Optimization of feeding frequency in *Labeo rohita* under different regimes of temperature at high stocking density

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Abstract
Rohu (*Labeo rohita*) is a freshwater fish extensively cultured in Asia. However, the optimal feeding regime for fish reared at high stocking density in relation to temperature has not yet established. The present study examined the feed intake, growth rate, feed utilization, body composition, activity of digestive enzymes, and levels of growth hormones to determine the optimal feeding frequency in relation to varying temperature. This study was conducted in two trials, trial I (high water temperature; summer) and trial II (low water temperature; winter). Each trial consisted of three groups, with fish stocked at a high density of 4.50 kg/m^3 in each group. The three groups (HF1, HF2, HF3) of trial I were fed according to 2% (two rations/day), 3% (three rations/day), and 4% (four rations/day) of their body weight, respectively. In case of trial II, its three groups (LF1, LF2, LF3) were also given feed according to 2% (two rations/day), 3% (three rations/day), and 4% (four rations/day) of their body weight, respectively. Each group in both trials had three replicates. The trial continued for 120 days, with 60 days for trial I (1st June-31st July) and 60 days for trial II (1st October-30th November). Fish exhibited better growth, higher levels of digestive enzymes activity, and hormones levels at higher temperature. Increase in weight gain was found to be in correlation with feeding frequency (4% > 3% > 2%) both during high and low temperature. The best feed conversion rate (FCR) was observed at a 2% feeding rate. Different temperatures and feeding frequencies did not affect the chemical composition of fish muscles. The levels of triiodothyronine, tetraiodothyronine, and growth hormone were higher at the 4% feeding rate at high temperature than other regimes. In conclusion, higher water temperatures (30.45±0.43°C) were effective in rearing rohu. Considering feeding rate, although a higher feeding rate (4%) resulted in maximum growth, but it reduced feed efficiency, as indicated by a higher FCR. Therefore, this study recommends a 2% feeding rate for rohu, administered twice a day at high stocking density, during both high and low temperature, which is also cost efficient and more sustainable strategy.
Keywords: Rohu; Feeding regime; Feeding ration; Feed conversion rate

1. Introduction

Aquaculture industry has expanded over the last three decades due to increased demand for fish and a decrease in capture fisheries (Board,1999; Brugere and Ridler, 2004). However, there have been several challenges in the advancement of aquaculture including concerns about the sustainability of the environment, profitability, and product quality (Watanabe, 2002). The sustainability of aquaculture practices can be enhanced by utilizing the optimized aquaculture model in terms of stocking density, disease prevention, environment management (water quality and waste output), and feeding (feed formula and feeding regime) (Wang *et al.,* 2004; Wang *et al.,* 2006, 2007; Tang *et al.,* 2015a, 2015b). Fish feed and feeding are essential components of aquaculture as feed makes up a 40-60% of the total cost of production in aquaculture (Ayinla, 2007). One significant factor limiting productivity is feed intake (Sun *et al.,* 2016).

The key factors that determine the profitability of fish farming are productivity, feed cost, fillet quality, and waste outputs, all of which may be strongly impacted by feeding management (Bureau and Hua, 2010). Feeding high quality diets with an optimal feeding regime will maximize growth of fish and

minimize waste production (Wang *et al.,* 2006, 2007). The feeding regime in fish farming is often characterized by the ration level, feeding operation, and feeding frequency, which are all easily quantifiable (Wang *et al.,* 2007). Feeding frequency refers to the number of times per day that fish are fed in the culture system (Riche and Garling, 2003). Feeding frequency can affect the survival rate, growth, feed utilization feed, disease resistance and waste outputs of a fish (Clichenner *et al.,* 2007; Silva *et al.,* 2007; Biswas *et al.,* 2010; Cho *et al.,* 2010).

Feeding rates depend on fish size and water temperature (Riche and Garling, 2003; Craig and Helfrich, 2002). Since fish is a cold-blooded animal, their body temperature, growth rate, food intake, feed conversion efficiency, stomach evacuation rate and other body processes are all influenced by the water temperature (Azevedo *et al.,* 1998; Handeland *et al.,* 2008). The growth and livability of fish is optimum within a defined range of temperature (Gadowaski and Caddell, 1991). The rates of physiological processes and temperature of the water are closely corelated which impacts the intake of food needed to fulfill the demand of chemical energy and substrates required for survival (Weidner *et al.,* 2020). As temperature influences the metabolic rate of fish, it also effects digestion of nutrients, assimilation and the utilization of spare energy into growth and reproduction, and feed intake (Neubauer and Andersen, 2019). When temperature of the surrounding water drops below the optimum level, it reduces feed intake and fish growth (Brett, 1979; Elliott, 1991; Jobling, 1994). During winter months (low temperature), fish can survive for long periods of time without food due to slower metabolic rate (Conde-Sieira and Soengas, 2017).

