic that deserves investigation concerning fish feeding behavior. Feeding rhythms are observed in some fish species that feed at specific times within 24 h. The variation in the ecology and physiology of fish nutrition indicates the diverse endocrine control of feeding in fish that involves species-specific mechanisms. Thus, the species-specific mechanism of internal timing causes separate and different appropriate feeding times for one species to another [14].

Scheduled feeding can adjust the internal biological clock, which in turn may change the behavioral feeding pattern of fish. Feeding memory may cause the fish to respond to feeding time, known as feeding anticipatory activity, which has been reported in a variety of animals [13, 15, 16]. Therefore, it can have the best feeding time and the most adaptation to peak times of appetite hormonal signals, such as NPY. Further knowledge on feeding rhythms can help improve feeding programs to be more adapted to the most appropriate feeding time for fish [14]. The ideal time for adaptation to environmental conditions and feeding regimens for appetite hormonal signals is of paramount importance, which can reflect the real feeding anticipation in fish [17]. In other words, it is possible to anticipate an appropriate feeding time based on feeding memory. The accordance of feeding times with real appetite in fish can affect the desired feeding performance, improvements in digestion and absorption, and growth indices. Therefore, NPY can be used as an appetite biomarker to detect and predict appropriate feeding times and thereby improve feeding times and frequency in fish.

The role of NPY in feeding regulation and stimulation has been reported in limited studies on *Danio rerio* [11], *Gadus morhua* [18], *Carassius auratus* [19], *Paralichthys olivaceus* [20], *Oreochromis niloticus* [21], *Siniperca chuatsi* [22], *Seriola quinqueradiata* [3], and *Ictalurus punctatus* [23, 24]. Aldegunde and Mancebo [25] reported the stimulatory role of NPY in the feed intake of rainbow trout. Yuan et al. [12] injected NPY into the brain of a Siberian sturgeon to measure the NPY gene expression levels at satiation and starvation times. They acknowledged the stimulatory role of NPY in the feed intake and appetite of fish. Therefore, it is assumed that if the trout get used to different feeding frequencies, they will adapt to feeding times after a while. This adaptation is based on the NPY expression as an appetite stimulant and the possibility of having feeding memory in this species. While the expression process of the NPY does not follow the feeding frequencies after adaptation, feeding memory is not established in trout. As a result of mismatching feeding frequencies with appetite, nutrient absorption will be decreased, and growth indices will decline. In this study, the relationship between feeding time preference in rainbow trout was investigated with scheduled feeding frequencies using the NPY, with a proven appetite stimulatory role in this species. A likely relationship between the NPY and appetite time, which can be used to predict an appropriate feeding time, can help improve feeding time and feed efficiency in this commercially valuable fish species.

## 2. Materials and Methods

**2.1. Experimental Design.** Rainbow trout samples (initial weight:  $38.72 \pm 2.99$  g) were adapted to the culture conditions for 2 weeks, during which fish were fed a commercial diet once a day (09:00) based on 5% of body weight. After the adaptation period, 288 fish were randomly stocked in three treatments with six replications in 18 polyethylene tanks (300 l, 16 fish/tank). An aeration system was designed in each tank and consisted of a central aeration pump and aeration stones. Water quality in the tanks was measured twice in the morning and afternoon. During the culture period, average water temperature ( $13.7 \pm 1.3^\circ\text{C}$ ), pH ( $7.5 \pm 0.2$ ), and dissolved oxygen ( $5.9 \pm 0.1$  ppm) were measured, and the photoperiod was set to 12 h light/12 h dark. The daily feeding rate of fish was calculated based on 5% of body weight. The calculated feed was divided into three treatments with different feeding frequencies, including one feeding time at 09:00 (T1), three feeding times at 09:00, 13:00, and 17:00 (T2), and five feeding times at 09:00, 11:00, 13:00, 15:00, and 17:00 (T3) every day. During the experimental period, fish were fed an extruded commercial diet (55% protein, 16% lipid, 7% ash, and 8.5% moisture) with a diameter of 2 mm. After 15 minutes of each feeding time, the remaining diet was collected by siphoning to correct feed intake [17]. The experimental fish were fed for 30 days.

