Effect of feed in the development of digestive System during ontogeny of major carp, *Labeo rohita*

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A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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2024

IN THE NAME OF ALLAH, THE MOST MERCIFUL THE MOST BENEFICENT

AND

THE MOST COMPASSIONATE

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Dedicated To:

“To my parents who made me”

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MASHOOQ ALI

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List of Abbreviations

Abbreviations	Full Names
µl	Microliter
µM	Micromole
SNF	Standardized nanoparticulate-feed
MRFs	Myogenic regulating factors
ALP	alkaline phosphatase
LF	Live feed
PUFA	Poly unsaturated fatty acid
SAFA	Saturated fatty acid
PLs	Post larvae's
cm^3	Centimeter cube
DO	Dissolved oxygen
EDTA	Ethylene diamine tetra acetic acid
g/dL	Gram per deciliter
$HCLO_4$	Perchloric acid
HDL	High density lipoprotein
h	Hours
LSD	Least significant difference
M	Molar
mg/L	Milligram per liter
min	Minutes
mM	Milli molar
MS222	Tricaine methanesulphonate
nmol	Nanomole
OD	Optical density
PBS	Phosphate buffer saline
Ppm	Parts per million

RPM	Rounds per minute
MUFA	Monounsaturated fatty acid
sec	Seconds
SGR	Specific growth rate
NWG	Net weight gain
DAH	Days after hatching
DPH	Days post hatching
EPA	Eicosapentanoic acid
DHA	Decosahexanoic acid
CP	Crude protein
CF	Crude Fats
FCR	Feed Conversion Ratio

ABSTRACT

Production of good quality fish seed is the major constrain in carps culture industry, which directly depends on the feed/nutrients provided during ontogeny. Due to the unavailability of larval-prepared feed, postlarvae (PL) are generally cultured in nursery earthen ponds on live feed, where high mortality and stunted growth of carp's seed are reported as the major issues. Rohu (*Labeo rohita*) is the abundantly cultured Indian major carp in most Asian countries including Pakistan. The present study was designed to study the impact of live feed and prepared larval feed on the ontogeny of the digestive system of *L. rohita* and addresses the major limitations with live feed. This study was conducted in four phases. In the first phase nursery ponds were prepared by conventional methods and live feed was produced. Results indicated month-wise variation in primary and secondary productivity of ponds; green algae dominated during March, April, and May, while Diatoms dominated the rest of the months. Similarly, rotifers, Cladocerans, and copepods dominated the overall zooplankton mass. The proximate composition of both phytoplankton and zooplankton indicated an increasing trend in CP (%), CF (%) and dry matter (%) from March to August. Among Fatty acids, n-3 and n-6 polyunsaturated fatty acids, Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) indicated an increasing trend from March to July and subsequently showed a decreasing trend. In the second phase of the study, based on the proximate composition of live feed, a 60% CP nanoparticulate larval diet (ND) was prepared and a 35-day feeding trial in a replicate of five was conducted to evaluate the impact of partial and total replacement of live feed (LF) with ND on rohu (*L. rohita*) during early rearing. Larvae, 3 days after hatch (3 DAH) were evenly distributed into 3 groups; T1 reared exclusively on LF, T2 on ND and T3 co-fed with both LF and ND (1:1). For ontogenetic changes, sampling was done at 3,10,15,20,25,30 and 35DAH from the experimental groups. All groups showed feed dependent increase in growth and the expression of genes involved in feed intake and growth with age DAH. The T3 group showed significantly higher WG, SGR, and expression of *IGF-1* followed by the T2 group while the highest expression of *ghrelin* and *GHS-R* were observed in a T3 followed by a T1 group. Furthermore, *leptin* showed the highest expression in a T2 followed by the T1 group. The intestinal enzymes showed variable trends with the highest activity of cellulase, amylase and protease in the T1, T2 and T3 groups respectively. The overall result of this trial showed the most significant effect of co-feeding followed by feeding ND, thus indicating the need for standardization of the prepared diet by determining the optimum requirement of CP (%) and

crude fat (%). Thus, in the third phase of the study, two independent experimental trials were conducted to standardize protein and fat levels in the larval feed of *L. rohita*. To optimize the dietary protein requirement, four experimental larval feeds having 45, 50, 55 & 60 % CP were prepared and a 35-day feeding trial in replicate of five was conducted with the same experimental set up. Among all the diets, P55 followed by P60 showed the most significant positive effect on all studied indices except amylase activity which did not show any significant difference among groups. Moreover, in all groups, GH showed a positive correlation, while MyoD and MYF5 showed a negative correlation with age DAH. To optimize the dietary lipid level, another experiment was conducted in the same experimental set up and rearing conditions except that 55% CP was fixed in experimental diets while fat (fish oil) was added at graded levels (4, 8, 12, 16 %), using fish oil as the major lipid source. Lipid at 8% followed by 12% inclusion level showed the most significant effect on all studied indices including growth, alkaline phosphatase and lipase activity. The ontogeny variation of protease activity showed a uniform increase up to 25DAH, followed by a surge at 30DAH and then slight but non-significant decrease in activity. The ontogenetic changes in GH gene expression showed a positive correlation with age DAH, i.e., expression uniformly increased with age DAH while MyoD and MYF5 expression showed a negative relation with DAH, i.e., uniformly decreased with age DAH. After understanding the ontogenetic variation in the digestive enzymes trend in response to live feed, ND and various protein and lipid levels a standardized nanoparticulate feed (SNF) with 55% CP and 8% fish oil were formulated and prepared and 35 days feeding trial in a replicate of 5 was conducted to compare the efficiency of SNF as compared live feed (LF). Results indicated a significant effect of both independent variables (feed and age DAH) on all studied indices. The comparative effect of LF and SNF indicated significantly higher survival (%), growth rate, activity of digestive enzymes and expression of MyoD, MYF5, GH, *IGF-1* and MEF2A in a group reared on SNF while, myostatin expression was significantly higher in a LF group compared to SNF group. Both lipase and proteases were seen to be enhanced with optimum lipid and protein level in the diet (experiment 3 and 4). Discussing this increase in proteases and lipase as the possible outcome of efficient utilization (in term of early maturation of digestive tissues/organ) of optimum protein and lipids, in experiment 5 although a high protein % was available in LF but the trypsin and chymotrypsin activity was significantly higher in SNF group. This inference may conclude a slight instantaneous impact of diet over direct release of certain digestive enzymes, however the major

change come from the maturation of that tissue. Further, the only slight change in proteas activity in experiment 5 (compared to experiment 3, P55) may be due to the early shift of larvae to gut peptidases (high trypsin and chymotrypsin activity with SNF). Overall, this study confirms the role of the extrinsic factor, larval feed on the regulation of ontogenetic changes in the release of digestive enzymes and their effect on survival and growth and indicates the most significant effect of SNF compared to live feed. Thus, the replacement of life feed with SNF could be recommended for improving the survival and growth during early rearing and to produce quality seed of *L. rohita*.

Introduction

With the expansion and intensification of aquaculture, the demand for quality seed and feed according to the developmental stage of species is continuously increasing. These two inputs play a vital role in the sustainable development of any culturable species (Islam, 1989; Shoko et al., 2023). In Pakistan, aquaculture is in the juvenile stage, mostly culturing of carp dominates this sector. Initially, in most countries, direct collection of carp spawn was practiced from natural sources, like rivers during the monsoon season (May-August). However, with the deterioration of natural habitat and intensification of aquaculture practices, the collection of carp seed from nature has declined, and the aquaculture venture is now almost fully dependent on hatchery-produced seeds. In Asia, generally, conventional ways of rearing larvae on zooplankton produced by fertilizing nursery ponds (organic and in organic fertilizer) are adopted in all carp hatcheries. However, many limitations have been reported with such a classical set-up like periodic and seasonal variation in live feed (species composition), nutrient imbalance and poor supply of essential nutrients (Cahu et al., 2003), presence of parasites and carnivores zooplankton which may feed on fish larvae (Marañón et al., 2013; Menden-Deuer and Lessard, 2000), and presence of oversize zooplankton (not consumable by fish larvae) etc. In addition, limitations to manipulate the quantity and quality of nutrients like amino acids in the body composition of rotifers (Srivastava et al., 2006), artemia (Aragão et al., 2004) as these nutrients are conservative (Rainuzzo et al., 1994). Secondly, no procedure is available to control or modify nutrients and supply them to fish in optimum amounts. Because of the absence of standardized larval feed and limitations associated with live feed, the mortality % in the nursery set-up is very high in carp (70-80%) during early ontogeny (Rao, 2003). Hence, the high mortality and stunted growth during early developmental stages (Coward et al., 2002; Stige et al., 2019) are the major constraints for the sustainable development of the carp industry, especially major carp and show the need for the preparation of nutritionally balanced larval feed.

One possible way to address these limitations is to formulate and prepare a larval feed as an alternative to live feed (Bengtson, 1993; Nordgreen, 2007). However, several issues related to compound diet, e.g., pellet size and poor formulation, i.e., the inclusion of complex protein that may not be utilized properly have been reported. Hence, acceptability, palatability, and digestibility are the prominent constituents of nutritional programming while dealing with fish

larvae (Southgate and Partridge, 1998; Nordgreen, 2007). During the past 30 years, special attention has given to larval feed-related issues like formulation and preparation methods (see reviews Teshima et al., 2000; Langdon, 2003; Pepin, 2023). Though, several larval feeds are commercially available, but are limited to certain marine species yet not available for carp species. Moreover, the cost of micro bound and microencapsulated larval feeds prepared by various new techniques, limited their practical use. Many investigators attempted to prepare larval feed for marine and carp species, but, still, no significant achievement has been reported (Herath and Atapaththu, 2013). In the last decade, the co-feeding (live feed and compound diet) strategy has been in practice with some compromising results in many species for growth and survival (Hamre et al., 2013). Still, no study can show a significant positive indicator of growth with compound/prepared diet alone in Indian major carp.

The major constraints in the preparation of larval feed of major carp are the lack /little information on 1) Nutritional requirement of larvae, especially protein and lipid requirements both in terms of quantity and quality. 2) physiological changes in the digestive tract in response to feed nature during early ontogeny, i.e., gut's enzymes secretion, hormonal crosstalk, expression of digestion and metabolism-related genes etc. 3) technical challenges of producing small particles feed of high nutritive quality and 4) adjusting particle size to the size of larvae, 5) texture of larval feed and its digestibility. Generally, naturally occurring live feed contains more than 90% water which makes the feed easy for larvae to consume. On the other hand, prepared feed comprises 60-90% dry matter which is challenging for the delicate digestive tube of fish larvae. Moreover, it is well documented that to meet the fast growth potential of larvae, enough dietary nitrogen of the right quality (soluble) and quantity is required (Conceição et al., 2003). However, it is documented that most of the protein in compound feed constitute non-soluble proteins (García-Ortega et al., 2001) which remains junk for the delicate larval digestive tube resulting in delayed growth (Kolkovski, 2001).

For the last three decades, the ontogeny of the digestive tract of fish larvae has been discussed in many studies. In addition to the scientific interest regarding developmental aspects of species, most of the investigators focused on the issues of commercial hatcheries and studied how the weaning process (switch from live feed to prepared diet) can be improved and reduce the bottlenecks in larvae culture (José Luis et al., 2008; Chakrabarti and Rathore, 2010; Hamre 2013). Thus extensive literature on the histology of the gastro-intestinal tract (GI) and digestive enzyme

profile during ontogeny is available (Ostaszewska et al., 2003), while information regarding the physiology of larval GI tract is very limited. Physiological and anatomical changes in the digestive system of any fish during larval–juvenile 'metamorphosis' are extremely crucial and depend on extrinsic (environmental) and intrinsic (genetic) factors (Burggren et al., 2020; Martínez et al., 2021). Generally, the digestive capabilities and intestinal capacity to hydrolyze nutrients are regulated by genetically set memory towards its natural diet (qualitatively and quantitatively fixed) and adaptive digestive capabilities (Kolkovski, 2001). Thus, determination of such adaptive changes in the digestive system in response to early diet is essential while dealing with larval diet. The present study was executed to assess and characterize the larval diets and determine the physiological, biochemical, and anatomical changes in the digestive system of *L. rohita* larvae, feeding on live feed and prepared Nano particulate diet.

In the fish life cycle, the larval period is considered one of the most crucial phases of development that is affected by both external and internal factors. During this period, the rapid organ development and morphological changes affect further fish development and their survival (Song et al., 2019; Zadmajid et al., 2019; Nowosad et al., 2021). Larvae of the family Cyprinidae start taking exogenous feed after reabsorption of the yolk sac (Balon, 1986; Elliott, 1989) and this period is considered critical because larvae may restrain foraging or may stop taking food, in case of low feed provision (Opuszyński et al., 1989; Foresti, 2000). Bryant and Matty (1981) suggested the provision of dry feed when the cyprinid larvae reach 5-15mg body weight. Cyprinid larvae are primarily given natural live feed initially until their digestive system develops (Hofer, Nasir Uddin, 1985). In both Catla (*Labeo catla*) and Rohu (*Labeo rohita*), a successful transition takes place from the incipient endogenous nutrition stage to the exogenous intake of feed with the completion of a process of differentiation/organogenesis (Sheriff and Altaff, 2018).

Generally, during ontogeny, different body segments grow at different rates following their role and physiological significance (Sheriff and Altaff, 2018). In cyprinid larval development, the Pre-flexion and flexion stages are the ones where the largest changes in growth occur (Nowosad et al., 2021). The preflexion stage starts with yolk sac absorption, with simultaneous morphometric growth, especially in the head, trunk, and tail regions. More growth in the head region indicates the development of branchial structures, followed by positive allometry of the trunk region suggesting the development of the digestive system (Sheriff and Altaff, 2018). Further, the development of morphometric features, musculature, paired fins, and caudal fin of *L. catla* and *L.*

rohita also occurs for active swimming. The flexion stage in rohu is marked by the shift in the growth coefficient of the above-mentioned structures. This type of shift is reported in many teleost's (Pena et al., 2003; Kupren et al., 2014; Nowosad et al., 2021). Moreover, because of the evolutionary behavior of *L. rohita* i.e., column feeding, the pattern of ontogenetic changes seemed to be related to the ecological changes, like early diet and habitat adaptation (Rodríguez-Mendoza et al., 2011). Certain complex factors might play an important role in the non-homogeneous growth trajectories including food density, fish mortality and environmental patterns. Moreover, the shift in foraging behavior occurs with age and development, e.g., phytoplankton dominates the gut content of rohu at the fries stage (Dewan et al., 1991) while zooplankton during pro-fries stages (Khan and Siddiqui, 1973: Bakhtiyar et al., 2018).

Regulation of feed intake, digestion, metabolism, and growth, have a crucial role in the development of fish larvae. Fish regulate these processes through various orexigenic (β-endorphin, *ghrelin*, and neuropeptide-Y) and anorexigenic (serotonin, cholecystokinin, *leptin*, amphetamine-regulated transcript bombesin, cocaine) signaling molecules and endocrine factors (Dar et al., 2020). However, certain external factors, mostly the nature/texture of feed/diet play a significant role (Bertucci et al., 2019). Fish brains have several feeding centers which regulate feeding by receiving a metabolic and neural peripheral signal related to the nutritional status and feed intake (Sobrino-Crespo et al., 2014).

Intrinsically digestion, growth and development during early ontogeny are regulated by the gut-brain axis (Goldstein et al., 2021), GH-IGF system, Myogenic regulatory factors (MRF's) system and other crosslinking hormones and enzymes (Fuentes et al., 2013). The brain regulates the digestion process via an autonomic nerve pathway. Feed intake stimulates signals in the gastrointestinal tract (GIT: mainly in enteroendocrine cells) that release gastrointestinal hormones, i.e., *ghrelin*, *leptin* etc. These hormones have multiple paracrine and endocrine roles and bind to receptors in the vagus nerve and/or central brain areas (i.e. hypothalamus), i.e., GHSR (*ghrelin* receptor) that govern feed intake and intestinal motility. In this manner, these hormones ultimately regulate food intake, digestive, absorptive and metabolic processes (Raka et al., 2019).

During ontogeny, the growth and development processes are regulated mainly by the GH-IGF system (Gilannejad et al., 2020); where hormones play a phenomenal role in somatic growth by targeting muscular tissue and bones, enhancing expression of genes encode for IGFs and IGF-binding proteins, myosin heavy chain isoforms, troponin T, myosin light chain etc. (Rehfeldt et

al., 2010). GH is produced in the pituitary (pars distalis), first reported in *Oreochromis mossambica*) (Farmer et al., 1976). Several neuropeptides and hormones govern the expression of GH like GHRH, GnRH, Thyroxine, *IGF-1*, IGF2, etc. (Holloway and Leatherland, 1998; Peng and Peter, 1997; Peter and Chang, 1999; Canosa et al., 2007; Gracia-Navarro et al., 2002). Additionally, *ghrelin* an endogenous ligand of GH secretagogue receptor (GHSR) (Edwards and Abizaid, 2017), is predominantly produced in endocrine cells of the stomach (Kaiya et al., 2008; Kojima and Kangawa, 2005; Sakata et al., 2004) also regulate the release of GH from the pituitary and the food intake (Unniappan and Peter, 2005). *Ghrelin* is the orexigenic signalling molecule that has been known to link feed intake, growth, and energy balance (Jönsson, 2013; Babaei et al., 2017).

Ghrelin is a peptide hormone that plays a significant role in meal initiation, energy homeostasis, growth (Kang et al., 2011; Jönsson et al., 2013), anti-inflammation, and glucose metabolism (Upton and Riley, 2013). It is an important molecule that regulates the motility and secretion of the intestine and exerts physiological effects through its specific receptor, the growth hormone secretagogue receptor (*GHS-R*), also known as the *ghrelin* receptor (Unniappan and Peter, 2005). Generally, the *ghrelin-GHS-R* axis connects with growth hormone (GH)-insulin-like growth factor-1 (*IGF-1*) to regulate fish growth (Zhang et al., 2016). *Ghrelin* is the only peripheral circulatory hormone that regulates metabolism through central and systemic administration: enhance appetite, food intake, growth and energy homeostasis (Jonsson and Holmgren, 2012; Castañeda et al., 2010).

The antagonistic relation of *ghrelin* with *leptin* is well documented (Babaei et al., 2017). *Leptin* is a 16-kDa peptide hormone that regulates feeding rate in response to energy status. It suppresses food intake by downregulating orexigenic signals (Babaei et al., 2017). Generally, it regulates energy expenditure by influencing the autonomic nervous system and neuroendocrine mechanisms (Angela and Donato, 2017). It inhibits appetite-stimulating neurons in the hypothalamic feeding centre (Elias et al., 1999; Ahima et al., 2000) and regulates growth (Murashita et al., 2011; Fuentes et al., 2012). The expression of the *leptin* gene has been reported in the liver of *Salmo salar* (Ronnestad et al., 2010), Crucian carp (Cao et al., 2011), *Takifugu rubripes* (Kurokawa et al., 2008), and *Oryzias latipes* (Kurokawa and Murashita, 2009).

Insulin-like growth factor 1(*IGF-1*) is the chief mediator of GH, playing a vital role in promoting cell growth and differentiation (Wood et al., 2005). It is generally produced in the liver

in response to the release of GH in bloodstream and stimulates systemic body growth. It also shows growth-promoting effects on almost every cell in the body, especially the liver, kidney, bone, skin, cartilage, skeletal muscle, nerve, and hematopoietic cells. Moreover, *IGF-1* also regulate the synthesis of cellular DNA. The higher expression of *IGF-1* mRNA in the muscles is related to enhance growth in *Oreochromis niloticus* (Eppler et al., 2007).

Myogenic regulating factors (MRFs) are the member of the family of transcription factors that are crucial in early development by regulating muscle differentiation (Weintraub, 1993). Prominent MRFs that regulate the ontogeny include MYF5 (Myogenic factor 5), MyoD (myoblast determination protein 1), myogenin and MRF4 (muscle regulatory factors). MYF5 and MyoD are considered determination genes that play vital roles in regulating skeletal muscle development and growth (Shi et al., 2022), while myogenin and MRF4 regulate tissue differentiation (Johnston, 2006; Chang, 2007; Brameld and Daniel, 2008). In mice, MYF5 plays a role during the earliest epaxial myogenesis, while MyoD participate in timely differentiation of hypaxially derived muscle (Hinits et al., 2009),). Members of the MRFs family, MyoD, MYF5, and myogenin have been identified in many fish species and found to be expressed in developing somites and skeletal muscles (Kobiyama et al., 1998; Chen et al., 2000; Tan et al., 2002) though not reported in any Indian carp species. Generally, MyoD and MYF5 play a common role in establishing myoblast identity, while myogenin is involved in terminal differentiation. According to Du et al. (2003), these genes induce the several muscle proteins and enzymes production, i.e., myosin, troponin, and creatine-kinase.

Recently, molecular tools have been used to understand the ontogeny of the digestive system (Galaviz et al., 2012; Srichanunet al., 2013; Mata-Sotres et al., 2016). Many investigators reported the myogenin expression and its prominent role in the differentiation of muscles in the embryo (Johansen and Overturf, 2005). MyoD and MYF5 synergistically regulate the expression levels of myogenic genes genome-wide by binding their consensus E-box sequences (Kim and Shendure, 2019). MyoD has been well-established as a master transcription factor in myogenic cell-lineage specification during development (Rudnicki et al., 1993; Davis et al., 1987; Weintraub et al., 1987). MyoD has a vital role in the cell arrest stage. The highest level of MyoD was detected at the early G1 phase of development and the lowest level was at G1 to S phase transition. MyoD and its cofactors play a critical role in myoblast cell cycle withdrawal. When MyoD is up-regulated, and MYF5 is down-regulated, cells exit their cycle into differentiation. The opposite

pattern is observed in quiescent non-differentiating myoblasts, with a high MYF5 and no MyoD (Kitzmann and Fernandez, 2001). MyoD and MEF2 family members function in a combined way to activate myogenesis.

Consistent with these observations, most of the skeletal muscle genes require both MyoD and myocyte enhancer factor-2 (MEF2) family members to activate their transcription (Dodou et al., 2003). On the other hand, Myostatin acts as a negative growth regulator that prevents satellite cell proliferation during muscle growth development (Garikipati and Rodgers, 2012). Myostatin acts as a growth suppressor that inhibits the proliferation and differentiation of satellite cells during muscle growth development (Prabu et al., 2020; Prabu et al., 2021). It is well documented that in fish species, dietary changes alter the relative expressions of muscle growth-related genes, such as MyoD and myostatin (Sudha et al., 2022).

In fish myogenesis, the formation of new muscle fibers continues well into adulthood (Koumans and Akster, 1995; Keenan and Currie, 2019). In adult fish, the growth continues because of the retention of MyoD expression. For instance, in rainbow trout on 180 DAH, intense myoblast proliferation was reported along with high MyoD expression, thus showing its contribution to hyperplastic skeletal muscle growth (Johansen and Overturf, 2005). The higher expression of MyoD and MYF5 was also reported in the embryonic stages of *L. rohita* (Sengupta et al., 2014). Generally, MYF5 determines the muscular lineage is the first MRF expressed in the embryo and is considered to be a 'specification' factor (Berkes and Tapscott, 2005). The MYF5 has been shown at 30 hours after fertilization in embryos of common carp *C. carpio* while decreased to only a slight expression after seven months (Kobiyama et al., 1998). MEF2A knockdown in zebrafish has significantly downregulated contractile proteins (troponins, myosin heavy and light chains, and α-tropomyosin) (Wang et al., 2006). In *L. rohita*, MEF2A expressed at all the stages, showing a higher expression after one month. Also, in *C. carpio,* a similar expression pattern of myogenin and MEF2A was observed by Kobiyama et al. (1998).

To determine nutritional-related problems, research for insight knowledge on the molecular mechanisms underlying the development of the digestive system and its regulation has been attempted in several fish species (Gisbert et al., 2013). The pattern of gene expression during early ontogeny, involved in the development of the digestive system, is the crucial molecular tool (Mata-Sotres et al., 2016; Srichanun et al., 2013) that can lead us to analyze the impact of various feed sources on the growth, survival, and overall physiology.

To understand the digestive physiology of larvae during ontogeny, the study of enzymatic activity is the most convenient way to assess (Rønnestad et al., 2013), while also helps in understanding the nutrition utilization capabilities of fish (Bolasina et al., 2006; Cara et al., 2007). The formation of digestive structures may not necessarily be expressed in terms of functionality, as secretion of some enzymes starts weeks after the formation of physical structures (Zambonino-Infante et al., 2009). Maturation and successful transformation usually change with feeding practices, species type, genetics, and rearing conditions etc. (Peňa et al., 2016). Hence, an insight into patterns of enzyme secretion in fish gut during ontogeny may help in developing an artificial diet compatible with the physiological capability of digestion. During ontogeny digestive enzymes activity changes progressively and develops independently (of other digestive enzymes), depending on fish species, diet, and temperature (Kolkovski, 2001). Depending on the species' requirement, digestive enzymes start releasing just after hatching. For instance, amylase, protease, lipase, trypsin, chymotrypsin and alkaline phosphatase was observed in larvae of many species (Murray et al., 2003; Oozeki and Bailey, 1995; Ozkizilicik et al., 1996; Martinez et al., 1999: Lahnsteiner, 2017; Frías-Quintana et al., 2019) including rohu larvae (Martinez et al., 1999; Tengjaroenkul et al., 2000; Cara et al., 2003; Bolasina et al., 2006; Chakrabarti et al., 2006). Ontogeny of the digestive system comprise stepwise development of pancreatic enzymes followed by secretion of intestinal enzymes and lastly stomach formation (Gisbert et al., 2009) with exception of no stomach formation in carps (Chakrabarti et al., 2006); loaches (Cobitidae), killifish (Cypridontidae), clingfish (Gobiesocidae) and gobies (Gobioidae) (Harder, 1975). The digestibility of nutrients reflects the growth of fish larvae (Wang et al., 2006). The availability of nutrients in feed modulates metabolism by altering the major digestive enzymes (Goswami et al., 2020), and morphological transformation of the digestive tract (Moraes et al., 2020).

One of the primary impacts of efficient protein utilization is expressed in terms of growth (Mitra and Mukhopadhyay, 2003). Proteolytic activity was observed higher at larval stages in omnivorous species including *L. rohita* larvae. Due to the lack of a true stomach, pancreatic secretions become more significant to compensate for digestion (Rønnestad et al., 2013). The most crucial enzyme in young fish is trypsin (Hjelmeland and Jorgensen 1985). Ribeiro et al. (2002) have deduced that trypsin is very much necessary for protein digestion till the development of a functional stomach, where trypsin accounts for 40%–50% of the protein digestion in the intestine (Eshel, Lindner, Smirnoff, Newton, and Harpaz, 1993). Generally, it hydrolyzes protein and

releases small peptides and free amino acids for intestinal absorption, thus trypsin activity is considered a valuable indicator of the fish's digestive capacity (Zamani et al., 2023). The role of trypsin in the cleavage of yolk proteins and food items, once exogenous feeding takes place has been reported by Gisbert et al. (2009). Trypsin activates various zymogens and a minor peak in its specific activity could significantly change protein digestion, including direct activation of chymotrypsin (Solovyev et al., 2023). Trypsin mostly hydrolyzes lysine and arginine residue of protein (Cara et al., 2007; Belitz et al., 2009), while chymotrypsin mostly breaks aromatic residues i.e., phenylalanine, tyrosine, and tryptophan; although the structure of both enzymes is very much same (Gisbert et al., 2013; Rønnestad et al., 2013). The differences in active sites allow these enzymes to act on a variety of proteins (García-Gasca et al., 2006; Lilleeng et al., 2007), as a diet with animal protein source possesses only a few aromatic residues while rich in lysine and arginine compared to plant source (Hamid et al., 2016). Hence, the secretion of trypsin and chymotrypsin changed significantly with nutritional condition and age in red drum larvae (Applebaum and Holt, 2003). The ratio of trypsin and chymotrypsin also signifies growth rate, where high trypsin secretion compared to chymotrypsin indicates a higher growth rate (Lazo et al., 2011). Live feed (*Daphnia carinata*) also imparts trypsin and chymotrypsin (Kumaret al., 2005).

The amylase is released from the exocrine tissue of the pancreas in fish (Bakke et al., 2011), which is released into the intestine and needs an alkaline pH (6 to 9) for activation (Solovyev and Izvekova, 2016). In many fish species, its role in the digestion of carbohydrates has been reported at larval stages (Rønnestad et al., 2013). A high amylase secretion in many fish species during the larval stage followed by a dynamic decrease-increase trend has been documented (Yúfera et al., 2018). High amylase is extremely beneficial for its practical significance (Bakke et al., 2011). Many investigators reported a higher amylase activity during the transformation stage from exogenous to endogenous nutrition in *L. rohita*, grass carp (*Ctenopharyngodon Idella*), silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*) (Tanaka 1973; Siebenallar 1984; Chakrabarti et al., 2006; Volkova, 1999) and red sea bream, *Pagrus major* (Khoa et al., 2019). Being cheap and easily obtainable, carbohydrate use in aquaculture is extremely welcomed, however, fish do not efficiently consume carbohydrates (Zhou et al., 2015). The carbohydrate digestion is limited to certain factors like fish type, age, type and nature of source etc. (Kamalam et al., 2017; Liu et al., 2018; García-Meilán et al., 2020). It has been shown that

high carbohydrate concentration induces amylase activity in yellow croaker (*Larimichthys crocea*) (Zhou et al., 2016) and Nile tilapia (Boonanuntanasarn et al., 2018).

The pancreas also secretes lipases to digest lipids into fatty acids (De Almeida et al., 2006; López-Vásquez et al., 2009). Lipases mainly act on ester bonds of triacylglycerol forming monoacylglycerol while forming free fatty acids (Campoverde et al., 2017). The lipases have a substrate-dependent regulation, since the activity seems to be stimulated by exogenous feeding (Hoehne-Reitan et al., 2001, Zambonino Infante et al., 2008). Lipase activity has been detected during larval development in several fishes (Oozeki and Bailey, 1995; Ozkizilicik et al., 1996; Martinez et al., 1999; Lahnsteiner, 2017). In some species, a relatively high activity has been detected in the first days of development and is usually associated with lipase-like enzymes that use lipids from the yolk sac (Córdova-Montejo et al., 2019). Subsequently, the activity increases and is associated with bile salts-dependent lipases (Zambonino-Infante and Cahu 2001). After hatching, lipase is typically associated with yolk lipid catabolism to provide energy for developing larvae before exogenous feeding starts (Martínez-Lagos et al., 2014). However, very low lipase activity has been detected in a freshwater fish, the walleye pollock (*Theragra chalcogramma*), at the start of exogenous feeding (Oozeki and Bailey 1995), while in turbot larvae, lipase activity has been reported to increase throughout early development (Cousin et al., 1987).

Two groups of digestive enzymes are found in enterocytes: cytosolic enzymes (mainly peptidases) found in the cytoplasm, and brush border membrane enzymes, which are linked to the cell membrane such as alkaline phosphatase (ALP) and aminopeptidase-N: APN. ALP, breaks the inorganic phosphates from various nucleotide and protein to be used for energy production while ANP plays role in nutrient transport through membranes into cells (absorption process) (Pereira et al., 2019; Teles et al., 2019; Alvarez-González et al., 2008). The activity commonly used as indicators of absorption of amino acids (Cara et al., 2003). ALP remove inorganic phosphate from certain organic phosphate esters such as hexose phosphates (like glucose-6 phosphate), glycerophosphate and the nucleotides derived from the diet and the digestion of nucleic acids by nucleases (Shaikila et al., 1993). Alkaline phosphatase activity has been detected during larval development in different marine fishes (Govoni et al., 1986; Zambonino Infante and Cahu, 1994; Moyano et al., 1996). The early detection of this digestive enzyme has also been described in other cichlids like zebra cichlid (*Archocentrus nigrofasciatus*, Mente et al., 2016) and *O. niloticus* (Pereira et al., 2019), and in other fish species like miiuy croaker (*Miichthys miiuy*, Shan

et al., 2008), sheatfish (*Silurus soldatovi*; Liu et al., 2010) and butter catfish (*Ompok bimaculatus*, Pradhan et al., 2013). As the alkaline phosphatase activity is found primarily in cell membranes where active transport takes place, it could be speculated that this enzyme is important in the absorptive process. and could be used as a general marker of nutrient absorption (Segner et al., 1995) and enterocyte differentiation (Smith, 1992).

Cellulose, a polymer of glucose residues connected by β-1,4 linkages, being a principal component of plant cell walls, is the most abundant carbohydrate in nature (Péreza and Samain, 2010). It consists of composite forms of highly crystallized microfibrils among amorphous matrixes, thus refusing access to hydrolyzing enzymes. The utilization of cellulose as a nutrient source requires the enzyme cellulase that cleaves β-1,4 glycosidic bonds in the polymer and releases glucose units (Barr et al., 1996). Cellulolytic bacteria and fungi have developed complex forms of cellulase systems which actively convert insoluble cellulosic substrates into soluble saccharides (Tomme et al., 1995). cellulase enzymes are active in a wide range of invertebrate taxa (Martin, 1983; Zinkler and Gotze, 1987). However, relatively few higher animals can utilize this resource efficiently (Goodenough and Goodenough, 1993). It is well-established that dietary manipulation modulates the gut microbiota of fish (Burr et al., 2005; Ringø et al., 2006; Navarrete et al., 2009; He et al., 2013). It is well documented that the intestinal microbiota plays an important role in nutrient digestion and absorption (Ramirez and Dixon, 2003; Merrifield et al., 2010; Dimitroglou et al., 2011; Ringø et al., 2022), influence the immune status, disease resistance, survival and feed utilization (Denev et al., 2009; Li et al., 2019; Luan et al., 2023). Fish are unable to produce cellulase endogenously, but they harbor microbial populations in their digestive tracts which help in the digestion of plant materials (Lindsay and Harris, 1980; Saha and Ray, 1998 Lesel et al., 1986; Bairagi et al., 2002). For instance, certain strains of *Bacilli* and *Sphingomonas* were proven to produce cellulase in moderate quantities and are considered good producers of protease and amylase (Haichar et al., 2007; He et al., 2009; Saha et al., 2006; Pham et al., 2021). Many investigators reported cellulase activity in herbivorous fish and suggested that enzymes are not synthesized by the fish themselves but produced by certain microorganisms (Saha et al., 2006; He et al., 2009).

