Partial Replacement of Fish meal with Fish Hydrolysate and Casein in larval diet: Effect on Early Rearing of *Labeo rohita*

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FISHERIES AND AQUACULTURE

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"In the Name of ALLAH, the most Beneficent, the most Merciful"

Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

Dua Laraib

CERTIFICATE

This dissertation "Partial replacement of fish meal with casein and fish protein hydrolyzate in larval diet : Effect on early rearing of *Labeo rohita*" submitted by **Ms. Dua Laraib,** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Fisheries and Aquaculture.

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Dedicated to:

This thesis is dedicated to my loving parents, siblings, respected supervisor and my beloved friends for their advice, patience, and faith in me.

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List of Abbreviations

EPA	Eicosa pentanioic Casein is a valuable source of protein in purified test diets for fish as well as for higher vertebrates' acid
FAO	Food and agriculture organization
EEZ	Exclusive economic zone
NEPAD	The new partnership for Africans development
DHA	Decosa hexanoic acid
PL	Postlarvae
DO	Dissolve oxygen
ppm	Parts per million
PPRI	Pakistan poultry research institute
NCP	National center for physics
GnRH	Gonadotrophin releasing hormone
FBW	Final body weight
IBW	Initial body weight
WG	Weight gain
WG%	Weight gain %
DWG	Daily weight gain
SR%	Survival rate %
SGR	Specific growth rate
PC	Protein casein
FPH	Fish protein hydrolysate
PH	Protein hydrolysate
CP	Crude protein
MT	metric tons

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ABSTRACT

A 35-day feeding experiment was conducted to assess the effect of partial replacement of fish meal with fish hydrolysate and casein on early rearing of *Labeo rohita*. The experiment was conducted in triplicates three-day post-hatch (dph) having an initial body weight of 0.40±0.07g were randomly divided into seven groups with a stocking density of 200 larvae /trough and 600 larvae /treatment. During the trial the postlarvae were fed seven different nano particulate isonitrogenous diets of Cp 50%. The groups P_0 (0%), PH_1 (4%) PH_2(6%) and PH_3(8%) having of fish hydrolysate substituted with fish meal and the other groups PC_0(0%), PC_1(5%), PC_2(10%) and PC_3 (15%) fed diet of casein substituted with fish meal respectively. Results demonstrated that PH_2 group of postlarvae has a significant ($p<0.05$) effect on growth performance (FBW, WG, WG%, DWG, SGR% and SR%) then followed by PH_1 group. The specific activity of digestive enzymes (amylase, lipase and protease) tented to increase ($p<0.05$) in PH_2 group as compared to all other groups. Additionally, Histological examination of intestine (villi's length, width and absorptive area) also followed the same results. Furthermore, the mRNA expression (Ghrelin and Myogenin) was observed to be highest in PH_2 group then followed by PH_1 group and least expression of both genes was observed in PH_3 group of postlarvae. Moreover, in groups fed diet having fish meal partially substituted casein. Results showed that PC_3 group of postlarvae has a significant ($p<0.05$) effect on growth performance (FBW, WG, WG%, DWG, SGR%) then followed by PC_2 group, PC_1 and control group. While SR% was significantly highest in PC_2>PC_1>Control and lowest in PC_3 group of postlarvae. In addition to Histological examination of intestine (villi's length, width and absorptive area) also followed the same results. Nevertheless, activity of digestive enzymes (amylase and protease) was increased ($p<0.05$) in PC_3 group as compared to all other groups. Whereas the activity of lipase enzyme showed comparable results in PC_2 and PC_3 group then followed by PC_1 group. Therefore, the expression of genes (Ghrelin and Myogenin) was observed to be highest in PC_3 group then followed by PC_2 group and least expression of both genes was observed in Control group of postlarvae. It was concluded that fish hydrolysate at moderate concentration showed a significant effect on *L. rohita* larvae while casein protein in combination with fish meal showed dose-dependent effects on various parameters

INTRODUCTION

Aquaculture is an organized rearing, it involves the propagation or protection of aquatic resources for recreational or public use (FAO, 2017). It is one of the rapidly growing food-production sectors and accounts for nearly half of all food fish and it is an alternative of wild fishes and plants (FAO, 2010). It is a source of socio-economic development since they create jobs all around the world. It is estimated that 660–880 million people depend entirely or partially on aquaculture for their subsistence (Allison *et al*., 2013). Fish has a high nutritional value and offers high-quality protein in addition to a diverse range of minerals and vitamins, such as vitamin A, D magnesium, and phosphorus. The necessary amino acids are present in fish protein, which raises the nutritional value of a diversified diet (Sujatha *et al*., 2013). Fish is a significant source of omega-3 fatty acids, particularly α-linolenic acid, eicosa-pentaenoic acid (EPA), and docosa-hexaenoic acid (DHA) (Tacon, 2013).

Aquaculture in world

The fish production systems in the world have contributed to the impressive growth of fish production within six decades, rising from 19 million metric tons (MT) in 1950 to 171 million MT in 2016 (Pauly, 2019). Global capture fisheries production peaked in 1996 at around 96 million MT. In contrast, aquaculture production has doubled every decade for the past 50 years to produce 80 million MT of food fish, 30.1 million MT of aquatic plants and 38,000 MT of non-food products in 2016 (Bush, 2019). In 2014, aquaculture overtook capture fisheries in the provision of fish for human consumption (Golden, 2017). As the global fastest growing food production sector, the future expansion of fish as food is expected to come from aquaculture in the next decades.

The fisheries and aquaculture sectors in Africa are increasingly contributing to food and nutrition security, foreign exchange, employment, and livelihood support services (Obiero, 2019). The New Partnership for Africa's Development (NEPAD) estimates that total fishery production in the region stands at 10.4 million tonnes comprising of 6.0 million tons from marine capture fisheries, 2.8 million tons from inland water fisheries, and about 1.6 million tons from aquaculture. Currently, more than 30% of the continent's population, or roughly 200millionpeople, consume fish as

the main protein source and micronutrition (AUC-NEPAD, 2014). Besides, 12.3 million people in Africa work in the fisheries and aquaculture sector, with 6.1 million (50%) being employed as fishers, 5.3 million (42%) as processors and 0.9 million (8%) as fish farmers (Obiero, 2019).

Aquaculture in Pakistan

Pakistan is an agricultural country and rich in natural water resources. Freshwater and marine water are the major resources for aquaculture. There are about 8,563,820km2 area is in the form of Rivers, Lakes, Ponds, and water lodging areas. (Jarwar, 2008). There are considered suitable for aquaculture and provide the basis for aquaculture development. While there is a great potential for coastal fisheries with about 1120 km coastline. In further, an open sea area, an Exclusive Economic Zone, of about 350 nautical miles in the control of the Pakistan government. Despite these huge resources for aquaculture potential, there is no any significant progress in the fisheries sector. The main reason is the aquaculture practice. Only in the freshwaters extensive and semi-intensive aquaculture systems have been adopted. On the other hand, coastal sources and deep-water marine sources are still not utilized for aquaculture purposes. Hence, from the marine aspect, we are dependent totally on natural availability (Minfal, 2012).

Pakistan is blessed with plenty of marine as well as inland aquatic resources. These fishery resources have immense potential for economic development in this region (Mohsin, 2017). Estimated figures show that about 50000 people are associated with aquaculture in Pakistan. In 2004, the aquaculture sector contributed 70000 tons of fish and the total fish production was 564105 tonnes in that year (Hayat, 2005). Capture fisheries production in Pakistani marine waters is decreasing with the passage of time. This is 3 because of overfishing which is continued in Pakistani marine waters. Due to a lack of planning, attention, and policy implementation, the marine sector is under an open access regime. Thus, currently, marine resources are under stress so overfishing must be controlled to conserve fishery resources and there must be a good alternative to produce fish food for human consumption (Mohsin, 2015). Aquaculture is the best the option that can not only help to eliminate hunger but also has a potential to uplift economic conditions. To our knowledge, available literature on the statistics of aquaculture in Pakistan is scarce and does not describe its potential for economic

development along with practical suggestions and steps for the betterment of this sector in this country. Thus, this research work is the first effort in this regard (Kalhoro,2013).

Indian major carps

Indian major carps are the most culturable fish species, contributing nearly 87% of the overall production of freshwater aquaculture (Ayyappan, 2003) and hence, carp culture is referred to as the backbone of aquaculture industry. The mainstay of freshwater aquaculture is rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*).These are the best cultivable and most preferred species in the inland water system because of their fast growth and high consumer acceptance (Saini *et al*., 2014).

Rohu (*Labeo rohita*)

Rohu fish (*Labeo rohita*) belongs to the family Cyprinidae and order Cypriniforms. It is populated in the subcontinent's rivers (Talwar & Jhingran, 1991). It is one of the dominant carp species for aquaculture which share more than 60% of total carp production. It contribute 3.7% of the major species yield in world aquaculture and under cluture condition highly variable growth is seen (FAO, 2020). These are the most popular aquaculture species in Pakistan, because of their high market value, manageability, nutritional value, and development potential (Abeda & Zahra, 2014). Among all Indian Major Carps rohu supplies the highest percentage of protein (Ahmad, 2012.) In little fraction, it also acts as a source of Calcium and Vitamin-A (Roos, 2003). Rohu is a herbivore, column-feeding that frequently prefers algae and submerged micro-vegetation (Alam *et al*., 2011).

Rohu larvae

In the early stages of life, it prefers zooplankton i.e., rotifers and Cladocera, with phytoplankton serving as the primary food source (Alam *et al.,* 2011). The intestinal tract of fish larvae is under-developed and simpler in organization which is congruent to lower production of digestive enzymes. Therefore, the larvae lack the required enzymes or enough quantity of enzymes to properly digest the diets (Deguara *et al.*, 2003).