To perform various activities, fish have an optimum range along with minimum and maximum temperatures (Beschta *et al.,* 1987). The optimum growing temperature for freshwater fish at which they grow quickly is in the range of 25-30^{o}C (Kausar and Sultan, 2006). An important freshwater fish cultured in Asia, mainly in Pakistan and India is rohu (*Labeo rohita*). Low temperature influences biological functions in fish. Therefore, growth rate of rohu and other cultured freshwater carps declines at low water temperature. By taking into account the impact of temperature, particularly low temperatures on fish performance, a farmer can maximize the profit by fully utilizing the production potential of local fish species. When temperature falls outside of the ideal range, the digestive processes and the digestion of nutrients and energy also decreases (Amin *et al.,* 2016). In addition to temperature, fish responds to stress through mechanisms aimed at maintaining physiological function and compensating for the stress (Helfman *et al.,* 2009). Once the stress is alleviated, fish can revert to their previous physiological state (Helfman *et al.,* 2009). Additionally, stress caused by excessive handling, diseases, and social interactions can diminish the appetite of fish, leading to a reduced interest in food (Eriegha and Ekokotu, 2017).

The optimization of feeding regime for the commercially important fish species has been studied intensively, such as Atlantic salmon (*Salmo salar*; Storebakken and Austreng, 1987), tilapia (*Oreochromis niloticus;* Tung and Shiau, 1991; Riche *et al.,* 2004), rainbow trout (*Oncorhynchus mykiss;* Ruohonen *et al.,* 1998; Bureau *et al.,* 2006) and channel catfish (*Ictalurus punctatus;* Peterson and Small, 2006). This study aimed at ascertaining the optimal feeding frequency for the feed rations and its impact on nutritional quality of rohu. It also investigated whether changes in temperature and feed ration affect the growth in this species.

2. Materials and methods

2.1. Experimental design

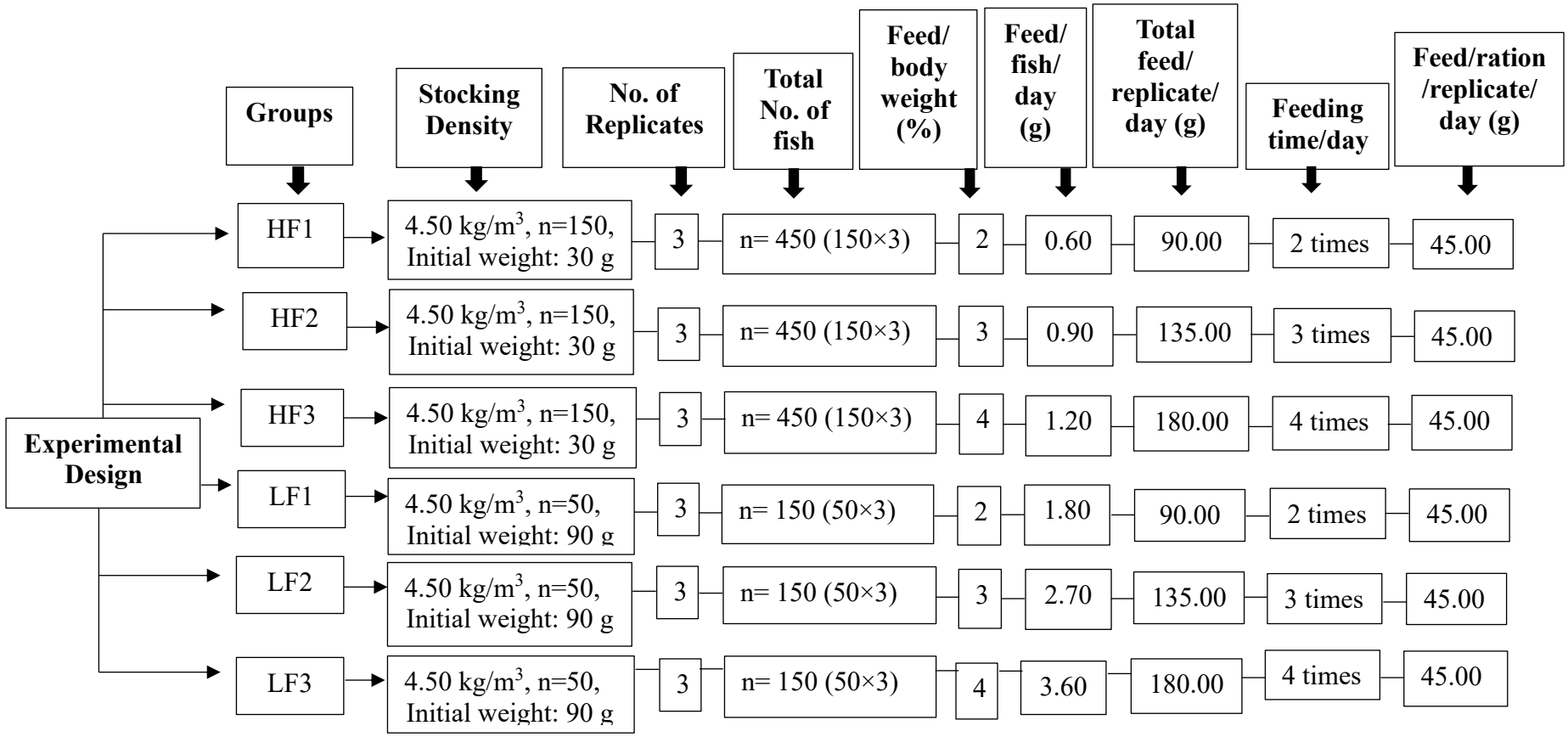
Fingerlings (n=2,700; initial weight= 30.00±0.69g) were procured from a local fish hatchery (Lahore, Pakistan) and transferred to Aquaculture facility, Lahore College for Women University. There was no mortality of fish during the transfer. This study commenced after the approval of Animal Ethics Committee of Department of Zoology, Lahore College for Women University (Approval #: Zoo/LCWU/930). Fish (n=2,700) were randomly distributed in 18 fiber glass tanks (water volume/tank= 1 m^3) as 18 groups. Each tank has water supplied from the same water sump, treated with UV filter and biofilters. All tanks had their own water supply. Fish were acclimatized for one week before commencement of trial. During acclimatization period, fish were fed with commercial floating feed of 2mm pellet size (Supreme Aqua Feeds, Pakistan) (Protein: 30.43±0.28 %, Starch: 23.00±0.34 %, Moisture: 11.17±0.14 %, Ash: 7.40±0.34 %, Fat: 5.67±0.08 %, Fiber: 3.97±0.14 %). Feed was given according to 2% of their body weight, twice a day. This study was divided into two trials.