Early feed both in terms of composition and availability directly induces the development and maturation of the digestive tract (Ma et al., 2005). Nowadays in most Asian countries, feeding live feed is in practice with all its limitations (imbalanced nutritional profile, missing essential

nutrients and presence of parasites), because formulated feed cannot be efficiently digested by larvae (People-Le-Ruyet et al., 1993; Hamre et al., 2013). Moreover, due to complex physiological and structural changes with age, feeding and nutritional requirements also change (Enberg et al., 2012).

Fatty acids (FAs) are essential biomolecules, play a vital role in many physiological processes and remain crucial during the early developmental process (Chaguaceda et al., 2020), where FA serve both as feed and fitness drivers (Sterner and Elser 2002; Raubenheimer et al., 2009). FA constitutes a major role in hormonal regulation and energy storage (Tocher, 2003) while also serving as a precursor of various bioactive molecules (e.g., eicosanoids) and cell membrane constituents (Stanley, 2000; Heckmann et al., 2008). Fish use MUFAs mostly as energy sources during early age however adult fish store MUFAs for growth during unfavorable or fasting periods (Tocher, 2003; De Roos and Persson, 2013). Among FA, Polyunsaturated Fatty Acids (PUFAs) like as EPA (eicosapentaenoic acid), ARA (arachidonic acid) and DHA (docosahexaenoic acid) are specifically important for growth and reproduction (Brett et al., 2009; Heckmann et al., 2008; Schlotz et al., 2012; Twining et al., 2016). DHA has an integral role in the membrane structure, especially neural membranes (Feller, 2008; Wassell and Stillwell, 2008; Tocher, 2010) and visual cell membranes (Brett and Muller-Navarra, 1997). The absence of DHA in a larval feed of herring impaired visual performance (Bell et al., 1995; Shields et al., 1999), while more DHA was correlated with enhanced growth via enhanced vision to take feed in cyclpoid larvae. Fish larvae usually take feed by sight (Conceica et al., 2010), EPA and ARA are important for eicosanoid production, while saturated fatty acids (SFAs) carry the sn-1 position of structural phospholipids (Rainuzzo, 1993; Tocher, 2003). The eicosanoid constitutes prostaglandins, thromboxanes and leukotrienes derived from ARA (Majed and Khalil, 2012). In fish 20:4n6; ARA, remain the chief source of eicosanoids (Tocher, 2010) which produce bioactive molecule involved in clotting of blood, immunity responses, inflammations, cardiovascular tone, renal and neural functions via a regulated dioxygenase enzyme-catalyzed oxidation of highly unsaturated fatty acid (HUFA): 20:4n6 (ARA) and 20:5n3 (EPA) (Schmitz and Ecker, 2008). During ontogeny, the requirement of FA also shifts qualitatively (Tocher, 2009), for instance, more omega-3 PUFAs (DHA) during organogenesis and development (Ballantyne et al., 2003; Tocher, 2010), more ARA and EPA (precursors of eicosanoids) for the physiological process during ontogeny of neural, immunity and reproductive systems (Tocher, 2003). The DHA and ARA are mostly required after maturity for

gonad maturation (Tocher, 2003). The limiting PUFAs are retained in the body while other FAs are used in metabolism (Abi-Ayad et al., 2000; Taipale et al., 2011). Moreover, selective FA is also retained during various ontogenetic shifts (Henderson and Tocher, 1987; Tocher, 2003).

In naturally diverse phytoplankton communities, low availability of long-chain polyunsaturated fatty acids (LC-PUFA) has been associated with the abundance of lipid-deficient cyanobacteria, which eventually may lead to a PUFA limitation of aquatic consumers (Müller-Navarra et al., 2000, Wacker et al., 2001). Fish require direct provision of LC-PUFAs as fish cannot synthesize PUFAs de novo and have a limited ability to form LC-PUFA from other PUFA compounds (Bell and Tocher, 2009; Tocher, 2003). PUFAs in the naturally available diet (live feed) vary substantially depending on the habitat (Lau et al., 2012; Hixson et al., 2015). The major primary source of LC-PUFAs is aquatic algae (Hixson et al., 2015; Ruess and Müller-Navarra 2019). Further, organisms at higher trophic levels comprise more portion of LC-PUFA compared to lower trophic levels (Strandberg et al., 2015; Kainz et al., 2004; Kainz et al., 2009; Mariash et al., 2011; Twining et al., 2016). The seston PUFA concentrations may also be affected by abiotic factors, such as temperature or light intensity, which are negatively correlated with the PUFA concentration in phytoplankton (Thompson et al., 1990; Thompson et al., 1992; Piepho et al., 2012).

Zooplankton often constitutes a higher proportion of omega-3 PUFA (Lau et al., 2012; Scharnweber et al., 2016). Generally, cyanobacterial bloom formation is presumably favored by global warming (Visser et al., 2016), which may also portray a risk of decline of PUFA in future. The fatty acid profile of an organism changes during the life course. Chaguaceda (2020) stated the quantitative impact of various factors responsible for variation in FA composition, like ontogenetic processes contribute 23%, diet 28% and, condition factors 1%, however, 48% of the FA variation remained unexplained by his model. Other factors reported previously were sex (Henderson et al., 1984), sexual maturity (Manor et al., 2012), temperature (Farkas et al., 1984), fasting (Abi-Ayad et al., 2000; Ballantyne et al., 2003; Taipale et al., 2011), pollutants (Janer et al., 2007), phenology (Blanchard et al., 2005; Rudchenko and Yablokov 2018; Keva et al., 2019), or possibly genetic variation. This may indicate a more significant effect on FA composition during the early development of organisms (Lane et al., 2011; Maazouzi et al., 2011; Iverson 2009). Ontogenetic patterns in single FAs did not always reflect dietary inputs but rather indicated FA regulation (or FA homeostasis) associated changes in FA requirements over ontogeny (Chaguaceda, 2020). DHA

mostly reduces during ontogeny in non-piscivores (Iverson et al., 2002; Maazouzi et al., 2011). Moreover, intraspecific bioconversion efficiencies of nutrients may change over ontogeny (Henrotte et al., 2011).

Larval feed mainly constitutes live feed; phyto-zooplankton including Copepods, Artemia, Moina, Rotifers, etc. Each candidate has several limitations and beneficial aspects, to serve as the optimum candidate for larval balanced diet. Copepods usually remain advantageous due to the abundance of PUFA and HUFA, essential for fish growth and development (Bell et al., 2003; Olivotto et al., 2006; Conceica et al., 2010), while their presence in the phospholipid fraction of copepods make it more bioavailable, compared to Artemia having neutral lipid fraction (Conceica et al., 2010). However, rotifers and artemia are still the most abundant live food utilized in feeding fish larvae (Sorgeloos et al., 2001; Kang et al., 2006). Artemia has a low n-3 HUFA and PUFA (Lavens et al., 2019), but larvae of freshwater species remain relatively less sensitive to variable FA (Bengtson et al., 1991). Moreover, *Moina macrocopa* compared to artemia is rich in protein and nutrients (Loh et al., 2012) and also an excellent live food for the early rearing of most fishes and crustaceans (Poynton et al., 2013; Ingram, 2009; Aguado et al., 2009; He et al., 2001). Furthermore, PUFAs greatly differ between cladocerans and copepods: cladocerans get most of the FAs from diet and only 2% is synthesized de novo (Goulden and Place, 1990). Cladocerans mostly accumulate eicosapentaenoic acid (EPA) and Arachidonic acid (ARA) while copepods predominately accumulate DHA (Farkas 1979; Persson and Vrede, 2006; Smyntek et al., 2008; Burns et al., 2011; Mariash et al., 2011).

There are many factors like temperature, season, feeding habits, water quality, and Food quantity that affect the body composition of zooplankton (Ismail et al., 2016). For instance, at low temperatures, EPA and mostly DHA content in the zooplankton increases (Farkas and Herodek 1964). However, literature indicated variation in response among species of zooplankton, e.g., in response to cold stress copepods readily adjust their n-3 HUFA and especially DHA content while *Daphnia magna* showed lower capacity to do so (Farkas et al., 1984). According to many investigators, zooplankton generally utilized PUFAs for adjustment to cold temperatures (Schlechtriem et al., 2006; Smyntek et al., 2008; Martin-Creuzburg et al., 2012; Sperfeld and Wacker, 2012).

Seasonal variation modulates the concentration of certain PUFAs that are distinct to plankton, (Ahlgren et al., 1990; Olofsson et al., 2012; Hartwich et al., 2013). During spring,

phytoplankton is often dominated by EPA-rich diatoms, whereas in summer the proportion of diatoms is usually lower Mancuso et al., 2021). Similarly**,** copepod (Acartia) showed higher MUFA in the winter and spring seasons while higher SAFA during summer and fall (Hartwich et al., 2013. Interestingly in live feed, more DHA was observed in warmer months which may be due to the high dinoflagellates (which often have high DHA content). Kainz et al. (2008) reported a maximum accumulation of PUFAs in macro-zooplankton during the spring season. However, Lau et al. (2012) observed FA variation and associated these variations with taxonomic differences and found no seasonal influences. Feeding nature also regulate FA profile; herbivorous zooplankton showed more HUFA than did seston, and carnivore contained more HUFA than herbivores. Similarly, carnivorous freshwater Cladocera could be distinguished from herbivorous Cladocera by a higher ARA and EPA content, and lower ALA + SDA content and n3:n6 ratios (Bret et al., 2009). However, accumulation of α-linolenic acid (ALA) was reported to almost a similar degree between copepods and Cladocera (Smyntek et al., 2008; Kainz et al., 2009).

The nature of water also affects the fatty acid profile of plankton. For instance, freshwater Cladocerans were notable for having much lower DHA (2%) and lowest n-3: n-6 ratios (i.e., 2.4–3.0). Similarly, EPA and DHA are often not detected in freshwater chlorophytes but are abundant in marine water (Peltomaa et al., 2018). Finally, food quantity also influences the transfer of energy and fatty acids considerably (Persson et al., 2007; Gladyshev et al., 2011). Copepods can feed selectively (Hansen, 2000) and thus may increase the ingestion of DHA-rich plankton. Another possibility would be the conversion of EPA into DHA (Persson and Vrede, 2006; Smyntek et al., 2008). Many investigators reported that zooplankton can synthesize de novo, several FA thus suggest a strong correlation between fatty acids in zooplankton and their food (e.g. Taipale et al., 2009; Gladyshev et al., 2010; Burns et al., 2011).

The most crucial phase in successful weaning of a species is the start of exogenous feeding (Trevino et al., 2011; Hamre et al., 2013), as the capacity of fish to hydrolyze compound feed may not meet the need for optimum growth and development (Garcia et al., 2001; Zambonino-Ifante et al., 2008). The nutritional requirements and digestion capability variate with age and species type, (Ruan et al., 2013; Zhang et al., 2016). Further, palatability and digestibility are still other important aspects of the larval diet. Fish larvae take feed according to their choice, which alters with the size of zooplankton (Bakhtiyar et al., 2011).

It is well documented that materials that are manufactured at Nanoscale have distinctive physical or chemical properties due to their small size, shape, high surface area to volume ratio and conductivity (Chau et al., 2007). Nanotechnology i.e., manufacturing, creation, manipulation and application of useful materials, systems and devices by controlling the size of matter at the nanometer scale (means less than 100 nm) (Masciangioli and Zhang, 2003; Roco, 2003) or range between 1 to 100 nm (Schenir, 2007). Nanotechnology could be applied to produce larval fish feed by improving the chemical, physical and nutritional quality of feed and other constituents during their manufacturing process. Higher absorption and assimilation of Nano forms of zinc (Zn), selenium (Se) and iron (Fe) and their positive impact on growth and immune responses and the antioxidant system of fish, is already anticipated (Carriquiriborde et al., 2004; Faiz et al., 2015; Kanwal, 2012; Jamil 2013; Khan et al., 2017; Kumar et al., 2023).

It is quite clear that developmental changes must be assessed to optimize the nutrients in the feed. However, it is difficult to assess the national requirements as many factors impart in the physiological performance of feed digestion, absorption and assimilation (Izquierdo and Lall 2004). The optimum level of macronutrients including protein, lipids, and carbohydrates are required in the weaning diets of the fish (Halver and Hardy, 2002).

Fish require protein as the most crucial component in the diet for optimum growth, survival, and reproduction (Wilson and Halver, 1986). The protein comprises amino acids which are required for the genesis of body protein and serve as an energy source (NRC, 1993; Kaushik and Médale, 1994; Francesca et al., 2020). Larval stage is the most crucial stage for growth in the form of protein deposition (Houlihan et al., 1995). The other significant point is the protein digestion capacity of fish larvae, as most fish lack stomach during early stages (Luizi et al., 1999) and have a low specific protease activity (Gawlicka et al., 2000; Rojas-Garcı´a and Rønnestad 2002). Larvae acquire some exogenous enzymes from the planktons body, but a very minute role has been observed of these enzymes in protein digestion (1–8%) (Munilla-Moran and Stark 1989; Kurokawa et al., 1998; Gawlicka et al., 2000) though, these enzymes significantly improved, the release of endogenous larval digestive enzymes (Munilla-Moran and Stark 1989; Beccaria et al., 1991), and ingestion (Kolkovski et al., 1997). While rearing fish larvae on a compound diet, certain factors like feed binders (affect particles nature) affect early protein digestion dilemma; (Person Le Ruyet et al., 1993; Lo´pez-Alvarado et al., 1994; Guthrie et al., 2000). For instance, weak binders favour digestion but increase leaching and Vice versa (Person Le Ruyet et al., 1993;

Guthrie et al., 2000), though binders' success also varies with species (Partridge and Southgate 1999).

The optimum dietary protein content is essential for efficient fish growth (Siddiqui and Khan, 2009), as beyond optimum level depression in growth was observed in *Carassius auratus* (Fiogbe and Kestemont, 1995); *O. niloticus* (Kaushik et al., 1995); *Perca fluviatilis* (Fiogbe, 1996); hybrid catfish (*Heterobranchus bidorsalis x Clarias anguillaris*) (Diyaware et al., 2009); *Heteropneustes fossilis* (Siddiqui and Khan, 2009); *H. niloticus* (Monentcham et al., 2010); *Clarias gariepinus* (Farhat and Khan, 2011). The optimum protein requirement varies concerning the nature of the species, like 48- 53% for carnivorous fish *S. trutta* larvae (, Arzel et al., 1995); 45-53% for *C. auratus* (Fiogbe and Kestemont, 1995); 48.5-49.4 for *P. fluviatilis*), (Fiogbé, 1996); 42-56% for *C. gariepinus* and *Heterobranchus logfiles*, Kerdchuen,(1992); 50-55% for hybrid catfish *H. bidorsalis x C. anguillaris*, (Diyaware et al., 2009), 30-36% for O. niloticus (Shiau, 2002) and 35- 42% for *C. carpio* (Tacon, 1987). Protein requirements also vary with the age of the fish, larvae need higher protein content than juvenile fish (Ha et al., 2018; Einen and Roem, 1997; Sweilum et al., 2005); For instance, crude protein requirement of olive flounder larvae was 55.4% CP whereas juvenile and grow out showed best growth on 46.4–51.2 and 40–44% CP respectively (Ha et al., 2018).

Lipid levels in the feed of fish larvae also play an important role Dietary lipids play a major role in providing a good source of concentrated energy, essential fatty acids and fat-soluble vitamins as the fishes have a limited ability to utilize carbohydrates as an energy source (Stickney et al., 1989; Tocher 2008; Izquierdo et al., 2000; National Research Council (NRC), 2011; Kamalam et al., 2017). The lipids attributed to high energy values, promotion of the absorption of fat-soluble vitamins, inhibition of dust, improvement of palatability, etc., are important for the feed industry (Yang et al., 2019). Feeding diets with high lipid contents exhibit unsatisfactory rearing results, like internal organ dysfunction (especially in the liver) and deposition of fat in viscera (Brown et al., 1996; Kestemont et al., 2003). The increasing inclusion of metabolizable nonprotein energy, mainly lipids, has been found to spare dietary protein from energy metabolism and to increase its utilization for fish growth. Such protein-sparing effects have been demonstrated in many fish species like salmon *S. salar* (Johnsen et al., 1991), rainbow trout *O. mykiss* (Garcia et al., 1981; Beamish and Medland 1986), carp *C. carpio* (Watanabe et al., 1987), hybrid striped bass *Morone saxatilis · Morone chrysops* (Nematipour et al., 1992). If a shortage in dietary lipid

supplements occurs, then metabolic disturbance may be observed in fish along with a decrease in the efficiency of feed protein and the lack of fat-soluble vitamins and essential fatty acids (Bonvini et al., 2015). However, high concentrations of lipids have been included in commercial diets, which have created controversy among some researchers and fish farmers (Trenzado et al., 2018). Excess dietary lipid supplements could bring about a diminution in feed consumption or feed ingestion rate by fish, thereby limiting the growth performance of the farmed fish (Ling et al., 2006). It is reported that an excess of dietary lipids could contribute to oxidative stress, accompanied by negatively affecting survival, growth, disease resistance, stress response, and lipid metabolism (Kjær et al., 2008).

Lipids requirements depend on the kind of species, the development stage, the culture conditions, the types of feed and the ingredients sources (Miller et al., 2005; Menoyo, 2004, 2006). For instance, juvenile *L. rohita* (0.43g) showed best performance at 9% lipid inclusion (Gandotra et al., 2017), while Mori *Cirrhinus mrigala at* 5 to 7% (Gumus and Ikiz, 2009), and African cichlids *Pseudotropheus* socolofi at 9% inclusion level (Erdogon et al., 2012).

The food selection and feeding affinity of Rohu showed that it is a zooplankton feeder at the post-larval stage and a phytoplankton feeder at the adult stage (Khan and Siddiqui, 1973). Freshwater aquaculture is dominated by carp, constituting three major carps. Rohu (*Labeo rohita* Hamilton 1822: Cypriniformes, Cyprinidae) is very popular due to its taste and public preference (FAO, 2019). Among the three major carps, Rohu is the most important species because of its high commercial value, good growth rate, consumer preferences and its easy weaning on artificial diets (Prasad et al., 2012). The main constraints to aquaculture are the feed cost, the emergence of diseases and the quality of the seeds, which ensure the sustainability of fish production. The high fecundity (2 lakh eggs/per kg), external fertilization and domestication of this species made it easy to undertake a breeding program intensively. However, the rearing success is extremely low during the early weaning stages, and good quality seed is thus a limiting factor in the conventional set-up.

Aims

To understand the digestive ontogeny of highly culturable indigenous fish species *L. rohita,* standardized the nutrients and prepared the highly palatable and digestible Nano-particulate larval feed. To achieve the set goal, the study was designed with the following objectives.

- ✓ To culture live feed by the conventional method in the earthen ponds

- ✓ To evaluate the species composition/nutrient profile of live feed
- ✓ To assess seasonal variation and relative abundance of live feed
- ✓ To determine the suitability of Nano-particulate prepared feed (as larval diet)
- ✓ To evaluate the impact of live feed and prepared diet on feed intake, ontogenetic release of digestive enzymes, and growth rate during early rearing
- ✓ To understand the impact of feed type (live feed and prepared diet) on the regulatory mechanism involved in the digestion of nutrients and growth.
- ✓ To standardize macronutrients (protein and fats) concentration in the larval diet
- ✓ To formulate a standardized feed for successful ontogeny of Rohu PLs

Materials and Methods

Experiment # 1: Mass Culture of Live Feed: Evaluation of Plankton Density and their Nutritive Value

Nursery pond preparation

Phytoplankton and zooplankton were cultured from March to October 2020 to 2022 in rectangular earthen ponds at Aquaculture and Fisheries Research Station, Quaid-i-Azam University. All ponds were prepared by following the conventional method, generally practiced in Pakistan and South Asia, for the rearing of Postlarvae (PLs) of carps species including Indian major carps. Briefly, all nursery ponds, rectangular in shape with an area of 120 m^2 and depth of 1.4 m were drained and sun-dried. Before filling with water, vegetation/weeds were removed and 1-2 inches of bottom soil of all ponds were removed and used for repairing dikes. Afterwards, ponds were fertilized by following Amir et al. (2020) method. Briefly, agricultural lime ($CaCO_3$) at a rate of 250 kg/ha was applied to the bottom soil with the aim of killing insects, germs and improving the P^H of the soil condition at the bottom. Additionally, cow dung and NPK (4:4:1) fertilizer at the rate of 3333.33 kg and 125 kg per hectare respectively, was spread across the bottom of the pond. Subsequently, ponds were half filled (0.6m) with stream water, permitting the sunlight to reach the bottom allowing the development of plankton community/live feed, then after 2-3 days more water was added by filling the pond up to 1.2 m. This level was maintained throughout the season. Rumali freshwater stream is adjacent to the Fisheries & Aquaculture facility. Its water was collected in a large concrete reservoir and from there through screening supplied to earthen ponds. The ponds were fertilized after every 15 days to maintain good plankton bloom throughout the experimental period. This technique was adopted to get live feed from a natural pond for the identification of phytoplankton and zooplankton composition and their proximate composition for the preparation of larval feed.

Water quality parameters

Water quality parameters such as temperature, ammonia, dissolved oxygen (DO) and P^H of all earthen ponds were regularly noted from March to October (Table 8).

Figure 1. Earthen pond preperation for the development of plankton community at fisheries and aquaculture research facility, quaid-i-Azam University, Isamabad.

using a multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA & test kit of ammonia for freshwater HI3824, ROMANIA).

Maintenance of plankton

Sampling of plankton was done on a regular basis for plankton assessment (composition and variation in nutritive profile) and for feeding to *L. rohita* PLs (PLs). The colour of pond water was also noted periodically as a marker of primary productivity (Table 8), while detailed productivity assessment was done using secchi disc reading and plankton density methods (unit: cm^3/45L) adopted by Alikuhni et al. (1951). Supplementary fertilization was done based on observed productivity and manuring ceased when the Secchi disk reading reached ≤30 cm or plankton density above 2.5 cm^3/L. Moreover, uniform productivity was maintained by following Rahman et al. (2006), and fortnightly supplementary manuring was done by adding 1250 kg/ha of decomposed cow manure, 31 kg/ha of urea, and 16 kg/ ha of triple superphosphate (TSP). The plankton density of 1.5-2.0 cm^3 per 45L was reported satisfactory for stocking PLs (Alikuhni et al., 1951).

Live feed/plankton collection and analysis

Plankton density was assessed on a weekly basis (Table 9) by collecting samples from 14 randomly selected spots across the earthen pond using a bolting silk tow net (standard No. 21) by early morning hours (5.00 am to 7.00 am). Briefly, water was stirred from each sampling location then 45L of water was sieved for the collection of plankton. The collected samples were preserved immediately in 4% formalin, simply by adding the sample to the glass tube having formalin. Thereafter supernatant was collected while the entire collection was transferred to a graduated tube and the readings of plankton volume after the mass settled at the bottom were taken. It was observed that zooplankton was settled quickly after fixation with formalin, however, phytoplankton took quite a few hours. Representative samples of plankton were repeatedly examined under the microscope for the assessment of the proportion of phytoplankton and zooplankton. Their ratio was determined from the relative bulk size.

Figure 2. Collection of phytoplankton and zooplankton from the earthen pond using plankton net, at fisheries and aquaculture research facility, quaid-i-Azam University, Isamabad.

Identification and relative abundance of phytoplankton and zooplankton

For the identification and variation in the relative abundance of phytoplankton and zooplankton from March to October, a detailed systematic study was conducted adopting the methodology mentioned by Manickam et al. (2019). Moreover, for identification of different species protocols and characteristics reported by different investigators; rotifers (Battish, 1992 & Dhanapathi, 2003), cladoceran (Michael and Sharma, 1988; Murugan et al., 1998), copepods (Sewell 1947; Reddy 1994; Dussart and Defaye 1995; Huys and Boxshall 1991; Williamson 1991) and ostracods (Victor and Fernando 1979) were adopted.

Briefly, each concentrated plankton sample was transferred to a plastic bottle and diluted to 100 ml with formalin and PBS (phosphate buffer solution) to obtain a 5% buffered formalin solution. Subsequently, 1 ml sample was put in the Sedgewick–Rafter (S–R) cell and left for 10 min to allow plankton to settle. The planktons in 10 randomly selected fields in the Sedgewick–Rafter (S–R) cell were identified.

For the detailed systematic differentiation of certain Zooplanktonic species, a drop of concentrate preserved in formalin was transferred on a glass slide and individual species of plankton were separated under a microscope. The separated species were placed on a glass slide and fixed with 20% glycerin. Subsequently, a drop of Eosin solution (stain) was added to the fixed species and left for 5-7 min. Afterwards, the slide was washed with distilled water 2 to 3 times and dried. The prepared slide was examined under microscope (OPTIKA Microscope, Italy). Each specimen was orientated in a dorsal, lateral or ventral view. using fine needles, to separate the parts of taxonomic importance and a picture was taken with a camera (C-PGS Series, OPTIKA, Italy) mounted on the microscope.

Proximate composition and fatty acid profile of live feed

For the evaluation of month-wise variation in the nutrition value of live feed, the proximate composition and fatty acid profile of the collected samples were conducted. Briefly, about 50g live feed (wet weight) each for proximate composition and fatty acid profile was collected month-wise (during early morning), dried, and used for analysis. Oversized plankton and insects were removed by repeated sieving using a plankton net (Table 12 and Table 14).

Proximate analysis

The proximate analysis, moisture, dry matter, ash content, crude fats and crude protein of live feed was conducted in collaboration with PPRI (Pakistan Poultry Research Institute) Islamabad by adopting the standard methods (AOAC, 2012). Soxhlet apparatus and micro Kjeldahl method were used for the determination of crude fats and crude protein respectively (Sutharshiny and Sivashanthini, 2011).

Dry matter and moisture content

For determination of moisture, a china dish was washed and placed in a hot air oven at 105ºC for 15 min. Then it was removed, cooled in a desiccator, and weighed on digital balance. Live feed after drying with filter paper was placed on a pre-weighed china dish and weighed again, the samples were placed in a vacuum oven for 5 h at 95ºC. After that following formula was used for the determination of dry matter:

$$\% \text{ dry matter} = \frac{\text{Weight of sample + Weight of a china dish after drying}}{\text{Weight of sample + Weight of a china dish before drying}} \times 100$$

$$\text{Percentage of moisture} = \frac{\text{Weight loses}}{\text{Original weight of sample}} \times 100$$

Crude ash

A crucible was washed and kept in a muffle furnace at 100ºC for an hour. Subsequently, cooled in a desiccator and weighed. After that 2.0 g sample was placed in the crucible and again placed in a muffle furnace at 600 ºC for 2- 4 h. The appearance of grey-white indicated the complete oxidation of organic matter in a sample. The sample was cooled and weighed quickly to prevent moisture absorption and crude ash% was determined by using the following formula.

$$\text{Crude ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Crude protein

For crude protein estimation, 1 g sample was digested with 5 g of a digestion mixture (w/w: 1g Na_2SO_4 and 10 g $CuSO_4$) and 30 ml of conc. H_2SO_4 and heated for 2 h at 250ºC on a hot plate until light green color appeared. After cooling, the final 250 mL volume was made in a volumetric flask by adding distilled water. After that the solution was made alkaline by adding 10 mL of 40% NaOH solution and the Kjeldahl apparatus was heated for 3 min. Then, with the addition of 10 mL boric acid (2%), liberated distillated (ammonia) was collected and titrated against H_2SO_4 (0.1 N)

by using 5 drops of methyl red as an indicator until the appearance of yellow color. Total protein was calculated by multiplying the amount of nitrogen with a conversion factor of 6.25, while %Nitrogen was calculated by using the following formula:

$$\textbf{\%Nitrogen} = \frac{\text{Normality of } H_2SO_4 \times \text{Volume of } H_2SO_4 \text{ used} \times 250 \times 0.014 \times 100}{\text{Weight of the sample}} \times 10$$

Whereby,

10 = Used volume of diluted mixture, 0.014 = Standard volume of H_2SO_4 (0.1 N) to neutralize 1mL of ammonia and 250 = Dilution of the digested mixture.

% Crude Protein = (%) Nitrogen x 6.25

Crude Fats

Crude fat in a sample was determined through the Soxhlet apparatus by using a hexane extraction method. Briefly, about 2.0 g of moisture-free, dry mass sample was taken in the pre-weighed thimble and kept correctly under the condenser of the apparatus. The receiving flask containing 150 mL of hexane was connected and the heater was turned on with a continuous supply of water. The extraction was completed in 120 min. Subsequently "thimble" was removed, dried, and weighed. The percentage of crude fat in the sample was calculated by using the following formula:

$$\text{Crude Fats (\%)} = \frac{\text{Weight of dried thimble after extraction} - \text{Weight of empty thimble}}{\text{Weight of the sample}} \times 100$$

Fatty acid analysis

Fatty acids analysis was conducted from the zooplankton mass isolated each month, using Gas chromatography mass-spectrometry (GC-MS) at the medicinal botanical center of Pakistan Council for scientific research (PCSIR) Laboratory, complex Peshawar. Zooplankton/live feed was collected. Each time 100g wet mass samples were collected (500g total) as a single sampling point. The wet mass was dried in an oven at 60°C overnight, and lipids extraction was conducted followed by FA analysis.

Extraction of total lipid

The extraction of total lipid, preparation of fatty acids methyl esters (FAMEs) and composition of FAMEs were analyzed by adopting previously adopted procedure (Afridi et al., 2018). Briefly, 10 g of dry mass was taken in a conical flask of Soxhlet apparatus and added n-hexane (Fisher Scientific, UK). The flask was heated for about 7 h and extracted oil was condensed with the help of a Rotary vacuum evaporator and shifted to glass vials. Extra hexane was evaporated by keeping vials overnight at ambient temperature (Poitevin, 2016).

Preparation and analysis of FAMEs on GC-MS

Poitevin (2016) procedure previously reported by Afridi et al. (2018) was adopted and FAMEs were prepared. The prepared FAMEs solution was filtered by membrane filter paper (pore size, 0.45 µm). The FAMEs composition was analyzed with a gas chromatography-mass spectrometer (GC-MS-QP2010, Plus Shimadzu, Kyoto, Japan) having 100 m CP Sil 88 capillary column (film thickness, 0.20 µm; i.d, 0.25 µm, Chrompack, Middleburg, Netherlands) and a flame ionization detector. Initially, at the time of sample injection, the column temperature for 1 min was set at 80°C, then increased at the rate of 2°C per min to 215°C and maintained this 30 min. The Inlet and detector temperatures were 220°C and 230°C respectively, while the mode of injection was split with a ratio of 100:1. The flow rate of 1 mL/min was set for H_2 carrier gas. Most of the fatty acid peaks were identified and quantified using FAMEs standard Mix SUPELCO (CRM47885, Bellefonte, USA) containing 37 components, used as an external standard (Gao et al., 2006; Wen et al., 2017). Their values are presented in terms of relative percent.

Statistical analysis

Data were analyzed using IBM SPSS statistics (software version 25, Inc. Chicago, USA). Before performing statistical analysis, Bartlett, and Shapiro-Wilk's tests were used to evaluate the homogeneity of variances and for normalizing the data. One-way ANOVA was conducted to indicate the significant seasonal variations in water quality parameters, proximate composition, and biochemical/Fatty acid composition. Duncan multiple range test was conducted to show intergroup comparison.

Experiment # 2: Comparative Effect of Live Feed and Prepared Diet and Cofeeding on Feed Intake, Digestive Enzymes Activity and Growth of *L. rohita* During Early Rearing

Experimental diets

Live feed

Live feed was collected from nursery ponds mentioned in chapter 1. Planktons were collected using plankton nets having mesh sizes: 20, 60 and 80 µm. Moreover, to avoid the oversized plankton inclusion, a larger-sized mesh plankton net was used before using the respective smaller-size plankton net as mentioned in Table 2. The gelatinous mass was repeatedly sieved to adjust the plankton size initially to avoid the inclusion of any oversized plankton in the calculated feeding mass. About 50g (wet weight) of live feed was collected, dried, and the proximate composition was determined (as discussed in chapter 1 in detail).

Nano-particulate feed formulation, preparation and characterization

A 60% crude protein (CP) ND, iso-proteinaceous to LF (60.7% dry mass based) was formulated and prepared. Briefly, all the dry ingredients mentioned in Table 1 were finely ground (electric grinder, MXBAOHENG Model HC-500), weighed, mixed well, and pelleted. The pellets were dried in an oven at 40°C and stored at 4°C. Afterwards, the nano-particulate feed was prepared by adding a 200 g finely ground prepared feed into a ball mill grinder (having a barrel with 24 small and 9 large balls) for conversion into fine form. X-ray diffraction analysis (XRD) of feed was performed (wavelength 0.7 to 2.3 Å) at the National Centre for Physics (NCP), Islamabad for analyzing the particle size and structure. Scanning electron microscope (JSM-6490-LA, Japan) 20,000 X, was used to determine the size, shape, and surface morphology of the particles of feed at the National University of Science and Technology (NUST), Islamabad.