Live feed problems

One of the challenges is live feed that is primarily used in commercial fish farming are rotifers and Artemia, which lack the biochemical profile that is needed by the fish larvae to survive and grow better into healthy fry (Sargent *et al.*, 1999).In live feed there are variations in the ratio and quantity of nutrients and deficiency of essential nutrients Live feed serves as a carrier of diseases to the larvae of fish and shellfish which directly or indirectly affect the growth and survival of juvenile (Hamre, 2016) therefore, maintenance of hygiene is very crucial during their production (Curnow *et al.*, 2006).Moreover, exacerbates issues around their future usage due to price and supply volatility since they need a lot of machinery, cost, and labour to produce the necessary amount of live food safely and continuously (Yanes-Roca, 2018).

Bottleneck of Larval rearing

In the nursery culture of rohu fish, the most common problems that are encountered are high mortality rate, stunted growth, and frequent outbreaks of diseases (Mitra & Mukhopadhyay, 2003). Consequently, supplementation in adequate quantity and quality is an important factor for better larval growth and survival. (Mitra *et al.,* 2007). Moreover, quantification of feed intake and diet digestibility is a major problem in larval nutrition studies (Conceicao *et al*., 2002). Subsequently, due to lack of information on the nutritional requirements of fish larvae and the development of digestive system are thought to play a major role in and limits the success of larval culture for many species (Hamlin *et al.,* 2000). To reduce costs and increase juvenile production, several researchers have tried to substitute live feed by inert diets, either a supplement or as primary dietary source (Lazo *et al*., 2011).

Feed characteristics

The success of larval culture depends upon on the composition and characteristics of the diet. The particle must be visible and appealing to the larvae which mean size, shape, colour, sinking properties and the attractants release are also important. The larvae must be able to digest the dietary particles and absorb and assimilate the nutrients i.e., Major factors include feed digestibility, feed binding characteristics, leakage, and nutrient bioavailability. Lastly, the nutritional profile of the feed must correspond to the needs of the fish larvae (Ozkizilcik *et al.*, 1996).

Protein

Protein is the main constituent of the fish body thus sufficient supply in the diet is needed for optimum growth (Ahmad *et al.,* 2004). Protein-energy ratio, protein digestibility, and quantity of non-protein energy in the diet also influence protein requirement (Bowyer *et al*., 2013). Considering that protein is the single most expensive ingredient in fish diets, it's critical to provide only the quantity that required for the growth and development of fish larvae. Its excess concentration is economically and biologically wasteful. Studies have indicated that having enough nonprotein energy sources in the diet, such fats and carbohydrate, can reduce the need for protein as an energy source (Cho & Kaushik, 1990). Protein digestibility in the body is greatly influenced by the amino acid balance because of its amino acid content, high-quality protein typically exhibits a higher digestibility. Mostly the high-quality proteins originate from animals, such casein from milk and fish meal (Huang *et al*.,2015).

Fish meal

Fish meal is the main protein source in diets fishes because of the well-balanced amino acid profile and the compositions of essential fatty acids, digestible energy, vitamins, and minerals. However, worldwide fish meal production has stalled by approximately 6–7 million tons in recent years, Additionally, aquaculture systems production costs have grown as a result of its increasing demand and rising values (Tacon & Metian, 2008). Fish meal has high phosphorus content that may cause environmental troubles if it is overused in aquatic feeds. Thus, it is crucial to restrict the quantity of fish meal in fish diets (Yang *et al*., 2011). Several research have been carried out on the whole or partial substitution of fish meal in the manufacture of diets for aquatic organisms (Teixeira *et al*., 2006).

Fish hydrolysate

Fish-protein hydrolysates (FPH) are produced from by-products of fish and shellfish. fish waste includes Skin, bones, head, scales, and viscera etc (Bui *et al*., 2014). It contains an appreciable quantity of protein (Kristinsson, 2000). Compared to the production of fishmeal, it is more cost-effective, safer, technologically easier, and environmentally friendlier (Hanafy & Ibrahim, 2004). The composition of a protein hydrolysate, produced from fish residue, may contain on average 78.75% crude protein, 3.42% fat, and 12.51% ash (Nilsang *et al*., 2005).It is a material rich in soluble small molecular peptides, highly palatable, digestible, and has a good balance of fatty acids and amino acids (Bauer *et al*., 2012). Smaller fractions of these peptides consist of biologically active peptides which act as growth and health promoters (Ha *et al*., 2019) that are readily absorbed by enterocytes at higher rates compared to proteins hence readily available for assimilation, the best overall growth performance (Zheng *et al.*, 2014).

Production

An alternative with great potential is the use of byproducts of the fish-processing industry, in the form of protein hydrolysate and fish of low-commercial value. The hydrolysis process is defined as the protein breakdown into peptides of various sizes. It can be achieved both chemically and biologically (Naylor *et al*., 2009). and chemical hydrolysis is more commonly in practice (Pigott *et al*., 1981).

Inclusion level

Hydrolysate was usually incorporated into diet in the range of 10 and 19% (Busta *et al.*, 1991). The inclusion of moderate levels of protein hydrolysates in larval diets promotes growth performance and feed utilization regulation of the lipid accumulation and fatty acid composition of fish larvae, but levels greater than 25–30% lead to poor growth and a low survival rate (Cahu *et al.*, 1999).

Effects on fish

The inclusion of fish protein hydrolysate at adequate levels in diets had positive impacts on growth, feed intake, nutrient utilization, immunological response, oxidative status, and disease resistance especially use in larval diet. These positive effects are due to FPH's bioactive components and more digestible and absorbable peptide profiles (Shukla *et al*., 2006). It contains a variety of lipids, including monounsaturated, polyunsaturated, saturated, and unsaturated fatty acids. These fatty acids are crucial to the dynamic development of fish (Jabeen *et al.*, 2011). Feeds having high levels of protein hydrolysed will have high protein leaching rates which lower protein levels in the diet below requirement, ingested by larvae. (Hamre*etal*.,2000).

Casein

Casein, a main milk protein is relatively cheap, accessible, non-toxic, and very stable (Katz *et al*., 2009) it is the most valuable component both quantitatively and nutritionally, representing approximately 80% of total milk's nitrogen (Grosclaude, 1988). Milk is acidified to produce acidic casein. It has a (0.7–0.9%) phosphorus content. covalently bound to the protein by a serine ester linkage so, casein is also known as phosphoprotein. Casein is a highly nutritious protein. All the essentials are present in it in high proportion except for cysteine. It is present in milk in intricate molecular clusters known as micelles. The micelles have a molecular weight of several hundred million Daltons and are composed of casein molecules, calcium, inorganic phosphate, and citrate ions. Casein is made up of numerous distinct casein components (as1-, as2-, b-, and k-casein), each of which has varying characteristics (Livney, 2010).

Casein is a valuable source of protein in purified test diets for fish as well as for higher vertebrates (Rtmsey *et al.*, 1975) it is a vital source of essential amino acids which are important for the growth and development of fish larva and its

Supplementation in the diet can enhance growth and survival rates (Cowey *et al*., 1971). Its protein is a slow-digesting protein (Plakas & Katayama, 1981). that can provide a sustained release of amino acids and lead to better feed conversion efficiency, as the fish larvae will be able to utilize the protein more efficiently (Tanaka *et al*., 1977).Enzymatic hydrolysis of casein releases bioactive peptides which are found to function as ion carriers, by enhancing minerals bioavailability (Shah, 2000), as immunomodulators, it is improving innate immune defence or as cell-growth factors, and by stimulating the activity of neonatal integral cells and thus promote the development of digestive tract (Meisel & Fitz Gerald, 2003). Due to its balance amino acid profile, it can promote the expression of amino acid and peptide transporters; this expression speeds up the transport of functional amino acids in the intestinal environment. The concentrations of aspartate, glutamate and lysine in the lumen are affected by different treatments of different protein sources. These changes can affect specific metabolites derivation and conversion pathways and modify physiological function and alter microecology balance in the gut (Weintraut, 2016)

Hypothesis

We hypothesized that by partial replacement of fish meal with casein and fish hydrolysate in the diet of *Labeo rohita* larvae, will have positive effect on growth performance, digestive enzymes, histological studies and on gene expression of (ghrelin and myogenin). so, to prove our hypothesis our research will focus on following objectives.

Aim and objectives

The aim of the study is to examine the effects of partial replacement of fish meal with fish hydrolysate and casein on *Labeo rohita* larvae.

To achieve the target following objectives were set.,

- To evaluate the detrimental inclusion level of fish hydrolysate
- To determine the effect of partially replacement of fish meal with fish hydrolysate and casein on gut morphology (villi height, villi width, and absorptive area) and activity of digestive enzymes i.e., amylase, lipase, protease activity.
- To determine the effect of fish hydrolysate and casein-based diet on the gene expression of Ghrelin and Myogenin

MATERIALS AND METHODS

Study area for experimental larvae

The experiment was conducted during the month of July-August (2022) at the outdoor facility of Fisheries and Aquaculture Research Station, Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad.

Fish breeding

Artificial propagation was done at Rawal hatchery to produce larvae of rohu fish. The well matured and healthy brooder was assessed prior to selection (Muir & Robert, 1985). Female fish with a distended abdomen, red-coloured, soft swollen vent and the male fish that release milt when mild pressure is applied to the abdomen. Following selection, they were transferred to the holding tank for acclimatization. A single dose of the hormone Ovaprim (Domperidone + GnRHa, Syndel, USA (0.2 ml) for male and (0.4 ml) for female) was administered in response to the better outcomes observed by (Kaui & Rishi, 1986). The dosage of Ovaprim was estimated and based on the weight of the brood fish, according to (Nandeesha *et al*.,1991). Brooders were kept in well arreated water of a circular tank for conditioning (8hr). The sex ratio of female and male brooders was kept to 1:2. Subsequently to injection, after 8hr breeding behaviour was observed the male started to follow the female fish. For spawning female's belly was pressed gently so, the eggs released out from a vent and were collected in plastic bowls the same approach was carried out again with the male, and milt was added into the bowls retaining eggs. Both milt and eggs were mixed using bird's feather for 2 minutes by following the wet fertilization system. Then eggs were rinsed with water for 10 min, they absorbed water and attained the size of 1 to 1.4 mm in diameter. After (4 hrs) the fertilized opaque eggs were seen. Following 24 hrs. hatching was seen at a water temperature of 28.5-29^{o}C.The newly hatched larvae were retained in circular tank for 3-4 days until yolk sac got absorbed.