2.1.1. Trial 1 (High water temperature; summer)

Nine out of 18 cohorts were randomly selected to commence three different feeding treatments at high water temperature. One group (HF1) having three replicates were fed at the feeding rate of 2% of their body weight (Table 1). The feeding rate in second (HF2) and third group (HF3) (each having three replicates) was 3% and 4% of biomass. Feeding times in HF1, HF2 and HF3 were two, three and four rations per day, respectively (Table 1). This feeding treatment started on June 01 and continued for 60 days, ending on July 31. Water temperature during the trial period was noted to be with the range of 30.12±0.41-30.45±0.43°C. Remaining nine cohorts of fish were fed at the rate of 2% of their body weight, twice a day until September.

This time period for the trial was selected following Fatima *et al.,* (2021), which investigated the growth of rohu over a period of 171 days, aiming to achieve a final stocking density of 57.81 kg/m^3. Furthermore, 60 days is the most recommended study period to detect the effect of any feeding alteration on the growth of fish. Fish husbandry conditions and health were excellently maintained throughout the entire trial period to minimize the mortality. Random weight check of fish was performed for each replicate after every 15 days to adjust the daily ration. This trial ended on July 31.

Table 1: Experimental design of feeding regime according to 2%, 3% and 4% body weight of rohu stocked at high density in both low and high temperature



2.1.2. Trial 2 (Low water temperature; winter)

The second trial involving remaining nine cohorts of fish started on October 01 and continued for 60 days, ending on November 30. Water temperature during this trial was noted to be within the range of 21.15±0.37-21.43±0.35°C. One group (LF1) having three replicates was fed at the feeding rate of 2% of their body weight (Table 1). The feeding rate in second (LF2) and third group (LF3) (each having three replicates) was 3% and 4% of biomass. Feeding times in LF1, LF2 and LF3 were two, three and four rations per day, respectively (Table 1). The difference in the initial weight of rohu in trial I (30±0.69 g) and trial II (90±1.21 g) occurred because all the fingerlings (n=2,700) were purchased simultaneously at the end of May. After one week of acclimatization, trial I was commenced in nine cohorts in June. Meanwhile, the remaining nine cohorts were fed with 2% feed until September to achieve conditions of low water temperature for the start of trial II. The fish gained weight (from 30±0.69 g to 90±1.21 g) over the four months (June-September) before the commencement of trial II, which resulted in the initial weight difference between the two trials.

2.2. Water quality parameters and survival rate

During both trials, water quality was very well maintained in all tanks to ensure the welfare of fish. A total 20% of water was exchanged every day from each tank. Water quality parameters were measured twice a day to ensure its standard quality levels throughout the trial period. Aeration pumps (120V/60Hz, Airmax SilentAir LR25, USA) were used to deliver air via diffuser grids. There was one rectangular diffuser grid in each tank (L ×W: 1 × 0.5 ft). Each diffuser grid was built by using the anti-microbial tubing (outer diameter: 25.4 mm; inner diameter: 12.7 mm; airflow: 2.2 m^3/h/meter) to generate microbubbles ensuring the good air saturation in water (>80%). All tanks were back washed on daily basis to drain solid waste settled at the bottom of tank. Water quality parameters including water temperature, dissolved oxygen and pH were monitored twice a day by using portable meters (HI98494, Hanna, USA). Ammonia and nitrite were monitored twice a week by using commercial kits (HI733, HI93708, Hanna, USA). Water temperature in all the groups of the trial I (high temperature) were in the range of 30.12±0.41-30.45±0.43 ^oC (Table 2). Other parameters in the trial I such as dissolved oxygen, pH, ammonia and nitrites were in the range of 4.25±0.31-4.31±0.37 mg/L, 8.35±0.10-8.42±0.12, 0.74±0.05-0.80±0.07 ppm and 0.12±0.02-0.14±0.03 mg/L, respectively. The physiochemical parameters of trial II (low temperature) such as temperature, dissolved oxygen, pH, ammonia and nitrites were in the range of 21.15±0.37-21.43±0.35 ^oC, 4.42±0.32-4.51 ± 0.34 mg/L, 8.15 ± 0.11-8.21 ± 0.13, 0.69±0.03-0.72±0.06 ppm and 0.11±0.04-0.14±0.03 mg/L, respectively. Fish in every tank were checked for any sign of disease, abnormal behavior and mortality twice a day. Survival rate observed was 100% due to well-maintained husbandry conditions over the study period.