**2.2. Experimental Design and Sampling on Day 31.** On day 31 of the experiment, each experimental treatment with six replications was divided into two groups. Group I with three replications from each treatment was fed based on the feeding times and frequencies designed for that treatment (fed group). Group II, with the other three replications from that treatment, was kept fasting (unfed group) and was not fed on day 31 (Table 1). In other words, each experimental treatment was divided into two groups, each with three replications for fed and unfed group. To measure the NPY mRNA expression levels, fish specimens in the treatments were sampled in both fed and unfed groups of all experimental treatments 15 min earlier than the scheduled feeding hours at 08:45, 10:45, 12:45, 14:45, and 16:45. In these hours, two fish were randomly sampled and sacrificed by overdosed methanesulfonate tricaine (MS-222, [23]). The sampled fish underwent biometry, including body length (0.01 cm accuracy) and wet weight (0.01 g accuracy). After that, brain and brainstem samples were isolated and transferred to microtubes, which were kept at  $-80^\circ\text{C}$  until the gene expression tests. The visceral index was measured by dissecting the ventral part and weighing the viscera.

**2.3. RNA Isolation, Cloning, cDNA Synthesis, and Real-Time PCR.** Total RNA from the brain tissue was extracted using a total RNA extraction kit (Cinnagen Co., Iran). Before the washing stage, potential genomic DNA contamination was removed using DNaseI based on the kit protocol. To ensure DNA removal, DNaseI-treated RNA samples were used as the template together with the target gene in the PCR reaction. To quantify the extracted RNA, a 1.20 dilution was

TABLE 1: Experimental design for the changes in the neuropeptide Y mRNA expression in *Oncorhynchus mykiss* at different feeding frequencies.

Treatments	Replicates	Scheduled feeding duration (30 days)					Groups	Replicates	Sampling on day 31				
		Feeding frequencies/feeding rate							Sampling times				
		9:00	11:00	13:00	15:00	17:00		8:45	10:45	12:45	14:45	16:45	
T1	6	5.0 %BW	×	×	×	×	T1 fed (feed based on previous days)	3	✓	✓	✓	✓	✓
							T1 unfed (starve)	3	✓	✓	✓	✓	✓
T2	6	1.7 %BW	×	1.7 %BW	×	1.6 %BW	T2 fed (feed based on previous days)	3	✓	✓	✓	✓	✓
							T2 unfed (starve)	3	✓	✓	✓	✓	✓
T3	6	1.0 %BW	1.0 %BW	1.0 %BW	1.0 %BW	1.0 %BW	T3 fed (feed based on previous days)	3	✓	✓	✓	✓	✓
							T3 unfed (starve)	3	✓	✓	✓	✓	✓

TABLE 2: The sequence and characteristics of primer used in real-time PCR.

Gene	Primer name	Primer sequence	Tm (°C)	Reference
NPY	F	CTCGTCTGGACCTTTATATGC	57	NM_001124266
NPY	R	GTTTCATCATATCTGGACTGTG	57	NM_001124266
B-actin	F	GATGGGCCAGAAAGACAGCTA	57	AJ438158
B-actin	R	TCGTCCCAGTTGGTGACGAT	57	AJ438158

prepared from the RNA, and the concentration and the 260/280 ratio were determined with a Bio-Photometer (Eppendorf, Germany). For the quality control, 3  $\mu$ l of the extracted RNA was run on 0.8% agarose gel, and the presence and quality of 18s and 28s rRNA bands were controlled in RNA samples. For electrophoresis on 1% agarose gel, 3  $\mu$ l of the RNA sample mixed with 6x loading dye was loaded into each well of the gel, and the sample was then electrophoresed with 70 V for 40 min [26]. The gel was kept in ethidium bromide for 10 min and then immediately transferred to a vessel containing distilled water for washing in 5 min. Finally, the sample was exposed to ultraviolet radiation by a GelDoc system (Bio-Rad, Iran), and then, photos were taken from the appeared bands. The cDNA was synthesized using 1  $\mu$ g of the purified RNA of the brain tissue as a template by a RevertAid™ First Strand cDNA Synthesis Kit with two oligo dT and random hexamer primers according to the manufacturer's instructions (Fermentas). The reverse-transcriptase enzyme M-MuLV and random hexamer as the primer were used to synthesize cDNA. The primers were designed using those specific to real-time (RT) PCR to evaluate the NPY gene expression. The sequences of the primers are listed in Table 2. After obtaining the optimum concentrations of the primers, the Q-RT-PCR reaction was carried out using Master Mix 2x (Cinnagen, Iran) in 15  $\mu$ l reactions with triple replications. For the reaction, the Thermocycler program consisted of initial denaturation (94°C, 2 min), denaturation (94°C, 15 s), primer annealing (58°C, 30 s), and elongation (72°C, 30 s). This was followed by a final melting stage (50-99°C), and then, the absorbance was measured with each increase of 0.5°C [27]. With the completion of the RT-PCR stages, relative mRNA levels were measured by the  $2^{-\Delta\Delta C_t}$  method [28].