Induced spawning

Sexually mature *L. rohita* brooders were breed at Fisheries and Aquaculture Research Station, Quaid-i-Azam University, Islamabad by adopting the method reported earlier (Ahmed et al., 2020). The ripeness of broodfish was accessed by several indicators like soft/distend belly with swollen genital opening and smooth pectoral fins (in females) while in males, secondary sexual characteristics were obvious as rough pectoral fin (Metwally et al., 2008) was observed. Moreover, by applying slight pressure on the abdomen, release of white colored milt confirmed the ripeness.

Table 1. Formulation and proximate analysis of the experimental feed for early rearing of *L. rohita*.

Feed Ingredients	**Inclusion level (g/100g)**	
Fish meal (66% CP)	42	
Soybean meal (48% CP)	05	
Casein (93.1% CP**)**	30	
Rice Polish (13% CP)	05	
Wheat bran (12% CP)	05	
Gluten 60 (60% CP)	05	
Vitamin and mineral premix	03	
Fish oil	05	
Proximate composition (%)	Prepared feed	Live feed
Crude protein	60.02	61.74
Crude lipids	07.90	09.74
Ash	08.70	09.69

Induced breeding was conducted by following the procedure commonly used for major carps (Ahmad et al., 2020). Briefly, after 2-3 h of conditioning, broodfish were weighed individually and injected Ovaprim (synthetic induced spawning agent: analog of LHRH + Dopamine antagonist Syndel, USA) intramuscularly at the rate of 0.5-0.7ml/kg of body weight (Female brooder), and 0.2 ml/kg (Male brooders) (Mohapatra et al., 2018; Ahmad et al., 2019). Later, fish were kept in a circular tank having a slow flowthrough system. For avoiding disturbance, a canvas screen was used to cover the tank. The water quality parameters like temperature and dissolved oxygen of the broodfish tank were measured frequently after injection to ovulation by using Multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA), and found to range between 28-30˚C and 5-6 mg/L respectively, while ammonia was less than 0.25 ppm. Fertilized eggs that appeared transparent and clear were incubated in a circulating tank till yolk sac absorption (postlarvae stage: PL).

Experimental design

A 35-days feeding trial was designed to compare the effect of live feed and Nano-particulate prepared feed on feed intake, digestive enzymes activity and growth performance of PLs of *L. rohita*. The experiment was conducted in a replicate of 5 small circular experimental

tanks (capacity: 8.0L), having a slight slope towards the drain in the center and supplied with aerated water. PLs 3 days after hatching (3DAH) were randomly stocked in 15 tanks at the rate of about 500 larvae/tank. The stocking density of 100 larvae/L was set by following Cahu et al. (1998). The tanks were randomly divided into 3 groups, T1 was reared exclusively on LF, T2 was reared on ND, and T3 was Co-fed with both LF +ND (1:1). All groups were provided with their respective diets for 35 days. Initially, PLs were fed continuously and then during the feeding trial, feeding frequency, and feeding rate were adjusted according to the size and body weight of the fries. Throughout the feeding trial, the prepared feed was provided in nano-particulate form, while live feed was sieved and provided according to the size of PL/DAH (Table 2).

Table 2. Mesh size, feeding rate and frequency during the experimental trials.

Plankton net mish(µm)	Feeding rate (%)	Feeding frequency
20-60*	50	Continuous feeding for 10 DAH
40-150*	30	6 times/day (10-20DAH)
60	10	3-4 Times/day (20-35DAH)

∗Represent mesh size, used to avoid any oversized plankton inclusion.

Sampling

Depending on size, PLs (3DAH), and then fries per tank were sampled at 10, 15, 20, 25, 30 and 35-days post-hatching by the slight opening of a drain of the tank in a small bucket (1L) having hand net (mesh size: 40µm). Sampling from experimental tanks was performed at 8:00 AM before feeding. The dead fries in experimental tanks were daily noted and the number of sampled fries was also considered for the calculation of survival at the end of the feeding trial. The number and wet weight of larvae/fries per tank sampled and used for the analysis of intestinal enzymes and the expression of appetite and growth-related genes, *Leptin*, *Ghrelin*, *IGF-1* (insulin-like growth factor-1, and *GHS-R* (growth hormone secretagogue receptors also called *ghrelin* receptor) are presented in Table 3.

Figure 3. Breeding of Rohu brooders: ovaprim injection, hand striping and fertilization to produce larvae at fisheries and aquaculture research facility, quaid-i-Azam University, Isamabad.

Table 3. Number and wet weight of PLs/fries of *L. rohita* sampled per tank for enzymatic analysis and genes expression assessment.

Sampling (DAH)	For Enzymes Activity		For Gene Expression Study		Sample
	Larvae/fries (No's)	Sample mass (mg)	Larvae/fries (No's)	Sample mass (mg)	
03	1500	600	250	100	Whole body
10	30	1700	20	900	Whole body
15	25	2000	15	800	Trunk
20	25	2500	15	1400	Trunk
25	20	2600	15	1500	Trunk
30	35	1100	25	800	GI tract
35	35	1300	25	900	GI tract

For digestive enzyme analysis, sample collection was performed by following López-Ramírez et al. (2011) procedure. Briefly up to 10 DAH, whole fries was taken and pooled (5 samples /group). Afterwards, fries were anaesthetized by immersing in buffered MS-222 (Sigma-Aldrich, St. Louis, MO, USA) solution (75mg/L) and dissected aseptically on ice pad, up to 25DAH abdominal portion was collected by removing the head and tail and then at 30DAH and 35DAH, GI-tract of fries was dissected out and pooled. Samples were immediately frozen in liquid nitrogen and stored at -20°C for further analysis. For analyzing the expression of appetite and growth-related genes (*Leptin*, *Ghrelin*, *IGF-1*, and *GHS-R*) similar sample collection procedure was adopted and samples of each group were collected and stored in RNAlater™ (Thermo Scientific CAT# AM7020) at 4 °C till further analysis.

Growth performance and survival

Growth performance data were collected on 10, 15, 20, 25, 30 and 35 DAH, while survival (%) was calculated at the end of the feeding trial. The initial wet weight of PLs (3DAH) was determined by taking the small number of larvae, weighed nearest 0.01 g by using a digital balance (GF- 300 digital scale, A&D company limited, USA), counted the number in that weight and then divide the weight by the total number of larvae. The procedure was repeated 4-5 times and an average weight of PLs was calculated. The average body weight of fries and advanced fries during and at the end of the feeding trial was recorded by weighing the number of fries collected in hand

net at different periods for sampling. The growth performance and survival were evaluated by adopting the following standard formulas.

$$\text{Average weight (g) of individual PLs /fries} = \frac{\text{Weight (g) of sampled larvae/fries}}{\text{Total number of sampled larvae/fries}}$$

$$\text{NWG (g)} = \text{FBW of fries} - \text{IBW of larvae}$$

$$\text{SGR (\%)} = [(\ln \text{Wf} - \ln \text{Wi}) \div \text{No. of days of the experiment}] \times 100$$

$$\text{Survival (\%)} = (\text{Number of fries survived} \div (\text{Total PLs stocked}) \times 100$$

Where,

BW=Body weight (g); NWG= Weight gain (g); SGR= Specific growth rate (% body weight/day); FBW= Final body weight (g); IBW= Initial bodyweight (g); *ln* Wf = Natural log of final body weight; *ln* Wi = Natural log of initial body weight.

Analysis of gut enzymes

Preparation of Enzyme solution

For enzymatic analysis, 0.5 g of the sample was homogenized using an electrical homogenizer (Model, VWR POWER 200) in 5ml chilled phosphate buffer (PBS) (0.1 M, P^H 7.5) and the homogenate was centrifuged at 10,000 rpm (Model Eppendorf centrifuge 5417R) for 10 min at 4ºC. The supernatant (Enzyme solution) was collected and stored at 4ºC until analysis.

The activity of intestinal enzymes, protease, amylase, and cellulase was performed following the standard protocol reported earlier (Amir et al., 2018).

Protease assay

The proteolytic enzyme activity was analyzed by the casein digestion method. Briefly, 1ml of enzyme solution was mixed with 5ml (0.65%) of casein solution and incubated at 37ºC for10 min. Afterwards, 5 mL of Trichloroacetic acid ($C_2HCl_3O_2$, 110 mM) was added and further incubated for 30 min at the same temperature.

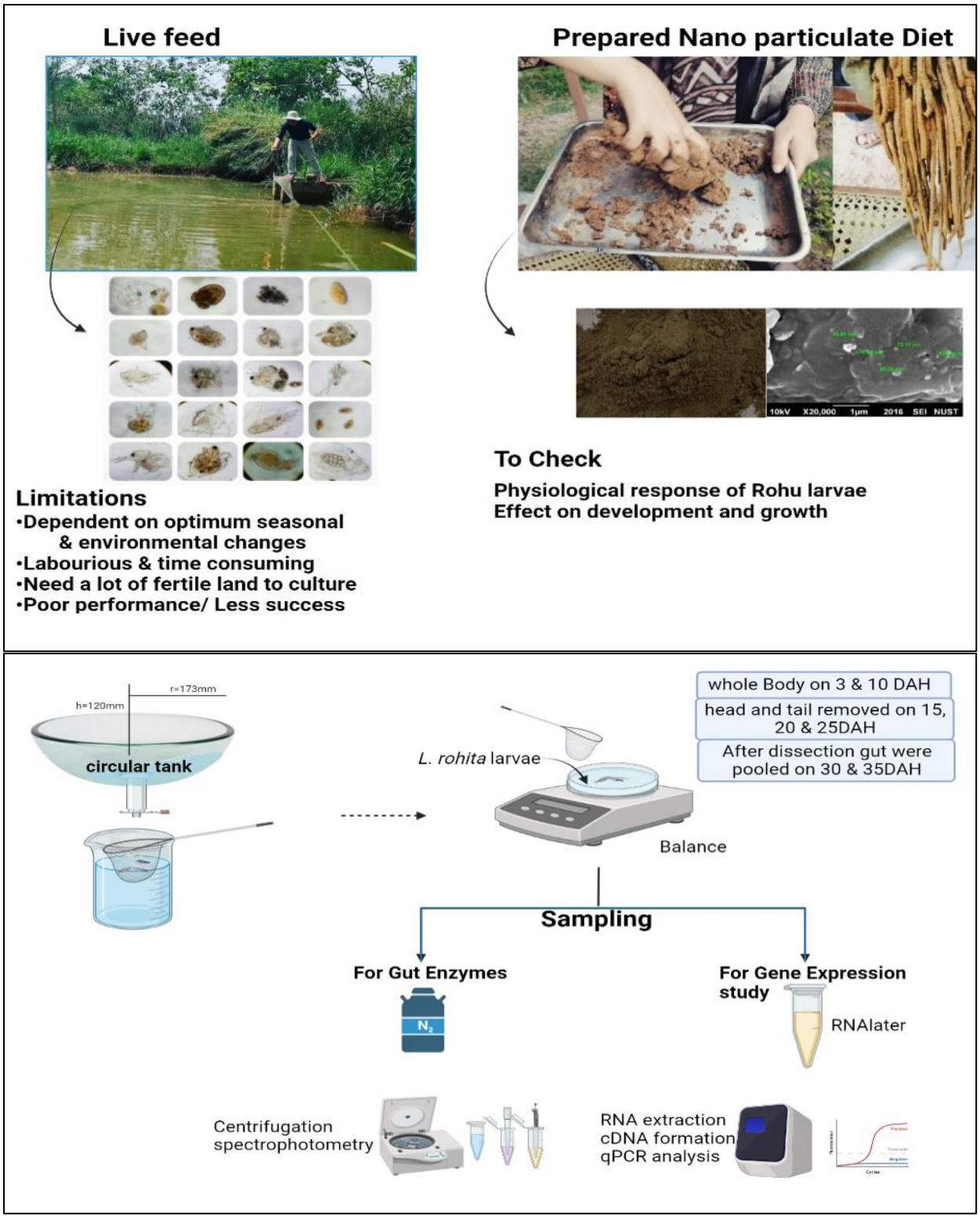

Figure 4. Graphical abstract of second experiment; feeding Rohu post-larvae, live feed and prepared nano-particulate feed.

Subsequently, the solution was kept at room temperature for cooling. Afterwards, the solution was filtered (Whatman filter paper #1, pore size 11µ), and 2 mL of filtrate was mixed with 1 mL Folin–Ciocalteu reagent (0.5 mM) and 5mL of Na2CO3 (500 mM) solution and incubated at 37ºC for 30 min. After cooling, absorbance was measured by a UV-Visible spectrophotometer at 660 nm. The tyrosine standard curve was used to determine protease activity and, expressed as a mole of tyrosine released/min/mg protein at 37°C.

Amylase assay

Amylase activity was measured by the 3, 5-Dinitrosalicylic acid (DNS) method (Bernfeld, 1955) which is based on the estimation of reducing sugars at 560 nm using maltose as the standard. Briefly, 0.5 mL of enzyme solution was incubated for 3-4 min at room temperature. Then, 500 µL of 1% starch solution was added and kept at ambient temperature for 3 min, followed by the addition of 1 mL of DNS reagent and incubated in a boiling water bath for about 5 min. After cooling at room temperature, 10 mL of reagent-grade water was added, and absorbance was noted by using a spectrophotometer set at 540 nm. The maltose standard curve was used to determine the activity and expressed as a mole of maltose released/min/mg protein at 37°C.

Cellulase assay

Cellulase activity was determined by adopting Denison and Koehn (1977) method with a few modifications. Briefly, 1 ml of enzyme solution was mixed with 1 ml of CMC (1% carboxymethyl cellulose) and 1 ml of citrate phosphate buffer (0.1 M) incubated at 50ºC for 30 min. Subsequently, 3 mL DNS (Di-nitro salicylic acid) reagent was added and continued incubation for a further 15 min. In the final step, 1 mL of sodium potassium tartrate (40%) was added and the whole mixture was allowed to cool down at ambient temperature. Afterwards, absorbance was noted by using a spectrophotometer at 540 nm and the production of reducing sugar (glucose) from CMC substrate resulting from cellulase activity was measured and expressed as mole mg of glucose liberated/min/ml of enzyme solution.

Expression of appetite and growth-related genes

The mRNA level of *IGF-1*, *Leptin*, *Ghrelin*, and its receptor *GHS-R* was determined by using Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Briefly, RNA from samples was extracted with the aid of Illustra RNA spin Mini Isolation Kit (GE Healthcare, Chicago, IL, USA) and quantified by NanoDrop^{TM} (ND-1000, UV-visible spectrophotometer, Thermo Scientific, USA). Later, the isolated RNA was converted into cDNA using a cDNA synthesis kit (Revert Aid First Strand cDNA synthesis kit) by following manufacturer instructions (Thermo Scientific, USA).

Primers were designed (Table 4) with Oligo Primer Analysis Software version 1.1.2 and manufactured from Humanizing Genomics Macrogen. The nucleotide sequence of the respective genes of *L. rohita* was obtained from the gene bank NCBI (www.ncbi.nlm.nih.gov)**.** The quality of cDNA and its compatibility with primers was checked by performing a simple PCR followed by gel electrophoresis of each sample in duplicate. Afterwards, qPCR was performed by following the protocol reported by Ahmad et al. (2020). Briefly, a 20 µL reaction mixture was prepared by mixing 0.4 µl of forward and 0.4 µl of reverse primer with 7.6 µl of syringe water, 1.6 µl of diluted cDNA, and 10 µl of SYBER green. The PCR conditions were optimized along with cycle numbers (initial denaturation at 95°C for 4 min followed by 40 cycles at 95°C for 15 seconds and subsequently, 62°C for 15 seconds). The efficiency of the PCR reaction for each gene was checked by plotting standard curves with serial dilutions (1/10, 1/100 and 1/1000) of the cDNA of a control sample. The mRNA levels of each gene were normalized to the expression of β-actin of *L. rohita***.** The relative variations in gene expression were calculated by the standard ΔΔCT method (Pfaffl 2001).

Statistical analysis

Data were analyzed using IBM SPSS statistics (software version 25, Inc. Chicago, USA). Before performing statistical analysis, Bartlett, and Shapiro-Wilk's tests were used to evaluate the homogeneity of variances and for normalizing the data. ANOVA followed by Duncan tests were used to analyze the effect of two independent variables (Age DAH and Feed) on growth performance, the activity of intestinal enzymes and expression of *IGF-1*, *Leptin*, *Ghrelin* and *GHS-R*. Moreover, the effect size was determined using Partial Eta Squared (η_p^2) estimates for the significance of a research outcome. Furthermore, the statistical program graph Pad Prism Version 9.0 was used for graphic presentation.

Table 4. Primers used to evaluate the mRNA expression of studied genes.

Name	Primers	start	stop	length	tm	GC%
β-actin	F-ATGAAGATCCTGACCGAGAGA	9	30	21	62	47.6
	R-CTCGAAGTCAAGAGCCACATAG	92	114	22	62	50
GHSR	F-GCATGCCTCTGGACTTGTATAG	124	146	22	62	50
	R-CGCTGAGAGCAGTGATGTTTA	235	256	21	62	47.6
IGF-1	F-TGTACTGTGCACCCGTAAAG	251	271	20	62	50
	R-CCTTACAGGAAGAGTGGCTATG	349	371	22	62	50
Leptin	F-ATCCAAGGTCTCGGGTCTAT	182	202	20	62	50
	R-TGAACAACTCGTAGCGTATCT	264	286	22	62	45.5
Ghrelin	F-AGCGAAGCAGAGTATGAGAAA	293	315	22	62	45.5
	R-GAGCTTAAGTGGAGAGAACTG	388	410	22	62	50
Myostatin	F-GTGTCCAAGAGAAACAGAGAGG	1622	1644	22	60	50
	R-CAATTGGATCGGTGCGTTTATT	1707	1729	22	60	40.9
MyoD	F-CTTTCGAGACCCTCAAGAGATG	36	58	22	59	50
	R-GCTTGGAGAGACTCAATGTAACTA	115	139	24	59	41.7
GH	F-GCCAGATCCTAAGCAGAACCG	220	59	22	60	45.5
	R-AAGCAAGCCAGCAGACGAAAG	235	256	21	60	50
Myogenin	F-GGCTTCGACCAAACAGGATA	251	271	20	59	50
	R-GCTCCTGGTGAGGAGACAAG	349	369	20	59	40.9
MYF-5	F-GCAGGCTGAAGAAGGTGAAC	182	203	21	58	50
	R-GGCTTCCTCAGGATCTCAAC	264	284	20	58	48
MEF2A	F-TGACTGTGAGATTGCCCTGA	293	313	20	58.8	49.1
	R-CGTGGGGTTCGTTGTATTCT	390	410	20	58	48

All primers are arranged in 5 to 3 direction.

Experiment # 3: Evaluation of Protein Requirement of *Labeo rohita* During Early Life Stage

Experimental feed

To optimize the dietary protein requirement of *L. rohita* during early rearing, five Nano-particulate diets having graded level of protein (45, 50, 55, and 60% CP) were formulated (Table 5) and prepared by the method mentioned in the experiment # 3 protocol. Fishmeal was used as chief protein source in the experimental feed, while the other ingredients were selected as mentioned in experiment # 2.

Table 5. Formulation (g/100g) and proximate analysis of the experimental feed with various protein inclusion levels.

Feed Ingredients	**Group based on CP**			
	P45	**P50**	**P55**	**P60**
Fish meal (66% CP)	42	42	42	46
Casein (93.1% CP)	10	15	22	26
SBM (48% CP)	10	10	10	05
Rice Polish (13% CP)	15	10	05	05
Wheat bran (12% CP)	10	10	08	05
Gluten 60 (60% CP)	05	05	05	05
Premix*	03	03	03	03
Fish oil	05	05	05	05
Proximate composition (%)				
Crude protein	45.18	49.99	54.89	59.98
Crude lipids	6.56	6.62	6.82	6.93
Ash	8.01	7.89	8.34	8.08

Table; * represent mineral and vitamin premix. CP; crude protein, SBM; Soya bean meal

Broodfish were induced bred and 3DAH PLs were obtained by method reported earlier. Experimental setup/condition was kept the same as mentioned above, including stocking density and sampling procedure. Shortly, 10,000 PLs (3DAH) were randomly stocked in 20 tanks. 5 tanks were randomly selected as one group. The four experimental groups based on crude protein

inclusion level labelled as P45, P50, P55 and P60 were fed their respective diets (Table 5) for 35 days.

Sampling

For the evaluation of temporal changes in growth rate, i.e., net weight gain (NWG) and SGR, larvae/fries were collected and weighed by the procedure mentioned above in the protocol of experiment # 2. Similarly, to assess the impact of dietary protein inclusion level on the digestion as well as on growth and muscle differentiation during early rearing of fish, sampling for intestinal enzymes (protease, amylase, trypsin, and chymotrypsin) and the mRNA level of myoblast determination protein 1(MyoD), Protein Coding gene, Myogenic Factor 5 (MYF5) and growth hormone coding gene (GH), was done by adopting procedure as reported in previous experiment.

The number and wet weight of larvae/fries per tank sampled and used for the analysis of intestinal enzymes (protease, amylase, trypsin, and chymotrypsin) and the expression of MyoD, MYF5 and GH are presented in Table 3.

Growth performance

Temporal change in body weight of PLs after feeding diet having different inclusion level of protein was calculated by standard formulas mentioned in experiment 2 protocol.

Digestive enzyme activity

All chemical used were of export quality (Sigma-Adrich Germany) and purchased from local market supplier. To get accurate results, all enzymatic activities were checked at 25 ˚C and P^H of different solutions were measured at room temperature. The enzyme solution was prepared, and amylase and protease assays were performed by method reported in Experiment # 2 materials and methods section. The other enzymes are mentioned below.

Trypsin assay

Trypsin activity was measured by following the protocols reported by Chakrabarti et al. (2006). Briefly, substrate was prepared by mixing 1 mM BAPNA (N-a-benzol.D, L-arginine p-nitroanalidine) and 0.1 mM SAPNA (Suc-Ala-Ala-Pro-phe, p-nitroanalidine) in 50 MmTris-HCl

and 20mM $CaCl_2$). The substrate was mixed with enzyme extract. Afterwards, mixture was incubated at 37 ℃ and absorbance was checked at 410 nm (Erlanger et al., 1961). Trypsin activity was measured as change in absorbance per minute per milligram of protein.

Chymotrypsin assay

Chymotrypsin assay was conducted with 1 mM BAPNA, *N*-*α*-benzoyl.D,L-arginine *p*-nitroanalidine, and 0.1 mM SAPNA, Suc-Ala-Ala-Pro-Phe *p*-nitroanalidine (in 50 mM Tris–HCl, P^H 7.5, 20 mM $CaCl_2$). Enzyme extract was incubated with respective substrates at 37°C and absorbance was recorded continuously under kinetic mode for 3 min at 410 nm (Erlanger *et al.,* 1961). Trypsin and chymotrypsin units of activity were expressed as change in absorbance per minute per milligram of protein. Activity units were calculated by the following equation:

$$\text{Activity units} = \frac{(Abs_{410}\text{min}) \times 1000 \times \text{mL of reaction mixture}}{\text{Extinction coefficient of chromogen} \times \text{mg protein in reaction mixture}}$$

The molar extinction coefficient of *para*-nitroanalidine liberated from BAPNA and SAPNA is

$\varepsilon_{410} = 8800\ cm^2/mg$

Expression of growth regulating genes

The mRNA level of MyOD, MYF5 and GH was determined by using Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Briefly, RNA from samples was extracted with the aid of same RNA spin Mini Isolation Kit (GE Healthcare, Chicago, IL, USA) as mentioned above and quantified by NanoDrop™ (ND-1000, UV-visible spectrophotometer, Thermo Scientific, USA). Afterwards, with the aid of cDNA synthesis kit (Revert Aid First Strand cDNA synthesis kit) and by following manufacturer instructions (Thermo Scientific, USA), the isolated RNA was converted into cDNA.

Primers for the selected genes were designed (Table 4) with Oligo Primer Analysis Software version 1.1.2 and manufactured from Humanizing Genomics Macrogen. The nucleotide sequence of the respective genes of *L. rohita* was obtained from the gene bank NCBI (www.ncbi.nlm.nih.gov)**.** The quality of cDNA and its compatibility with primers was checked by

performing a simple PCR followed by gel electrophoresis of each sample in duplicate. Afterwards, qPCR was performed by following the protocol reported by Ahmad et al. (2020). Briefly, a 20 µL reaction mixture was prepared by mixing 0.4 µl of forward and 0.4µl of reverse primer with 7.6 µl of syringe water, 1.6 µl of diluted cDNA, and 10 µl of SYBER green. The PCR conditions were optimized along with cycle numbers (initial denaturation at 95°C for 4 min followed by 40 cycles at 95°C for 15 seconds and subsequently, 62°C for 15 seconds). The efficiency of the PCR reaction for each gene was checked by plotting standard curves with serial dilutions (1/10, 1/100 and 1/1000) of the cDNA of a control sample. The mRNA levels of each gene were normalized to the expression of β-actin of *L. rohita***.** The relative variations in gene expression were calculated by the standard ΔΔCT method (Pfaffl 2001).

Statistical analysis

The same statistical tools mentioned in experiment 2 were followed.

Experiment 4: Evaluation of Optimum Dietary Requirement of Lipid During Early Rearing of *L. rohita*

Experimental feed

To evaluate an optimum dietary requirement of lipid during early rearing of *L. rohita*, four iso-proteinious 55% CP experimental diets having graded levels of lipid (4, 8, 12, 16 %), using fish oil as the major source were prepared (Table 6). Diets were prepared and converted into Nano-particulate form by the method reported in initial experimental procedure in detail (Experiment # 2; Section, Materials and methods).

Table 6. Formulation (g/100g) and proximate analysis of larval feed with various fats inclusion level in feed for *L. rohita* PLs.

Feed Ingredients	**Group based on fats inclusion level**			
	CF4	CF8	CF12	CF16
Fish meal (66% CP)	53	53	53	53
Casein (93.1% CP)	15	16	17	18
SBM (48% CP)	10	09	05	03
Rice Polish (13% CP)	06	03	03	02
Wheat bran (12% CP)	05	04	03	03
Gluten 60 (60% CP)	05	05	05	05
Premix*	03	03	03	03
Fish oil	03	07	11	13
Proximate composition (%)				
Crude protein	54.72	54.63	54.31	54.29
Crude lipids	4.07	8.22	12.23	16.22
Ash	8.11	8.19	8.12	8.01

Table; * represent mineral and vitamin premixes.

Experimental design and rearing conditions were same as reported for the evaluation of optimum dietary protein requirement except that 50% CP was fixed in experimental diets while lipid was added at graded levels. Briefly experiment was conducted in a replicate of 5 in small circular experimental tanks (capacity: 8.0L). PLs 3DAH were randomly stocked in 20 tanks at the rate of about 500 larvae/tank (stocking density:100 larvae/L). The tanks were randomly divided

into 4 groups based on dietary lipid inclusion level, CF_4, CF_8, CF_{12} and CF_{16} and reared on nano-particulate isonitrogenous(50%CP) diets having lipid inclusion level, 4, 8, 12 and 16% respectively. All groups were provided with their respective diets for 35 days. Initially, PLs were fed continuously and then during the feeding trial, feeding frequency, and feeding rate were adjusted according to the size and body weight of the fries.

Sampling

For the evaluation of temporal changes in growth rate, intestinal enzymes and mRNA level of myoblast determination protein 1(MyoD), Protein Coding gene, Myogenic Factor 5 (MYF5) and growth hormone coding gene (GH), sampling was done at 3, 10, 15, 20, 25, 30 and 35-days post-hatching by adopting the procedure mentioned above. The number and wet weight of larvae/fries per tank sampled and used for the analysis of intestinal enzymes (lipase, protease, amylase, and Alkaline phosphatase) and the expression of MyOD, MYF5 and GH as presented in Table 3. Samples collected for enzymes assay were immediately frozen in liquid nitrogen and then stored at -20°C for further analysis, while samples collected for gene expression analysis were stored in RNAlater^{TM} (Thermo Scientific CAT# AM7020) at 4 °C till further analysis.

Growth performance

Growth performance data was collected on 3, 10, 15, 20, 25, 30 and 35 DAH, while survival (%) was calculated at the end of the feeding trial. The initial wet weight of PLs (3DAH), and body at different time period was determined by the method reported earlier while the growth performance and survival were evaluated by adopting the standard formulas also mentioned in the experiment # 2, materials and methods section.

Digestive enzyme assay

The intestinal enzymes amylase and protease activity in different groups of fish was determined by the method reported earlier in detail (Experiment # 2, Section; Materials and methods).

Lipase assay

Lipase activity was determined by the titrimetric method of Mukundan et al, (2013). Crude enzyme extracts (3 ml) of the various tissue preparations were added separately to reaction mixtures consisting of 5 ml of tributyrin emulsified with PVA (0.1 M) and 5 ml of 0.05 M calcium chloride and incubated at 37 °C for 1 h. The reaction was terminated by the addition of 20 ml of 80% ethanol. Blank determinations were conducted in a similar manner, except that the extracts were introduced after termination of the reaction. Lipase activity was estimated by titration with standard 0.1 M NaOH using thymolphthalein as indicator. Specific enzyme activity was expressed as µmol butyric acid liberated as /mg protein/ min.

Alkaline phosphatase (ALP) assay

Alkaline phosphatase activity was estimated following the method of Bramley (1974) with slight modification using p-nitrophenyl phosphate (p-NPP) in alkaline medium. The enzyme solution, with buffer (1 M sodium carbonate, P^H 10.4), 0.5 µM EDTA (adjusted to P^H 10.4 with 1N NaOH) and 3 µM magnesium acetate was incubated at 30 °C. The reaction was stopped by adding 0.5% (w/v) EDTA in 0.5 M NaOH after 30 min. Free p-nitrophenyl was measured at 420 nm. Protein content of supernatant solution was measured following the method of Lowry et al, (1951). Enzyme activity expressed as µg p-NP liberated/ mg protein/h.

Expression of growth regulating genes

The mRNA level of MyOD, MYF5 and GH was determined by using Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and protocol reported earlier.

Statistical Analysis

The same statistical tools mentioned in experiment 2 were followed.

Experiment # 5: Post-standardization Feeding Trial in Comparison to Feeding Live Feed

After figuring out the optimum level of lipids and protein in the diet of *L. rohita* larvae, a standardized nano-particulate feed (SNF) was formulated to conclude our hypothesis of balance larval feed provision. Despite the efficient growth with prepared feed (compare to live feed alone) in the 1st trial lower survival rate was a major limitation with prepared diet.

Experimental feed

Standardized nano-particulate feed

Standardized nano-particulate feed (SNF) with 55% CP and 8% fish oil feed was formulated using the same dry ingredients (Table 7). The dry ingredients were fine grinded and feed was prepared and then converted into Nanoform by the method reported earlier. XRD and SEM (Scanning electron microscopy) of SNF were conducted at NCP, Islamabad.

Table 7. Formulation and proximate composition of SNF.

Feed Ingredients	Inclusion level (g/100g)
Fish meal (66% CP)	46
Soybean meal (48% CP)	05
Casein (93.1% CP)	23
Rice Polish (13% CP)	05
Wheat bran (12% CP)	06
Gluten 60 (60% CP)	05
Premixis*	03
Fish oil	07
Proximate composition (%)	
Crude protein	55.02
Crude Fats	8.42
Ash	08.01

Table 6. *Premixes include vitamin and mineral premixes. CP: Crude protein

Live feed

Live feed composition changes significantly during the year, due to changes in primary productivity and manuring. That is why special emphasis was kept on maintaining a uniform zooplankton community in the nursery ponds following the protocols mentioned in experiment 1. Live feed was collected both for biochemical assessment and as feed in the experimental trial. During experimental trial, planktons were collected using plankton nets having mesh sizes: 20, 60

and 80 µm. Moreover, to avoid the oversized plankton inclusion, a larger-sized mesh plankton net was used before using the respective smaller-size plankton net as mentioned in Table 1. The gelatinous mass was repeatedly sieved to adjust the plankton size initially to avoid the inclusion of any oversized plankton in the calculated feeding mass. About 30 g (wet weight) of live feed was collected, dried, and the proximate composition and fatty acids profile was conducted.

Proximate and biochemical analysis of feed

Proximate composition

About 30 g sample of each, standardized nano-particulate feed and live fed to 3DAH, 10DAH, 15DAH, 20DAH, 25DAH, 30DAH and 35DAH PLs was collected and their proximate composition (crude protein, crude fat, ash and % dry matter) was conducted in collaboration with PPRI (Pakistan Poultry Research Institute) Islamabad by adopting the standard methods (AOAC, 2012). The Soxhlet apparatus and micro Kjeldahl method were used for the determination of crude fats and crude protein respectively (Sutharshiny and Sivashanthini, 2011). The detailed procedure for the estimation is mentioned in experiment 1.

Fatty acid profile

Fatty acid profile of standardized nano-particulate feed and the live feed fed to PLs of 3DAH, 10DAH, 15DAH, 20DAH, 30DAH and 35DAH was conducted in collaboration with PCSIR by adopting procedure reported earlier.