Table 2: Variations (Mean ± SE) in water quality parameters indicating temperature (°C), dissolved oxygen (mg/L), pH, ammonia (ppm) and nitrate (mg/L) at high and low temperature and also at different feeding frequencies groups

High temperature					
Groups	**Water temperature (°C)**	**Dissolved oxygen (mg/L)**	**pH**	**Ammonia (ppm)**	**Nitrites (mg/L)**
HF1	30.12 ± 0.41	4.25 ± 0.31	8.39 ± 0.09	0.74±0.05	0.12±0.02
HF2	30.32 ± 0.37	4.31 ± 0.37	8.35 ± 0.10	0.80±0.07	0.14±0.03
HF3	30.45 ± 0.43	4.29 ± 0.33	8.42 ± 0.12	0.79±0.08	0.15±0.04
Low temperature					
Groups	**Water temperature (°C)**	**Dissolved oxygen (mg/L)**	**pH**	**Ammonia (ppm)**	**Nitrites (mg/L)**
LF1	21.30 ± 0.33	4.51 ± 0.34	8.21 ± 0.13	0.69±0.03	0.13±0.02
LF2	21.15± 0.37	4.47 ± 0.37	8.19 ± 0.09	0.72±0.06	0.14±0.03
LF3	21.43 ± 0.35	4.42 ± 0.32	8.15 ± 0.11	0.71±0.05	0.11±0.04

2.3. Sampling and analysis

At the end of each trial, five fish were randomly sampled from each replicate of all feeding frequency groups (15 fish from three replicates of each group). A total of 90 fish were euthanized at terminal sampling out of 2,700 fish used in both trials (trial I and II) following the designated limit of 5% of population approved by Animal Ethics Committee. Remaining 2,610 fish were humanely released in nearby lake, administered by Department of Fisheries, Pakistan for stocking purpose (Release Approval #: DOF/27858/2022). Before sampling, fish were fasted for 24 hours. On sampling day, randomly selected fish were euthanized using clove oil (0.8 ml/L of water, Sigma-Aldrich, USA). This dose of clove oil is standard to euthanize fish in a very humane way which took less than ten minutes to euthanize sampled fish.

Blood was collected from the caudal vein in to the tube coated with clot activator for plasma collection. Blood samples were centrifuged (Centurion scientific Ltd., UK) at 5,000 rpm for 15 minutes and plasma was collected in separate Eppendorf tubes and stored at -20 ° C. The total body weight and total body length were measured before dissection. Fish were dissected and muscle samples were collected and stored at – 20°C for chemical composition analysis following the guidelines of Association of Official Analytical Chemists (AOAC, 2005). Muscle samples were dehydrated in hot air oven at 80°C until a consistent dry weight was reached. These dried samples were processed for further chemical analysis. The Kjeldahl apparatus (PCSIR Laboratories, Pakistan) was used to determine the crude protein, while crude lipids were identified using the Folch method (Folch *et al.,* 1957) using the Soxhlet apparatus (PCSIR Laboratories, Pakistan). The ash content in muscles was determined using muffle furnace (PCSIR Laboratories, Pakistan).

The whole intestinal samples were weighed, rinsed with deionized water using tissue homogenizer (PCSIR Laboratories, Pakistan) in 0.86 % sterile normal saline solution (tissue: saline= 1:9). This mixture was centrifuged at 5000 rpm for 15 minutes. Supernatant was collected and stored at -20°C. Each sample was analyzed in three replicates for all analyses. Condition factor (K), specific growth rate (%) (SGR), hepatosomatic index (HSI), viscerosomatic index (VSI), fish weight gain, survival rate, feed conversion rate (FCR) was measured by using the given formulae.

$$\text{Condition factor (\%)} = \frac{\text{Total body weight (g)}}{\text{Total body length (cm)}^3} \times 100$$

$$\text{Specific growth rate (\%)} = \frac{\text{Ln final weight (g)} - \text{Ln initial weight(g)}}{\text{Time interval in days}} \times 100$$

$$\text{Hepatosomatic index (\%)} = \frac{\text{Liver weight (g)}}{\text{Total body weight (g)}} \times 100$$

$$\text{Feed conversion ratio} = \frac{\text{Weight of feed consumed (g)}}{\text{Weight gain (wet weight; g)}}$$

$$\text{Survival rate (\%)} = \frac{\text{Final number of fish}}{\text{Initial stocking density}} \times 100$$

$$\text{Visceroosomatic index (\%)} = \frac{\text{Viscera weight (g)}}{\text{Total body weight (g)}} \times 100$$

2.4. Digestive enzymes assays

To measure the activity of digestive enzymes, supernatant of processed whole intestine samples was utilized. Commercial ELISA kits were used for the activity of lipase (U/L) (Sigma Aldrich, USA, CAT No. MAK046, EC 3.1.1.3) for amylase (U/L) (Sigma Aldrich, USA, CAT No. MAK009A, EC 3.2. 1.1.) at 570 nm and 405 nm wavelength respectively. The activity of protease was determined following Walter (1984). Casein 1% w/v was used as substrate in 0.2 M phosphate buffer at pH 7.0. One unit of protease indicates the amount of enzyme that releases 1 µg/ml/min of tyrosine determined at 660 nm of wavelength.