2.4. *Growth and Feeding Performances.* At the end of the experiment, percentage weight gain (PWG), daily weight gain (DWG), specific growth rate (SGR), viscerosomatic index (VSI), feed conversion ratio (FCR), and survival rate (SR) were calculated based on the following standard equations:

$$\begin{aligned}
 \text{PWG} &= \frac{[\text{Final wet weight(g)} - \text{Initial wet weight(g)}] \times 100}{\text{Initial wet weight(g)}}, \\
 \text{DWG} &= \frac{\text{Final wet weight(g)} - \text{Initial wet weight(g)}}{\text{Experiment duration(day)}}, \\
 \text{SGR} &= \frac{[\ln \text{ final weight(g)} - \ln \text{ initial weight(g)}] \times 100}{\text{Experiment duration(day)}}, \\
 \text{VSI} &= \frac{\text{Viscera weight(g)}}{\text{Fish wet weight(g)}} \times 100, \\
 \text{FCR} &= \frac{\text{Feed intake(dry weight, g)}}{\text{Total wet weight gain(g)}}, \\
 \text{SR} &= \frac{\text{Final fish number}}{\text{Initial fish number}} \times 100.
 \end{aligned}
 \tag{1}$$

2.5. *Statistical Analysis.* The findings are presented as mean  $\pm$  standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) to determine the significance levels of experimental treatments at different sampling times at a probability level of 5%. Significant differences in mean values between treatments were compared using Duncan's post hoc test. The fed and unfed groups at each sampling time and each experimental treatment were compared by the *t*-test.

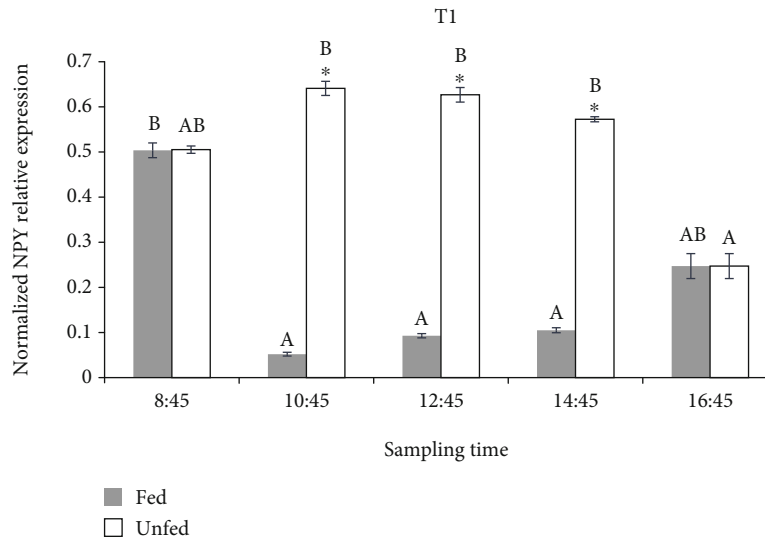


FIGURE 1: NPY mRNA expression levels in the fed and unfed groups in treatment 1 (fed once a day at 09:00; mean  $\pm$  SE values of triplicates ( $n = 3$ )). A different superscript in the same-colored bars denotes a statistically significant difference ( $P < 0.05$ ). Asterisks represent significant differences between the groups at the same time point ( $t$ -test).