Experimental design

Feeding trial was designed and conducted in a replicate of 5 in small circular experimental tanks (capacity: 8.0L), having slight slope towards the drain in center and supplied with aerated water. PLs (3 Days after Hatching, DAH) were randomly stocked in 10 tanks at the rate of about 500 larvae/tank. The stocking density:100 larvae/L was set by following Cahu et al. (1998). The tanks were randomly divided into 2 groups, first group was reared exclusively on LF, while the second group was reared on SNF. Both groups were provided with their respective diets for 35 days following the same feeding strategy as mentioned in experiment 2. Throughout the feeding trial, prepared feed was provided in nano-particulate form, while live feed was sieved and provided according to the size of larvae/DAH (Table 2).

Growth performance and survival

To determine the temporal change in growth performance on SNF compared to LF, NWG and SGR was determined using the standard formulae, mentioned in experiment 2. Survival percent was determined at the end of trial.

Sampling

Same sampling procedure was adopted for enzymatic analysis and gene expression studies as mentioned in experiment 2. Numbers of PL/fry sampled and the sampling points has been presented in figure below.

Enzymatic assay

All chemical used were of export quality (Sigma-Adrich Germany) and purchased from local market supplier. To get accurate results, all enzymatic activities were checked at 25 ˚C and P^H of different solutions were measured at room temperature. The enzyme solution was prepared, and same procedure was followed for cellulase, amylase and protease assays as mentioned in Experiment # 2; trypsin, chymotrypsin in Experiment # 3; and ALP and lipase activity in Experiment # 4.

Biochemical analysis of PLs/fry

Fatty acids were analyzed from muscle tissue using Gas chromatography mass spectrometry (GC-MS) at medicinal botanical center of Pakistan council for scientific research (PCSIR) Laboratory, complex Peshawar, following the same protocol mentioned above.

Genes expression

The mRNA level of MEF2A, MyoD, MYF5 and GH, Myogenin, myostatin, *IGF-1*, was determined by using Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the protocol reported earlier. Primers of the above-mentioned genes are given in Table (4).

Histology

Post larvae/fries after feeding different feeds (live or prepared feed) was collected at the end of trial and fixed in 10% formalin (for 24h) for studying morphological changes in the digestive tract. The fixed samples were then dehydrated through graded alcohols (50%, 70%,

80%, 95% and absolute ethyl alcohol), cleared in xylene and subsequently embedded in paraffin. Serial sections (6 µm) were cut from each block and mounted on acid-washed slides. Sections was de-waxed, rehydrated and stained with hematoxylin and eosin. All prepared slides were observed under light microscope (OPTIKA, Italy). The villus height, villus thickness and muscular thickness of each slice was measured.

Statistical analysis

The same statistical tools mentioned in experiment 2 were followed.

RESULTS

Experiment 1

Water quality

The water quality of the earthen pond was assessed periodically (Table 8). Significant changes in water quality parameters were observed during the studied period. Notable variations in temperature of water were observed from March to October (studied period 2020-2022). The average temperature of water in March was noted to be about 21°C, with a significant increase in temperature each month till July (33°C) and significant decrease thereafter. Dissolved oxygen also periodically altered with significantly high dissolved oxygen during March, April, and May. However, a significant drop in dissolved oxygen was observed till August and a re-raised in September. The P^H of water during the study period increased steadily during the early month with a significant rise in May followed by significantly the highest P^H in July and August (8.6) and a sudden fall in September and October. Other parameters like Total Dissolved Solids (TDS), alkalinity, and ammonia concentration followed a similar trend (Table 8) with few exemptions. Moreover, color of water was also noted; that was transparent to mild yellowish in March with addition of green tint with every month and a bright green colour by June, brownish green in July and brownish in August having a slight reddish tint. In September the water tuned brownish and slightly transparent toward the end of October.

Productivity of nursery pond

The productivity of earthen nursery ponds was estimated by assessing the plankton concentration periodically (Table 9). Seasonal change in plankton community was evident throughout the study period; phytoplankton concentration was observed to be inversely related to the zooplankton concentration in the nursery ponds. Initially phytoplankton were predominantly observed soon after the initial manuring with a comparative low relative biomass of zooplankton. The dry mass (DM) was significantly lower (0.009 g/cm^3) during March, where 73% of the mass was comprised of phytoplankton. The dry mass (DM) increased from March to August (peak value 0.07 cm^3) and a slight decline after that till November (0.05 g/cm^3). The month-wise ratio between phytoplankton and zooplankton also changed: from May, the zooplankton appeared to

Table 8. Month-wise changes in water quality parameters in the earthen nursery ponds.

Parameters	March	April	May	June	July	August	September	October
DO (mg/L)	6.20 ± 0.15^{a}	6.17 ± 0.20^{a}	6.16 ± 0.32^{a}	5.90 ± 0.39^{b}	5.77 ± 0.12^{c}	5.58 ± 0.09^{d}	5.94 ± 0.32^{b}	6.09 ± 0.09^{ab}
pH	7.16 ± 0.14^{d}	7.17 ± 0.15^{d}	7.52 ± 0.09^{c}	8.14 ± 0.13^{b}	8.68 ± 0.05^{a}	8.69 ± 0.012^{a}	7.53 ± 0.01^{c}	$7.\ 18\pm0.01^{d}$
Temperature (°C)	21.00 ± 0.35^{f}	23.71 ± 0.26^{e}	25.53 ± 0.39^{d}	29.67 ± 0.26^{c}	33.00 ± 0.23^{a}	32.73 ± 0.27^{b}	24.67 ± 0.26^{e}	20.60 ± 0.35^{f}
TDS (mg/L)	154.36 ± 1.8^{e}	176.73 ± 5.3^{de}	180.00 ± 3.31^{d}	201.57 ± 1.56^{b}	202.57 ± 1.86^{b}	208.79 ± 2.2^{a}	188.57 ± 1.5^{c}	175.57 ± 1.5^{de}
Alkalinity (ppm)	142 ± 0.22^{f}	150 ± 0.30^{de}	167 ± 0.78^{d}	173 ± 1.33^{c}	200 ± 4.35^{b}	267 ± 5.03^{a}	166 ± 2.31^{d}	162 ± 1.22^{e}
Transparency (cm)	30 ± 0.31^{a}	29 ± 0.36^{b}	29 ± 1.70^{b}	28 ± 0.99^{c}	28 ± 1.035^{c}	27 ± 1.77^{d}	28 ± 1.39^{c}	30 ± 0.89^{a}
Ammonia (mg/L)	0.016 ± 0.01^{d}	0.021 ± 0.02^{cd}	0.024 ± 0.02^{c}	0.028 ± 0.02^{b}	0.037 ± 0.01^{a}	0.038 ± 0.03^{a}	0.027 ± 0.02^{b}	0.023 ± 0.01^{c}
Color of water	Light green	Green	Green	Green	Brownish green	Brownish	Yellowish-brown	Brown

Table 8. Results were analyzed via using One-way ANOVA followed by post hoc pair wise inter group comparisons (n=5). Mean values with different superscripts within same rows are significantly different at $p < 0.05$ and show comparison among the recorded values from March to October.

Table 9. Month-wise productivity and relative abundance of phytoplankton and zooplankton in earthen nursery ponds.

Month	Phytoplankton (%)	Zooplankton (%)	DM(g/cm^3) of sediment	Predominant species
March	73 ± 0.3^{a}	27 ± 1.5^{f}	0.009 ± 0.001^{e}	Green algae/Rotifers
April	62 ± 1.4^{b}	38 ± 0.6^{e}	0.03 ± 0.0021^{e}	Green algae/Rotifers
May	54 ± 0.7^{c}	46 ± 1.1^{d}	0.05 ± 0.0041^{d}	Green algae/copepods
June	42 ± 0.9^{d}	58 ± 0.6^{ab}	0.06 ± 0.0251^{c}	Diatoms/Cladocerans
July	38 ± 1.6^{de}	62 ± 1.8^{a}	0.06 ± 0.0301^{b}	Diatoms/Cladocerans
August	37 ± 1.8^{e}	63 ± 0.9^{a}	0.07 ± 0.0411^{a}	Diatoms/Cladocerans
September	45 ± 1.8^{d}	55 ± 1.8^{b}	0.06 ± 0.0031^{c}	Diatoms/Cladocerans
October	51 ± 0.7^{c}	49 ± 1.2^{c}	0.05 ± 0.0044^{d}	Diatoms/Rotifers

Table 9. Results were analyzed via using One-way ANOVA followed by post hoc pair wise inter-month comparisons (n=5). Mean values with different superscripts within same column are significantly different at $p < 0.05$ and show comparison among the recorded values from March to October.

dominate significantly with a peak commonly inclusion during July (62%) and August (63%). Further, among phytoplankton, green algae dominated the nursery ponds community during March, April, and May. Afterwards, diatoms dominated over green algae.

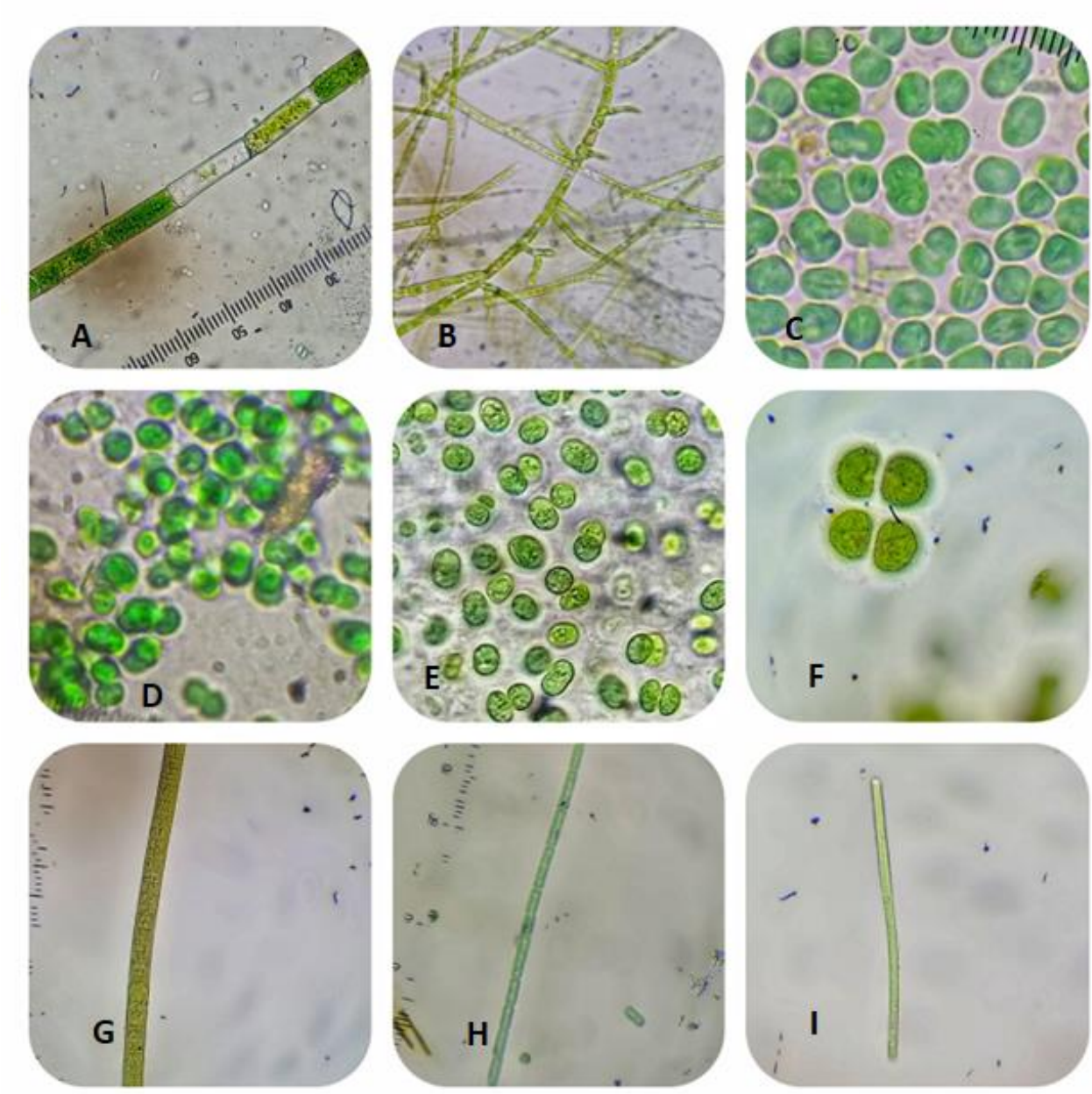

Figure 5. Diversity of Blue green algae ***(a) Rhizoclonium pachydermum (b) stigeoclonium attenuatum (c) Synechocystis aquatilis(d) Synechocystis salina (e) Aphanothe ceconferta (f) Cryptococcum Brasiliense (g) Oscillatoria sancta (h) Phormedium tenue (i) Phormedium corium.***

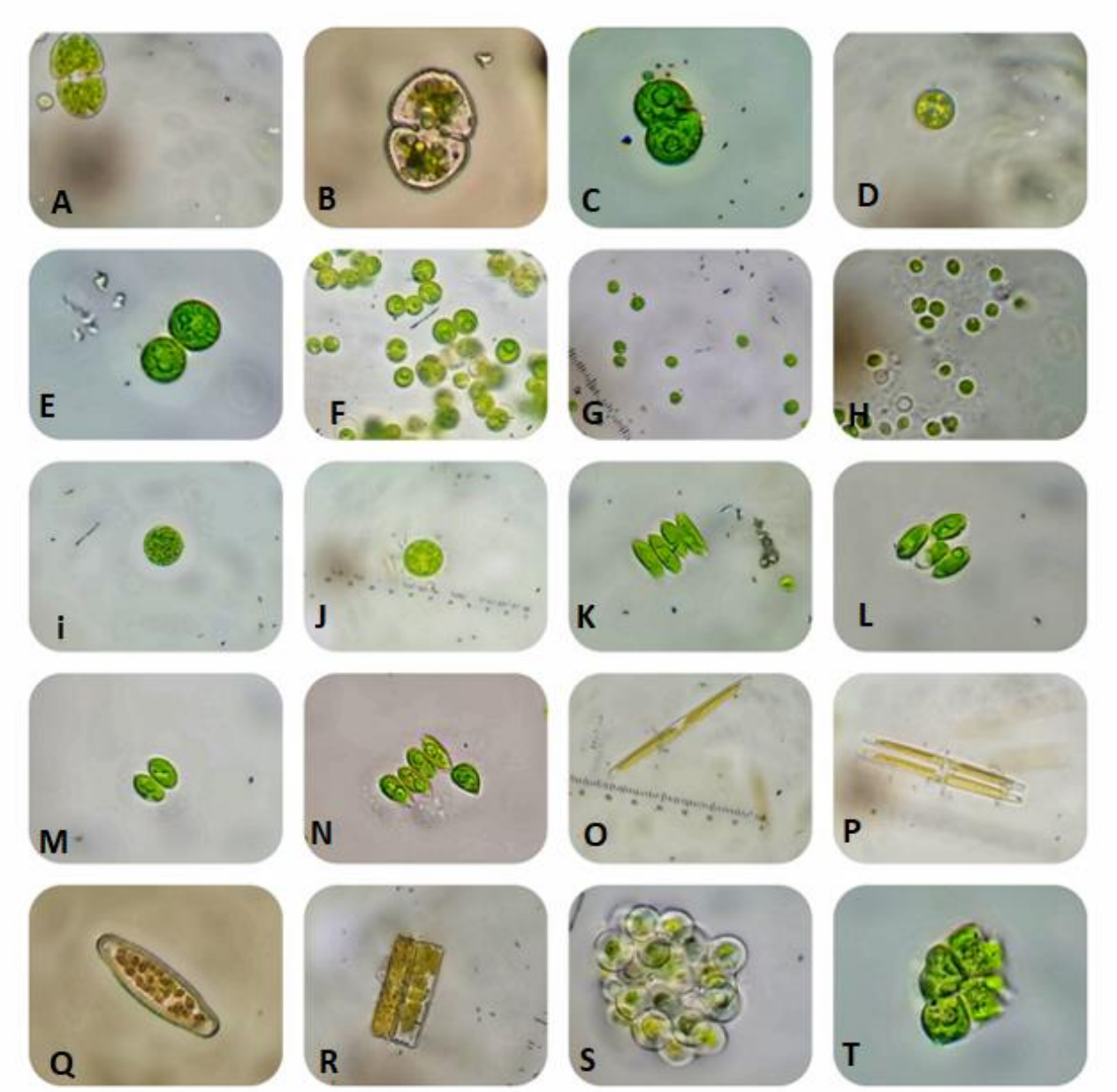

Figure 6. Green algae and diatoms: (a)Cosmarium granatum (b)Cosmarium phaseolus (c)Cosmarium ornatum (d) Dictyochloropsis splendida (e) Chlorococum infusionum (f) Chlorella vulgaris (g) Chlorella pyrenoidosa (h) Chlorella variabilis (i) Chlorococcum macrostigmatum (j) Chlorococcum citriforme (k) Scenedesmus arcuatus (l) Scenedesmus bijuga (m) Scenedesmus bijugatus (n) Scenedesmus armatus (o) Nitzschia agnita (p) Nitzschia obtuse (q) Amphora ovalis (r) Navicula bottnica (s) Eudorina elegance (t) Pediastrum tetras.

Among the zooplankton species rotifers (during March, April and October) and Cladocerans (during June, July, August and September) were the most prevalent, while copepods dominated during May.

Relative abundance of phytoplankton/zooplankton species

Phytoplankton and zooplankton were identified and noted periodically. The DM concentration as well as their species composition altered significantly during the study period (Table 10).

Table 10. Relative abundance of Phytoplankton in the earthen nursery ponds.

Species (% composition)	March	April	May	June	July	August	September	October
Rhizoclonium pachydermum	2±0.03	3±0.01	4±0.01	5±0.01	4±0.02	4±0.11	3±0.71	2±0.03
stigeoclonium attenuatum	2±0.01	3±0.02	3±0.01	3±0.01	5±0.33	5±0.21	2±0.63	2±0.05
Synechocystis aquatilis	3±0.02	2±0.07	2±0.01	2±0.01	4±0.43	4±0.31	3±0.65	1±0.07
Synechocystis salina	3±0.03	2±0.01	2±0.01	1±0.01	3±0.33	3±0.04	2±0.44	1±0.32
Aphanothe ceconferta	2±0.01	3±0.04	5±0.01	5±0.01	5±0.01	5±0.06	2±0.23	2±0.33
Cryptococcum brasiliense	1±0.01	4±0.05	4±0.01	4±0.01	4±0.61	4±0.07	1±0.76	1±0.07
Oscillatoria sancta	3±0.02	5±0.02	5±0.01	6±0.01	5±0.07	5±0.07	2±0.43	1±0.54
Phormedium tenue	3±0.04	3±0.06	2±0.01	1±0.01	2±0.07	2±0.01	3±0.33	1±0.76
Phormedium corium	2±0.02	1±0.01	3±0.01	3±0.01	3±0.08	3±0.09	2±0.43	2±0.44
Blue green algae	21±3.7	25±4.1	30±2.1	30±3.1	35±2.5	35±2.4	20±2.9	13±1.4
Cosmarium granatum	2±0.01	1±0.07	1±0.01	-	-	-	3±0.24	6±0.23
Cosmarium Phaseolus	2±0.01	1±0.02	2±0.01	-	-	1±0.02	3±0.22	2±0.87
Cosmarium ornatum	2±0.05	2±0.08	1±0.01	-	-	1±0.31	2±0.32	2±0.88
Dictyochloropsis splendida	3±0.06	3±0.04	2±0.01	-	-	2±0.61	3±0.65	1±0.89
Chlorococum infusionum	3±0.06	4±0.08	2±0.01	-	-	-	1±0.23	1±0.23
Chlorella vulgaris	10±0.2	8±0.01	6±0.01	1±0.01	-	4±0.06	5±0.21	5±0.01
Chlorella pyrenoidosa	10±0.3	8±0.04	-	1±0.10	1±0.08	2±0.07	3±0.21	6±0.11
Chlorella variabilis	6±0.12	5±0.02	5±0.21	-	-	2±0.07	4±0.76	9±0.11
Chlorococcum macrostigmatum	5±0.09	4±0.01	3±0.11	1±0.03	1±0.05	-	2±0.76	8±0.56
Chlorococcum citriforme	4±0.05	3±0.01	3±0.31	1±0.05	1±0.03	2±0.08	5±0.09	3±0.65
Scenedesmus arcuatus	3±0.01	3±0.03	2±0.31	-	1±0.03	-	2±0.21	2±0.06
Scenedesmus bijuga	4±0.04	1±0.01	2±0.11	-	-	-	1±0.45	4±0.43
Scenedesmus bijugatus	2±0.02	1±0.02	1±0.01	-	-	1±0.02	3±0.87	3±0.66
Scenedesmus armatus	3±0.04	7±0.16	2±0.05	1±0.04	1±0.01	-	3±0.97	3±0.01
Green algae	59±1.3	50±2.0	40±2.9	5±0.5	5±0.09	15±2.1	40±2.5	55±2.8
Nitzschia agnita	2±0.03	2±0.01	3±0.05	5±0.32	5±0.88	3±2.31	3±0.99	2±0.12
Nitzschia obtuse	1±0.07	3±0.02	4±0.09	9±0.21	8±2.05	7±2.51	4±0.65	1±0.01
Navicula bottnica	5±0.07	6±0.12	5±0.08	15±1.6	10±4.1	11±4.1	10±2.3	7±0.98
Eudorina elegance	4±0.09	5±0.11	7±0.06	16±4.1	16±3.2	14±3.0	10±1.6	10±2.7
Pediastrum tetras	6±0.05	6±0.04	7±0.07	10±2.1	10±2.0	11±2.0	9±4.11	9±3.01
Amphora ovalis	2±0.05	3±0.03	4±0.04	5±1.0	5±0.31	4±2.04	4±0.01	3±0.31
Diatoms	20±1.4	25±2.0	30±2.7	65±3.5	60±2.9	50±2.6	40±1.5	32±1.2

Each value represents a relative percent of that species, - represent absence of that species.

Green algae dominated the early months with a slow decline with rise in temperature/season, while blue green algae and diatoms species showed an opposite trend (increase from May to August). Further it was noted that during the rohu breeding season, diatoms dominated the overall species composition (Table 10) followed by Blue-green algae while a lower percentage of green algae.

Table 11. Relative abundance of zooplankton species in the earthen nursery ponds.

Zooplanktons	March	April	May	June	July	August	September
Asplanchna spp.	19±1.2	11±1.0	4±0.03	3±0.02	1±0.01	4±0.12	7±0.92
Brachionus spp.	17±2.7	14±1.6	5±0.66	5±0.02	2±0.02	8±0.32	13±2.1
Keratella spp.	13±1.6	9±0.32	1±0.02	1±0.77	5±0.72	11±1.2	22±3.2
Other	05±1.9	6±1.02	1±0.02	1±0.52	1±0.66	2±0.71	6±0.42
Total Rotifers	54±2.2	40±3.2	11±1.1	10±2.3	9±1.12	25±1.4	48±1.2
Daphnia spp.	08±0.28	13±1.3	18±1.3	21±1.1	17±2.2	15±1.3	9±1.12
Ceriodaphnia spp.	04±0.12	6±0.22	8±0.98	7±1.25	13±1.1	6±0.92	5±0.92
Moina spp.	07±1.2	8±0.09	12±1.4	20±1.7	22±1.2	18±1.1	3±0.62
Other	-	1±0.22	1±0.02	-	2±1.00	1±0.19	3±0.82
Cladocerans	19±1.1	28±4.1	39±3.2	48±2.3	54±1.9	40±4.2	30±1.2
Mesocyclops spp.	11±0.2	13±1.2	21±1.0	16±1.2	17±1.7	11±3.0	9±1.12
Diaptomus spp.	04±0.6	5±1.71	9±1.33	6±1.25	8±1.00	08±1.2	5±0.52
Nauplii	03±0.1	6±1.45	11±1.7	3±0.12	5±0.32	4±0.15	2±0.42
Other	03±0.8	2±1.26	4±1.02	5±1.26	5±0.29	2±0.21	3±0.72
Copepods	21±1.1	26±1.4	45±4.2	30±1.9	35±4.2	25±1.6	19±0.4
Cypris spp.	04±1.2	3±0.66	2±0.33	3±0.55	3±0.07	2±0.01	-
Cyprinodopsis spp.	01±1.0	1±0.02	2±0.01	5±1.21	2±0.42	1±0.32	01±0.2
Other	-	-	-	1±0.44	-	-	-
Ostracoda	05±1.0	4±0.99	4±0.88	9±1.27	5±0.27	3±0.12	01±1.0
Other clads	01±1.8	2±0.17	1±0.02	3±0.12	2±0.42	2±0.36	02±1.3

Each value represents % presence.

Species-wise occurrence indicated *Chlorella vulgaris, Chlorella pyrenoidosa, Chlorella variabilis, Aphanothe ceconferta, Cryptococcum Brasiliense, Oscillatoria sancta, Navicula bottnica, Eudorina elegance, Pediastrum tetras* as the predominant phytoplankton species across all the months (Fig 5). Significant alteration in the zooplankton community (Table 11) was also observed species (of genus) *Asplanchna spp., Brachionus spp., Daphnia spp.* and *Moinea Sp.* with dominating the zooplankton community, numerically. Overall, among zooplankton rotifers and Cladocerans were the most prevalent one, where rotifers were prevalent during March, April and

September (at somewhat low temperature), while Cladocerans were dominated during May, June , July and August (at high temperature).

Figure 7. Various zooplankton species observed in the earthen nursery pond during March to October.

Owing to the reproductive maturation period of rohu, June and July months remain crucial, hence the species dominate these months are also important to analyze. The prominent species during June and July were *Rhizoclonium pachydermum, stigeoclonium attenuatum, Synechocystis aquatilis, Synechocystis salina, Aphanothe ceconferta, Cryptococcum Brasiliense, Oscillatoria*

sancta (bluegreen algae); *Nitzschia obtuse, Navicula bottnica, Eudorina elegance, Pediastrum tetras* (diatoms). Zooplankton dominates during these months including Cladocerans followed by copepods, Ostracoda and least presence of rotifer. Overall, rotifers are dominated by *Asplanchna spp., Brachionus spp.,* and *Keratella spp*.; Cladocerans by *Daphnia spp. Ceriodaphnia spp., Moina spp.;* Copepods by *Mesocyclops spp. Diaptomus spp., Nauplii;* while *Cypris spp., Cyprinodopsis spp.* constitute the major percent of ostracods (Figure 8). The significant species of zooplankton which dominate these waterbodies during Rohu breeding season are *Ceriodaphnia, Moina, Mesocyclops and Daphnia species.*

Proximate and biochemical analysis of phyto/zooplankton

The proximate composition of both phytoplankton and zooplankton indicated a increasing trend in % CP, % CF and % dry matter from March to August (Table 12).

Table 12. Proximate composition of live feed collected from nursery ponds.

Month	Plankton	Proximate composition (%)				
		CP	CF	Crude Ash	Moisture	DM
March	Zooplankton	53.7±18	8.04±02	7.69±10	78.29±12	21.71±1
	Phytoplankton	43±120	3.8±012	6.1±121	80±761	20±182
April	Zooplankton	57.04±1	8.11±72	7.59±09	77.3±12	22.7±11
	Phytoplankton	43±169	3.9±812	6.2±111	80±123	20±123
May	Zooplankton	58.14±1	9.24±31	7.02±02	76.4±10	23.6±11
	Phytoplankton	46±129	4.1±402	6.1±077	78±111	22±122
June	Zooplankton	61.74±4	9.54±88	7.09±17	74.2±17	25.8±17
	Phytoplankton	47.9±12	4.3±121	6.3±119	78±887	22±128
July	Zooplankton	61.74±2	9.74±12	7.01±12	74.2±12	25.8±19
	Phytoplankton	50±127	4.3±002	6.1±122	76±665	24±188
August	Zooplankton	60.74±2	9.84±22	6.94±12	75.4±15	24.6±19
	Phytoplankton	48±199	4.4±201	5.9±332	77±128	23±009
September	Zooplankton	59.74±1	8.74±10	7.29±72	77.9±10	22.1±08
	Phytoplankton	46±188	3.9±177	6.2±232	78±123	22±109
October	Zooplankton	58.74±3	8.84±32	7.43±61	78.9±11	21.1±09
	Phytoplankton	44±223	3.9±121	6.1±125	79±129	21±101

***CP: Crude protein, CF: Crude fats, DM: Dry matter, values in the table represent % presence in the plankton sampled.**

Table 13. Fatty acid profile of live feed.

Fatty acid composition	March	April	May	June	July	August	September	October
C8:0	01	-	-	-	-	01	01	01
C10:0	02	-	-	-	01	01	01	03
C11:0	02	01	-	-	-	02	01	02
C12:0	03	02	02	02	02	05	02	02
C13:0	03	03	02	03	02	01	02	04
C14:0	04	05	06	06	06	06	06	05
C15:0	03	04	04	02	07	07	07	05
C16:0	10	12	13	15	20	21	20	12
C17:0	02	01	02	05	03	03	03	02
C18:0	05	06	07	07	06	05	04	02
C20:0	-	02	04	05	03	03	03	02
C14:1c	04	05	01	01	02	01	-	-
C16:1c	09	08	04	04	04	03	05	06
C17:1	06	04	03	03	04	02	02	04
C18:1c	08	05	05	05	04	06	08	10
C18:1n9T	15	15	10	02	01	01	02	05
C20:1	08	08	12	05	05	06	08	12
18:2n−6	-	01	02	01	02	02	03	02
18:3n−6	01	-	01	02	01	01	-	-
20:2n−6	01	01	02	03	02	03	02	02
20:3n−6	02	01	03	04	05	03	03	02
20:4n−6	03	04	03	09	12	15	06	05
18:3n−3	01	01	01	-	-	01	-	01
20:3n−3	02	01	02	-	-	01	01	02
20:5n−3	04	05	06	06	07	07	04	04
22:5n−3	03	01	01	02	04	04	02	03
22:6n−3	03	04	04	07	07	08	04	02

Each value represent % presence of FA with an average standard deviation 0±0.1 to 0±0.25, C8:0: Caprylic acid methyl ester, C10:0: Capric acid methyl ester, C11:0: Undecanoic acid methyl ester, C12:0: Lauric acid methyl ester, C13:0: tridecanoic acid methyl ester, C14:1c: Myristoleic acid methyl ester, C14:0: Myristic acid methyl ester, 7-HAD: 7-Hexadecenoic acid methyl ester, C15:0: Pentadecanoic acid methyl ester, C16:1c: Palmitoleic acid methyl ester, C16:0: Palmitic acid methyl ester, C17:0: Margaric acid methyl ester, C17:1: Heptadecanoic acid methyl ester, C18:1c: Oleic acid methyl ester, C18:2c: Linoleic acid methyl ester, C18:1n9T: Elaidic acid methyl ester, C18:0: Stearic acid, DA22:1 (n-9) : Docosanoic acid methyl ester/ Erucic acid, C20:5n3: EPA: 5,8,11,14,17-Eicosapentaenoic acid methyl ester, C22:6n3 DHA: 4,7,10,13,16,19-Docosahexaenoic acid methyl ester, C20:3n6: 7,10,13-Eicosasatrienoic acid methyl ester, C20:2: 11,14-Eicosadienoic acid methyl ester, C20:1: Eicosanoic acid methyl ester.

There was observed a significantly increasing trend in term of %CP, %CF and over all dry matter (DM) from March to August (Table 12), then a slight decrease thereafter, while an inverse trend was observed with the moisture content (significantly high moisture content in March and October). However, only slight variation observed in crude ash content. Results also indicated relatively high CP %, CF % and ash in zooplankton throughout the study period while a high moisture content was observed in phytoplankton.

Table 14. Seasonal variation in major fatty acid groups in live feed.

Fatty acid composition	March	April	May	June	July	August	September	October
∑SAFA	30 ± 2^{f}	36 ± 5^{e}	40 ± 5^{d}	45 ± 6^{c}	50 ± 6^{b}	55 ± 5^{a}	50 ± 4^{b}	40 ± 5^{d}
∑MUFA	50 ± 3^{a}	45 ± 2^{b}	35 ± 2^{cd}	20 ± 2^{e}	20 ± 5^{e}	19 ± 2^{e}	25 ± 2^{d}	37 ± 2^{c}
∑EPA	4 ± 0.1^{d}	5 ± 0.5^{c}	6 ± 0.2^{b}	6 ± 0.1^{b}	7 ± 0.2^{a}	7 ± 0.1^{a}	4 ± 0.1^{d}	4 ± 0.3^{d}
∑DHA	3 ± 0.2^{d}	4 ± 0.2^{c}	4 ± 0.1^{c}	7 ± 0.2^{b}	7 ± 0.3^{b}	8 ± 0.4^{a}	4 ± 0.3^{c}	2 ± 0.4^{e}
∑n−3 PUFA	13 ± 2^{e}	12 ± 0.3^{f}	14 ± 2^{d}	15 ± 2^{c}	18 ± 2^{b}	21 ± 6^{a}	11 ± 4^{g}	12 ± 2^{f}
∑n−6 PUFA	7 ± 1^{g}	8 ± 1.4^{f}	11 ± 2^{e}	20 ± 6^{c}	22 ± 1^{b}	24 ± 2^{a}	14 ± 5^{d}	11 ± 2^{e}
∑PUFA	20 ± 2^{f}	20 ± 1.0^{f}	25 ± 5^{d}	35 ± 6^{a}	30 ± 2^{b}	26 ± 5^{c}	25 ± 7^{d}	23 ± 1^{e}

Each value represents % presence of Fatty Acid.