2.5. Hormones assays

The levels of thyroid stimulating hormone (TSH; µIU/mL) from blood plasma were measured using ELISA (Calbiotech, USA, CAT No. TS227T) having a sensitivity of 0.05 µIU/ml. For the determination of triiodothyronine (T3; ng/ml) levels in blood plasma, ELISA (Calbiotech, USA, CAT No. T3379T) kit was used. Thyroxine (T4; µg/dl) levels from plasma was measured using ELISA kit (Calbiotech, USA, CAT No. T4224T). The absorbance value of all these hormones was read at 450 nm using an ELISA microplate reader (RT-6,000) (Rayto, China)

2.6. Statistical analysis

For the statistical analyses, SPSS v.29 software was used. Data were presented as Mean± SE for all the parameters. To check the homogeneity of variance of data, Levene test of homogeneity was performed. Two-way ANOVA was used to determine the effect of two independent variables i.e. water temperature and feeding frequency on dependent variable i.e. all the parameters measured in the study. To reject the null hypothesis, 0.05 probability level was used. The statistical analysis conducted through two-way ANOVA elucidates the significant ($P<0.05$) or insignificant ($P>0.05$) influence of water temperature and feeding frequency independently, as well as the combined impact of their interaction (temperature*feeding frequency), on various parameters examined in the study. Along each parameter, degree of freedom (df) was mentioned which was calculated as the sample size minus the number of restrictions. In case of temperature, df was 1 as there were two temperatures. In case of feeding frequency, it was 2 as three feeding frequencies were employed in the study. For the combined effect of temperature*feeding frequency, df was 2 (1×2).

3. Results

3.1. Growth

The total body length (df_1, F=729.167), total body weight (df_1, F=507162.500), specific growth rate (df_1, F=437546.341), condition factor (df_1, F=5270.068) and viscerosomatic index (df_2, F=2157.167) were found to be significantly different (P<0.05) at two different temperatures except hepatosomatic index (df_1, F=4.808, P>0.05) (Table 3). Total body length (df_2, F=171.500), total body weight (df_2, F=1416304.167), condition factor (df_2, F=1567.004), specific growth rate (df_2, F=123894.270) and viscerosomatic index (df_2, F=354.667) differed significantly (P<0.05) between different feeding frequencies. However, an insignificant difference (df_2, F=0.324, P>0.05) was observed in hepatosomatic index between different feeding frequencies. Other than this, temperature*feeding frequency showed a significant effect (P<0.05) on total body length (df_2, F=15.167), total body weight (df_2, F=40512.500), condition factor (df_2, F=141.484), specific growth rate (df_2, F=4572.283) and viscerosomatic index (df_2, F=32.667) except hepatosomatic index (df_2, F=1.934, P>0.05). The survival rate of fish both in low and high temperature against all feeding frequency groups were 100%.

Table 3: Analysis of total body length (cm), total body weight (g), condition factor (%), hepatosomatic index (%), specific growth rate (W), viscerosomatic index (%) and FCR (Mean ± SE) at two temperatures in three feeding frequencies groups (2%, 3% and 4%).

Groups	TBL (cm)	TBW (g)	SGR (W)	K (%)	HSI (%)	VSI (%)	FCR	Survival rate (%)
HF1	8.23±0.21	64.25±1.21	410.61±1.31	2.29±0.23	1.67±0.04	8.05±0.16	1.65±0.05	100.00±0.04
HF2	8.28±0.19	68.50±1.18	417.01±1.28	2.30±0.19	1.51±0.02	8.10±0.20	2.20±0.04	100.00±0.03
HF3	8.32±0.22	70.00±1.23	419.10±1.30	2.35±0.20	1.77±0.03	8.13±0.19	2.25±0.06	100.00±0.04
LF1	13.79±0.38	110.55±1.60	462.13±1.27	2.38±0.15	1.22±0.05	7.90±0.18	1.92±0.03	100.00±0.02
LF2	13.85±0.41	111.00±1.64	465.29±1.32	2.41±0.14	1.24±0.02	7.93±0.21	2.37±0.04	100.00±0.03
LF3	14.10±0.23	115.15±1.71	468.96±1.38	2.55±0.12	1.20±0.06	8.02±0.17	2.32±0.02	100.00±0.04

*TBL: Total body length, TBW: Total body weight, SGR: Specific growth rate, K: Condition factor, HSI: Hepatosomatic index, VSI: Viscerosomatic index, FCR: Feed conversion rate

3.3. Proximate composition of muscles

The moisture content (df_1, F=74.667), crude protein (df_1, F=94.500), crude fat (df1, F=1512.00) and crude ash (df_1, F=1429.167) differed significantly (P<0.05) between two temperature trials (Figure 1). A significant difference (P<0.05) in the content of moisture (df_2, F=10.500), crude protein (df_2, F=518.000), crude fat (df_2, F=1221.500) and crude ash (df_2, F=325.500) was observed between different feeding frequencies groups. Effect of temperature*feeding frequencies calculated by two-way ANOVA also showed a significant effect (P<0.05) on moisture (df_2, F=736.167), crude protein (df_2, F=1442.000), crude fat (df_2, F=234.500) and crude ash (df_2, F=631.167).