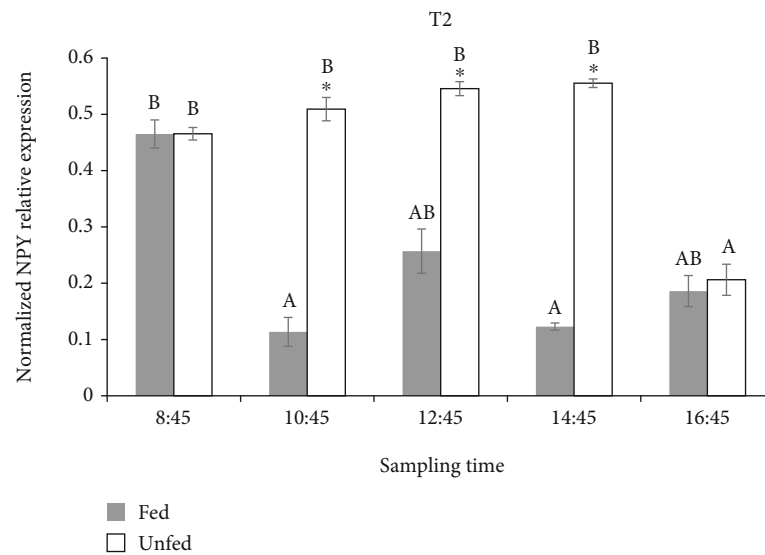


FIGURE 2: NPY mRNA expression levels in the fed and unfed groups in treatment 2 (fed thrice a day at 09:00, 13:00, and 17:00; mean  $\pm$  SE values of triplicates ( $n = 3$ )). A different superscript in the same-colored bars denotes a statistically significant difference ( $P < 0.05$ ). Asterisks represent significant differences between the groups at the same time point ( $t$ -test).

### 3. Results

**3.1. The NPY mRNA Expression Levels.** Figures 1–5 compare the results of changes in the NPY mRNA expression levels within and between experimental treatments. When compared to the other sampling times, the NPY mRNA expression level was highest in T1 (fed once a day) of the fed group at 08:45 hrs (Figure 1). In other words, the NPY was expressed downwardly after feeding from 09:00 up to 16:45, and its expression increased again at 16:45. Thus, the NPY mRNA expression was not significantly different between these two hours ( $P \geq 0.05$ ), but it was significantly

different from the other times ( $P < 0.05$ ). In T1 of the unfed group, the NPY mRNA expression level at 10:45 was not significantly different with 08:45, 12:45, and 14:45 ( $P \geq 0.05$ ). A comparison of NPY data between fed and unfed groups by the  $t$ -test revealed that the NPY mRNA expression level was significantly higher in unfed groups at all times except at 08:45 and 16:45 ( $P < 0.05$ ).

In T2 of the fed group (fed thrice a day), maximum levels of the NPY mRNA expression were observed at 08:45, 12:45, and 16:45 (Figure 2). In other words, the NPY mRNA expression decreased after feeding (09:00 and 13:00), with the lowest expression levels at 10:45 and

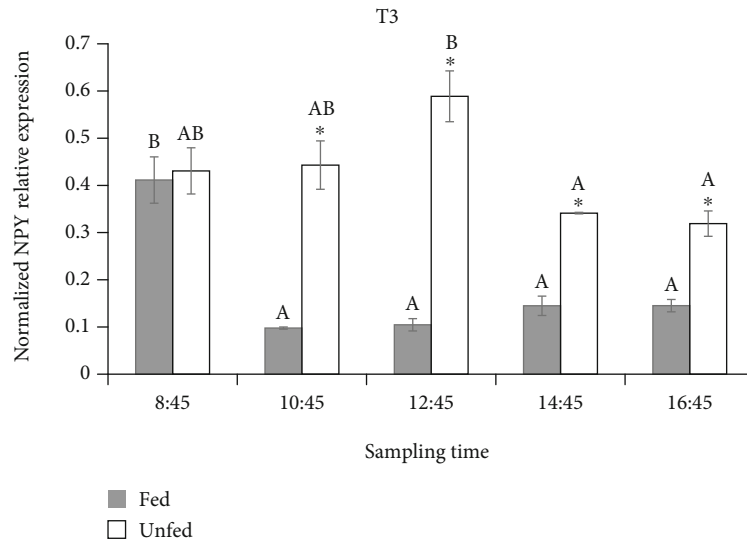


FIGURE 3: NPY mRNA expression levels in the fed and unfed groups in treatment 3 (fed five times a day at 09:00, 11:00, 13:00, 15:00, and 17:00; mean ± SE values of triplicates ( $n = 3$ )). A different superscript in the same-colored bars denotes a statistically significant difference ( $P < 0.05$ ). Asterisks represent significant differences between the groups at the same time point ( $t$ -test).