Significant variation was observed in the fatty acid profile of live feed during the studied months (Table 13). The fatty acid profile indicated that in live feed saturated fatty acid (SAFA) gradually increased from March to August with peak value in August while unlike SAFA, MUFA decreased during July and August, while their peak were observed in March and April. Being the most crucial requirement of *L. rohita* PLs development, Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) over all n-3 and n-6 polyunsaturated fatty acids (PUFA) have been analyzed, mentioned in Table 14. The PUFA concentration indicated increasing trend from march to July, and subsequently showed decreasing trend.

Experiment 2

Characterization of nano-particulate feed

Scanning electron microscope (Figure 8B) and XRD pattern (Figure 8A; Table 15) of Nano-particulate prepared larval feed indicated that the sizes of particles ranged from 53.85nm to 80.00nm with smooth surface area (Table 15, Figure 8).

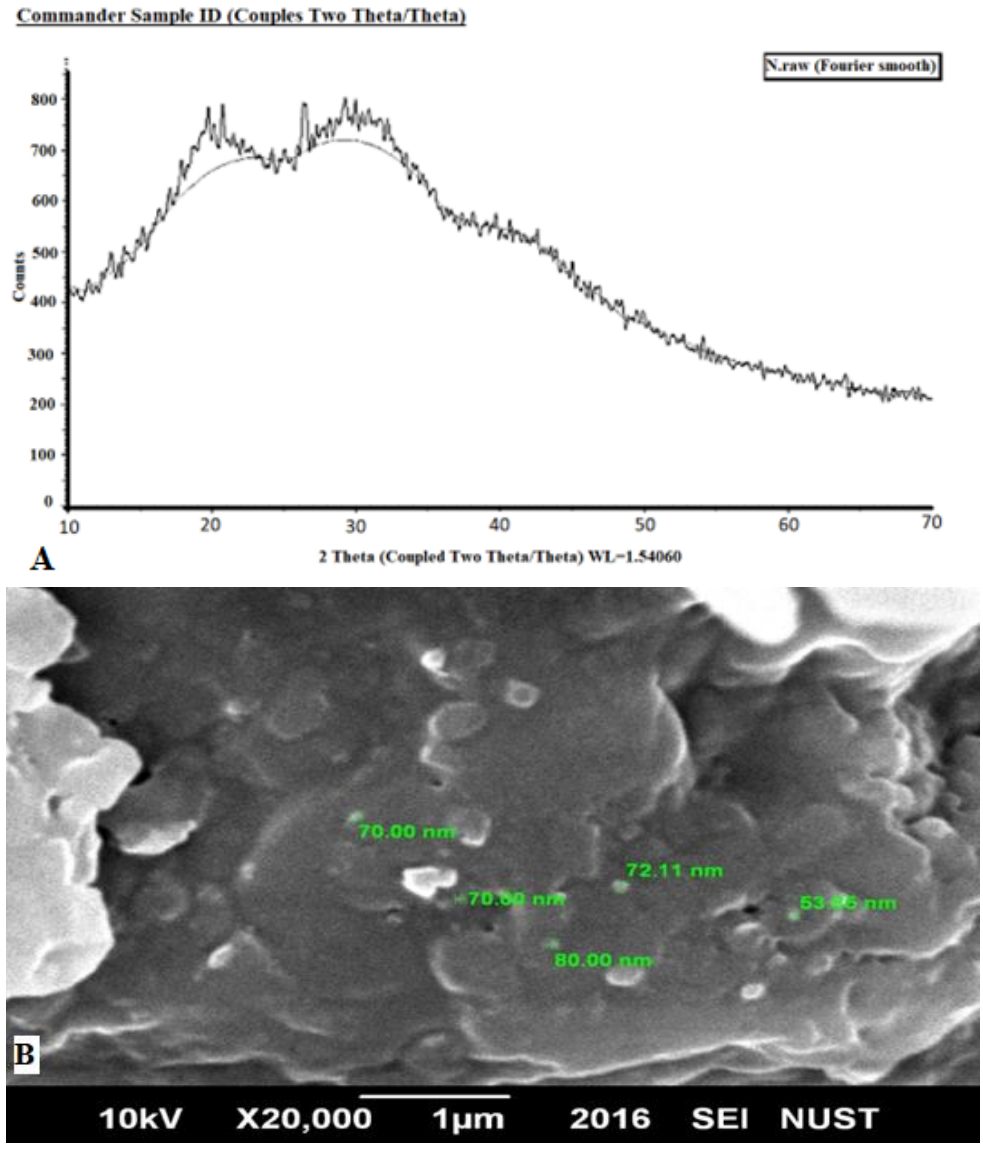

Figure 8. XRD pattern (A) and scanning electron microscope image (B) of Nano-particulate prepared feed.

Table 15. Position of peaks and size of nano-particulate prepared feeds observed through XRD.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Average Size (nm)	55.23, 62.34, 77.23	69.93, 78.94, 71.13	59.23, 60.84, 79.23	54.23, 55.34, 62.30
Position of Peaks	19.69, 20.71, 26.38	22.39, 27.01, 23.33	19.99, 20.71, 26.99	18.69, 18.71, 21.38

Growth performance and survival rate

The Live feed (LF), Nano-particulate feed (ND), and Co-feeding (LF+ ND) significantly affected the growth performance of *L. rohita* at their early life stages (Table 16, Figure 9). Analysis of results by using two-way ANOVA indicated that growth performance was significantly affected by both independent factors, i.e., feed (NWG, $F_{2,84}$=621.3, *P<0.001*, η_p^2= 0.937; SGR, $F_{2,84}$=344.4, *P<0.001*, η_p^2=0.905), and age DAH (NWG, $F_{6,84}$=10587, *P<0.001*, η_p^2=0.958; SGR, $F_{6,84}$=8077, *P<0.001*, η_p^2=0.998) and their interaction, feed × age DAH (NWG: $F_{12,104}$=159.4, *P<0.001*, η_p^2=0.999; SGR, $F_{12,104}$=16.53, *P<0.001*, η_p^2=0.697). The partial eta-squared (η_p^2) values of both variables (feed and age DAH) indicated a similar and large effect on NWG and SGR while their interaction reflected a larger effect on NWG and a medium effect on SGR.

Simple main effects analysis indicated the most significant effect of co-feeding (T3, LF +ND) as compared to LF and ND alone. All pairwise comparisons indicated that NWG and SGR showed an increasing trend with age DAH, however, T3 showed the highest values in periods, followed by T2, while the T1 group showed the lowest values. Moreover, at the end of the feeding trial, the T3 group showed the highest survival (%) (Figure 10) followed by the T2 and the T1 group.

Intestinal enzymes activity

The effect of Live feed, Nanoparticle feed and co-feeding on the activity of intestinal enzymes of *L. rohita* during early rearing is shown in Table 17. Two-way ANOVA indicated a significant effect of feed on protease activity ($F_{2,84}$=1400, *P=0.001*, η_p^2=0.971), amylase activity ($F_{2,84}$=66.86, *P<0.001*, η_p^2= 0.614) and cellulase activity ($F_{2,84}$=1736, *P<0.001*, η_p^2= 0.976). Similarly, another independent factor, age DAH also showed a significant effect on intestinal enzymes activity (Protease activity, $F_{6,84}$ =9728, 249, *P<0.001*, η_p^2= 0.999; amylase, $F_{6,84}$=570.2,

Table 16. Growth performance of *L. rohita* PLs reared on live feed, nano-particulate prepared feed and co-feeding for 35 days.

		Feed type					
	Age (DAH)	**T1**(g)	**T2**(g)	**T3**(g)	**P-value**	**F-value**	**SEM**
	03	0.0004^{Ga}	0.0004^{Ga}	0.0004^{Ga}	Ns	-	-
	10	0.1069^{Fb}	0.128^{Fa}	0.131^{Fa}	0.010	14.06	0.0034
NWG	**15**	0.1724^{Ec}	0.208^{Eb}	0.227^{Ea}	<0.001	49.85	0.0064
	20	0.2572^{Dc}	0.271^{Db}	0.299^{Da}	<0.001	90.14	0.0048
	25	0.4524^{Cb}	0.492^{Ca}	0.494^{Ca}	<0.001	300.7	0.0052
	30	0.6696^{Bc}	0.762^{Bb}	0.923^{Ba}	<0.001	1408.7	0.0280
	35	0.8612^{Ac}	1.015^{Ab}	1.286^{Aa}	<0.001	192.32	0.0467
	P-value	<0.001	<0.001	<0.001			
	F-value	11694.52	3378.04	2865.21			
	03	**-**	**-**	**-**	-	-	-
	10	4.07^{Fb}	4.31^{Fa}	4.32^{Fa}	0.0010	14.65	0.0358
SGR	**15**	4.66^{Ec}	4.90^{Eb}	5.01^{Ea}	<0.001	52.38	0.040
	20	5.16^{Dc}	5.23^{Db}	5.35^{Da}	<0.001	79.66	0.021
	25	5.86^{Cb}	5.97^{Ca}	5.98^{Ca}	<0.001	320.9	0.013
	30	6.35^{Bc}	6.51^{Bb}	6.75^{Ba}	<0.001	1330	0.043
	35	6.66^{Ac}	6.87^{Ab}	7.162^{Aa}	<0.001	228.33	0.055
	P-value	<0.001	<0.001	<0.001			
	F-value	6.994	5.604	5.243			

Results were analyzed via using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (PLs weight values in g) with different subscripts in same rows are significantly different at $p < 0.05$) and show comparison between groups, however superscript letters in same column shows age wise difference within group.

T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared feed, T3= PLs co-fed live and prepared feed, ns stands for statistically nonsignificant.

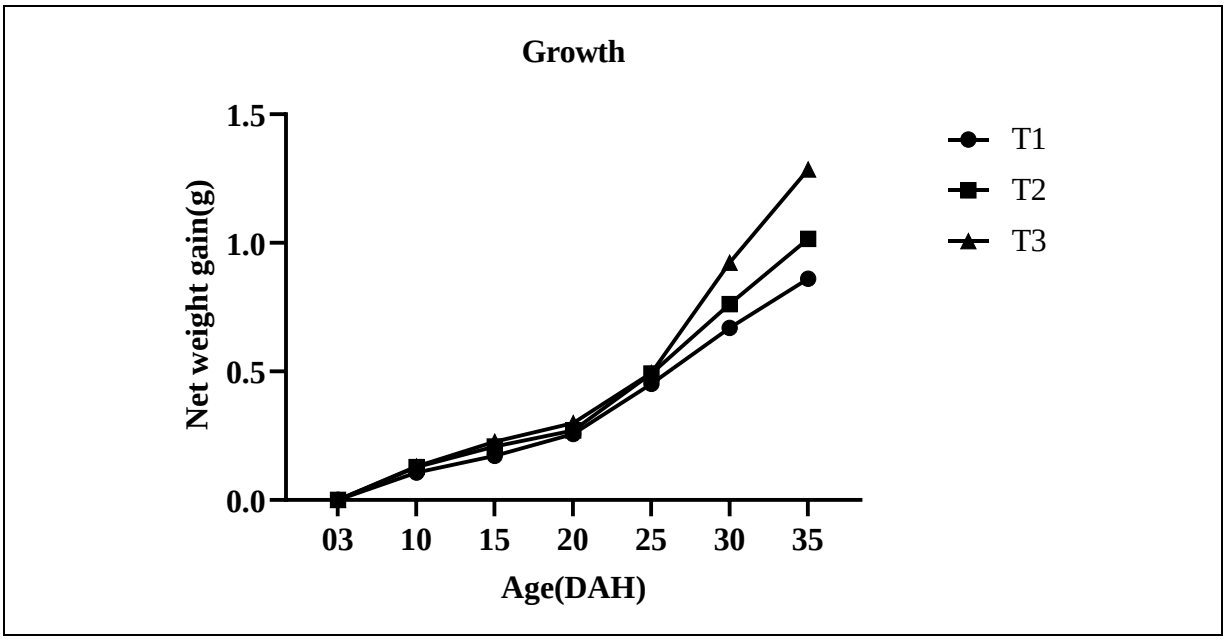

Figure 9. Comparative weight gain of *L. rohita* PLs after 35 days feeding trial: T1= PLs reared on live feed, T2= PLs reared on nano-particulate prepared diet, T3= PLs co-fed live feed and prepared diet.

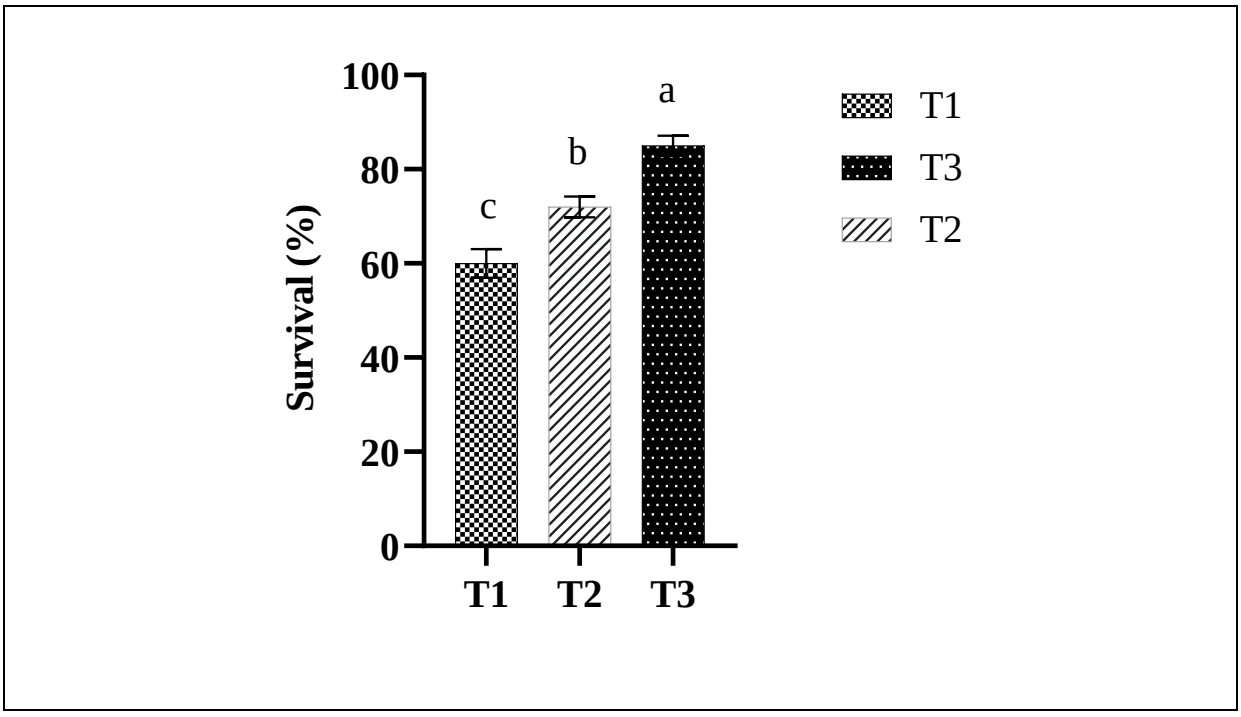

Figure 10. Survival percentage of L. rohita PLs after 35 days feeding trial: T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared diet, T3= PLs co-fed live feed and prepared diet.

Table 17. Protease, amylase and cellulase activity in *L. rohita* PLs, while rearing on live feed, nano-particulate prepared feed and co-feeding for 35 days.

		Feed type					
	Age (DAH)	**T1**	**T2**	**T3**	**P-value**	**F-value**	**SEM**
Protease	**03**	0.0201^{Ga}	0.0201^{Ga}	0.0201^{Fa}	ns	-	-
	10	0.081^{Fb}	0.079^{Fc}	0.086^{Ea}	<0.001	48.067	0.0009
	15	0.102^{Eb}	0.10^{Eb}	0.18^{Da}	<0.001	688.992	0.0099
	20	0.199^{Da}	0.17^{Dc}	0.19^{Db}	<0.001	298.162	0.0032
	25	0.268^{Cb}	0.24^{Cc}	0.30^{Ca}	<0.001	70.190	0.0071
	30	0.412^{Bb}	0.30^{Bc}	0.54^{Ba}	<0.001	648.988	0.0273
	35	0.380^{Ab}	0.36^{Ac}	0.49^{Aa}	<0.001	385.573	0.0144
	P-value	<0.001	<0.001	<0.001			
	F-value	10858.6	4243.2	2314.5			
Amylase	**03**	0.49^{Ba}	0.49^{Ca}	0.49^{Ca}	=0.540	0.649	0.030
	10	0.29^{Ea}	0.28^{Fb}	0.29^{Fa}	<0.0019	5.656	0.002
	15	0.31^{Db}	0.33^{Ea}	0.30^{Fc}	<0.001	66.29	0.003
	20	0.38^{Cc}	0.42^{Da}	0.39^{Eb}	<0.001	52.32	0.006
	25	0.58^{Ac}	0.64^{Aa}	0.60^{Ab}	<0.001	150.9	0.008
	30	0.47^{Bc}	0.55^{Ba}	0.51^{Bb}	<0.001	114.2	0.010
	35	0.38^{Cb}	0.49^{Ca}	0.42^{Db}	=0.003	9.605	0.017
	P-value	<0.001	<0.001	<0.001			
	F-value	575.1	102.4	528.3			
Cellulase	**03**	0	0	0	ns	-	-
	10	0.10^{Fb}	0.08^{Fc}	0.10^{Fa}	<0.001	145.29	0.035
	15	0.14^{Eb}	0.11^{Ec}	0.19^{Ea}	<0.001	383.63	0.009
	20	0.21^{Db}	0.17^{Dc}	0.24^{Da}	<0.001	400.19	0.007
	25	0.27^{Ca}	0.21^{Cc}	0.26^{Cb}	<0.001	567.65	0.007
	30	0.30^{Ba}	0.25^{Bc}	0.29^{Bb}	<0.001	299.84	0.006
	35	0.34^{Aa}	0.27^{Ac}	0.30^{Ab}	<0.001	541.36	0.008
	P-value	<0.001	<0.001	<0.001			
	F-value	8299.98	4524.96	5058.37			

Results were analyzed by using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (Enzymes activity: U mg -1) with different subscripts in same rows are significantly different (P< 0.05) and show comparison between groups, however superscript letters in same column shows age wise difference within group.
T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared feed, T3= PLs co-fed live and prepared feed, ns stands for statistically nonsignificant.

P<0.001, η_p^2=0.976 and Cellulase, $F_{6,84}$=16986, *P<0.001*, η_p^2= 0.999). Moreover, the most significant interaction (*P<0.001*) between feed × age DAH (Protease, $F_{12,104}$=276.6, ηp2= 0.975;

Amylase, $F_{12,104}$=8.570, η_p^2= 0.550, and Cellulase, $F_{12,104}$=165.4, η_p^2= 0.959) indicated how both independent variables interact and modulate the intestinal enzymes activity (Figure 11). The partial eta-squared (η_p^2) values reflected the large and almost similar effect size of both variables (feed and age DAH) and their interaction on protease and cellulase activity. However, age (DAH) accounted for a large effect size compared to feed and feed× age (DAH) interaction showing a lesser but still significant proportion of variation in amylase activity.

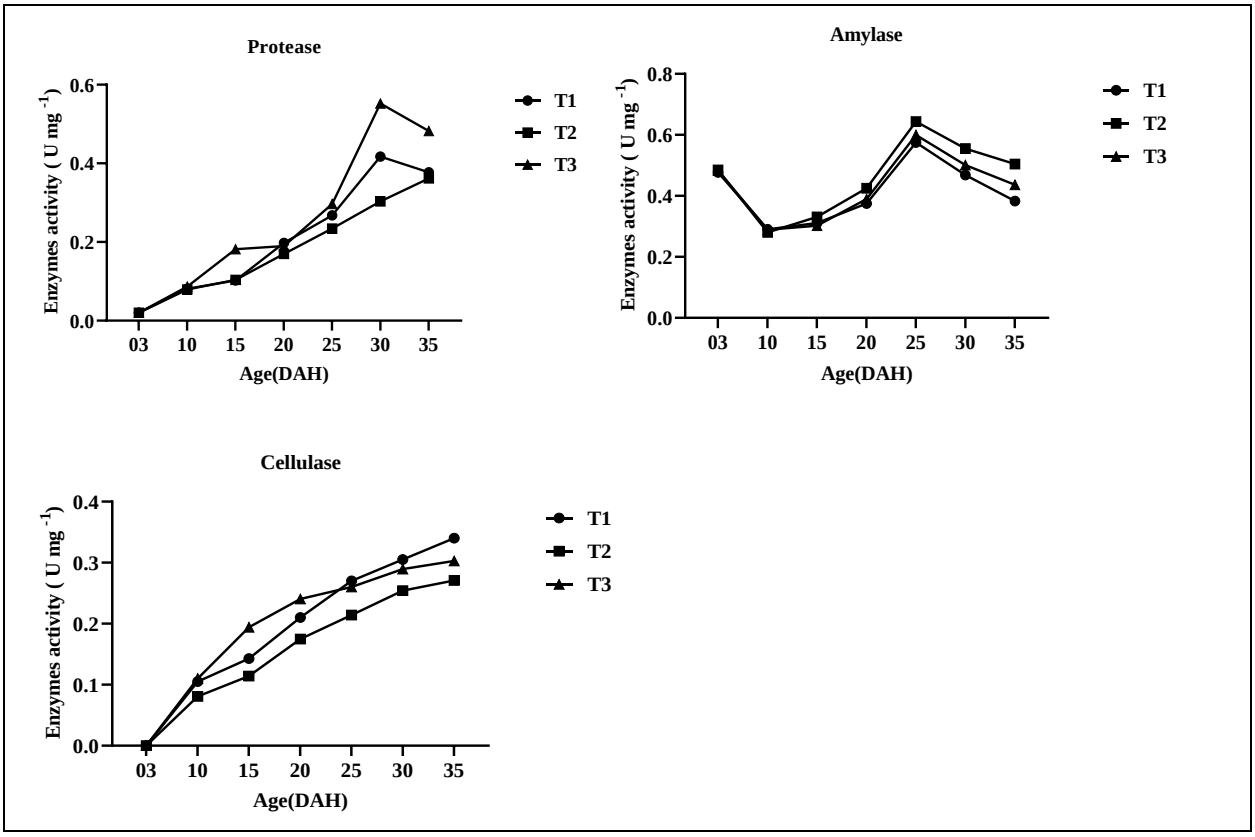

Figure 11. Comparative activity of intestinal enzymes during early rearing of *L.* on live feed and prepared diet. T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared diet. T3= PLs co-fed live feed and prepared diet.

Simple main effects analysis revealed the most significant effect of LF on cellulase activity while ND and Co-feeding showed the most significant effect on amylase and protease activity respectively. All pairwise comparisons indicated a variable trend of intestinal enzymes DAH in response to different feeds. For instance, there was no significant effect of feed on protease activity at 3 DAH (*P=0.999*), however in the T3 group (in response to Co-feeding), it showed the most significant increasing trend with age DAH. Similarly, feed type did not show any significant effect on intestinal amylase activity at 3DAH, however, it was decreased at 10DAH and then showed the most significant increasing trend with age DAH in the T2 group as compared to other groups. The

intestinal cellulase activity initially (3DAH) was not detected, however, after that, it showed an increasing trend with age DAH in all groups with the highest activity in the T3 group up to 20DAH, an afterword from 25DAH to 35DAH, the T1 group showed the highest activity.

Expression of growth and appetite-regulating genes

The effect of different feeds at the molecular level, i.e., expression of growth and appetite-regulating genes at age DAH is shown in Table 18. Two-way ANOVA indicated a significant effect (Table 18) of feed nature on the expression of *ghrelin* ($F_{2,84}$=40233, *P<0.001*, η_p^2= 0.999), *GHS-R* ($F_{2,84}$=188.2, *P<0.001*, η_p^2=0.818), *leptin* ($F_{2,84}$=2746, *P<0.001*, η_p^2=0.985) and IGF ($F_{2,84}$=5027, *P<0.001*, η_p^2=0.992). Like feed, age DAH also showed a significant effect on the expression of all studied genes (*ghrelin*, $F_{6,84}$=73348, *P<0.001*, η_p^2= 1.000; *GHS-R*, $F_{6,84}$=2496, *P<0.001*, η_p^2= 0.994; *leptin*, $F_{6,84}$=6752, *P<0.001*, η_p^2=0.998 and *IGF-1*, $F_{6,84}$=33228, *P<0.001*, η_p^2=1.000). Moreover, the most significant interaction (*P<0.001*) between feed nature × age DAH on genes expression (*ghrelin*, $F_{12,104}$=3633, *P<0.001*, η_p^2= 0.998; *GHS-R*, $F_{12,104}$=9.563, *P<0.001*, η_p^2= 0.577; *leptin*, $F_{12,104}$=207.9, *P<0.001*, η_p^2=0.967 and *IGF-1*, $F_{12,\ 104}$=703.544, *P<0.001*, η_p^2=0.990) indicated how both independent variables interact and affect the dependent variable. The partial eta-squared (η_p^2) values reflected the large and almost similar effect size of both variables (feed and age DAH) and their interaction on the expression of all studied genes except *GHS-R* where feed × age DAH interaction η_p^2 value of 0.577 indicated medium effect size.

Simple main effects analysis revealed a most significant positive effect of co-feeding on *ghrelin*, *GHS-R*, and IGF gene expression (Figure 12), while *leptin* expression showed an increasing trend with age DAH with ND.

All pairwise comparisons indicated the increasing trend with age DAH in the expression of *ghrelin* and *GHS-R*, moreover, both genes showed the highest expression in T3 followed by T1.

Table 18. Comparative effect of larval feed type on early *ghrelin*, *GHS-R*, *IGF-1* and *leptin* on expression in *L. rohita* PLs, while rearing on live feed, nano-particulated prepared feed and co-feeding for 35 days.

		Feed type					
	Age (DAH)	**T1**	**T2**	**T3**	**P-value**	**F-value**	**SEM**
	03	0.130^{Ga}	0.128^{Gab}	0.120^{Gb}	0.067	3.42	0.002
	10	0.74^{Fa}	0.50^{Fc}	0.70^{Fb}	<0.001	704.23	0.025
	15	1.51^{Ea}	0.80^{Eb}	1.49^{Ea}	<0.001	1359.8	0.099
Ghrelin	**20**	1.70^{Db}	1.20^{Dc}	2.30^{Da}	<0.001	3994.1	0.118
	25	2.10^{Cb}	1.60^{Cc}	2.90^{Ca}	<0.001	11238	0.140
	30	2.40^{Bb}	1.80^{Bc}	3.60^{Ba}	<0.001	40030	0.199
	35	2.80^{Ab}	2.10^{Ac}	4.10^{Aa}	<0.001	190216	0.221
	P-value	<0.001	<0.001	<0.001			
	F-value	25938	5810	285592			
	03	0.08^{Ga}	0.08^{Ga}	0.08^{Ga}	0.995	0.0050	0.0008
	10	0.5^{Fc}	0.6^{Fb}	1.0^{Ba}	<0.001	429.54	0.064
	15	1.2^{Eb}	1.0^{Ec}	1.4^{Ea}	<0.001	139.34	0.412
GHS-R	**20**	1.7^{Db}	1.7^{Db}	2.2^{Da}	<0.001	257.33	0.063
	25	2.5^{Cb}	2.3^{Cc}	2.9^{Ca}	<0.001	285.84	0.068
	30	3.2^{Bb}	3.0^{Bc}	3.8^{Ba}	<0.001	436.44	0.088
	35	4.0^{Ab}	3.7^{Ac}	4.4^{Aa}	<0.001	211.71	0.079
	P-value	<0.001	<0.001	<0.001			
	F-value	59880	1248.9	1950.9			
	03	0.506^{Ga}	0.507^{Ga}	0.508^{Ga}	0.778	0.257	0.0014
	10	0.80^{Fc}	0.81^{Fb}	1.1^{Fa}	<0.001	1962.1	0.037
	15	1.00^{Eb}	1.04^{Ec}	1.4^{Ea}	<0.001	459.6	0.053
IGF-1	**20**	1.4^{Dc}	1.45^{Db}	1.6^{Da}	<0.001	81.62	0.028
	25	1.5^{Cc}	1.9^{Cb}	2.0^{Ca}	<0.001	482.66	0.064
	30	2.4^{Bc}	2.8^{Bb}	3.3^{Ba}	<0.001	1543	0.098
	35	3.0^{Ac}	3.7^{Ab}	4.8^{Aa}	<0.001	2963	0.191
	P-value	<0.001	<0.001	<0.001			
	F-value	4467	830.2	4608			
	03	0.09^{Ga}	0.09^{Ga}	0.093^{Fa}	0.433	0.897	0.0006
	10	0.2^{Fb}	0.5^{Fa}	0.1^{Fc}	<0.001	592.6	0.045
	15	0.4^{Eb}	0.6^{Ea}	0.2^{Ec}	<0.001	268.3	0.044
Leptin	**20**	0.6^{Db}	0.8^{Da}	0.4^{Dc}	<0.001	413.7	0.044
	25	0.9^{Cb}	1.0^{Ca}	0.6^{Cc}	<0.001	245.9	0.052
	30	1.3^{Bb}	1.7^{Ba}	1.0^{Bc}	<0.001	368.7	0.071
	35	1.5^{Ab}	2.3^{Aa}	1.2^{Ac}	<0.001	1361.5	0.125
	P-value	<0.001	<0.001	<0.001			
	F-value	148.35	5095.7	633.7			

Two-way ANOVA for overall statistical analysis of two independent variables *Feed type* and *Age* and dependent variable *Ghrelin* expression followed by One-way ANOVA for intergroup and intra group comparison where n=5. LSD post hoc test shows a pairwise comparison between and within groups. Means (gene expression relative to β-Actin) with different lowercase superscripts in same rows are significantly different at $p < 0.05$ and show comparison between groups, however uppercase superscript letters in same column shows age wise difference within group. T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared feed, T3= PLs co-fed live and prepared feed, *ns* stands for statistically nonsignificant.

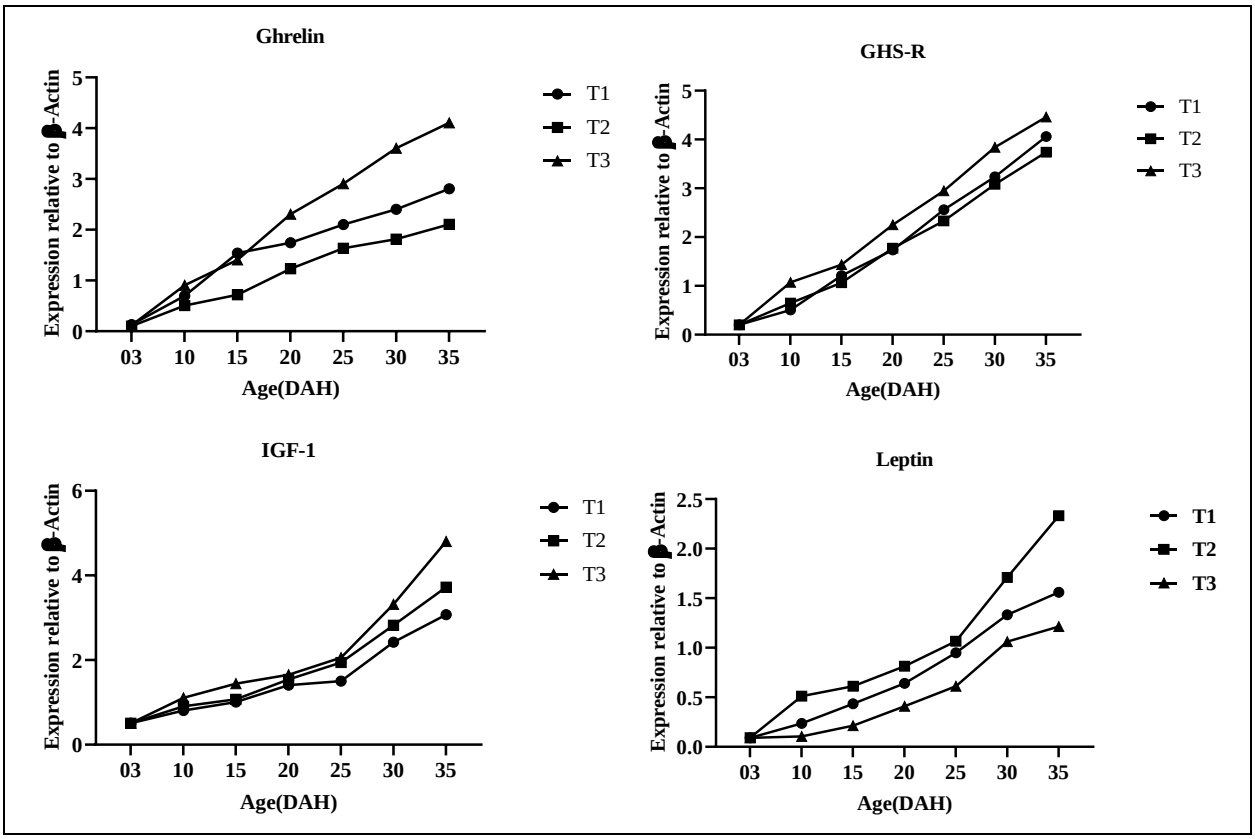

Figure 12. Comparative genes expression of *Ghrelin* and GHR-R, and *IGF-1* and *leptin* during early rearing of *L. rohita* PLs on live feed and prepared diet. T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared diet, T3= PLs co-fed live feed and prepared diet.