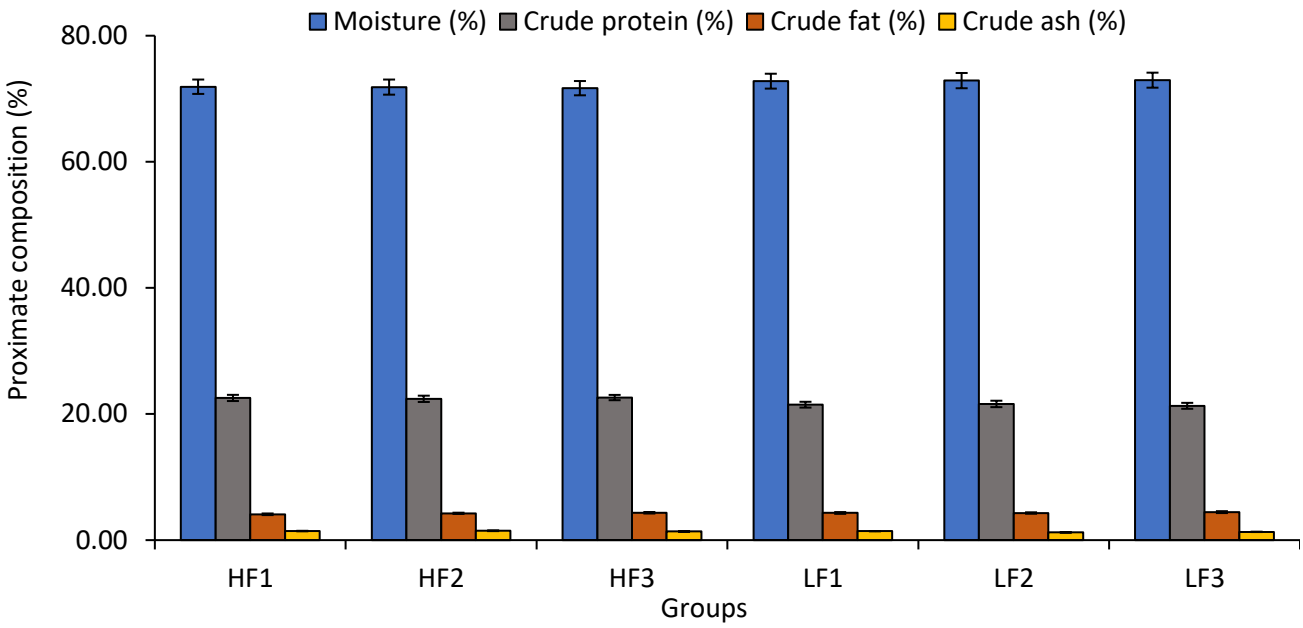


Fig.1: Analysis (Mean±SE) of chemical composition (%) of muscles between three feeding frequencies groups (2%, 3% and 4%) at high and low temperatures.

3.4. Digestive enzymes activity

The activity of amylase (df_1, F=403368.000), protease (df_1, F=373748.667) and lipase (df_1, F=53428.667) were significantly different (P<0.05) at different temperatures (Figure 2). A significant variation (P<0.05) in the activity of amylase (df_2, F=2570.167), lipase (df_2, F=4623.500) and protease (df_2, F=1107.167) was also observed between different feeding frequencies. Effect of temperature*feeding frequencies had a significant impact (P<0.05) on the activity of amylase (df_2, F=171.500), lipase (df_2, F=176.167) and protease (df_2, F=57.167).