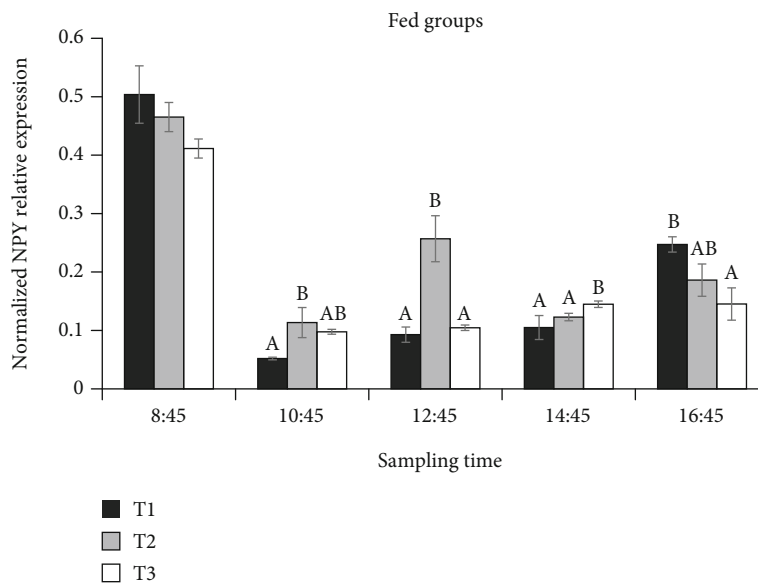


FIGURE 4: Changes in the NPY mRNA expression levels between the three experimental treatments in fed groups at different sampling times (mean ± SE values of triplicates ( $n = 3$ )). T1: fed once a day at 09:00; T2: fed thrice a day at 09:00, 13:00, and 17:00; T3: fed five times a day at 09:00, 11:00, 13:00, 15:00, and 17:00. A different superscript denotes a statistically significant difference between the experimental treatments at each sampling time ( $P < 0.05$ ).

14:45. No significant differences were found between 08:45, 12:45, and 16:45 ( $P > 0.05$ ). In T2 of the unfed group, the NPY mRNA expression was maximized at 14:45 without significant differences between 10:45 and 12:45. Similar to the trend of T1, a comparison of NPY data between fed and unfed groups by the  $t$ -test indicated that the two groups were significantly different in the NPY mRNA expression levels at all hours, except at 08:45 and 16:45 ( $P < 0.05$ ).

In T3 (fed 5 times a day) of the fed group, the NPY mRNA expression level rose significantly at 08:45 compared

to the other sampling times ( $P < 0.05$ ), in which the NPY mRNA was expressed with no significant differences. In T3 of the unfed group, the NPY mRNA expression level peaked at 12:45. In other words, upward and downward changes in NPY were observed from 08:45 to 12:45 and from 12:45 to 16:45, respectively. A comparison of NPY data between fed and unfed groups in T3 by the  $t$ -test showed that the NPY mRNA expression level was significantly higher in unfed groups at all hours ( $P < 0.05$ ), except for a nonsignificant difference between the two groups at 08:45 ( $P > 0.05$ , Figure 3).



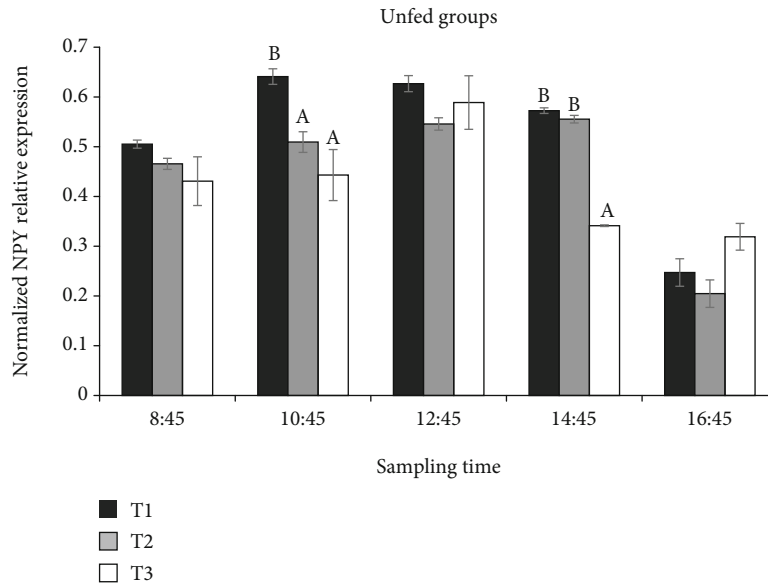


FIGURE 5: Changes in the NPY mRNA expression levels between the three experimental treatments in unfed groups at different sampling times (mean  $\pm$  SE values of triplicates ( $n = 3$ )). T1: fed once a day at 09:00; T2: fed thrice a day at 09:00, 13:00, and 17:00; T3: fed five times a day at 09:00, 11:00, 13:00, 15:00, and 17:00. A different superscript denotes a statistically significant difference between the experimental treatments at each sampling time ( $P < 0.05$ ).