The *IGF-1* expression also followed similar increasing expression with age DAH in all groups (Figure 12), but the highest expression was observed in the T3 group followed by the T2 group. Moreover, *leptin* also showed an increased expression with age DAH, however, the T2 group showed the highest expression followed by the T1 group, while the T3 showed the lowest expression.

Furthermore, Pearson's correlation tests revealed that the net weight gain of all groups showed a positive correlation with the expressions of *ghrelin*, *GHS-R*, IGF and *leptin* (Figure 13).

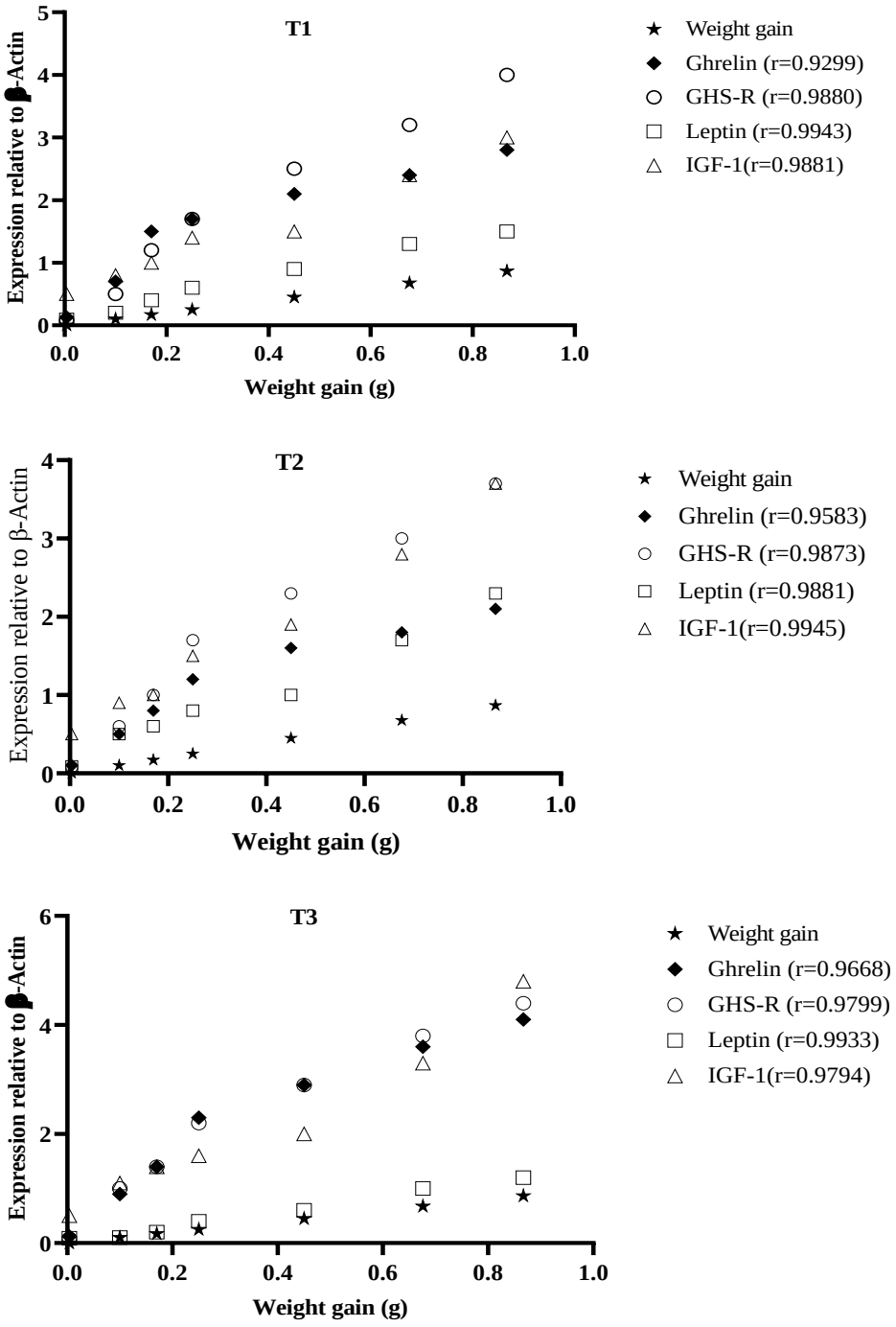

Figure 13. Correlation between weight gain and expression of *ghrelin*, *GHS-R*, *leptin* and *IGF-1* during early rearing of PLs of *L. rohita* for 35 days. T1= PLs reared on live feed, T2= PLs reared on Nano-particulate prepared feed while T3= PLs co-fed live and prepared feed.

Table 19. Two-way ANOVA values of the studied genes, enzymes and growth of *L. rohita* PLs reared on live feed, nano-particulated prepared feed and co-feeding for 35 days.

Enzymes activity	Independent fators	DF	F-Value	P-Value	% of total variation
	Feed Type	2	1400	<0.01	4.335
Protease	Age (DAH)	6	9728	<0.01	90.39
	Feed type × Age (DAH)	12	276.6	<0.01	5.140
	Feed Type	2	66.86	<0.01	3.574
Amylase	Age (DAH)	6	570.2	<0.01	91.43
	Feed type × Age (DAH)	12	8.570	<0.01	2.749
	Feed Type	2	1736	<0.01	3.230
Cellulase	Age (DAH)	6	16986	<0.01	94.84
	Feed type × Age (DAH)	12	165.4	<0.01	1.847
	Feed Type	2	40233	<0.01	14.26
Ghrelin	Age (DAH)	6	73348	<0.01	78.00
	Feed type × Age (DAH)	12	3633	<0.01	7.727
	Feed Type	2	1516	<0.01	2.421
GHS-R	Age (DAH)	6	20502	<0.01	96.30
	Feed type × Age (DAH)	12	77.03	<0.01	0.7381
	Feed Type	2	3.157	<0.01	4.621
IGF-1	Age (DAH)	6	20.83	<0.01	91.48
	Feed type × Age (DAH)	12	0.439	<0.01	3.862
	Feed Type	2	2746	<0.01	11.30
Leptin	Age (DAH)	6	6752	<0.01	83.39
	Feed type × Age (DAH)	12	207.9	<0.01	5.134
	Feed Type	2	621.3	<0.01	1.861
Growth	Age (DAH)	6	10587	<0.01	95.15
	Feed type × Age (DAH)	12	159.4	<0.01	2.865

Experiment # 3

Growth performance and survival rate

Protein inclusion level in the larval feed of *L. rohita* significantly affected the growth performance during early ontogeny (Table 20, Figure 14). Statistical analysis of the growth data, using two-way ANOVA suggest significant effect of the two independent variables, i.e., protein inclusion level (NWG, $F_{[3,112]}$=1558.9, *P<0.001*,η_p^2=0.990; SGR, $F_{[3,80]}$=26.31, *P<0.001*, η_p^2= 0.811), and age DAH (NWG, $F_{[6,112]}$=26000, *P<0.001*, η_p^2= 0.977; SGR, $F_{[5,80]}$=18450, *P<0.001*, η_p^2= 0.993) and their interaction, dietary protein inclusion level × age DAH (NWG: $F_{[18,112]}$=324.42, *P<0.001*, η_p^2= 0.981; SGR, $F_{[15,80]}$=33.30, *P<0.001*, η_p^2= 0.450).

Table 20. Growth performance of *L. rohita* PLs rearing on nano-particulate feed having varying levels of crude protein

Growth Indices	Age (DAH)	Groups based on protein inclusion level (%)						
		P45	P50	P55	P60	P-value	F-value	SEM
NWG (g)	03	0.0004^{Ga}	0.0004^{Ga}	0.0004^{Ga}	0.0004^{Ga}	0.993	0.030	0.0001
	10	0.119^{Fc}	0.13^{Fbc}	0.14^{Fa}	0.13^{Fab}	<0.001	17.562	0.0020
	15	0.16^{Ed}	0.173^{Ec}	0.239^{Ea}	0.212^{Eb}	<0.001	158.08	0.0076
	20	0.23^{Dd}	0.246^{Dc}	0.31^{Da}	0.297^{Db}	<0.001	214.91	0.0075
	25	0.435^{Cd}	0.460^{Cc}	0.512^{Ca}	0.491^{Cb}	<0.001	138.10	0.0068
	30	0.643^{Bc}	0.655^{Bc}	0.92^{Ba}	0.90^{Bb}	<0.001	1674.8	0.0302
	35	0.819^{Ad}	0.849^{Ac}	1.275^{Aa}	1.059^{Ab}	<0.001	441.03	0.0424
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	8309.1	13138.4	17127.2	3055.2			
SGR (%)	03	-	-	-	-	-	-	-
	10	6.996^{Fb}	7.039^{Fab}	7.179^{Fa}	7.122^{Fab}	0.008	5.561	0.0228
	15	7.32^{Eb}	7.448^{Eb}	7.845^{Ea}	7.706^{Ea}	<0.001	34.30	0.1390
	20	7.82^{Db}	7.88^{Db}	8.16^{Da}	8.123^{Da}	<0.001	18.79	0.1399
	25	8.59^{Cb}	8.66^{Cab}	8.788^{Ca}	8.74^{Ca}	0.004	6.492	0.0275
	30	9.08^{Bb}	9.09^{Bb}	9.52^{Ba}	9.499^{Ba}	<0.001	48.39	0.0368
	35	9.38^{Ac}	9.42^{Ac}	9.92^{Aa}	9.70^{Ab}	<0.001	45.302	0.0342
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	987.24	518.01	913,70	665.41			

Table 10. Results were analyzed via using two-way ANOVA by taking two independent variables *Protein inclusion level* and *Age* followed by post hoc pairwise inter and intra group comparisons (n=5). Means (PLs weight values in g) with different subscripts in same rows are significantly different at $p < 0.05$) and show comparison between groups, however superscript letters in same column shows age wise difference within group.

P45= PLs reared on nano-particulate feed with 45% CP, P50= PLs reared on nano-particulate feed with 50% CP , P55= PLs reared on nano-particulate feed with 55% CP, P60= PLs reared on nano feed with 60% CP.

The partial eta-squared (η_p^2) values of protein inclusion level and age DAH showed a similar and large effect on dependent variables, NWG and SGR while their interaction indicated a larger effect on NWG and a medium effect on SGR.

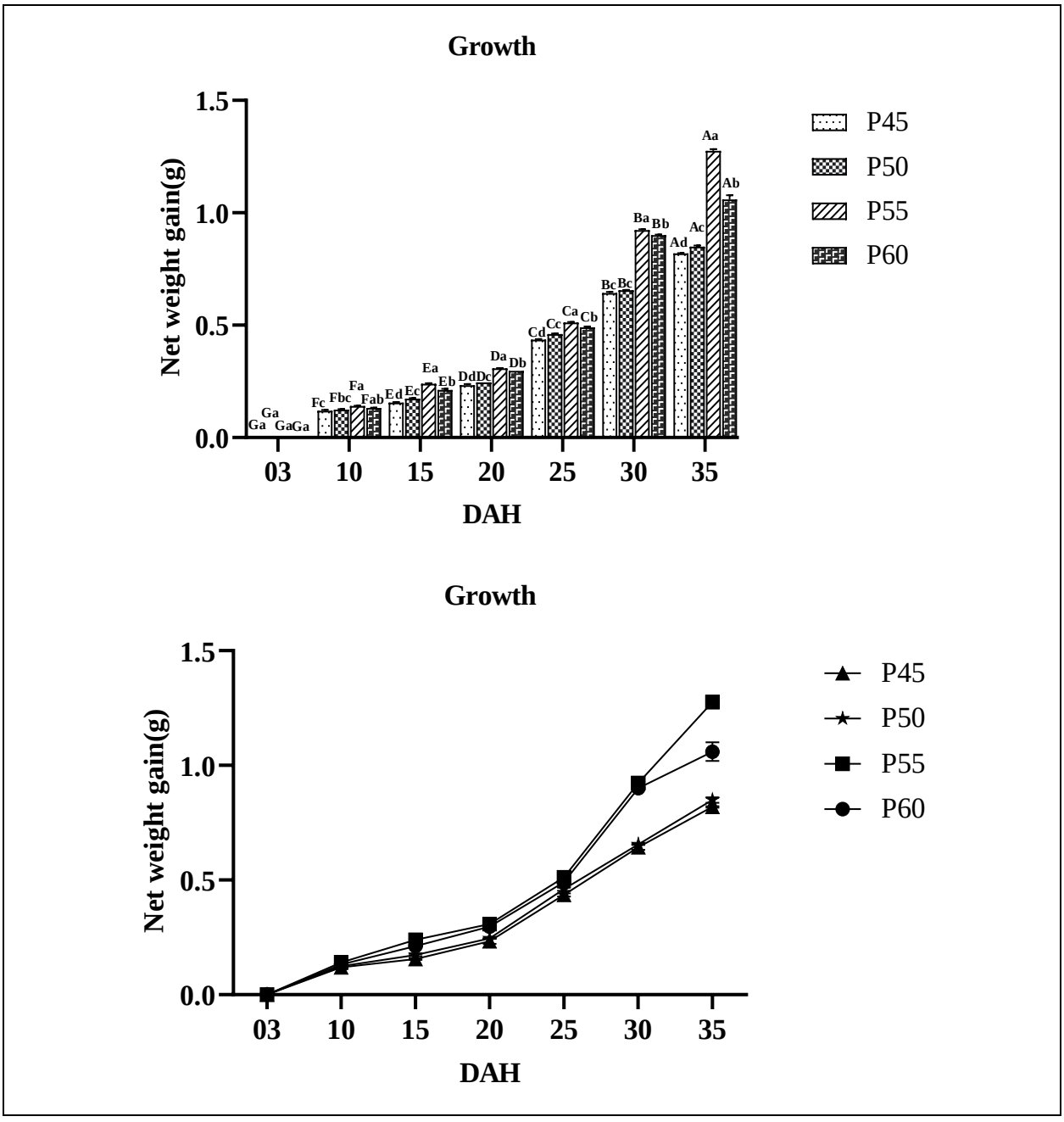

Figure 14. Growth performance of *L. rohita* PLs reared on Nano-particulate diets having varying inclusion levels of crude protein.

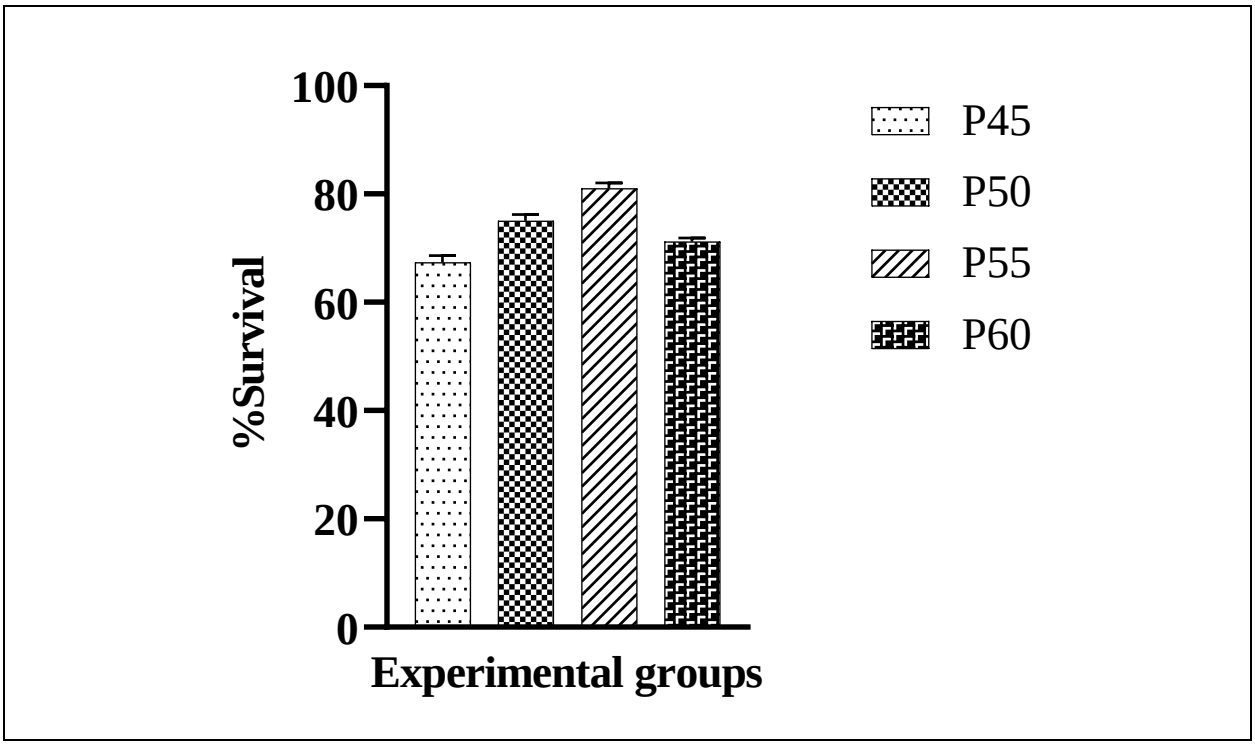

Figure 15. Survival percent of *L. rohita* PLs reared on nano-particulate diet having varying levels of crude protein.

All pairwise comparison indicated the highest NWG in P55 group (fed 55% CP diet) followed by P60 (fed 60% CP), while P45 group reared on 45% CP diet showed the lowest NWG. Moreover, P55 and P60 groups showed statistically similar but higher value of SGR as compared to other groups. The NWG and SGR of all groups showed positive relation with DAH, i.e., increase with age. In addition, it was noted that the NWG difference became more significant among the groups after 25DAH.

Protein inclusion level in the larval feed also significantly affects the survival % of *L. rohita* during early ontogeny (Figure 15). The protein inclusion level showed a curvilinear relationship with survival (%), i.e., a positive correlation up to 55%CP. However, a decreasing trend was noted with inclusion level over 55% crude protein inclusion level. All pairwise comparison indicated the highest survival (%) in the P55 group followed by P50 group while P45 group showed the lowest survival.

Intestinal enzyme activity

During early ontogeny the protein content of diet significantly modulates the release of digestive enzymes in *L. rohita* (Table 21; Figure 17 and 18). Two-way ANOVA indicated a

significant effect of percent CP in the larval feed on protease activity ($F_{[3,112]}$=2772, *P<0.001*, η_p^2=0.987), Trypsin activity ($F_{[3,112]}$=4023.2, *P<0.001*, η_p^2=0.990) and chymotrypsin activity ($F_{[3,112]}$=862.48, *P<0.001*, η_p^2=0.959), while, no significant effect was observed on amylase activity ($F_{[3,112]}$=1.466, *P=0.2614*, η_p^2=0.051) among groups. However, the other independent factor, age DAH have shown a significant effect on all the studied enzymes including amylase activity ($F_{[6,112]}$ =17215, *P<0.001*, η_p^2=0.999); total Protease activity ($F_{[6,112]}$ =36814, *P<0.001*, η_p^2=0.999); Trypsin ($F_{[6,112]}$=197225, *P<0.001*, η_p^2= 1.00), and chymotrypsin activity ($F_{[6,112]}$=23918, *P<0.001*, η_p^2=0.999). Moreover, the significant interaction (*P<0.001*) between dietary protein inclusion level × age DAH on Protease, $F_{[18,112]}$=562.2, η_p^2=0.989; trypsin, $F_{[18,112]}$=657.96, η_p^2=0.991; and chymotrypsin, $F_{[18,112]}$=390.29, η_p^2=0.984; indicated that results are the combined effect of both variables. While non-significant interaction on amylase (amylase, $F_{[18,112]}$= 0.5219, *P= 0.945*, η_p^2=0.073) has been observed.

The partial eta-squared (η_p^2) values indicated the large and almost similar effect size of both variables (protein inclusion level and age DAH) and their interaction on protease and trypsin and chymotrypsin. However, age (DAH) accounted for a large effect size compared to feed and feed× age (DAH) interaction showing a lesser but still significant proportion of variation in amylase activity.

Table 21. Intestinal enzymes activity in *L. rohita* PLs reared on nano-particulate diets having varying levels of crude protein.

Gut Enzymes	Age (DAH)	Groups based protein inclusion level (%)						
		P45	P50	P55	P60	P-value	F-value	SEM
	03	0.020^{Ga}	0.020^{Fa}	0.020^{Fa}	0.020^{Ga}	0.989	0.040	0.00007
	10	0.078^{Fd}	0.081^{Ec}	0.087^{Ea}	0.082^{Fb}	<0.001	814.36	0.00076
	15	0.104^{Ec}	0.102^{Dc}	0.186^{Da}	0.152^{Eb}	<0.001	1025.4	0.0081
Protease	**20**	0.169^{Dc}	0.186^{Cb}	0.189^{Db}	0.198^{Da}	<0.001	157.93	0.0024
	25	0.234^{Cc}	0.252^{Bb}	0.296^{Ca}	0.261^{Cb}	<0.001	50.748	0.00547
	30	0.303^{Bd}	0.417^{Ac}	0.573^{Aa}	0.493^{Ab}	<0.001	4354.5	0.0228
	35	0.36^{Ad}	0.421^{Ac}	0.476^{Ba}	0.4575^{Bb}	<0.001	1481.88	0.0100
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	4426.4	10676	7262.7	88672			
	03	12.03^{Ga}	12.05^{Ga}	12.96^{Fa}	12.09^{Ga}	0.249	0.861	0.8469
	10	14.41^{Fc}	16.12^{Fb}	17.29^{Fa}	17.61^{Fa}	<0.001	53.56	0.3018
	15	22.64^{Ec}	41.68^{Eb}	43.27^{Ea}	42.85^{Ea}	<0.001	1573.03	1.9914
Trypsin	**20**	50.49^{Dc}	67.84^{Db}	70.06^{Da}	70.06^{Da}	<0.001	1014.60	1.8874
	25	143.49^{Cc}	162.84^{Cb}	168.82^{Ca}	168.29^{Ca}	<0.001	598.52	2.3723
	30	222.48^{Bc}	289.74^{Bb}	302.34^{Ba}	301.46^{Ba}	<0.001	1419.28	7.5874
	35	310.46^{Ad}	355.69^{Ac}	397.7^{Aa}	362.45^{Ab}	<0.001	1058.95	7.1394
	P-value	0.001	0.001	0.001	0.001			
	F-value	85115	178523	24523	61958			
	03	16.37^{Fa}	16.09^{Fa}	16.37^{Ea}	16.77^{Fa}	0.367	1.147	0.2854
	10	27.78^{EFd}	33.63^{EFc}	38.84^{Ea}	37.78^{EFb}	<0.001	755.41	1.0001
	15	43.22^{Ed}	54.81^{Ec}	64.81^{Ea}	62.62^{Eb}	<0.001	548.88	1.9481
Chymotrypsin	**20**	212.94^{Dc}	252.52^{Db}	276.52^{Da}	272.94^{Da}	<0.001	503.39	5.8272
	25	531.4^{Cd}	692.66^{Cc}	792.66^{Ca}	728.96^{Cb}	<0.001	4650.5	22.124
	30	678.0^{Bc}	882.96^{Bb}	964.96^{Ba}	978.01^{Ba}	<0.001	261.95	27.801
	35	1846.6^{Ac}	2240.3^{Ac}	3300.3^{Aa}	2834.93^{Ab}	<0.001	461.21	128.19
	P-value	0.001	0.001	0.001	0.001			
	F-value	23044	23384	1963.87	24135			
	03	0.456^{Ca}	0.459^{Ca}	0.456^{Ca}	0.458^{Ca}	0.902	0.189	0.0013
	10	0.2842^{Ga}	0.2846^{Ga}	0.2844^{Ga}	0.2806^{Ga}	0.236	1.569	0.0007
	15	0.3194^{Fa}	0.3128^{Fa}	0.3129^{Fa}	0.3119^{Fa}	0.337	1.215	0.0005
Amylase	**20**	0.3656^{Ea}	0.3663^{Ea}	0.3644^{Ea}	0.3635^{Ea}	0.486	0.852	0.0006
	25	0.5844^{Aa}	0.5840^{Aa}	0.5832^{Aa}	0.5816^{Aa}	0.258	1.479	0.0005
	30	0.4756^{Ba}	0.4730^{Bab}	0.4737^{Bab}	0.4716^{Bb}	0.009	5.403	0.0004
	35	0.3864^{Da}	0.3880^{Da}	0.387^{Da}	0.3866^{Da}	0.920	0.163	0.0009
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	7438.95	2196.63	6918.16	3874.56			

Results were analyzed by using two-way ANOVA; %*protein inclusion level* and *Age* followed by post hoc pairwise inter and intra group comparisons.

Means (Enzymes activity: U/mg) with different subscripts in same rows are significantly different (P< 0.05) and show comparison between groups.

however, superscript letters in same column shows age wise difference within group.

P45= PLs reared on feed 45% protein, P50= PLs reared on feed 50% protein, P55= PLs reared on feed 55% protein, P60= PLs reared on feed 45% protein.

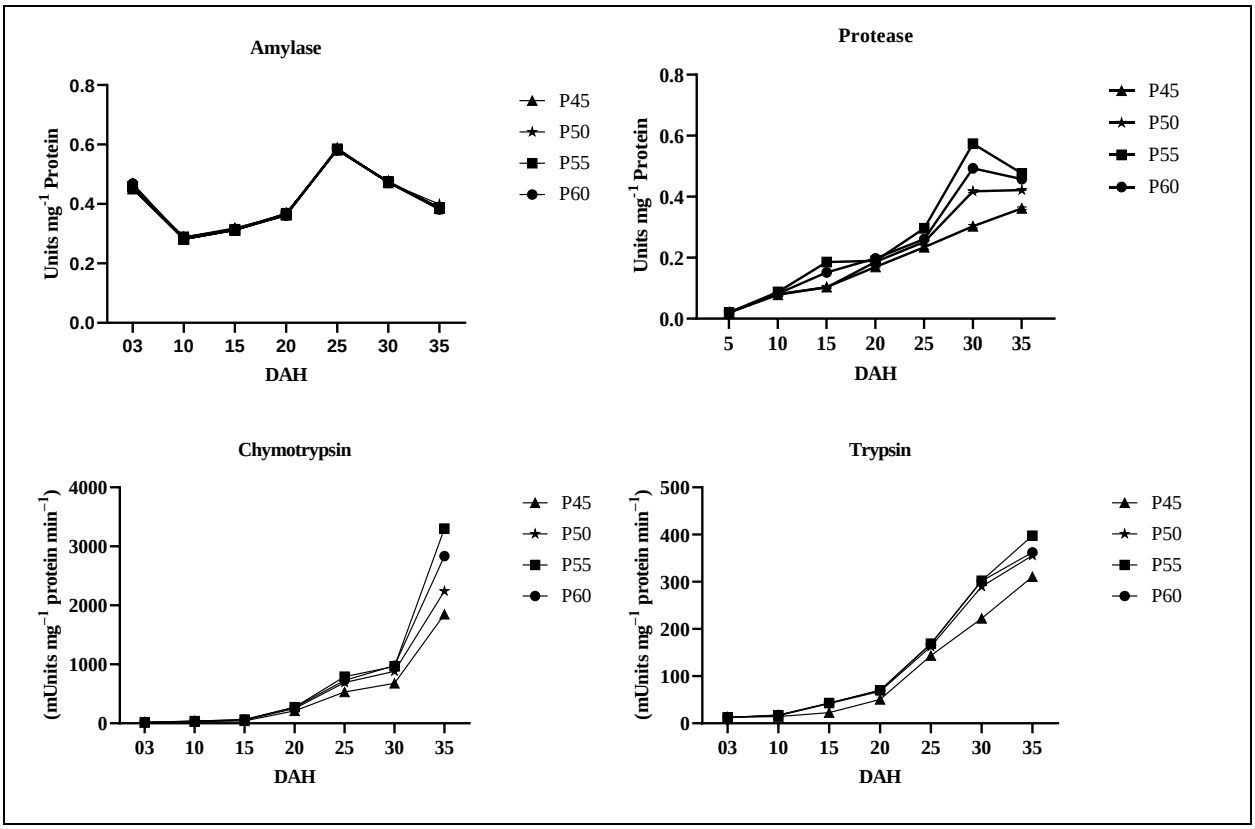

Figure 16. Pattern of intestinal enzyme activity during early ontogeny while rearing on diet with various protein inclusion levels.

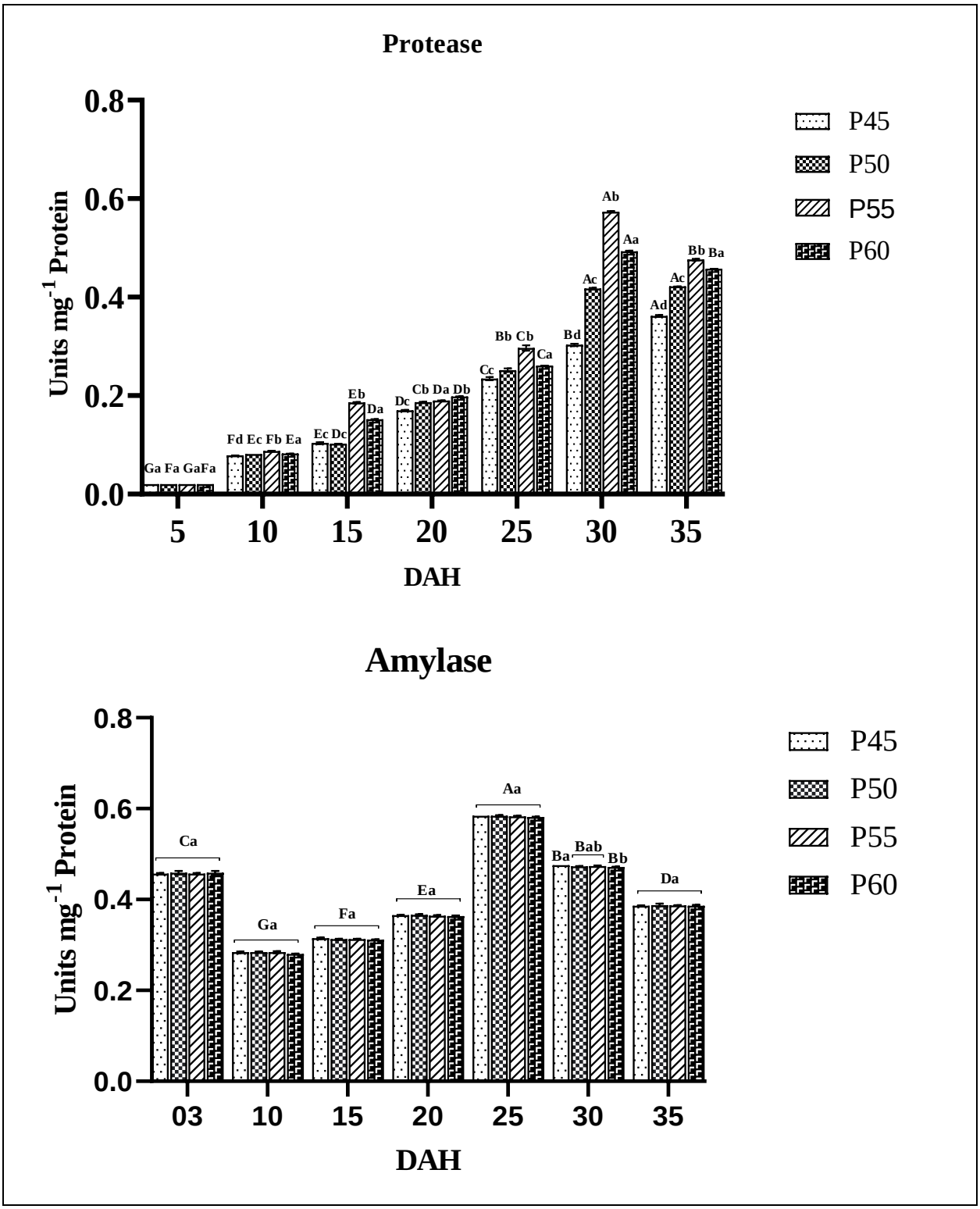

Figure 17. Bar graph showing inter and intra group variation in protease, amylase activity during early ontogeny of *L. rohita* PLs.