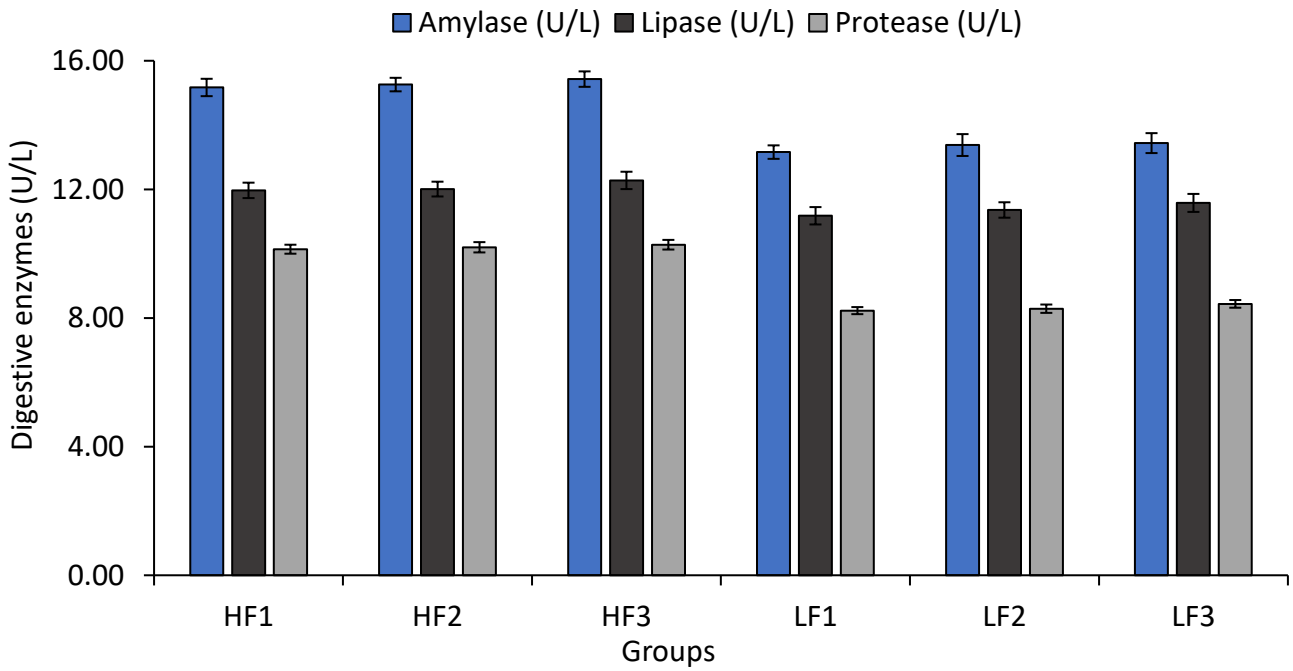


Fig.2: Analysis (Mean±SE) of digestive enzymes activity including amylase (U/L), lipase (U/L), protease (U/L) at two different temperatures and on different feeding frequencies (2%,3% and 4%).

3.5. Profile of hormones

The levels of T3 (df_1, F=421.167), T4 (df_1, F=378.000), TSH (df_1, F=466.667) and GH (df_1, F=94.500) differed significantly (P<0.05) between high and low temperature groups (Table 4). In addition to this, the activity of T3 (df_2, F=57.167), T4 (df_2, F=71.167), TSH (df_2, F=126.000) and GH (df_2, F=56.000) showed a significant variation (P<0.05) between different feeding frequencies. Effect of temperature*feeding frequencies on these hormones also showed a significant impact (P<0.05) on T3 (df_2, F=8.167), T4 (df_2, F=10.500), TSH (df_2, F=4.667) while GH differed insignificantly (df_2, P>0.05).

Table 4: Analysis (Mean±SE) of hormones i.e., T3 (ng/ml), T4 (ug/dl**)**, TSH (uIU/ml) and GH (ng/ml) at two temperatures in three feeding frequencies groups (2%, 3% and 4%).

Groups	T3 (ng/ml)	T4 (ug/dl)	TSH (uIU/ml)	GH (ng/ml)
HF1	0.80±0.01	7.14±0.14	1.80±0.08	0.08±0.01
HF2	0.84±0.02	7.16±0.11	1.83±0.05	0.10±0.01
HF3	0.85±0.03	7.20±0.13	1.87±0.07	0.12±0.02
LF1	0.75±0.01	7.09±0.10	1.74±0.09	0.05±0.01
LF2	0.78±0.02	7.11±0.12	1.77±0.06	0.07±0.01
LF3	0.77±0.03	7.12±0.13	1.79±0.07	0.09±0.02

4. Discussion

The present study determined the optimum feeding schedule for rohu in high-density environments at different temperatures. Based on the data of this study, it may be concluded that rohu grows better at higher temperatures than at lower ones. In this study, total body weight, specific growth rate, and

condition factor were the highest with a 4% feed ration given four times a day at higher temperatures. Despite a slight reduction in feed intake at lower temperatures, rohu still achieved a significant weight gain. This was further supported by growth hormone assays, which showed an increased activity at higher temperatures. Previous research also indicated that rohu kept at lower temperatures (20-22°C) gained significantly lesser body weight in contrast to those kept at higher temperatures. The fish kept at 24-26 °C showed the highest weight gain (Kausar and Salim, 2006). Previous study has also shown that rohu raised in polyhouses, where the average temperature was 19°C, grow more quickly than those raised in outdoor tanks, where the average temperature was 14.8°C (Khan *et al.,* 2004). The water temperature has a significant effect on fish performance (Houlihan *et al.,* 1993; Britz *et al.,* 1997; Azevedo *et al.,* 1998) with weight gain increasing as water temperature rises (Kausar and Salim, 2006). Fish undergo a variety of biochemical and physiological alterations when exposed to cold temperatures. Increased intracellular lipid concentration, improved tissue capillarity, higher enzyme concentration, and elevated mitochondrial concentration are some of these alterations (Burel *et al.,* 1996). The weight gain observed at lower temperatures can partially be attributed to increased body fat accumulation, which is inversely related to the feeding level (Burel *et al.,* 1996).

Numerous other studies have documented a similar pattern of increasing fish growth in response to temperature increase and reduced growth in low temperature. In tilapia, daily weight gain significantly increased at temperatures ranging from 27°C to 32°C, with feed consumption peaking at 32°C within this range (Pandit and Nakamura, 2010). Similarly, in Atlantic salmon (*Salmo salar*), feed intake increases three times approximately as temperatures increases from 2°C to 6°C (Brett, 1979). Temperature also affects the feeding behavior as feed intake increases with temperature and decreases when temperature declines. This correlation between temperature and feeding has been observed in different fish species such as goldfish (*Carassius auratus*; Nadermann *et al.,* 2019), sockeye salmon (*Oncorhynchus nerka*; Brett, 1971), perch fry (*Perca flavescens;* Smirnov and Smirnova, 2019), channel catfish (*Ictalurus punctatus*; Buentello *et al.,* 2000), Indian major carp (*Cirrhinus cirrhosus;* Sharma *et al.,* 2017), walking catfish (*Clarias batrachus*; Ahmad *et al.,* 2014), cobia (*Rachycentron canadum*; Nguyen *et al.,* 2019) and Atlantic salmon (*Salmo salar*; Folkdeal *et al.,* 2012; Hevroy *et al.,* 2012). Previous data suggest that fish experience a loss of appetite and eventually cease feeding at temperatures significantly below their species-specific critical maximum temperature (Shafland and Pestrak, 1982).