Figures 4 and 5 illustrate the results of changes in the NPY mRNA expression levels between the three experimental treatments in fed and unfed groups at different sampling times. Significant differences in the NPY mRNA expression levels were found in the fed groups of the three experimental treatments at all hours ( $P < 0.05$ ), except for a nonsignificant difference between the treatments at 08:45. In experimental treatments, the peak of the NPY mRNA expression occurred at 08:45. The comparison of the NPY mRNA expression indicated this level was also higher after 08:45 (i.e., at 16:45) than those measured at the other sampling times. The unfed groups of the three treatments were not significantly different in the NPY mRNA expression levels at 08:45, 12:45, and 16:45 ( $P > 0.05$ ). However, significant differences were recorded between the treatments at 10:45 and 14:45 ( $P < 0.05$ ).

**3.2. Growth Performances and Survival Rates.** Table 3 represents the results of growth performance, FCR, and survival rates measured in fish fed with different feeding frequencies after 31 days. The highest and the lowest body weight gain (BWG) and specific growth rate (SGR) belonged to T2 and T1, respectively ( $P < 0.05$ ), but no significant differences were observed between T2 and T3 ( $P > 0.05$ ). In the three treatments, the uppermost and lowermost measurements of visceral indices were obtained for T2 and T1, respectively. No significant difference was observed between the treatments in the feed intake ( $P > 0.05$ ). Maximum and minimum FCR values were calculated for T1 ( $1.91 \pm 0.30$ ) and T2 ( $1.02 \pm 0.09$ ), respectively, but there were no significant differences between T2 and T3 ( $P > 0.05$ ). T2 and T1 showed the highest ( $100\% \pm 0.00$ ) and the lowest ( $92.40 \pm 5.80$ ) survival rates ( $P < 0.05$ ), respectively, but T2 and T3 were not significantly different in this parameter.

TABLE 3: Growth performance, FCR, and survival rate of *Oncorhynchus mykiss* fed with the different feed frequencies after 31 days (mean  $\pm$  SE values of six replicates ( $n = 6$ )).

	Feed frequency		
	T1	T2	T3
IW (g)	$38.34 \pm 2.42$	$38.47 \pm 2.78$	$39.37 \pm 3.01$
FW (g)	$46.61 \pm 2.98^a$	$54.08 \pm 3.21^b$	$53.30 \pm 3.52^b$
PWG (%)	$21.57 \pm 3.48^a$	$40.57 \pm 4.32^b$	$35.38 \pm 3.70^{ab}$
DWG (g/fish)	$0.27 \pm 0.06^a$	$0.52 \pm 0.07^b$	$0.46 \pm 0.07^b$
SGR (%)	$0.65 \pm 0.13^a$	$1.13 \pm 0.15^b$	$1.01 \pm 0.12^b$
VSI (%)	$2.71 \pm 0.31^a$	$3.62 \pm 0.31^b$	$3.25 \pm 0.31^{ab}$
FI (g)	$15.79 \pm 0.98$	$15.92 \pm 1.01$	$14.34 \pm 1.00$
FCR	$1.91 \pm 0.30^b$	$1.02 \pm 0.09^a$	$1.03 \pm 0.07^a$
Survival rate (%)	$92.4 \pm 5.89^b$	$100 \pm 0.00^a$	$94.4 \pm 4.60^{ab}$

IW: initial weight; FW: final weight; PWG: percentage weight gain; DWG: daily weight gain; SGR: specific growth rate; VSI: viscerosomatic index; FI: feed intake; FCR: feed conversion ratio; T1: fed once a day at 09:00; T2: fed thrice a day at 09:00, 13:00, and 17:00; T3: fed five times a day at 09:00, 11:00, 13:00, 15:00, and 17:00. A different superscript in the same row denotes statistically significant differences ( $P < 0.05$ ).

## 4. Discussion

It is necessary to find appropriate feeding times and frequencies to improve feed efficiency, reduce mortality, and decrease faeces to maximize growth and minimize the environmental consequences of aquaculture [13]. A series of neural networks and neurotransmitters are involved in the regulation of feed intake. The NPY hormone plays an appetite regulatory role in vertebrates and stimulates feed intake in fish when injected at specific doses [7]. In rainbow trout, the NPY level is higher at starvation than at satiation [24].