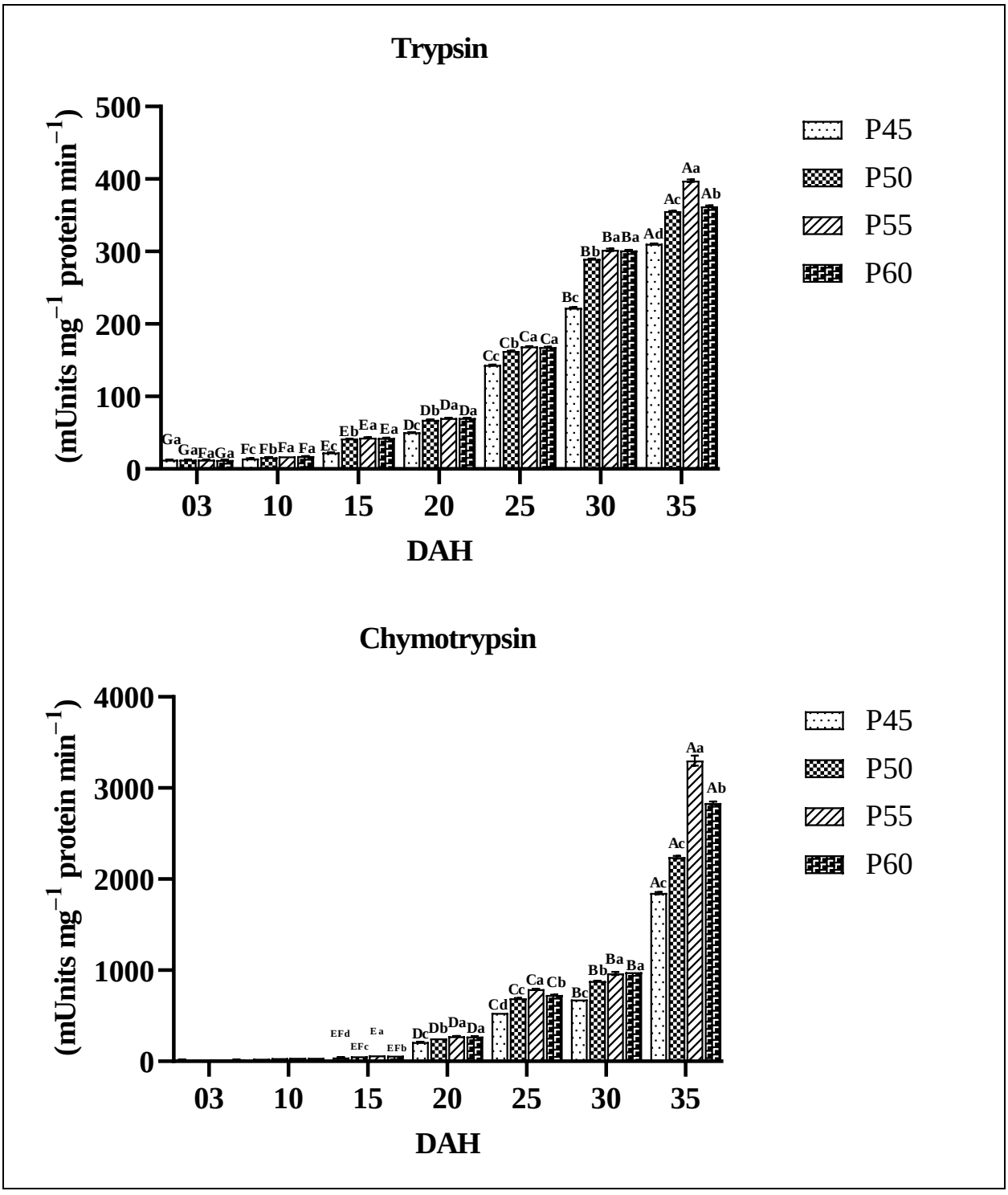

Figure 18. Bar graph showing inter and intra group variation in trypsin and chymotrypsin activity during early ontogeny of *L. rohita* PLs.

All pairwise comparison indicated that P55 followed by P60 group had the highest activity of protease and chymotrypsin, while trypsin activity was statistically similar in both group (P55 and P60). The amylase activity of all groups did not show any significant difference.

Table 22. Relative expression of MyoD, MYF5 and GH in *L. rohita* PLs during early ontogeny rearing on nano-particulate diets having varying levels of crude protein.

Genes expression	Age (DAH)	Groups based on protein inclusion level (%)						
		P45	P50	P55	P60	P-value	F-value	SEM
	03	1.125^{Aa}	1.124^{Aa}	1.124^{Aa}	1.124^{Aa}	0.997	0.015	0.0065
	10	1.131^{Ad}	1.150^{Ac}	1.22^{Aa}	1.179^{Ab}	<0.001	354.88	0.0073
	15	1.046^{Bc}	1.107^{Bb}	1.16^{ABa}	1.157^{Ba}	<0.001	43.910	0.0117
MYF5	**20**	0.946^{Cc}	1.019^{Cb}	1.139^{Ba}	1.131^{Ca}	<0.001	65.434	0.0192
	25	0.88^{Db}	0.94^{Db}	1.041^{Ca}	0.90^{Db}	<0.001	25.945	0.0153
	30	0.822^{Ec}	0.89^{Eb}	1.019^{Ca}	0.90^{Db}	<0.001	64.546	0.0168
	35	0.746^{Fd}	0.757^{Fc}	0.902^{Da}	0.80^{Eb}	<0.001	1329.6	0.0141
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	461.59	289.28	81.080	5112.4			
	03	1.409^{Aa}	1.405^{Aa}	1.407^{Aa}	1.405^{Aa}	0.486	0.851	0.0011
	10	1.202^{Bb}	1.22^{Bb}	1.406^{Ba}	1.208^{Bb}	<0.001	12.465	0.0232
	15	1.006^{Cb}	1.20^{Ba}	1.20^{Ca}	1.128^{Ba}	<0.001	16.642	0.0209
MyOD	**20**	0.903^{Dc}	0.923^{Cb}	1.104^{Da}	1.01^{Cab}	<0.001	10.150	0.0225
	25	0.906^{Db}	0.87^{Cb}	1.017^{Ea}	0.906^{CDb}	<0.001	24.889	0.0139
	30	0.89^{Db}	0.75^{Dc}	1.019^{Ea}	0.899^{Db}	<0.001	63.214	0.0225
	35	0.799^{Ec}	0.698^{Dd}	0.907^{Fa}	0.845^{Db}	<0.001	193.57	0.0177
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	259.07	118.702	298.033	81.331			
	03	0.502^{Ga}	0.496^{Fa}	0.500^{Ga}	0.503^{Ga}	0.775	0.371	0.0093
	10	0.556^{Fd}	0.602^{Ec}	0.806^{Fa}	0.70^{Fb}	<0.001	1799.7	0.0221
	15	0.704^{Ed}	0.786^{Dc}	1.026^{Ea}	0.903^{Eb}	<0.001	196.36	0.0282
GH	**20**	0.901^{Dc}	1.10^{Cb}	1.20^{Da}	1.20^{Da}	<0.001	1322.5	0.0281
	25	1.031^{Cd}	1.142^{Bc}	1.485^{Ca}	1.294^{Cb}	<0.001	184.26	0.0396
	30	1.115^{Bd}	1.161^{Bc}	1.56^{Ba}	1.31^{Bb}	<0.001	1936.1	0.0397
	35	1.20^{Ad}	1.25^{Ac}	1.60^{Aa}	1.50^{Ab}	<0.001	7203.4	0.0385
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	516.49	5641.91	2771.7	24774			

Two-way ANOVA for overall statistical analysis of two independent variables *Age* and *protein inclusion level* and dependent variable *MYF5/MyoD/GH* expression followed by One-way ANOVA for intergroup and intra group comparison where n=5. LSD post hoc test shows a pairwise comparison between and within groups. Means (gene expression relative to β-Actin) with different lowercase superscripts in same rows are significantly different at $p < 0.05$ and show comparison between groups, however uppercase superscript letters in same column shows age wise difference within group. P45: PLs reared on feed with 45% crude protein, P50: PLs reared on feed with 50% crude protein, P55: PLs reared on feed with 55% crude protein, P60: PLs reared on feed with 60% crude protein.

It was also noted age DAH had a curvilinear relationship with protease and amylase activity, i.e., showing increasing trend (positive correlation) up to 30DAH and 25 DAH respectively and then showed decreasing trend (Figure 16). The trypsin and chymotrypsin showed positive correlation with age DAH (continuous increase trend); however, trypsin showed a uniform increasing pattern while abrupt increase was noted after 30DAH in case of chymotrypsin. Overall, the developmental pattern of digestive enzymes in response to CP inclusion level in larval diet showed a decline in

amylase and protease activity after 30DAH, unlike trypsin and chymotrypsin which showed increasing trend.

Expression of genes regulating myogenesis

Myogenesis comprises recruitment of new muscles fibers and growth of the existing one. The protein inclusion level in the larval feed of *L. rohita* significantly affects the regulation of myogenesis at molecular level, i.e., expression of myogenic regulatory factors (MRFs) including MYF5 and MyoD genes responsible for myoblast determination and protein coding, respectively and GH gene during early ontogeny (Table 22, Figure 25 and 24).

Two-way ANOVA have shown a significant effect of protein inclusion level (Table 23) in larval diet on the expression of MYF5 ($F_{[3,112]}$=288.43, *P<0.001*, η_p^2= 0.900; Fig.), MyoD ($F_{[3,112]}$=81.98, *P<0.001*, η_p^2= 0.711; Fig.), and GH ($F_{[3,112]}$=2521.5, *P<0.001*, η_p^2= 0.984; Fig.) during early development (Figure 19 and 20). Like feed, age DAH also showed a significant effect on the expression of all studied genes (MYF5 ($F_{[6,112]}$=1024.6, *P<0.001*, η_p^2= 0.983; Fig.), MyOD ($F_{[6,112]}$=514.6, *P<0.001*, η_p^2= 0.966; Fig.), and GH ($F_{[6,112]}$= 8783.5, *P<0.001*, η_p^2= 0.941; Fig.). Moreover, the most significant interaction (*P<0.001*) between protein inclusion level × age DAH on genes expression (MYF5 ($F_{[18,112]}$=20.44, *P<0.001*, η_p^2= 0.699; Fig.), MyoD ($F_{[18,112]}$=9.74, *P<0.001*, η_p^2= 0.966; Fig.), and GH ($F_{[18,112]}$=120.9, *P<0.001*, η_p^2= 0.941) indicated how both independent variables interact and affect the dependent variable.

The partial eta-squared (η_p^2) values indicated the large and almost similar effect size of dietary protein inclusion level and age DAH on the expression of GH and MYF5, while medium effect of dietary protein inclusion level and larger effect size of age DAH on MyoD. Moreover, the η_p^2 values significant interaction between dietary protein inclusion level and age DAH indicated large effect size on GH gene expression while medium on MyoD and MYF5 genes expression. All pairwise comparisons indicated that P55 followed by P60 group had the highest expression of all genes. Moreover, in all groups, GH showed significant positive correlation (P45, R^2=0.8948*, P=0.0013*; P50, R^2=0.7630, *P=0.0102*; P55, R^2=0.7633 ,*P=0.0102*; P60, R^2=0.7821, *P=0.0082*) while MyoD (P45, R^2=0.6526, *P=0.0279*; P50, R^2=0.8679, *P=0.0022*; P55, R^2=0.7607, *P=0.0105*; P60, R^2=0.7597, *P=0.0106*) and MYF5 (P45, R^2=0.9285, *P=0.0005*; P50, R^2=0.9468, *P=0.0002*; P55, R^2=0.8407, *P=0.0037*; P60, R^2=0.8358, *P=0.004*) indicated

significant inverse relation (negative correlation, r) with age DAH. Furthermore, it was also noted that during ontogeny, the expression of the studied genes increased/decreased in a uniform fashion. For instance, MYF5 expression increased (non-significant) initially till 10DAH followed by a significant uniform decline till 35DAH. Like MYF5, MyoD expression also remained high in P55 till 10DAH and then showed a uniform decline. Moreover, MyoD expression showed a uniform decline in P55 and P60 groups while zigzagging (Figure 24) trend in P50 and P45groups. Unlike MRFs expression, GH expression enhanced with age in each group quite uniformly.

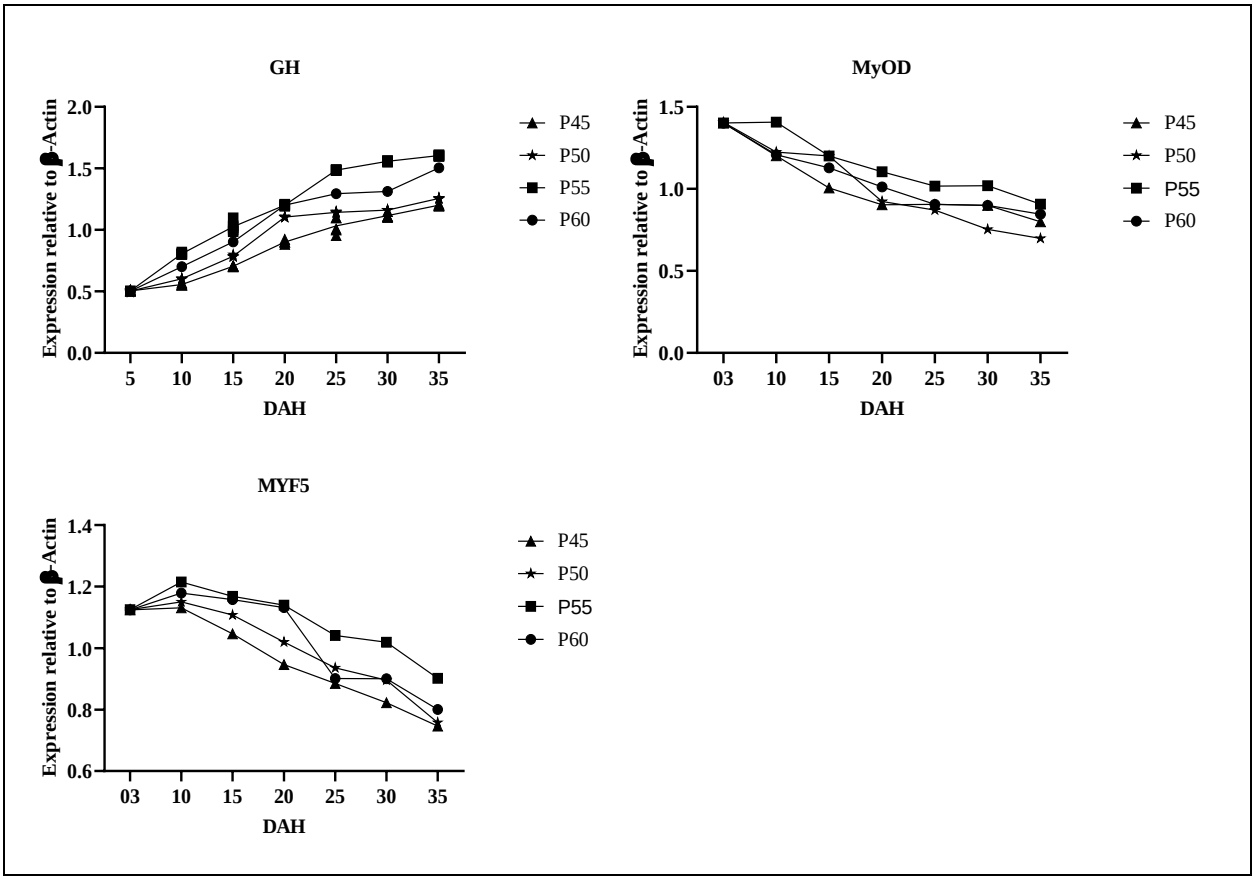

Figure 19. Pattern of expression of genes in *L. rohita* PLs while rearing on graded level of protein concentration in the nano particulate feed.

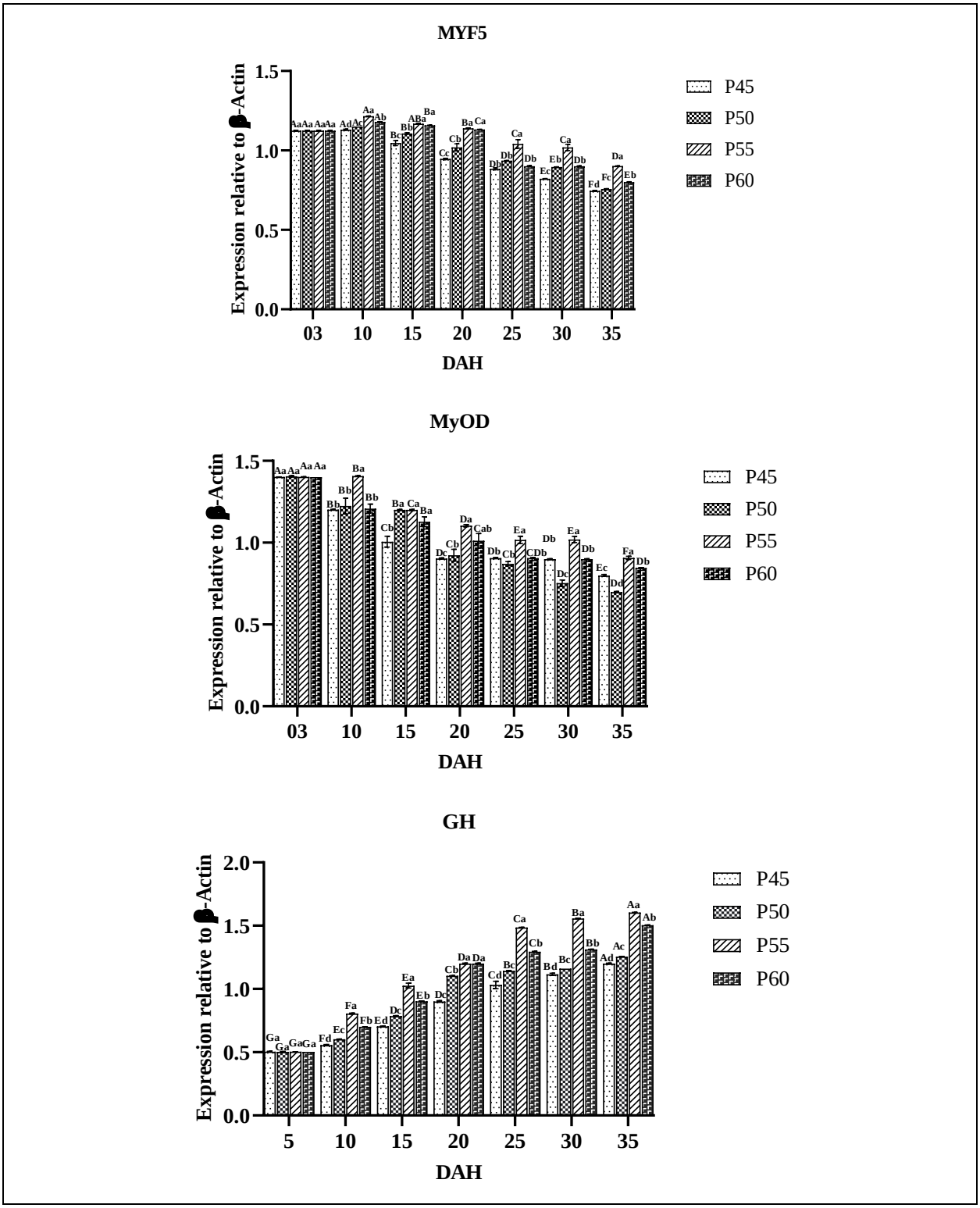

Figure 20. Bar graph showing inter and intra group variation in expression of MYF5, MyoD and GH, while rearing on feed having different inclusion level of protein during early ontogeny of *L. rohita* PLs.

Table 23. Two-way ANOVA values of the studied genes, enzymes and growth of *L. rohita PLs* reared on nano feed with different protein concentration for 35 days.

Dependent Factors	Independent Factors	DF	F-Value	P-Value	% of total variance
Protease	Protein level	3	2772	<0.001	3.473
	Age (DAH)	6	36814	<0.001	92.25
	Protein level × Age (DAH)	18	562.2	<0.001	4.227
Trypsin	Protein level	3	4023.2	<0.001	1.017
	Age (DAH)	6	197225	<0.001	98.01
	Protein level × Age (DAH)	18	657.96	<0.001	0.9637
Chymotrypsin	Protein level	3	862.48	<0.001	1.693
	Age (DAH)	6	23918	<0.001	93.65
	Protein level × Age (DAH)	18	390.29	<0.001	4.579
Amylase	Protein level	3	1.466	0.2614	0.006
	Age (DAH)	6	17215	<0.001	99.87
	Protein level × Age (DAH)	18	0.5219	0.9415	0.009
MyoD	Protein level	3	81.98	<0.001	7.416
	Age (DAH)	6	514.6	<0.001	85.78
	Protein level × Age (DAH)	18	9.74	<0.001	3.790
MYF5	Protein level	3	288.43	<0.001	12.63
	Age (DAH)	6	1024.6	<0.001	82.72
	Protein level × Age (DAH)	18	20.44	<0.001	3.249
GH	Protein level	3	2521.5	<0.001	12.64
	Age (DAH)	6	8783.5	<0.001	83.98
	Protein level × Age (DAH)	18	120.9	<0.001	3.174
Growth	Protein level	3	1558.9	<0.001	2.795
	Age (DAH)	6	26000	<0.001	93.62
	Protein level × Age (DAH)	18	324.42	<0.001	3.522
SGR	Protein level	3	26.34	<0.001	2.733
	Age (DAH)	5	18454	<0.001	96.11
	Protein level × Age (DAH)	15	33.30	<0.001	0.5203

Experiment # 4

Growth performance and survival rate

Dietary fats inclusion level in the larval feed (CF%) of *L. rohita* significantly affects the growth performance during early ontogeny (Table 24). Statistical analysis of the growth data, using two-way ANOVA suggest significant effect of the two independent variables, i.e., CF inclusion level (NWG, $F_{[3,112]}$=897.8, *P<0.001*, η_p^2= 0.960; SGR, $F_{[3,80]}$=16.42, *P<0.001*, η_p^2= 0.294), and age DAH (NWG, $F_{[6,112]}$=18650 , *P<0.001*, η_p^2=0.998; SGR, $F_{[5,80]}$=22978, *P<0.001*, η_p^2=0.993) and their interaction, dietary CF inclusion level × age DAH (NWG, $F_{[18,112]}$=192.6, *P<0.001*, η_p^2=0.969; SGR, $F_{[15,80]}$=28.13, *P<0.001*, η_p^2=0.739).

Table 24. Growth performance of *L. rohita* PLs during rearing on Nano-particulate diet having varying levels of crude fats.

Growth indices	Age (DAH)	Groups based on protein inclusion level (%)						
		CF4	CF8	CF12	CF16	*P*-value	F-value	SEM
NWG(g)	03	0.0042^{Ga}	0.004^{Ga}	0.0043^{Ga}	0.004^{Ga}	0.993	0.030	0.0001
	10	0.119^{Fb}	0.136^{Fa}	0.132^{Fa}	0.117^{Fb}	<0.001	19.343	0.0021
	15	0.174^{Ec}	0.235^{Ea}	0.208^{Eb}	0.175^{Ec}	<0.001	142.25	0.0059
	20	0.245^{Dc}	0.308^{Da}	0.297^{Db}	0.245^{Dc}	<0.001	1162.0	0.0066
	25	0.453^{Cc}	0.511^{Ca}	0.491^{Cb}	0.452^{Cc}	<0.001	190.58	0.0059
	30	0.668^{Bb}	0.92^{Ba}	0.91^{Ba}	0.67^{Bb}	<0.001	1247.3	0.0285
	35	0.856^{Ac}	1.258^{Aa}	1.06^{Ab}	0.85^{Ac}	<0.001	213.46	0.0387
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	20077	3181.89	3629.2	19281.8			
SGR (%body weight/day)	03	-	-	-	-			
	10	$6.98F^{ab}$	7.152^{Fa}	7.105^{Fa}	6.927^{Fb}	0.008	5.566	0.0283
	15	7.453^{Eb}	7.828^{Ea}	7.664^{Ea}	7.425^{Eb}	<0.001	16.23	0.0434
	20	7.882^{Db}	8.163^{Da}	8.107^{Da}	7.843^{Db}	<0.001	15.086	0.0388
	25	8.64^{Cab}	8.792^{Ca}	8.731^{Cab}	8.601^{Cb}	0.021	4.385	0.0233
	30	9.123^{Bb}	9.524^{Ba}	9.497^{Ba}	9.090^{Bb}	<0.001	31.08	0.0532
	35	9.431^{Ac}	9.908^{Aa}	9.687^{Ab}	9.397^{Ac}	<0.001	32.73	
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	530.37	522.32	586.64	545.669			

Results were analyzed via using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (PLs weight values in g), with different subscripts in same rows are significantly different at $p < 0.05$) and show comparison between groups, however superscript letters in same column shows age wise difference within group. CF4= PLs reared on 4% crude fats feed, CF8= PLs reared on 8% crude fats feed, CF12= PLs reared on 12% crude fats feed, CF16= PLs reared on 16% crude fats feed.

The partial eta-squared (η_p^2) values of CF inclusion level and age DAH showed a similar and large effect on dependent variables, NWG and SGR while their interaction indicated a larger effect on NWG and a medium effect on SGR.

All pairwise comparison indicated the highest NWG in a CF8 group (fed 8% CF diet) followed by CF12 (fed 12% CF), while CF4 group reared on 4% CF diet showed the lowest NWG. Moreover, CF4 and CF16 groups showed statistically similar and lower value of SGR as compared to other groups. The NWG and SGR of all groups showed positive relation with DAH, i.e., increase with age. In addition, it was noted that the NWG difference became more significant among the group after 25DAH, especially in CF8 and CF12 compared to CF4 and CF8 (Figure 26).

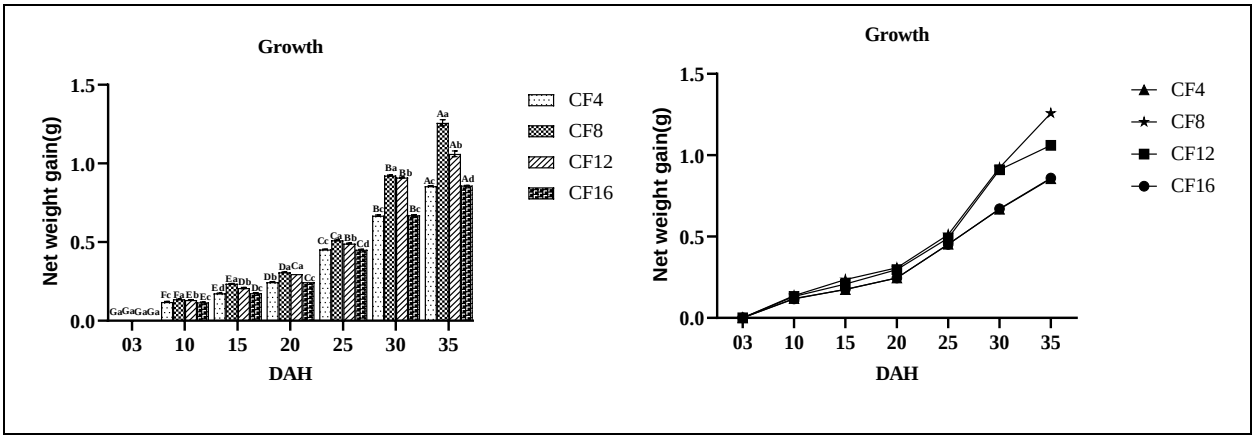

Figure 21. Growth assessment of *L. rohita* PLs while feeding on graded level of lipids

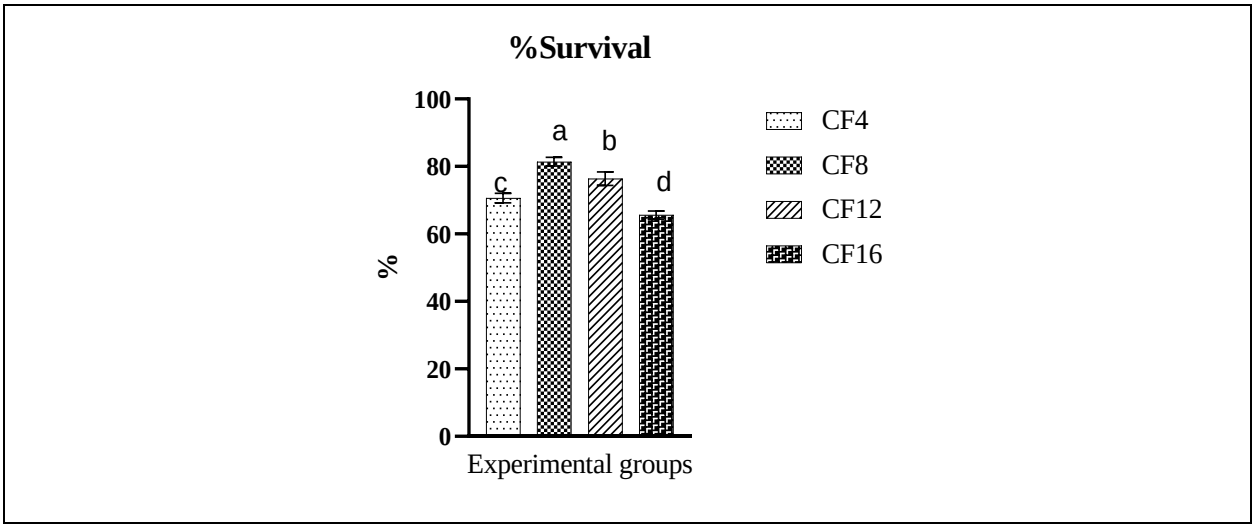

Figure 22. Survival rate of *L. rohita* PLs while feeding on graded level of lipids.

CF inclusion level in the larval feed also significantly affects the survival % of *L. rohita* during early ontogeny (Figure 27). High mortality was observed at both lower and higher CF inclusion level in the larval diet of rohu: 35.4% in CF16 while 29.6% in CF4. All pairwise comparison indicated the highest survival (%) in CF8 (81.4%) followed by CF12(76.4%).

Intestinal enzyme activity

During early ontogeny it was observed that CF content of diet significantly modulates the release of digestive enzymes in *L. rohita* (Figure 28,29 & 31). Two-way ANOVA indicated a significant effect of percent CF in the larval feed on lipase activity ($F_{[3,112]}$=35.89, *P<0.001,* η_p^2= 0.092), protease ($F_{[3,112]}$=753.9, *P<0.001*, η_p^2=0.927), amylase ($F_{[3,112]}$=1392 , *P<0.001*, η_p^2=0.970) and alkaline phosphatase ($F_{[3,112]}$=3567, *P<0.001*, η_p^2=0.990). Similarly, the other independent factor, age DAH has also shown a significant effect on all the studied enzymes including lipase activity ($F_{[6,112]}$= 75182, *P<0.001*, η_p^2= 1.00), protease ($F_{[6,112]}$=33479, *P<0.001*, η_p^2=0.999), amylase ($F_{[6,112]}$=10605 , *P<0.001*, η_p^2=0.998) and alkaline phosphatase ($F_{[6,112]}$=24389, *P<0.001*, η_p^2=0.999). Moreover, the most significant interaction (*P<0.001*) between percent CP × age DAH (Lipase, $F_{[18,112]}$=3.247, η_p^2=0.343; total Protease, $F_{[18,112]}$=63.14, η_p^2=0.915; amylase, $F_{[18,112]}$=194.9, η_p^2=0.970 and ALP, $F_{[18,112]}$=301.8, η_p^2=0.998) indicated how both independent variables interact and modulate the intestinal enzymes activity of *L. rohita* larvae. The partial eta-squared (η_p^2) values indicated the large and almost similar effect size of both variables (CF inclusion level and age DAH) and their interaction on protease, ALP, and amylase. However, age (DAH) accounted for a large effect size compared to feed and feed× age (DAH) interaction showing a lesser but still significant proportion of variation in amylase activity. On the other hand, the lipase activity in the gut increased with age and the partial eta-squared (ηp2) values indicate the effect size mainly due to age DAH, suggesting no significant role of CF content in the feed.

Simple main effects analysis revealed the most significant effect of CF% of all studied gut enzymes. All pairwise comparisons indicated no significant change in lipase activity initially in each group till 25DAH, although a significantly high lipase activity was observed on 30 and 35DAH in both CF8 and CF12 compared to CF4 and CF16 (Table 25). Protease activity was significantly high in experimental groups CF4, CF8, and CF12 while only in CF16 the protease activity was lower.

Further no significant difference in protease activity was observed among CF4, CF8 and CF12, however protease activity was significantly lower in CF16. Ontogeny trend of protease activity show the same comparative difference all along the sampling points. Amylase followed

the same pair wise difference as protease; CF4, CF8 and CF12 with no significant difference in between, remained significantly lower in CF16.

Table 25. Activity of intestinal enzymes in *L. rohita* PLs during rearing on nano-particulate diets having varying levels of crude fats.