Experimental larvae

The newly hatched larvae of *Labeo rohita,* three days old, were collected from the Rawal Fish Seed Hatchery in Islamabad. They were then transported to the research

site within a limited time frame of 30 minutes, using oxygenated plastic bags. Subsequently, the larvae were carefully transferred to prerequisite water troughs at the research site.

Experimental design

The three-day old hatchling of *Labeo rohita* were equally distributed across 21 troughs (200 larvae/trough or 600/treatment). The water trough was randomly divided into 7 groups and fed their respective diets. After 2 weeks larvae were transferred to a glass aquarium.

Water quality

During *L. rohita* post-larvae rearing, several water quality parameters were monitored using the Multi-parameter Hanna HI (9147) device. These parameters included temperature, Dissolved Oxygen (DO), pH, and total ammonia levels. The DO levels and temperature were recorded daily at 8:00 AM and 4:00 PM, while pH and total ammonia were measured after every week or two. The water temperature during experiment was the in range of 25°C to 28°C. Dissolved Oxygen levels ranged from 5.0 to 6.0 mg/L, pH was within the range of 7.5 to 8.0, and total ammonia levels were maintained below 0.5 mg/L. The water quality parameters remained within the acceptable range for the successful rearing of *L. rohita*.

Preparation of Fish protein hydrolysate

Fish hydrolysate was prepared by chemical method (Jialin & Lied, 2001). Fish viscera from the local market (Bhara kahu market) were ground up and added (85% formic acid) at a rate of 25ml/1000gm. The pH of the hydrolysate was kept at 4 or lower to prevent microbial activity and kept at room temperature. For three days the floating oil was manually poured from the hydrolysate and for up to one week the hydrolysate was manually stirred to ensure proper mixing. After 25 days, base (NaOH)was used to neutralize the acidic silage. It was incorporated into the experimental diets after knowing its proximate composition

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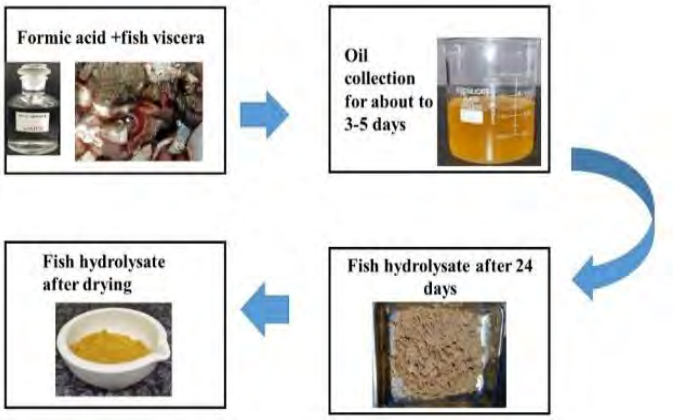

Fig:1 Preparation of fish hydrolysate

Feed formulation and preparation

The feed ingredients listed in table 1 were thoroughly ground and combined according to the formulation. Mix the dry grounded ingredients and water was added to prepare the dough. The glutinous mixture was processed using a hand pelletizer to make pellets. then dried in an oven (SANFA DHG-9053A) for 24 hours at 60 degrees Celsius and store Stored at 4 degrees until needed. These pellets were ground again into powder using an electric grinder (MXBAOHENG Model HC-500). Seven isonitrogenous diets (50%) were prepared one for control and six experimental diets that contain three different levels of hydrolysate (4% ,6%,8%) and casein protein (5%,10%,15%).

The larva could not take the larger size particles, so the powdered feed was then reduced to nano size at the NCP (National Centre for Physics in Islamabad). The larva was continuously fed 75% of their body weight, and after 10 days of trial, they switched to 50% of their body's weight. A 12:12 hour photoperiod and a 5 ppm DO were maintained throughout the experiment, the water temperature was between 27°C and 28°.

Proximate analysis

In collaboration with the Pakistan Poultry Research Institute (PPRI) in Islamabad, a proximate analysis was conducted on the muscle ash content, crude fats, and crude protein from 15 fries collected from each tank, using standard methods (AOAC, 2000). Utilizing the Soxhelt apparatus and the micro Kjeldhal technique,

respectively, crude fats and protein were measured (Sutharshiny & Sivashanthini.,2011).

Growth parameters

Fish Fry were sampled from each tank at the end of the trial. Then weighted using a microbalance (METTLER TOLEDO) and examined for growth characteristics. In accordance with the established procedure described by castell views, the following matrices were calculated: net increase in (%), specific growth rate (SGR) and survival rate in (%.).

$$\boldsymbol{Average\ weight\ of\ post\ larvae} = \frac{total\ \ weight\ of\ post\ larvae}{total\ number\ of\ \ post\ larvae}$$

$$\boldsymbol{weigth\ gain} = Fbw\ of\ fry - Ibw\ of\ fry$$

$$\boldsymbol{Weigth\ gain}\% = \frac{Fbw\ of\ fry - Ibw\ of\ post\ larvae}{Ibw\ of\ post\ larave} \times 100$$

$$\boldsymbol{Specific\ growth\ rate}\ = \frac{Fbw(g) - Ibw(g)}{trial\ days} \times 100$$

$$\boldsymbol{survival\ rate}\ = \frac{Number\ of\ larvae\ survived}{total\ number\ of\ larvae} \times 100$$

$$\boldsymbol{DWG} = \frac{Final\ weigth - initial\ weigth}{number\ of\ experimental\ days}$$

Histological studies

Three normal specimens were randomly chosen for histology at the end of study. Fry was fixed in 10% formalin solution and dehydrated with ethanol, after washing with xylene samples were embedded in paraffin wax. Each block was sliced into sagittal sections, which were mounted on glass slides, dried 60% and then stained with haematoxylin-eosin (Drury & Wallington, 1980) Slides were then examined using a digital microscope and photographed using an AIPTEK digital camera. The villi height, width, and thickness at various gut sections were measured using the image j version 20 software.

Estimation of intestinal enzymes

Fry was starved for 24 hours after trial and then sampled for assessment of intestinal enzymes. 45 fry/treatment 15/aquarium) were taken and aseptically dissected at low temperature (on ice) using clove oil, by cutting off the head and tail, a whole fry was collected as a sample. to obtain enough samples for analysis. They were then thoroughly cleaned with chilled distilled water, placed in an ice-cooled petri dish, and weighed. Add 1M phosphate buffer and homogenize it for 5 minutes then sample was centrifuged (10000rpm) for 10min at 4 degrees Celsius. The supernatant, which served as the enzyme extract, was used to measure the activities of amylase, protease, lipase.

Amylase activity

The 3,5-dinitro salicylic acid (DNS) technique was used to measure the amylase activity (Bernfeld, 1955). Here maltose sugar was used as a reference substance. For 3–4 minutes, an enzyme solution (0.5 mL) was incubated at room temperature and added 1% starch solution of 500 µL, after letting it sit at room temperature for 3 minutes, the DNS reagent (1 mL) was added, and the mixture was incubated for 5 minutes over a pot of boiling water. Reagent grade water (10 ml) was added after the sample had cooled to room temperature, and the sample's absorbance at 540 nm was assessed using a spectrophotometer.

Protease activity

The (Cupp-Enyard, 2008) methodology was used for protease analysis,1ml of a 0.65% casein solution was combined with an enzyme solution. and the solution was incubated at 37 degrees for a period of 10-minutes. Then, 5 ml of A solution of 110 mM TCA was added., and the incubation process was repeated for another 30 minutes at the previously mentioned temperature, after being chilled at 37^0C. then the solution was filtered using watt-man filter paper. filtrate of 2 ml was then added with 500 mM sodium carbonate and 5 mL of 5Mm foline-ciocalteu reagent. The mixture was incubated at 37 ^{0}C for 30 minutes then the absorbance at 600 nm was measured using an ultraviolet-visible spectrophotometer.

Lipase activity

The specific activity of lipase was assessed using the technique outlined by (Lott *et al.*,1986).0.25ml of supernatant, 1ml of phosphate buffer (pH 7), and (0.25ml) of olive oil were combined and incubated at 30^0C for 15 minutes. The reaction mixture was then given a thorough shake, and 2 ml of ATC reagent and 10 ml of chloroform were added after it had been left to stand for 10 minutes. A 2ml pipette of chloroform layer was then removed. Lipase colouring agent (1 ml) was added. The colour was measured at 550 nm.

Gene expression analysis

for analysis of gene expression of growth and feed intake related gene (Ghrelin and myogenin) for few tissue samples of fry from each group were fixed in RNA latter and stored at 4^0C until further analysis.

Isolation of RNA

Gene expression was analysed by real time PCR. (APPLIED biosystem, foster citty, CA, USA). In bried, tissue samples were removed from RNAlatter, homogenized and (0.5ml) chilled TRLzol reagent was added. Then the incubation was done at ambient temperature for 5 minuties before being applied to chloroform (0.1ml) and vigorously agitated for 15sec.the sample was again incubated for 5 min at room temperature before being centrifuged at 12000rpm for 5 min at 4. Afterwards the upper colourless aqueously layer was separated and transferred to new micro centrifuged. the separated aqueous phase was vortexed briefly with chilled iso prophy alcohol (absolute) prior to centrifugation the samples were again incubated for 10 minuties at room temperature and hen centrifuged at 12000rpm for 10 mins. The pellets were washed in 75 % ethanol (0.5ml) (prepared in DEPC treated water) and liquid phase was discarded. After that air drying of pellets was done and then dissolved in $50^0\mu$ of nuclease-free water. The isolated RNA was stored at -80 ^{0}C analysis.