Moreover, the NPY mRNA expression levels increased in the brain and intestine of *Megalobrama amblycephala* after starvation, and secondary feeding could return the gene expression level to the control level [29]. The present results indicated that the NPY mRNA expression levels were maximized before the first-time feeding at 08:45 in all three treatments, which decreased significantly after feeding at 10:45. At the other feeding times, the NPY mRNA expression levels declined after each feeding time. In T1 and T2 of this study, changes in the NPY mRNA expression shifted from a downward trend at 10:45 to an upward trend at 12:45 after first-time feeding, possibly resulting from restarvation that suggests the need for refeeding in the midday. In a study on *Ictalurus punctatus*, a reduction occurred in the NPY gene expression in the hypothalamus 2 h after feeding and then increased after about 4 h [23].

Feeding rhythms are observed in some fish species that feed based on an internal timing mechanism at specific times during the day. As a result, there are species-specific appropriate feeding times or, in other words, peak appetite times or real appetite. On the other hand, when food is presented on a regular and predictable basis, the behavioural and swimming patterns of fish may be altered, leading to feeding time responses. Accordingly, fish may change their feeding times, known as feeding anticipatory activity (feeding memory), which has been reported in a variety of animals [13, 15]. In addition to movement/swimming activity, physiological variables can also predict the feeding time in fish. In mammals, increases in corticosterone secretion, body temperature, gut movement, hormones involved in appetite regulation (e.g., ghrelin), and some hormones involved in the digestion and metabolism process have been reported as physiological indicators of appetite [30]. Therefore, the ideal conformation of time with environmental conditions and feeding regimens is of paramount importance for hormonal signals of appetite, which can reflect the real feeding prediction in fish.

The results of the present study show that the effect of feeding memory (scheduled feeding times) cannot be reliable on the NPY expression in rainbow trout because the T1 of the fed group represents two peaks of the mRNA expression at 08:45 and 16:45, which are significantly different from the other times. Therefore, it can be argued that, despite feeding at 09:00 for 30 days, the increased NPY expression at around sunset can indicate no effect of feed memory on the NPY mRNA expression. Based on the results of T1 and T2 in the fed groups, the NPY levels shift from the downward trend resulting from feeding and the signal of satiation sent to the brain to an upward trend about 4 h after feeding each time. These changes can suggest a restarvation message and the ineffectiveness of the feeding memory. Thus, the consistency between feeding memory and real appetite (times of rising NPY expression and feeding time preference) is an influential factor that increases feeding efficiency. As a result, it improves food digestion and absorption rates, ultimately improving fish growth. In the experimental species, this conformation between feeding time and times of rising NPY mRNA expression was observed in the morning, noontime, and evening, leading to the improvements in the results of

feeding and fish growth (Table 3). In this study, therefore, the best feeding time of rainbow trout at three feeding rounds seems to correspond with the times of rising NPY mRNA expression (appetite) in the morning, noontime, and before sunset.

The examination of results obtained from the NPY mRNA expression in the three experimental treatments with different feeding frequencies in fed and unfed groups resulted in some observations on the feeding time preference in rainbow trout. In T1 of the fed groups, the NPY mRNA expression rose before feeding (at 08:45), declined after feeding at 09:00, and then increased again at 16:45. The increased NPY expression at the hours close to sunset can witness the feeding time preference and real need of fish for a further feeding time besides the morning feeding, which indicates fish appetite in the morning and afternoon. Given the NPY mRNA expression levels at these hours (08:45 and 16:45), the increased NPY expression level at 16:45 is lower than that occurred in the early morning, suggesting that the cultured fish were fed at lower levels in the afternoon than in the morning. In other words, although fish require feeding in the afternoon and before sunset, the required amount of diet is lower at these times than in the morning. In T1 and T2, the NPY level at 16:45 was not significantly different between fed and unfed groups. In other words, the NPY expression level was almost constant whether the fish was fed or not fed on that day. Moreover, the three treatments were not significantly different in terms of the mRNA expression levels at 16:45 in unfed groups. Regardless of fish feeding frequencies on that day, it can be speculated that the NPY expression level is an almost constant value, suggesting that the fish probably needs to receive a fixed amount of diet at any condition before sunset. The feeding memory in trout is weak, and NPY expression is regulated by the biological clock or other factors, as seen by the increase in NPY expression in T2 of the fed group compared to T1 and T3 at 12:45. When compared to 10:45 in T1, the rise in NPY expression at 12:45 is modest. This outcome can demonstrate the feeding schedule to some extent. While in the T2, these changes are more noticeable. In other words, in this treatment, feeding memory is seen more clearly, whereas during the T3, the process of alterations is identical to that of the T1, with the exception that during this treatment, feeding took place at this time (13:00). As a result, this therapy has not been found to feed memory. Due to the absence of a clear trend in NPY expression in trout (particularly between treatments 2 and 3), we can doubt the existence of feeding memory in this species.