The main aspect of the study was to determine the ideal number of daily feedings and optimum feeding frequency in order to promote fish growth. Feeding a ration equal to 4% of body weight four times a day resulted in better growth amongst all. Growth hormone assays further supported the improved growth at the 4% feed ration. Even though the 4% feed ration caused the maximum growth, substantial weight gain was also observed at different feeding levels. Numerous studies have shown that higher feeding frequencies promote better growth in a variety of fish species, including rohu, which exhibits maximum growth when fed three times daily as compared to others (Biswas *et al.,* 2006; Abid and Ahmed, 2009; Aga *et al.,* 2017), as well as when fed six times a day (Choudhury *et al.,* 2002). Similarly, three times a day feeding had a beneficial effect on growth in pangas catfish and silver carp polycultures compared to once and twice a day feeding (Khan *et al.,* 2009). There has been similar reporting of maximum growth at higher feeding frequency compared to lower feeding frequency in tilapia (Bhujel *et al.,* 2007), Siamese fighting fish (James and Sampath, 2004), red swordtail (James and Sampath, 2003), channel catfish (Andrews, 1976), broadhead catfish and common carp (Mollah and Tan, 1982; Charles *et al.,* 1984).

This study examined how temperature and different feeding levels affect the chemical composition of rohu. The findings revealed that both factors significantly influenced the protein, ash, and fat content. Specifically, at higher temperatures with a 4% feed ration, the protein content was significantly higher as compared to other feeding levels and lower temperatures. According to earlier research, the feed ration had a major impact on the total protein content of the animal, with feed rations containing 4% to 6% showing higher contentof protein (Khandan *et al.,* 2019). Similar results have been observed in mrigal carp (Khan *et al.,* 2004). Additionally, previous research showed that tilapia raised at 22°C needed more dietary protein than those raised at 28°C and 34°C (Zeng *et al.,* 2021). Spotted seabass reared at 27°C and 33°C had protein requirements of 46.8% and 44.9%, respectively, based on weight gain (Cai *et al.,* 2020). These results imply that when temperature drops below the optimum range, need for dietary protein increases in to maintain growth, energy balance, and overall health in fish.

Water temperature exerts a significant impact on the function of digestive enzymes. The results of this study indicate that quantity of feed and temperature have a significant effect on the activity of these enzymes. At lower temperatures, there was a decrease in the activity of the digestive enzymes. Higher temperature boosts digestive enzyme activity, which can speed up nutrient digestion and support improved growth (Shcherbina and Kazlauskene, 1971). Digestive enzyme properties differ according to climate and habitat. The development of isozymes (capable of functioning at various temperatures), structural variations, changed secretion rates, and other phenotypic alterations can result from genetic adaptation to temperature (Gelman *et al.,* 2008). The impact of water temperature on digestive enzyme activity is specific to each species and typically corresponds to the temperature ranges of their native environments. Optimum temperature for the activity of digestive enzymes in tilapia, common carp (Watanabe *et al.,* 1996), seabass (Pereira *et al.,* 2018), three spine sticklebacks (Hani *et al.,* 2018), walking catfish (Ahmad *et al.,* 2014) and Indian major carp (Sharma *et al.,* 2017) was 25°C, 25°C, 8–24°C, 4–20°C, 10–30°C and 18–28°C, respectively.

The activity of thyroid hormones, which respond to temperature variations to control overall metabolism, is intimately linked to the effects of feeding frequency and temperature on digestive activity of fish. For fish larvae and juveniles to grow and develop properly, thyroid hormones are essential (Suchiang and Gupta, 2011). Thyroid activity has been shown in the past to vary in response to different environmental stimuli such as intensive rearing conditions (Rafatnezhad *et al.,* 2008). Fish and other animals have been shown to have lower thyroid hormone concentrations when their feed intake is less (Suchiang and Gupta, 2011). Thyroid hormones are also usually associated with higher metabolic rates and are lowered in order to preserve energy during fasting (Suchiang and Gupta, 2011). The T3, T4, and TSH hormones were examined in this study to check the impact of feeding frequency and temperature on these hormones. Although various feeding levels were tested at both high and low temperatures, the minimum feed ration used was 2%, which is considered standard for fish as used for other warm water species such as rohu (Minahal *et al.,* 2024), tilapia (Fatima *et al.,* 2021; Komal *et al.,* 2024) and striped catfish (Liaqat *et al.,* 2024a; Liaqat *et al.,* 2024b). As the minimum feeding rate was 2%, the fish did not experience starvation or stress from reduced feed, leading to no major fluctuations in profile of thyroid hormones.

Conclusion

The results of this study show that rohu achieved better growth rates at higher temperature as compared to lower temperature ranges, due to elevated metabolism and increased feed intake. Among the different feed ration levels tested, 4% feed ration resulted in the highest growth and weight gain. However, the most efficient FCR was observed

at high temperatures with a 2% feed ration, indicating better feed efficiency. The relatively uniform growth across different feeding frequencies suggests that a 2% feed frequency is suitable for various culture systems, balancing cost-efficiency without negatively impacting growth rates.

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