Quantification of RNA

Nano drop ND1000(thermos scientific USA) was used to evaluate the quality and quantity of RNA. The quantification of total RBA concentration in sample was

performed by measuring absorbance at 260nmwhile to check the purity of samples the Nd at 260 and 280 was used with estimated values between 1.9 and 2.0.

Synthesis of c DNA

Each isolated RNA was reverse transcribed to c DNA by using the method as reported earlier by (Amir *etal* ,2019) in brief, the reaction mixture of 20 µLwas prepared by mixing 8µL RNA,4µL of dNTPs,1µLof MMLV-RT,2.5µL random primer,0.5 µL and 3 µL DEPC water. The reaction mixture was incubated in water bath at 37 degrees for an hour and then in pcr machine (BIO-RADT100TM thermal cycler) at 55^0C for 5 min. Nano drop ND1000 (Thermo scientific, USA) was used evaluate the concentration of synthetic cDNA in each sample. The prepared cDNA was immediately stored at temperature of -20^0C.

***µ*Primer designing**

Primer shown in table 4 were self-designed and oligo primer analysis software version1.1.2 was used for primers designing and manufacturing from humanizing genomics macrogen. The nucleotide sequence of corresponding genes of *L.rohita* was obtained from gene bank NCBI (www.ncbi.nlm.nih.gov). The quality of cDNA and its compatibility with primer was checked by performing a simple PCR followed by gel electrophoresis of each sample in duplicate. Afterwards, qPCR was performed protocol previously reported by (Ahmad *et al*., 2020). briefly, 20µL reaction mixture was prepared by mixing 0.4µL of reverse and 0.4µL of 4µLwith 7.6µL of syringe water,10µL of SYBER GREEN and 1.6µL of diluted cDNA. The pcr condition were optimized along with cycle numbers (initial denaturation 95^0C or 4 min followed by 40 cycles at 95^0C for 15 seconds and subsequently, 62^0C for 15 sec). the efficacy of PCR reaction for each gene was measured by slope of standard curve using serial dilutions of cDNA of control sample. Them RNA levels of each gene were compared with the expression of beta actin reference gene of *Labeo.rohita*. the relative variation in gene expression were calculated by standard CT (Pfaff, 2001).

Table A: Target genes and Primer sequences

Gene	Sequence	Amp Length	TM	GC%	Accession No.
B-actin	F<AAGGGAGGTATTGTGGGTAAAC R<GTTGTCCTGGCACTCAATCT	120	57.69 57.81	45.45 50	XM_051108628.1
Ghrelin	F< TCTGCTCTTATGTGCTCTTTCC R<GTGGTGGTCTTCGATCCTTAAC	119	58.13 58.74	45.45 50	XM_051113064.1
Myogenin	F<TGAGGTCCCTGACGTCTATT R<CCATCACCCTCCTCGTTTATTT	99	57.47 58.11	50 45.45	XM_051122763.1

Statistical analysis

For the statistical analysis of the normalized data, Statistics 8.1 was used. The one-way (ANOVA) variance followed by LSD was applied to compare the experimental groups. Additionally, the significance level was set at ($p>0.05$). Graph Pad Prism Version 8.0 was used for graphs.

RESULTS

Growth performance

Growth performance of *Labeo rohita* larvae fed a diet having fish meal partially substituted with casein and fish hydrolysate after 35 days of feeding trial is presented in Table 2 and 3. The average initial body weight (0.0004g) of control and experimental groups showed a non-statistical difference ($p>0.05$). At the end of trial, the growth performance was significantly increased by partial replacement with fish hydrolysate in diets. Statistical analysis by adopting one-way ANOVA showed significant difference in FBW ($n=3$, $F_{3, 26=}274$, $p=0.001$), WG ($n=3$, $F_{3,26}=337$, $p=.001$), WG% ($n=3$, $F_{3,26}=25.5$, $p=0.001$), DWG ($n=3$, $F_{3\ 26}=337$, $P=0.001$) and SGR ($n=3$, $F_{3,26=}3.22$ $P=.01$), SR% ($n=3$, $F_{3,26}=4.5$, $p=0.03$) among all the experimental groups. Although pairwise comparison of different groups indicated significant increase in weight gain of PH_2 group (6% hydrolysate in diet) of larvae, then followed by PH_1(4%) and control diet. However, minimum growth was observed in PH_3 group (8%). Additionally, in diet having fish meal partially substituted with casein protein, a significant ($p<.05$) increase was observed in dose dependent manner on FBW($n=3$, $F_{3\ 26}=548$, $p=0.001$), WG ($n=3$, $F_{3,26}=544$, $p=0.001$), WG% ($n=3$, $F_{3,26}=544$, $p=0.001$), DWG ($n=3$, $F_{3,\ 26}=377$, $p=0.001$) and SGR% ($n=3$, $F_{3,26}=7.8$, $p=0.005$) among all experimental groups. Pairwise comparison of results showed highest weight gain in PC_3 group of larvae followed by PC_2> PC_1>Control. But SR% was highest in PC_2 (10%) ($n=3$, $F_{3,26}=10.7$, $p=0.03$) and lowest in PC_3(15%) group of post larvae.

In comparative analysis of WG of post larvae fed a diet having fish meal partially substituted with casein and fish hydrolysate. One-way ANOVA showed a significantly highest WG in PC_3(15%) group of post larvae ($n=3$, $F_{6,14}=308$, $p=0.001$), then followed by PC_2(10) and PH_2(6%) group, PC_1(5%) and minimum in PH_3(8%) and control group.

Gut histology

Histological observations of the gut of *Labeo rohita* larvae after feeding a diet having partially substituted fish meal with fish hydrolysate and casein for 35 days are presented in table 4 and 5. Results showed that substitution had significant effect ($p<0.05$) on gut morphology of larvae. One-way ANOVA showed a significant

difference in villi height (n=3, $F_{3,26}$=3213, p=0.001) villi width (n=3, $F_{3,26}$=160, p=0.001) and its absorptive area (n=3, $F_{3,26}$=1607, p=0.001) in PH_2 (6%) group of post larvae, followed by PH_1(4%), control and then minimum in PH_3 (8%). Furthermore, in diet having fish meal partially substituted with casein protein, significant difference was observed in PC_3 (15%) fed groups of larvae that displayed the maximum villi height (n=3, $F_{3,26}$=12365, p=0.001), villi width (n=3, $F_{3,26}$=1681, p=0.001) and its absorptive area (n=3, $F_{3,26}$=1.3×10^7, p=0.001) of intestine then followed by PC_2 (10%), PC_3 (5%) and least in control group.

Intestinal enzymes

Specific activities of digestive enzymes of *Labeo rohita* larvae fed a diets of partially substituted fish meal with fish hydrolysate and casein after 35 days are presented in table 6 and 7. This partial substitution of fish meal had a significant effect (p>0.05) on digestive enzymes. The statistical analysis by using One-way ANOVA showed significant highest protease enzyme activity in PH_2 (6%) group of larvae (n=3, $F_{3,26}$=317, p=0.001) then followed by PH_3(8%), PH_1(4%) and least in control group. Similarly, amylase enzyme activity (n=3, $F_{3,26}$=561, p=0.001) and lipase enzyme (n=3, $F_{3,26}$=4809, p=0.001) was also highest in PH_2 (6%) group of larvae and lowest in PH_3 (8%). Moreover, the diet having fish meal substituted with casein the significantly highest protease (n=3, $F_{3,26}$=99.7, p=0.001) and amylase activity (n=3, $F_{3,26}$=3397, P=0.001) was observed in PC_3(15%) group of larvae then followed by PC_2(10%) and PC_1(5%), while low in control diet. While lipase enzyme activity was highest in control diet then followed by PC_2(10%) and PC_1(5%) minimum in PC_3(15%).

Gene expression

The comparative analysis of Gene expression of Myogenin in diet having partial substitution of fish meal with fish hydrolysate and casein are presented in Fig:4. One way ANOVA showed significant differences (P<0.05) in comparative expression level of Myogenin and Ghrelin among all experimental groups of rohu larvae. Pair-wise comparison showed significant upregulation in relative expression of Myogenin gene was observed in PC_3 group of post larvae then followed by PC_2 and PH_2>PH_1>PC_1 and control diet. Furthermore, in case of Ghrelin the gene expression was observed to be highest in PC_3 group then followed by a comparable gene expression in PC_2= PH_2 group

and PC_1=PC_2 >control. While lowest expression was observed in PH_3 group of post-larvae

Table 1: Feed formulation of 50% of crude protein for larvae of *labeo rohita*

Ingredients	**Control**	**PH_1**	**PH_2**	**PH_3**	**PC_1**	**PC_2**	**PC_3**
Fish meal	50	46	44	42	45	40	35
Soya bean meal	15	15	15	15	15	15	15
Fish hydrolysate	----	4	6	8	-----	----	----
Casein	----	----	----	-----	5	10	15
Gluten	15	15	15	15	15	15	12
Rice bran	6	6	6	6	6	6	8
Wheat bran	6	6	6	6	6	6	7
Premixes [a]	2	2	2	2	2	2	2
Fish oil	4	4	4	4	4	4	4
CMC	2	2	2	2	2	2	2
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Proximate composition

Parameter	**control**	**PH_1**	**PH_2**	**PH_3**	**PC_1**	**PC_2**	**PC_3**
N×6.25%[b]	48.5	48.9	49.0	47.5	49.1	48.7	49.2
Lipid%	11.0	11.6	12.3	11.6	11.6	10.0	11.3
Ash%	8.12	8.34	7.89	7.92	8.42	7.94	7.75

[a] Composition of mineral premix kg− 1: manganese, 53 g; zinc, 40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; selenium, 70 mg; cobalt, 70 mg and calcium carbonate as carrier up to 1 kg.
Composition of vitamin premix kg− 1: vitamin A, 8,000,000 IU; vitamin D3, 2,000,000 IU; vitamin E, 7000 mg; vitamin K3, 1500 mg; vitamin B1, 700 mg; vitamin B2, 3500 mg; vitamin B6, 1000 mg; vitamin B12, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid, 7000 mg.
[b]Crude protein

Table 2: Effect of partial replacement of fish meal with graded level of fish protein hydrolysate on growth performance larvae of *Labeo rohita* after 35 days of feeding trial.