In T2 and T3 of the fed groups, the mRNA expression level decreased after each feeding time. However, the results of mRNA expression in T2 fed thrice a day revealed a decrease in the NPY mRNA expression after the first-time feeding at 10:45, after which it increased again around 4 h after feeding at 12:45, i.e., 15 min before feeding again. After feeding at 13:00, the NPY level declined again at 14:45, but it rose anew at 16:45. Accordingly, it seems that it is not appropriate to refeed the T3 (with five times of feeding a day) at 10:45 and 14:45 due to the reduced hormone level after each feeding time at these hours and then its elevation again. In

contrast, feeding three times a day is desirable as the NPY mRNA expression level peaks before feeding hours (08:45, 12:45, and 16:45). Chen and Purser [31] studied the motion and desire of fish for food acceptance and reported a reduction in fish desire to feed with an increase in feeding frequency. This was also seen in the FCR of the experimental treatments, with a higher value in T3 than in T2. Although these two were not significantly different, the other growth performances lead to the deduction that the two treatments may show a significant difference in this index during a more cultural period. In addition, better results of growth performance were obtained in the treatment with three feeding times a day. Although no significant difference was observed between T3 and T2, a comparison of the studied results of growth performances and the NPY expression emphasizes the agreement of feeding time with real appetite in rainbow trout. These results are indicative of the highest feed efficiency at the peak time of the NPY mRNA expression. Moreover, the comparison of changes in the NPY in unfed groups of the three experimental treatments revealed that the NPY expression peaked in all treatments almost at noontime, which may suggest the need for a feeding time between the early and last hours of the day. Additionally, it is also possible that the lack of feed intake in the fish of unfed groups resulted in starvation and thus stimulated the hypothalamic control system, leading to the NPY mRNA expression in later hours of feeding. It appears that either the frequency of feeding or the volume of feed affects the significant negative trend between 12:45 and 14:45 in the NPY expression alterations in the T3 unfed groups as compared to other experimental treatments. In other words, unfed fish stop searching after a while, and as a result, the central nervous system orders a decrease in appetite. In this case, the habit of feeding more frequently can affect the decision-making time of the central control system. However, there is not a solid case to be made in this case. This topic was added to the discussion, based on the comment of the respected reviewer.

According to the results of growth performances, feeding, and measurements of the NPY mRNA expression levels in different treatments and times, it can be concluded that the results of both indexes were somehow in agreement with each other. As such, better results of growth and feeding performances were obtained in T2 with three-time feeding a day than those in T1 and T3. Furthermore, the NPY mRNA expression levels at different times lead to the conclusion that rainbow trout feeding requirements are higher before noon than during the rest of the day. According to the results of the NPY mRNA expression in the fed groups, feeding once a day cannot meet the true feeding requirements of fish based on NPY expression. However, feeding three times a day with an interval of about 4 h can be appropriate for rainbow trout (weighing  $38.72 \pm 2.99$  g on average), which probably conforms to the real appetite of this species. Feeding five times a day will waste the cost and increase the cultural system pollution in the long term. The trout appear to have no feeding memory or are very poor if present. Therefore, the best feeding frequencies/times for this species are when feeding is done at the peak of appetite. It can be stated

that the expression of NPY as the initial biomarker of appetite stimulation in trout is not affected by scheduled feeding frequency. In trout, NPY expression and appetite peak are influenced by other internal or external factors that need more investigation.

### Data Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author on request.

### Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

### Conflicts of Interest

The authors declare that they have no conflict of interest.

### Authors' Contributions

Mozhdeh Nahayat run the experiment and lab work. Mohammad Zakeri supervised the study, designed the experiment, and wrote the paper. Amir Parviz Salati analyzed the data and methodology. Ahmad Qasemi analyzed the data and carried out real-time PCR.

### Acknowledgments

The authors gratefully acknowledge the Khorramshahr University of Marine Science and Technology for their financial support for this research project.

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