Parameter	Control	PH_1	PH_2	PH_3	F value	P value
IBW (mg)	0.40 ± 0.07^{a}	0.42 ± 0.01^{a}	0.41 ± 0.01^{a}	0.42 ± 0.01^{a}	…	Ns
FBW (g)	1.42 ± 0.01^{c}	1.65 ± 0.01^{b}	1.73 ± 0.01^{a}	1.34 ± 0.01^{d}	274	0.001
WG (g)	1.41 ± 0.01^{c}	1.64 ± 0.05^{b}	1.73 ± 0.01^{a}	1.34 ± 0.01^{d}	337	0.001
WG%(g)	352238 ± 6224^{c}	400113.7 ± 14619^{b}	431591 ± 6556^{a}	333950 ± 3939.5^{c}	25.5	0.001
DWG (g)	0.040 ± 0.01^{c}	0.047 ± 0.07^{b}	0.049 ± 0.06^{a}	0.038 ± 0.01^{d}	337	0.001
SGR $\%d^{-1}$	22.6 ± 0.05^{a}	22.7 ± 0.05^{ab}	22.8 ± 0.07^{a}	22.62 ± 0.03^{b}	3.22	0.01
SR%	67 ± 0.6^{b}	70 ± 0.8^{ab}	72 ± 1.7^{a}	66 ± 1.3^{b}	4.5	0.03

Data is presented as Mean+SE (n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different levels of fish hydrolysate groups as presented in rows. The values having different upper-case letters show the significant difference ($p<0.05$).

Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% of fish hydrolysate-based diet. **IBW**=initial body weight, **FBW**=final body weight=weight gain, **WG%**=weight gain percent, **DWG**=daily weight gain, **SGR=**specific growth rate, **SR%**=survival rate.

Table 3: Effect of partial replacement of fish meal with graded level of casein protein on growth performance *Labeo rohita* larvae after 35 days of feeding trial**.**

Parameter	Control	PC1	PC2	PC3	F value	P value
IBW (mg)	0.41±.008[a]	0.41±.008[a]	0.41±0.01[a]	0.40±0.005[a]	..	Ns
FBW (g)	1.38±0.7[d]	1.55±0.8[c]	1.75±1.0[b]	1.86±1.0[a]	548	0.001
WG (g)	1.37±0.01[d]	1.55±0.01[c]	1.74±0.01[b]	1.86±0.01[a]	544	0.001
WG%(g)	328500±1585.9[d]	370694.7±6168[c]	410642±10462[b]	451603.4±5997.8[a]	544	0.005
DWG(g)	0.039±0.04[d]	0.044±0.02[c]	0.050±0.01[b]	0.053±0.01[a]	377	0.001
SGR %d^{-1}	22.53±0.02[c]	22.6±0.04[bc]	22.7±0.07[ab]	22.8±0.02[a]	7.86	0.005
SR%	67±0.6[b]	74±0.8[a]	73±1.4[a]	69±0.5[b]	10.7	0.03

Data is presented as Mean+SE(n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different dietary levels of casein groups as presented in rows. The values having different upper-case letters show the significant difference ($p<0.05$).

Control= given 50% cp of FM based diet, PC_1=5%casein, PC_2=10% casein, PC_3=15%casein of casein-based diet. **IBW**=initial body weight, **FBW**=final body weight=weight gain, **WG%**=weight gain percent, **DWG**=daily weight gain, **SGR=**specific growth rate, **SR%**=survival rate.

Table 4: Effect of partial replacement of fish meal with graded levels of fish hydrolysate on specific activities digestive enzymes of *Labeo rohita* larvae after 35 days of feeding trial.

Digestive enzymes	**Control**	**PH_1**	**PH_2**	**PH_3**	**F value**	**P value**
A**mylase**(U/mg**)**	0.58±0.05^{c}	0.73±0.05^{b}	0.79±0.04^{a}	0.40±0.04^{d}	504	0.001
Lipase(U/mg**)**	1.12±0.01^{c}	1.16±0.01^{b}	1.22±0.01^{a}	1.1±0.04^{d}	4809	0.001
Protease(U/mg**)**	0.19±0.01^{c}	0.21±0.04^{b}	0.23±0.03^{a}	0.14±0.07^{d}	317	0.001

Data is presented as Mean+SE(n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different levels of fish hydrolysate groups as presented in rows. Intestinal enzymes were significantly affected by different levels of fish hydrolysate. The values having different upper-case letters show the significant difference ($p<0.05$).

Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% of fish hydrolysate.

Table 5: Effect of partial replacement of fish meal with graded level of casein protein on specific activities digestive enzymes *Labeo rohita* larvae after 35 days of feeding trial.

Digestive enzymes	**Control**	**PC_1**	**PC_2**	**PC_3**	**F value**	**P value**
A**mylase (**U/mg)	0.58 ± 0.01^{c}	0.69 ± 0.01^{b}	0.74 ± 0.01^{a}	0.72 ± 0.01^{a}	94	0.001
Lipase (U/mg)	1.11 ± 0.01^{c}	1.13 ± 0.01^{bc}	1.16 ± 0.02^{a}	1.15 ± 0.01^{ab}	8.56	0.007
Protease (U/mg)	0.19 ± 0.01^{d}	0.21 ± 0.01^{c}	0.23 ± 0.04^{b}	0.26 ± 0.01^{a}	26.7	0.001

Data is presented as Mean+SE(n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different levels of casein protein groups as presented in rows. Intestinal enzymes were significantly affected by different levels of casein. The values having different upper-case letters showed the significant difference ($p<0.05$).

Control= given 50% cp of FM based diet, PC_1=5%casein, PC_2=10% casein, PC_3=15% of casein.

Table 6: Effect of partial replacement of fish meal with graded levels of fish hydrolysate on intestinal Villi height and villi's width Labeo *rohita* larvae after 35 days of feeding trial**.**

Parameter	Control	PH_1	PH_2	PH_3	F value	P value
Villi height(µm)	186 ± 0.9^{d}	223.6 ± 0.6^{b}	276.6 ± 0.6^{a}	201.4 ± 0.5^{c}	3213	0.001
Villi width(µm)	76 ± 0.9^{d}	107 ± 0.7^{b}	145 ± 0.8^{a}	85.4 ± 0.5^{c}	1607	0.001
Absorptive area(µm)	8554 ± 1.7^{c}	13457 ± 2^{b}	16777 ± 1.5^{a}	$1154\pm.8^{d}$	2.4×10^{7}	0.001

Data is presented as Mean+SE(n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different levels of fish hydrolysate groups as presented in rows. Intestinal villi height and villi's width were significantly affected by different levels of fish hydrolysate. The values having different upper-case letters show the significant difference ($p<0.05$)

Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% fish hydrolysate.

Table 7: Effect of partial replacement of fish meal with of graded level of casein protein on villi height and villi's width *Labeo rohita* larvae after 35 days of feeding trial.

Parameter	Control	PC_1	PC_2	PC_3	F value	P value
Villi height(µm)	186 ± 0.9^{d}	243 ± 0.8^{c}	276 ± 0.6^{b}	395 ± 0.5^{a}	12365	0.001
Villi width(µm)	76 ± 0.9^{d}	85 ± 0.7^{c}	93 ± 0.5^{b}	145 ± 0.8^{a}	1681	0.001
Absorptive area(µm)	8554 ± 1.7^{d}	14891 ± 1.1^{c}	16777 ± 1.2^{b}	18992 ± 0.5^{a}	1.3×10^{7}	0.001

Data is presented as Mean+SE(n=3). One way ANOVA followed by LSD post hoc test that demonstrates a pairwise comparison among different level of casein protein groups as presented in rows. Intestinal villi height and villi's width were significantly affected by different levels of casein. The values having different upper-case letters show the significant difference ($p<0.05$)

Control= given 50% cp of FM based diet, PC_1=5%casein, PC_2=10% casein, PC_3=15% of casein in diets

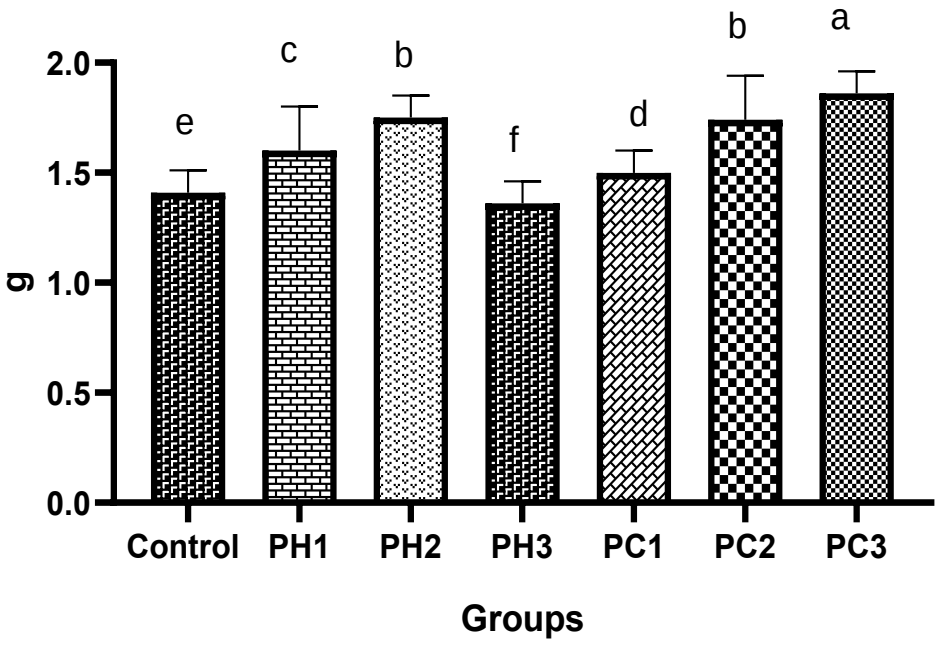

Fig 2: Comparative effect of WG of *Labeo rohita* larvae after feeding a diet having fish meal partially substituted with fish hydrolysate and casein protein. Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% hydrolysate & PC_1=5%casein, PC_2=10% casein, PC_3=15%casein in diets.

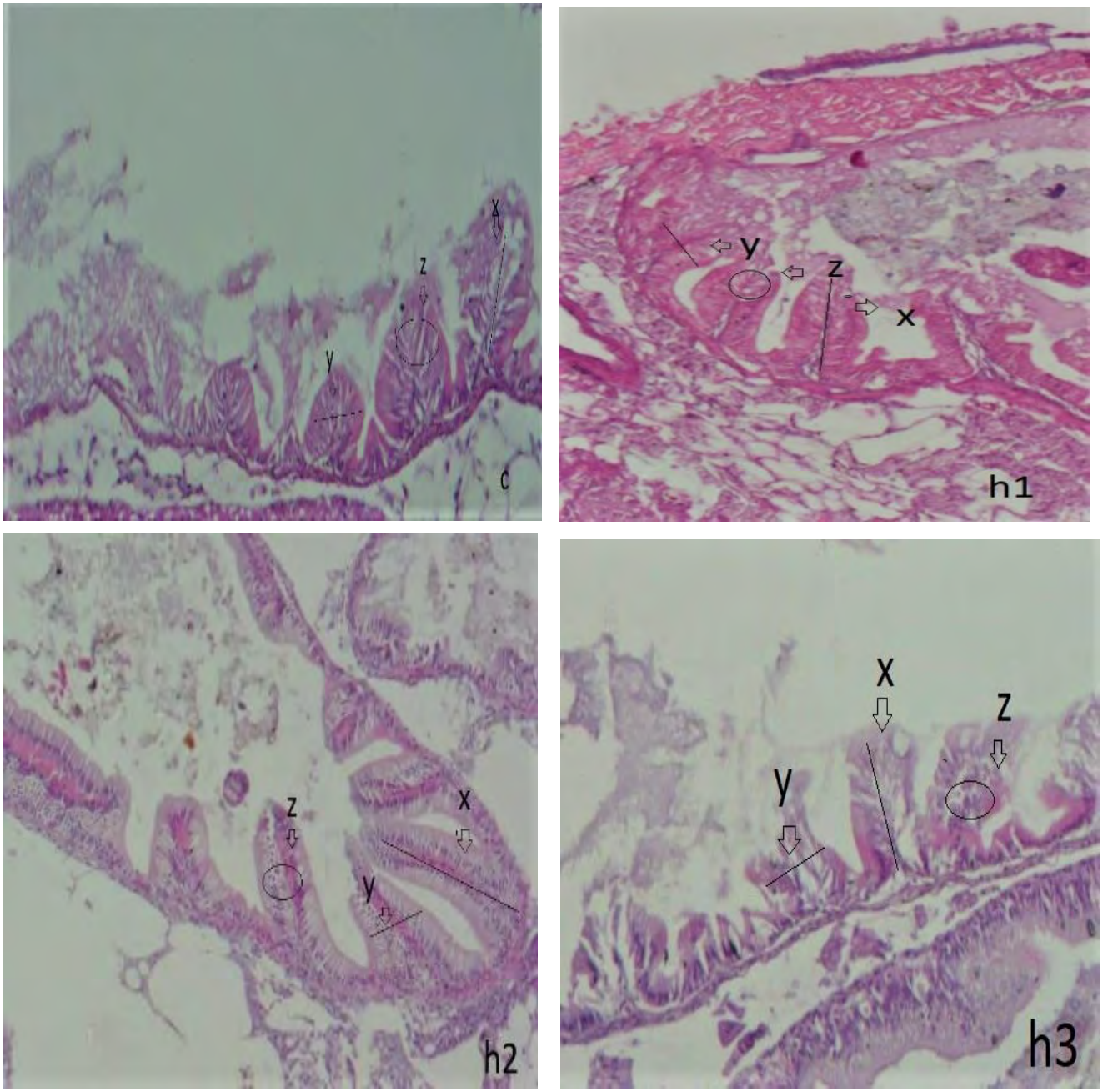

Fig:3 Gut histology of *Labeo rohita* larvae after 35 days feeding a diet having partial substation of fish meal with fish hydrolysate .**x**=villi length, **y**=villi width and **z** =absorptive area.

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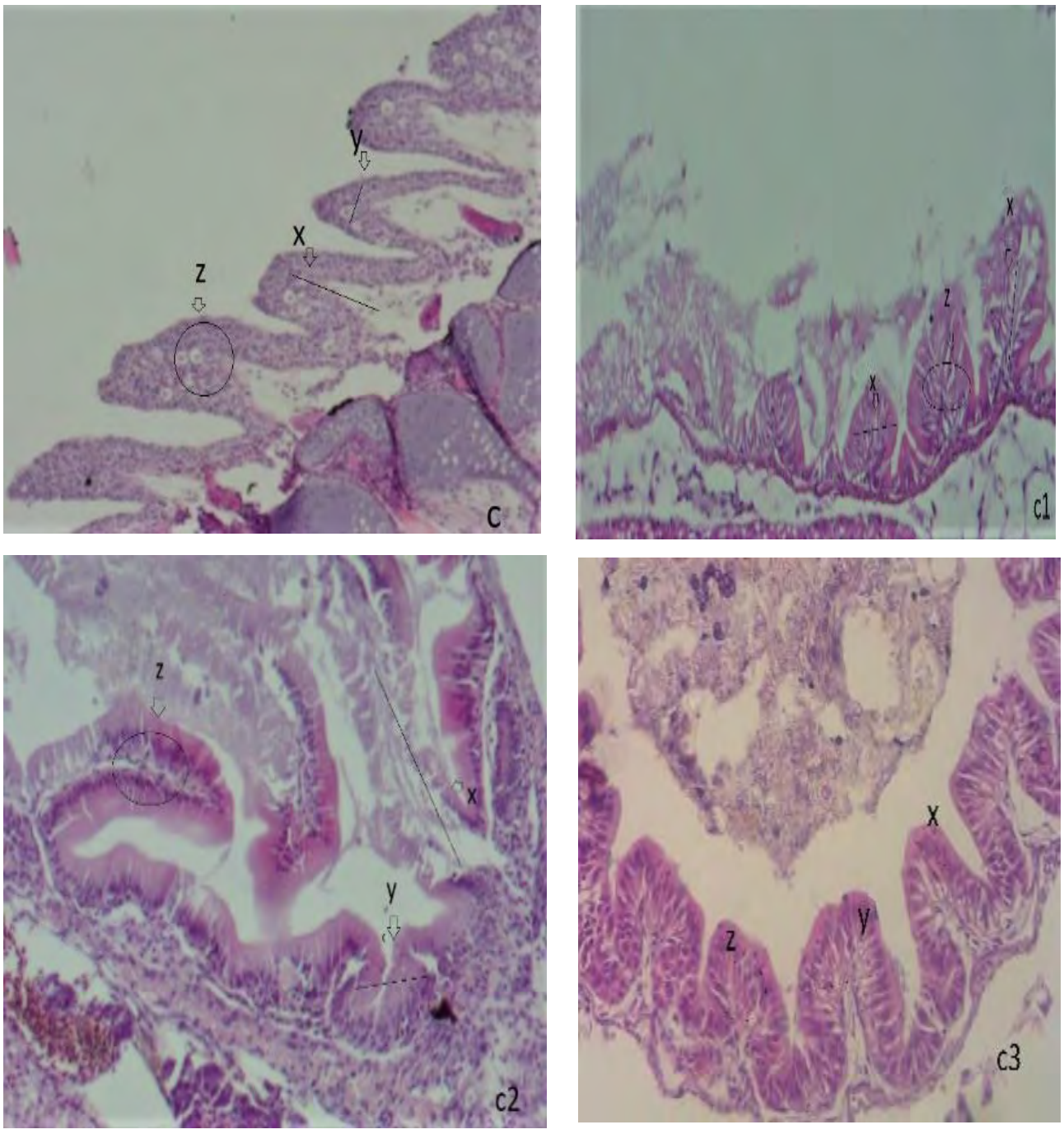

Fig:4 Gut histology of *Labeo rohita* larvae after 35 days feeding a diet having partial substation of fish meal with casein=villi length, **y**=villi width and **z** =absorptive area

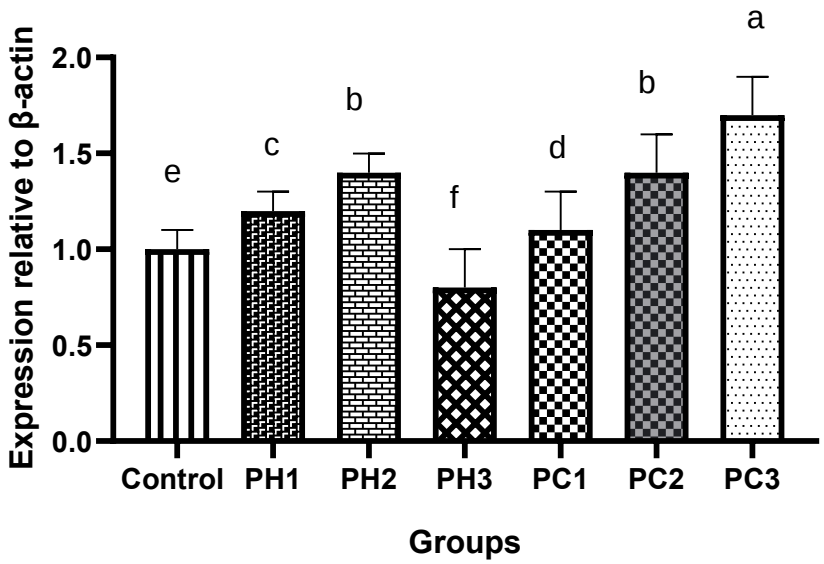

Fig:**5** Comparative analysis of gene expression of myogenin in *Labeo rohita* larvae after feeding a diet having fish meal partially substituted with fish hydrolysate and casein. Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% of hydrolysate & PC_1=5%casein, PC_2=10% casein, PC_3=15% of casein in diets.

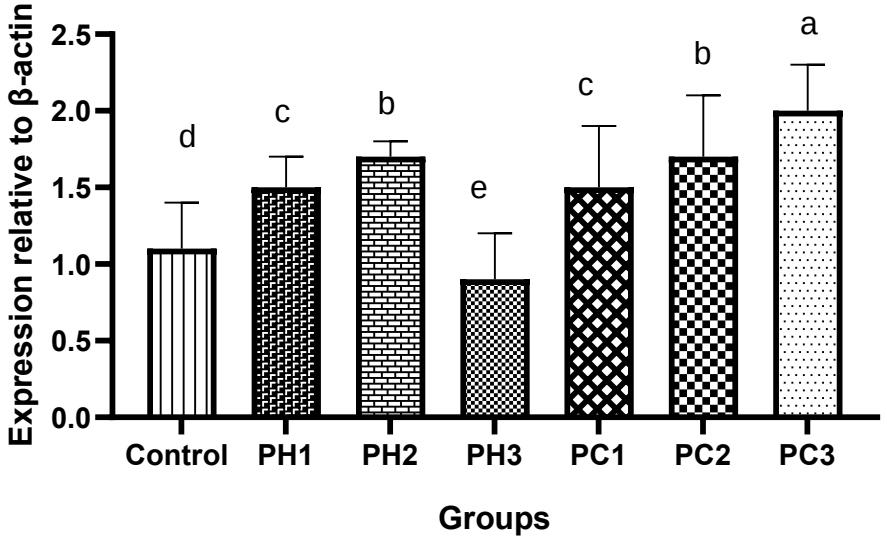

Fig:**6** Comparative analysis of gene expression of Ghrelin in *Labeo rohita* larvae after feeding a diet having fish meal partially substituted with fish hydrolysate and casein. Control= given 50% cp of FM based diet, PH_1=4% hydrolysate, PH_2=6%hydrolysate, PH_3=8% of hydrolysate & PC_1=5%casein, PC_2=10% casein, PC_3=15% of casein in diets

DISCUSSION

The larval stage is a crucial time in the lives of many species, and success in larva rearing depends mainly on access to a suitable diet (Giri *et al*., 2002*)*. If the diet lacks essential nutrients, mortality can reach as high as (70-80%) during the early stages of development, particularly during the transition from the yolk sac to the first feeding stage, and growth may be hindered (Ringo & Birkbeck, 1999*)*. The unavailability of cheap prepared larval feed in Asia leads to the use of live food organisms for the rearing of fish and shellfish (Kolkovski, 2001). However, this method faces many challenges such as the deficiency of essential nutrients, presence of pathogens and microorganisms, and variations in the ratio and quantity of nutrients (Hamre, 2016). To reduce costs and increase juvenile production reliability, aquaculture research has focused on replacing live food with artificial diets for the early stages of fish development (Zambonino Infante & Cahu, 2001). The availability of proper foods that easily palatable and contain the necessary amount of nutrients to promote the growth and development of fish is a major factor in the early rearing larval stages in captivity. The feed take of larvae depends on the texture and more specifically on the size of the feed particles. A larvae cannot take the larger feed particles because of their small sized mouth, so the feed was converted into nanoparticulated size in the currently reported study. Additionally, the quantity and kind of nutrients needed by fish during early ontogeny vary depending on their age and organogenesis (Malla & Banik, 2015), Evaluating the dietary protein needs is crucial for supplying an adequate amount of nutrients to cultured species to achieve optimal growth (Khan & Maqbool, 2017).

In recent years, research has focused on developing diets for larvae using more digestible protein sources like fish protein hydrolysates (Hamre *et al*., 2013*)*.In the current study fish protein hydrolysates has been used to enhance the growth and survival of fish and reduce the dependence on fish meal and casein, a protein derived from milk, is commonly used as a protein source in animal nutrition, including the nutrient requirements studies of fish and other vertebrates (Deshimaru, 1982), it provides essential amino acids that are crucial for the growth and development of fish larvae and can improve growth and survival rates when added to their diet (Cowey *et al*., 1971). In the present study, we investigated nutritional programming as a strategy

to improve the metabolic utilization of dietary protein in rohu larvae (an herbivorous freshwater fish).

Protein hydrolysates have been recognized as good supplements for feeds, (Cordova, Murueta & Garcıa-Carreno, 2002). The incorporation of FPH in artificial larval diets. has been suggested as an alternative approach to overcoming the limited digestive capacity of fish larvae (Dabrowska *et al*., 1979; Dabrowski, 1984; Govoni *et al*., 1986). Pre-hydrolysed protein has been used in artificial diets to improve protein availability and thus enhance the growth of marine larval and juvenile fish (Turbot, Scophthalmus maximus: Oliva-Teles *et al*. 1999) Gilthead seabream, *Sparus aurata*: Cahu & Zambonino-Infante, 2001; Common carp, *Cyprinus carpio*: Carvalho *et al*., 2004). Moreover, Refstie *et al*, (2004) found that the dietary substitution of 10% to 15% of fish meal by a commercial enzymatically treated FPH positively affected the growth performance of Atlantic salmon. More recently, Liang *et al*, (2006) reported that the dietary addition of 15% FPH prepared from pollock by-products supported higher growth in Japanese sea bass compared with higher and lower inclusion levels. Furthermore, Tang *et al*, (2008) have shown that including up to 10% fish protein hydrolysate (FPH) in the diet of yellow croaker (*Pseudosciaena crocea*) improved the growth and immunological parameters of this fish. Likewise, Lian *et al*, (2005) and Chotikachinda *et al*, (2013) found positive effects of FPH supplementation in the diet of the studied species. (Cordova-Murueta and Garcıa-Carreno, 2002) reported that juvenile Pacific white shrimp fed feeds supplemented with fish protein hydrolysate at 3 and 9% had better growth performance than those fed feeds supplemented with fish protein hydrolysate at 0 and 15%.

In the present study, PH_2 group of post larvae fed a larval diet having (6%) of fish meal substituted with fish hydrolysate showed statistically high FBW, WG, WG%, DWG, SGR% and SR% than all other groups. Our results are in line with the findings of (Refstie *et al*., 2004; Hevroy *et al*., 2005*)*, Turbot on Scophthalmus maximu; Oliva-Teles *et al*., 1999; Gilthead seabream, *Sparus aurata*: Cahu & Zambonino-Infante 2001; Common carp, *Cyprinus carpio*: Carvalho *et al.*, 2004) and (Berge & Storebakken, 1996; (Khosravi *et al*., 2015); (Refstie *et al.,* 2004), on shrimp (Hernández *et al*., 2011) and abalone (Goosen *et al.,* 2014) also observed a positive effect on growth resulting from dietary FPH. However, all these researchers studied the effects of protein

hydrolysate on larval fish species. In the above studies, though moderate supplementation of pre hydrolysed proteins in artificial diets has been shown to improve larval growth. Furthermore, it may due to the fact that these partially hydrolysed proteins and dipeptides and tripeptides are absorbed more efficiently into the bloodstream, improving utilization of the protein. (Fairclough *et al.,* 1980; Zhanghi & Mattews, 2010) these Hydrolysed protein and dietary amino acids have shown to up-regulate the production of intestinal PepT1 mRNA, increasing PepT1's intestinal transport ability and facilitating small peptide absorption (Osmanyan *et al.*, 2018).Moreover and its positive effects were also due to improved palatability and high digestibility, leading to the stimulation of digestive enzymes and more efficient nutrient absorption for biomass production (Hevroy *et al*., 2005 & Zheng *et al*., 2013). The positive effects on the growth rate and the final mass of salmon juveniles, fed on a diet containing 5 to 8% FPH, are mentioned by (Berge & Storebakken, 1996).

Moreover, PH_3 group of post larvae fed larval diet, (8%) of fish hydrolysate incorporated in diet by partially replacing fish meal have minimum FBW, WG, WG%, SGR%, SR%, Our results are in agreement with findings reported by (Carvalho *et al*., 2004) also found that a dietary excess of di- and tripeptides detrimentally affected the performance of common carp larvae in early feeding stages. Excessive dietary FPH inclusion can lead to decreased growth performance and larval survival (Cahu, 1999; Espe *et al*., 1999; Kvale *et al*., 2009). Higher replacement levels of fish meal by FPH can leads to decreased growth in aquaculture species (Carvalho *et al*., 2004; Hernandez *et al*., 2013; Ospina-Salazar *et al*., 2016). It may due to a fact that in diet that is rich in hydrolysate would result in a sudden release of nutrients, including amino acids and peptides, in the intestine, which may cause transporter mechanisms to become saturated (Kolkovski & Tandler, 2000). Pre-hydrolysed protein sources are added to larval diets to increase A.A availability, but leaching can occur due to increased solubility and lowered molecular weight. Rapid loss of nutrients from feed particles before ingestion by larvae has been reported, especially for free amino acids.

The digestive enzymes present in the gut of fish play a crucial role in the breakdown and absorption of nutrients. It's essential to understand the developmental changes that occur during the early stages of a fish's life as it provides insights into their ability to process different food components (Buddington & Doroshev, 1986; Rathore

et al., 2005**).** Lack of digestive enzymes in fish larvae results in poor nutrient utilization during the transition from endogenous to exogenous feed (Degura *et al*., 2003). Adequate and proper quality nutrition is crucial for better growth and survival of larvae (Mitra & Mukhopadhyay, 2009).

Protease activity is linked to protein content of diet (Xiong *et al*., 2012). Protease enzyme is the quantity of enzyme giving an increase absorbance of 1.0 at 440nm. In present study, the PH_2 group of post larvae fed larval diet having (6%) fish meal substituted with hydrolysate had significantly highest protease activity (0.23±0.01U/mg) than other groups. protein. Similar to results obtained by (Aguila *et al*., 2007) who observed a significantly high level of total protease in the digestive gland of Mexican four-eyed octopus (*Octopus maya*) fed FPH. This may be due to the fact that our diet contains 50% crude protein. (Lopez-Lopez *et al*., 2005) reported that there is correlation between protease activity and dietary crude.

Amylase is responsible for carbohydrate digestion (Kumar & Chakravarty, 2018). Its activity could be regarded as an indicator of pancreas maturation in fish larvae (Cahu *et al.,* 2004). In current study the activity of amylase was highest in PH_2 (6%) group of post larvae as compared to all other groups. This might be due to differential maturational time of pancreas by graded level of dietary AA might be one reason for variation in amylase activity in the larvae of different dietary groups. Our findings are in contrast to (Yang *et al.,* 2021) and (Xu & Zhou, 2005) who observed towering activity of amylase enzyme by feeding a diet containing hydrolysate in *L. vannamei* and hybrid grouper respectively

Lipase enzymes are responsible for lipid digestion (Sheridan, 1989). In our study, the activity of lipase enzyme was higher in PH_2 (6%) group of post larvae as compared to all other groups. and same findings have been described by (Klomklao *et al*., 2006) who studies the effect of fish silage in *L. rohita* fingerlings, this is may be due to the fact that the group with high fish hydrolysate concentration in their diet also had higher lipid content and, as a result, higher will be its lipase enzyme activity. The possible influence of dietary AA on the augmentation of the enzyme activity might be due to increased need of digestion of lipid to meet the increased energy and fatty acid requirement during rapid larval development (Johnston *et al*., 2006).

The histological examination of the structures within the digestive system is considered an effective means of evaluating the nutritional status of fish larvae (Gisbert *et al.,* 2004). A recent study shows that PH_2(6%) group of post larval have improved in the height and width of the villi, as well as in their absorptive area. The increased height, width and absorptive area of the villi results in an improvement in the integrity of the apical brush border and the absorptive area, which may lead to a better uptake of nutrients (Salze *et al., 2008;* Dimitroglou *et al.,* 2009; Daniels *et al.,* 2010). In PH_3(8%) post larva group had observed that there was decrease in villi height, width, and absorptive area its low level has high plasticity impact on size and structure of GI tract of rohu larvae (Yu *et al*., 2012). Further study is needed to determine the effects of fish hydrolysate on tissue of GI tract.

A sufficient supply of dietary amino acids is a prerequisite for high growth rates. Compound feeds usually have high nutrient density, high relative protein content and are based on ingredients that have good amino acid balance as far as sustaining growth in fishes is concerned.

In the current investigation, labeo rohita larva readily accept formulated diet, the study showed that PC_3 (15%) group of post larvae showed maximum FBW, WG, WG%, DWG, SGR%, as compared to other groups. Our findings are consistent with observation made by (Rumsey & Ketola,1975) reported the significant improvements in growth of Atlantic salmon by supplementing casein with mixtures of amino acids to simulate the improvement in protein utilization. Moreover, similar finding was observed by (Carvalho, SaL, Oliva-Teles & Bergot, 2004) on common carp larvae. Furthermore, the study of (Teshima & Kanazawa, 1984) indicates that larval growth depends on adjustment to the nutritional needs the highest larval growth was obtained with diet containing 60% casein and 10% starch in artificial diet. But current study contrasted with (Sen *et al*., 1978) first-feeding common carp larvae were found to be unable to survive and grow up satisfactorily on diets with native casein as the only nitrogen source, contrarily to juveniles that seem to use this protein efficiently may be due to Casein diets improved digestibility may stem from individual, higher-quality energy ingredient*s* (Ogino & Chen, 1973; Asgard & Austreng, 1985; Eid & Matty, 1989) and also in our study casein was used in mixture with fish meal due to balanced amino acid profile of both enhanced the overall growth of rohu larvae. While our results

are in contrast with (Lim *et al.*, 1979; Deshimaru, 1982) also reported that casein protein in diet decreased growth rate through an increase in protein degradation as the mortality rate was very high with the casein diet. Therefore, casein is likely to be a poor protein source for crustaceans as well. (Goolish *et al*., 1999) also reported the much poorer survival registered with a casein-based diet (23% at day14) might be partly attributed to the kind of casein used. (Fyhn, 1989) also found that high level of casein had detrimental effect on larvae growth and survival. (Szlaminska *et al*., 1993) also suggested that, despite the digestive potential of larvae, the hard texture of the native casein would limit its utilization by preventing the penetration of larval proteolytic enzymes and then reducing protein degradation.

The development of digestive enzymes during early stages of various species has been documented by various researchers (Pradhan *et al*., 2013), little is known about the effect of diets on the intestinal enzymes of *L. rohita* larvae. Nutrition has a significant impact on the adjustment of the digestive system (Fernandez *et al*., 2001), and changes in intestinal enzyme activity may be linked to both the feeding habits of the fish and the biochemistry of the feed (Kuzmina *et al*., 1996).

In the current study, it was observed that the protease activity in PC_3(15%) group of post larvae was higher (0.470 ± 0.01 U/mg) as compared to all other groups. Our findings are parallel with the study of (Kuzmina, 1996) who discovered a high capability of non-carnivorous fish to effectively utilize plant-based protein sources. This suggests that *L. rohita* larvae possess a strong proteolytic ability, enabling them to use protein in their diet for tissue formation and growth, which is critical during their rapid growth phase. The connection between growth and protein deposition emphasizes the significance of the larvae capability to effectively digest protein. The activity of amylase enzyme was high in PC_3(15%) group of post larvae as compared to control group reared on larval feed. The high amylase activities assayed in young larvae was also reported by (Zambonino Infante & Cahu, 1994). And the activity of lipase enzyme was significantly low in PC_3 (15%) group of post larvae reared on larval feed as compared to all other groups.

The relationship between feed components and digestive tract development is important in understanding the nutritional needs of fish during early life stages. This knowledge can guide nutrition practices. Gut morphology of fish changes greatly

during early development and is influenced by nutrition and diet (Pradhan *et al*., 2014). The study found that the height, width, and absorption of villi was highest in the PC_3 (15%) group of post larvae, leading to increased nutrient absorption and digestion (Yu *et al*., 2012).

Ghrelin the only known orexigenic gut hormone and plays an important role in energy balancing by regulating the food intake, weight of body and maintains glucose homeostasis (Dieguez *et al*., 2010; Pusztai *et al*., 2008). Ghrelin is predominantly produced in endocrine cells in the stomach (or in the intestine in species that lack a proper stomach) as well as in many other organs and tissues to a lesser extent (Jonsson, 2013). In current reported study, the expression of ghrelin was significantly affected by partial replacement of fish meal with fish protein hydrolysate and casein in diet. Therefore, in overall comparative analysis of all diets the maximum expression was observed in PC_3 group of post larvae then followed by PC_2 and PH_2 group as compared to Control. These findings suggests that these possibles positive feeding behaviour caused by experimental diets was controlled by these endocrine factors i: e ghrelin.

The study of (Shrestha *et al.* 2009; Sakurai *et al*. 2006) demonstrate the fact that ingested feed stimulates CCK expression in digestive tract first, (Stanley *et al*. 2005) then the CCK stimulates ghrelin expression in digestive tract. Consequently, it increases body mass, by stimulating GH releasing peptide that promotes growth, and together with its effect of reducing fat utilization, also promote fat storage (Lotfi *et al*., 2013; Tschop *et al.*, 2000).

Myogenesis is the generation of muscular tissue during embryonic development from stem cells, by fusion of myoblasts into multinucleated fibres (myotubes) (Johnston, 2006). This process is highly conserved in all vertebrates and requires the synchronized participation of four myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin and MRF4 (Massari and Murre, 2000). Therefore, in overall comparative analysis of all diets the maximum expression was observed in PC_3 group of post larvae then followed by PC_2 and PH_2 group and minimum expression was observes in PH_3 group fed diet as compared to Control. This might be due to fact that the expression of genes involved in myogenesis is also influenced by feed intake as reported by (Alami-Durante *et al*., 2010).

Conclusion

From the present study it is concluded that fish hydrolysate at (6%) substituted with fish meal have significant effects on growth performance, on digestive enzymes, on gut histology and on gene expression of myogenin and Ghrelin. While casein protein at (15%) substituted with fish meal have positive impact on various-studied, parameters.

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