Enzyme Activity	Age (DAH)	Groups based protein inclusion level (%)						
		CF4	CF8	CF12	CF16	P-value	F-value	SEM
	03	1.0146^{Ga}	1.015^{Ga}	1.017^{Ga}	1.012^{Ga}	0.389	1.071	0.0011
	10	6.126^{Fa}	6.12^{Fa}	6.14^{Fa}	6.124^{Fa}	0.115	2.313	0.0043
	15	9.37^{Ea}	9.376^{Ea}	9.379^{Ea}	9.368^{Ea}	0.992	0.031	0.0128
Lipase	**20**	11.06^{Da}	10.99^{Da}	10.99^{Da}	10.99^{Da}	0.945	0.123	0.0396
	25	14.76^{Ca}	14.77^{Ca}	14.77^{Ca}	14.75^{Ca}	0.988	0.042	0.0195
	30	15.34^{Bb}	15.96^{Ba}	15.41^{Bb}	15.34^{Bb}	0.016	5226	0.0136
	35	16.45^{Ab}	17.84^{Aa}	16.80^{Aab}	16.54^{Ab}	<0.001	31.231	0.0414
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	26581	15041	23672	15082			
	03	2.83^{Ga}	2.792^{Ga}	2.83^{Ga}	2.82^{Ga}	0.876	0.241	0.1721
	10	5.033^{Fb}	7.034^{Fa}	5.1^{Fb}	5.096^{Fb}	<0.001	2466.9	0.1951
	15	6.03^{Eb}	8.204^{Ea}	6.089^{Eb}	6.05^{Eb}	<0.001	4499.8	0.2135
ALP	**20**	8.843^{Dd}	11.29^{Da}	11.044^{Db}	9.29^{Dc}	<0.001	1106.4	0.2452
	25	10.07^{Cc}	14.83^{Ca}	14.2^{Cb}	10.43^{Cc}	<0.001	582.07	0.4958
	30	11.35^{Bc}	15.12^{Ba}	15.35^{Ba}	12.2^{Bb}	<0.001	618.34	0.4049
	35	12.63^{Ac}	16.57^{Aa}	16.45^{Aa}	13.65^{Ab}	<0.001	1536.8	0.3961
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	12318	30162.6	6693.8	2771.51			
	03	0.458^{Ca}	0.459^{Ca}	0.458^{Ca}	0.458^{Aa}	0.996	0.020	0.0009
	10	0.285^{Ga}	0.285^{Ga}	0.277^{Gb}	0.261^{Fc}	<0.001	112.32	0.0005
	15	0.318^{Fa}	0.316^{Fa}	0.308^{Fb}	0.294^{Ec}	<0.001	40.149	0.0008
Amylase	**20**	0.368^{Ea}	0.368^{Ea}	0.352^{Eb}	0.326^{Dc}	<0.001	85.000	0.0009
	25	0.586^{Aa}	0.583^{Aa}	0.557^{Ab}	0.427^{Bc}	<0.001	2579.6	0.0007
	30	0.509^{Ba}	0.509^{Ba}	0.479^{Bb}	0.422^{Bc}	<0.001	249.051	0.0082
	35	0.388^{Da}	0.391^{Da}	0.388^{Da}	0.366^{Cb}	<0.001	74.888	0.0023
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	7847.52	2396.9	2367.4	1859.5			
	03	0.205^{Ga}	0.0205^{Ga}	0.0205^{Ga}	0.0206^{Ea}	0.996	0.021	0.0009
	10	0.0875^{Fa}	0.0877^{Fa}	0.0878^{Fa}	0.0855^{Db}	<0.001	21.12	0.0003
	15	0.1043^{Ea}	0.1043^{Ea}	0.102^{Ea}	0.0897^{Db}	<0.001	19.88	0.0007
Protease	**20**	0.1877^{Da}	0.1866^{Da}	0.186^{Da}	0.1693^{Cb}	<0.001	28.09	0.0019
	25	0.2859^{Ca}	0.2863^{Ca}	0.285^{Ca}	0.2132^{Bb}	<0.001	410.4	0.0072
	30	0.5717^{Aa}	0.5637^{Aa}	0.567^{Aa}	0.4816^{Ab}	<0.001	72.61	0.0088
	35	0.5466^{Ba}	0.5420^{Ba}	0.546^{Ba}	0.4765^{Ab}	<0.001	779.3	0.0068
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	22099.9	54885.5	32329.9	2242.59			

Results were analyzed by using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (Enzymes activity: U/mg) with different subscripts in same rows are significantly different (P< 0.05) and show comparison between groups, however superscript letters in same column shows age wise difference within group. CF4= PLs reared on 4% crude fats feed, CF8= PLs reared on 8% crude fats feed, CF12= PLs reared on 12% crude fats feed, CF16= PLs reared on 16% crude fats feed.

While the ontogeny trend was quite unique; an initial high amylase activity followed a decline followed by a steep surge on 20DAH and again decrease after 25DAH in all the experimental

groups. ALP activity, however remained significantly different among the groups; CF8>CF12>CF16>CF4. Finally, a uniform ontogeny trend of ALP activity was noted.

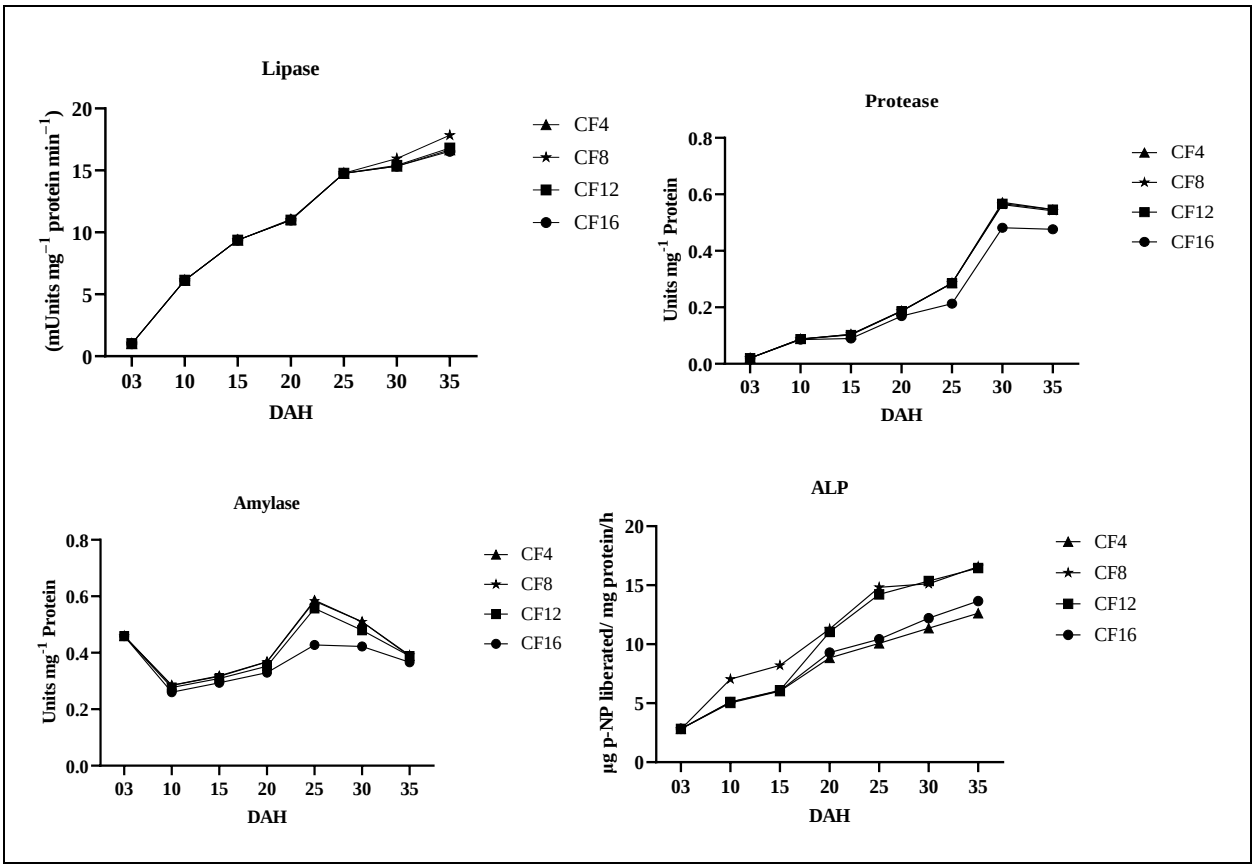

Figure 23. Pattern of development of protease and lipase in *L. rohita* PLs during rearing on Nano-particulate diets having varying levels of crude fats.

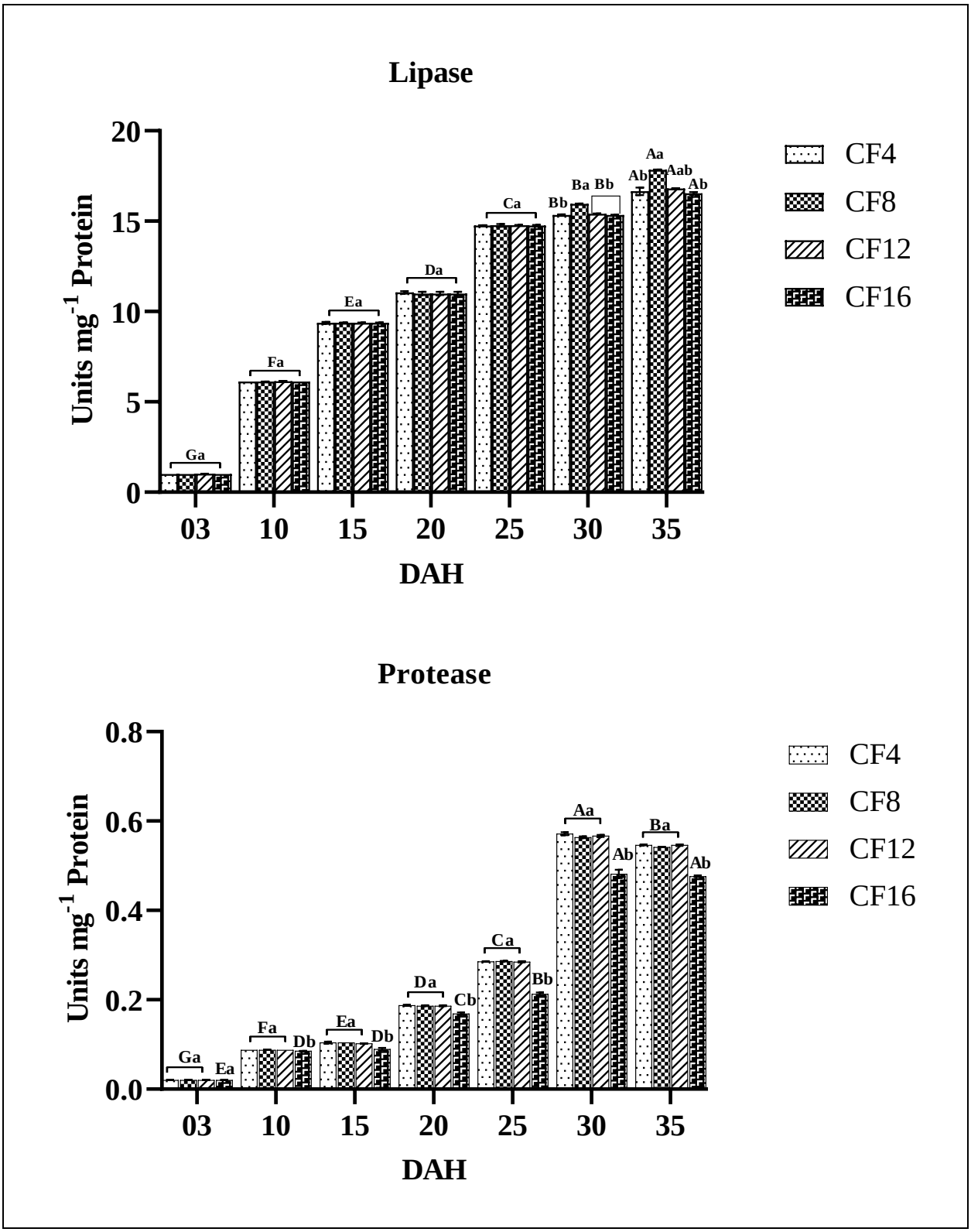

Figure 24. Bar graph showing pattern of development of protease and lipase in *L. rohita* PLs during rearing on nano-particulate diets having varying levels of crude fats.

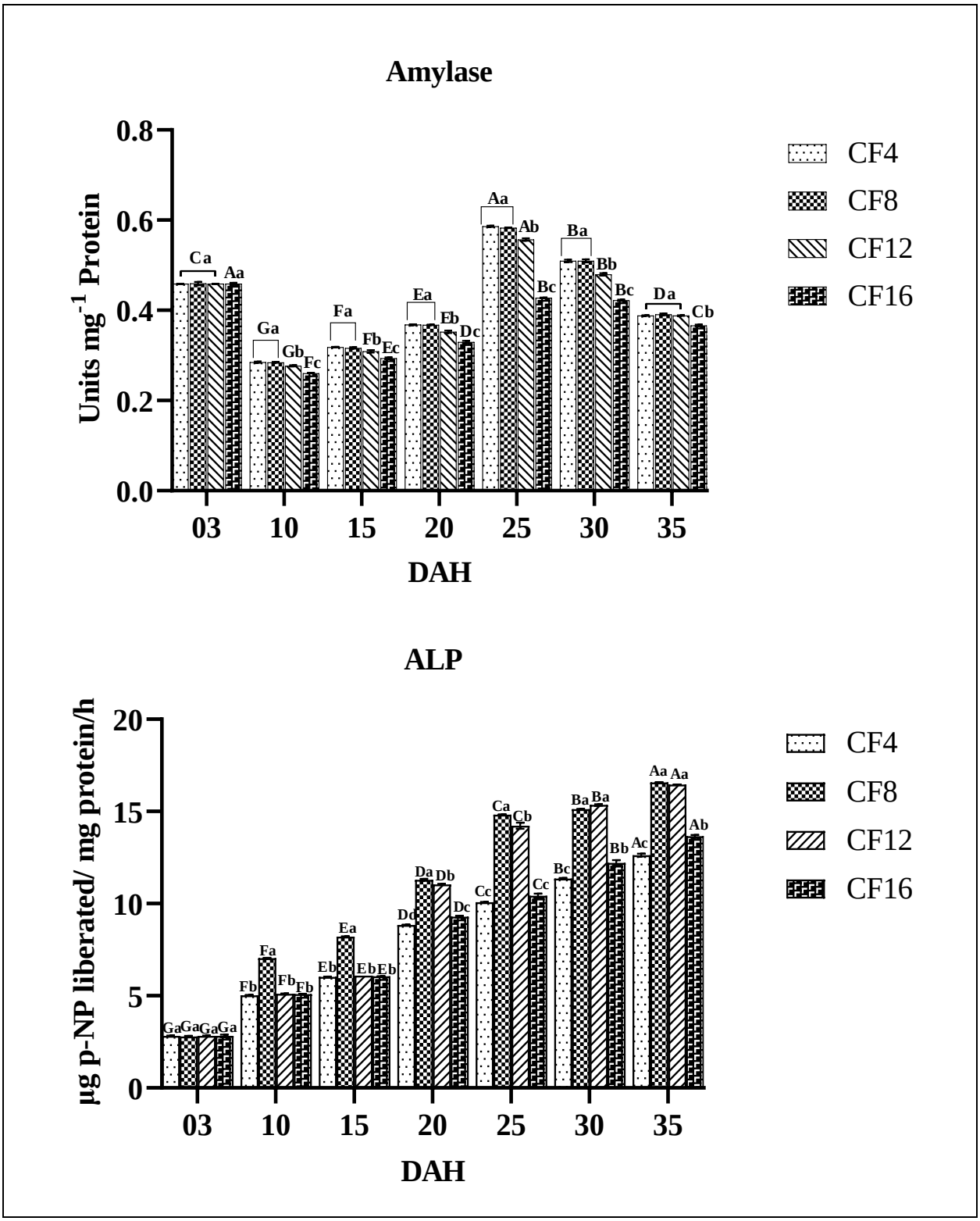

Figure 25. Bar graph show pattern of development of amylase and ALP in *L. rohita* PLs during rearing on nano-particulate diets having varying levels of crude fats.

Expression of genes regulating myogenesis

CF level in the larval feed also govern muscles formation and growth of muscles by maneuvering the expression of genes responsible for muscles differentiation and organogenesis MyoD and growth of myotube GH (Table 26 & 27) however, not affecting MYF5, significantly. Two-way ANOVA revealed a significant effect of %CF level in larval feed on the expression of MyOD ($F_{[3,112]}$=489.3, *P<0.001*, η_p^2=0.929 ; Fig.), and GH ($F_{[3,112]}$=3356, *P<0.001*, η_p^2=0.990; Figure 26)

however no significant effect on MYF5 ($F_{[3,112]}$=1.296, *P=0.279*, η_p^2=0.034) and accounted only for 0.0075% of the total variance. Unlike feed, age DAH showed a significant effect on the expression of all studied genes; MYF5 ($F_{[6,112]}$=8579, *P<0.001*, η_p^2=0.998), MyOD ($F_{[6,112]}$=2585, *P<0.001*, η_p^2= 0.993), and GH ($F_{[6,112]}$=11360, *P<0.001*, η_p^2=0.998). Never the less, the most significant interaction (*P<0.001*) between CF% × age DAH on genes expression, MyOD ($F_{[18,112]}$=48.23, *P<0.001*, η_p^2= 0.886), and GH ($F_{[18,112]}$=169.6, *P<0.001*, η_p^2=0.965) indicated how both variables interact and affect muscles growth, although non-significant interaction was evident in case of MYF5 ($F_{[18,112]}$=1.826, *P=0.303*, η_p^2=0.227).

The partial eta-squared (η_p^2) values indicated the large and almost similar effect size of dietary fats inclusion level and age DAH on the expression of GH and MyoD, while medium effect of dietary CF inclusion level and larger effect size of age DAH on MYF5. Moreover, the η_p^2 values significant interaction between dietary CF inclusion level and age DAH indicated large effect size on GH gene expression while medium on MyoD and lower on MYF5 genes expression.

Interestingly, all pairwise comparisons indicated no significant CF% on MYF5 expression at any sampling point (Figure 31 & 32). However, significantly high expression of MyoD and GH was observed on 35DAH in CF8 followed by CF12. Further MyoD expression was least in CF16 while GH expression was least in CF4. Another important observation is the trend of expression during ontogeny: MYF5 and MyoD decreases after an initial high expression while a reverse trend of GH expression till 35 DAH.

Table 26. Relative expression of MyOD, MYF5 and GH in *L. rohita* PLs during rearing on nano-particulate diets having varying levels of crude fats.

	Age (DAH)	CF4	CF8	CF12	CF16	P-value	F-value	SEM
	03	1.124^{Aa}	1.124^{Aa}	1.125^{Aa}	1.125^{Aa}	0.915	0.171	0.0008
	10	1.035^{Ba}	1.03^{Ba}	1.04^{Ba}	1.035^{Ba}	0.971	0.078	0.0007
	15	1.03^{Ba}	1.014^{Ca}	1.014^{Ca}	1.016^{Ca}	0.122	2.246	0.0028
MYF5	**20**	0.93^{Ca}	0.93^{Da}	0.931^{Da}	0.93^{Da}	0.221	1.636	0.0011
	25	0.88^{Da}	0.89^{Ea}	0.89^{Ea}	0.897^{Ea}	0.118	2.281	0.0019
	30	0.825^{Ea}	0.82^{Fa}	0.82^{Fa}	0.82^{Fa}	0.751	0.406	0.0008
	35	0.74^{Fa}	0.74^{Ga}	0.746^{Ga}	0.745^{Ga}	0.773	0.374	0.0009
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	764.366	5200.27	4791.73	7852.92			
	03	1.403^{Aa}	1.405^{Aa}	1.402^{Aa}	1.401^{Aa}	0.145	2.063	0.0007
	10	1.206^{Bc}	1.402^{Ba}	1.296^{Bb}	1.21^{Bc}	<0.001	3990.1	0.0183
	15	1.02^{Cc}	1.20^{Ca}	1.124^{Cb}	1.20^{Ba}	<0.001	44.635	0.1791
MyOD	**20**	0.902^{Dc}	1.15^{Ca}	1.08^{Db}	0.927^{Cc}	<0.001	112.99	0.0245
	25	0.90^{Dbc}	1.04^{Da}	0.91^{Eb}	0.87^{Dc}	<0.001	118.84	0.0155
	30	0.895^{Db}	1.043^{Da}	0.89^{Eb}	0.77^{Ec}	<0.001	82.249	0.0226
	35	0.791^{Ec}	0.898^{Ea}	0.845^{Fb}	0.698^{Fd}	<0.001	876.51	0.0176
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	545.99	341.935	4153.13	1086.0			
	03	0.493^{Ga}	0.493^{Ga}	0.492^{Fa}	0.494^{Fa}	0.568	0.697	0.0006
	10	0.55^{Fc}	0.81^{Fa}	0.666^{Eb}	0.60^{Ec}	<0.001	85.336	0.0227
	15	0.70^{Ed}	1.03^{Ea}	0.907^{Db}	0.747^{Dc}	<0.001	254.76	0.0301
GH	**20**	0.90^{Dc}	1.216^{Da}	1.204^{Ca}	1.102^{Cb}	<0.001	1694.2	0.0289
	25	1.036^{Cd}	1.484^{Ca}	1.297^{Bb}	1.106^{Cc}	<0.001	11614	0.0401
	30	1.122^{Bc}	1.56^{Ba}	1.312^{Bb}	1.134^{Bc}	<0.001	2138.5	0.0409
	35	1.21^{Ad}	1.622^{Aa}	1.516^{Ab}	1.255^{Ac}	<0.001	7485.3	0.0396
	P-value	<0.001	<0.001	<0.001	<0.001			
	F-value	4657.2	1589.6	3096.3	9718.01			

Two-way ANOVA for overall statistical analysis of two independent variables *Feed type* and *lipids inclusion level* and dependent variable *MYF5/MyoD/GH* expression followed by One-way ANOVA for intergroup and intra group comparison where n=5. LSD post hoc test shows a pairwise comparison between and within groups. Means (gene expression relative to β-Actin) with different lowercase superscripts in same rows are significantly different at $p < 0.05$ and show comparison between groups, however uppercase superscript letters in same column shows age wise difference within group. CF4: PLs reared on feed with 4% crude fats, CF8: PLs reared on feed with 8% crude fats, CF12: PLs reared on feed with 12% crude fats, CF16: PLs reared on feed with 16% crude fats.

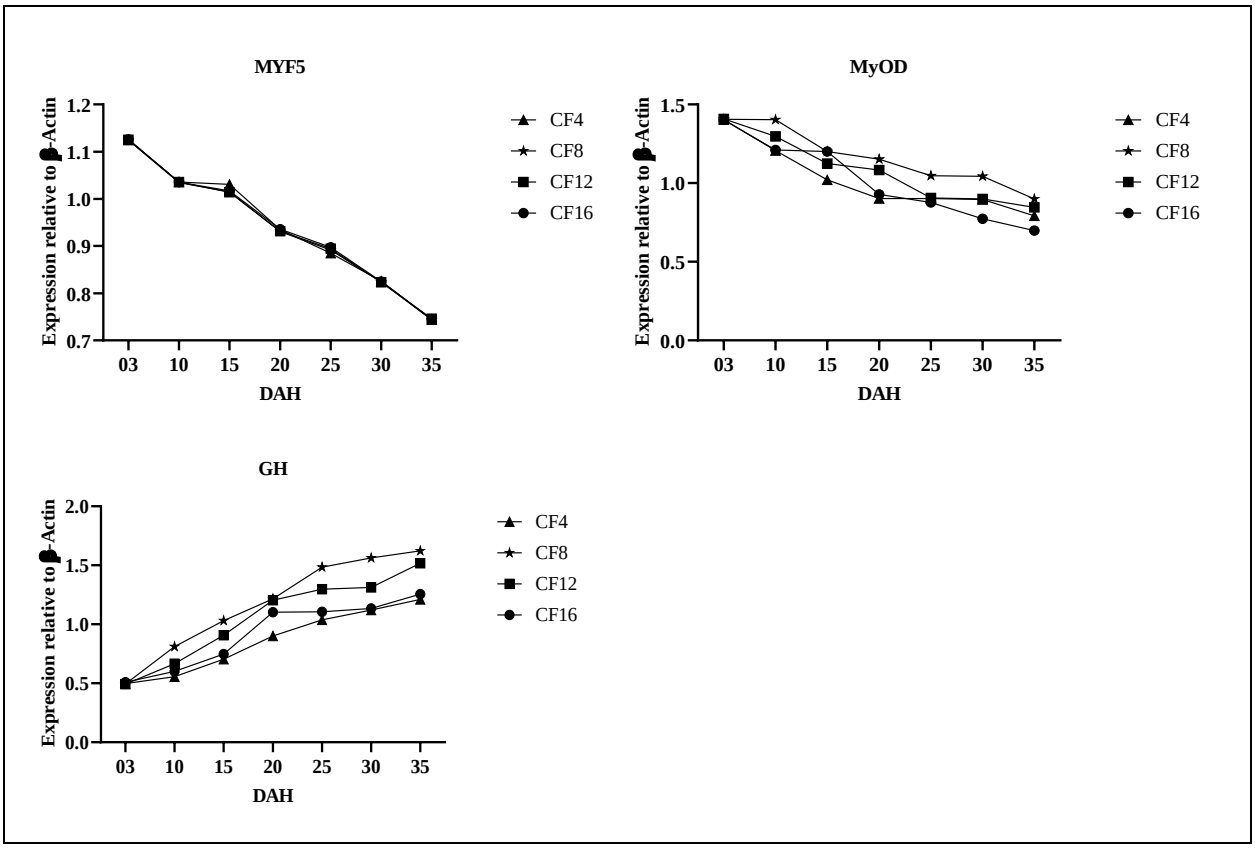

Figure 26. Pattern of expression of MyoD, MYF5 and GH in *L. rohita* PLs during rearing on nano-particulate diets having varying levels of crude fats.

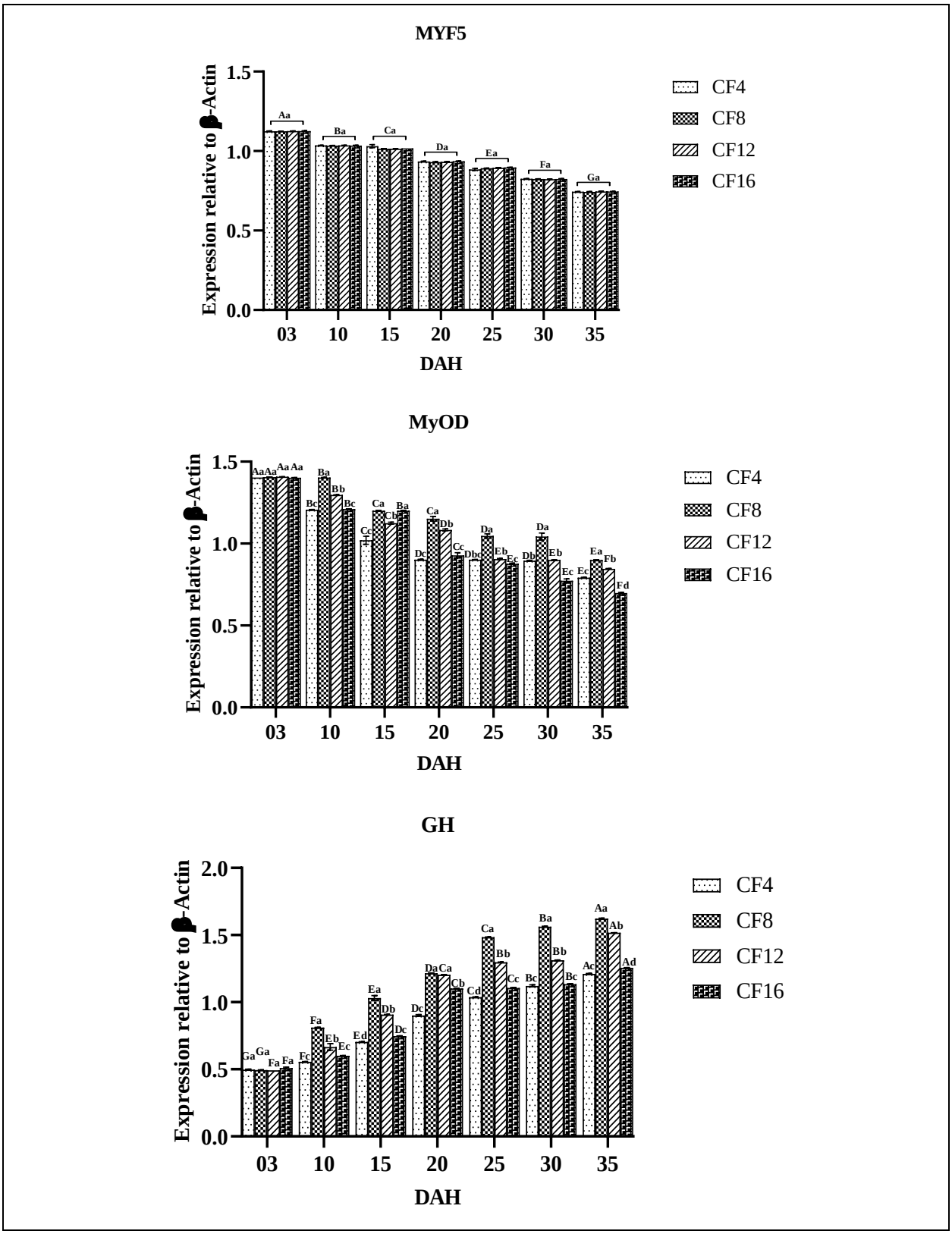

Figure 27. Trend of expression of MYF5, MyoD and GH while rearing on diets with graded level of crude fats, during early ontogeny of *L. rohita* PLs.

Table 27. Two-way ANOVA values of the studied genes, enzymes and growth of *L. rohita* PLs reared on graded level of crude fat for 35 days.

Dependent factors	Independent Factors	DF	F-Value	P-Value	% of total variance
Lipase	Lipids level	3	35.89	<0.001	0.0437
	Age (DAH)	6	75182	<0.001	99.76
	Lipids level × Age (DAH)	18	3.247	<0.001	0.129
ALP	Lipids level	3	3567	<0.001	6.583
	Age (DAH)	6	24389	<0.001	90.31
	Lipids level × Age (DAH)	18	301.8	<0.001	3.341
Amylase	Lipids level	3	1392	<0.001	4.93
	Age (DAH)	6	10605	<0.001	89.95
	Lipids level × Age (DAH)	18	194.9	<0.001	4.96
Protease	Lipids level	3	753.9	<0.001	0.6634
	Age (DAH)	6	33479	<0.001	98.73
	Lipids level × Age (DAH)	18	63.14	<0.001	0.5586
MyOD	Lipids level	3	489.3	<0.001	8.174
	Age (DAH)	6	2585	<0.001	86.37
	Lipids level × Age (DAH)	18	48.23	<0.001	4.834
MYF5	Lipids level	3	1.296	0.279	0.007532
	Age (DAH)	6	8579	<0.001	99.71
	Lipids level × Age (DAH)	18	1.826	0.303	0.06366
GH	Lipids level	3	3356	<0.001	12.37
	Age (DAH)	6	11360	<0.001	83.74
	Lipids level × Age (DAH)	18	169.6	<0.001	3.751
Growth	Lipids level	3	897.8	<0.001	2.279
	Age (DAH)	6	18650	<0.001	94.69
	Lipids level × Age (DAH)	18	192.6	<0.001	2.933
SGR	Lipids level	3	16.42	<0.001	0.354
	Age (DAH)	5	22978	<0.001	96.318
	Lipids level × Age (DAH)	15	28.13	<0.001	2.415

Experiment 5

Experimental feed

Live feed in the month that coincide the PLs rearing season of *L. rohita* was dominated by diatoms and Cladocerans mentioned in detail in experiment 1. Species composition of phytoplankton and zooplankton along with their proximate composition has already been mentioned in experiment 1, however the most significant thing observed was the biochemical/fatty acid composition of these zooplankton species during these coinciding days. Table 28 shows the FA composition of SNF and live feed collected during the experimental days periodically. It was evident from the assessment Table (28) that overall, SAFA and PUFA increased while MUFA decreased during the study period, suggesting variation in FA profile of live feed.

Table 28. Fatty acid analysis of the feed given during the 35days feeding trial.

FA type	SNF	LF (DAH)							<LF>
		03	10	15	20	25	30	35	
∑SAFA	33±0.75^{e}	55±0.91^{ab}	57±0.50^{a}	55±0.33^{ab}	49±0.68^{d}	51±0.4^{cd}	55±0.74^{ab}	56±0.75^{a}	53±0.85^{bc}
∑MUFA	30±0.67^{a}	23±0.3^{bc}	18±0.78^{de}	16±0.95^{e}	18±0.21^{e}	16±0.9^{e}	24±0.91^{b}	23±0.24^{bc}	20±0.95^{cd}
∑n-6PUFA	12±0.54^{a}	7±0.01^{c}	9±0.21^{b}	9±0.38^{b}	12±0.24^{a}	10±0.11^{b}	7±0.41^{c}	7±0.55^{c}	9±0.81^{b}
∑n-3PUFA	23±0.25^{a}	13±0.64^{e}	14±0.45^{e}	17±0.11^{d}	18±0.65^{c}	20±0.02^{b}	12±0.66^{f}	11±0.62^{g}	16±0.75^{d}
∑PUFA	35±0.28^{a}	20±0.69^{e}	24±0.23^{d}	26±0.53^{c}	31±0.57^{b}	31±0.12^{b}	20±0.66^{e}	20±0.13^{e}	26±0.34^{c}
n-3/n-6	1±0.85^{a}	1±0.76^{b}	1±0.64^{c}	1±0.64^{c}	1±0.452^{d}	1±0.856^{a}	1±0.73^{b}	1±0.56^{c}	1±0.73^{b}
∑EPA	8±0.61^{a}	3±0.21^{e}	4±0.11^{d}	4±0.51^{c}	5±0.21^{bc}	5±0.41^{b}	2±0.21^{f}	2±0.22^{f}	4±0.435^{c}
∑DHA	12±0.43^{a}	6±0.01^{f}	7±0.31^{d}	8±0.01^{c}	9±0.01^{b}	9±0.02^{b}	7±0.01^{e}	6±0.01^{f}	8±0.11^{c}

One-way ANOVA was conducted, each value represent percent presence. superscripts describe significant variation in a row; where SAFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: poly unsaturated fatty acid, EPA: Eicosapentaenoic acid methyl ester, DHA: Docosahexaenoic acid methyl ester.

SNF was formulated (Table 31) after evaluating protein and fats level in larval diet of *L. rohita* mentioned above. The particle nature was observed as in Nano-form with a crystalline property, analyzing XRD pattern and SEM results. The same formulation and preparation technique ensured the consistent FA profile of SNF throughout the feeding trial.

Proximate and biochemical composition of feed

The FA composition of both feeds suggest two important observations regarding the hypothesis of this study. First, MUFA and PUFA level in the SNF was significantly higher than the live feed in all samples analyzed, at each sampling point of the study. Secondly, it was also noted that there occurs significant alteration in FA profile of live feed. SAFA was significantly higher in live feed during the initial days that coincide rohu larva hatching.

Table 29. biochemical composition experimental feed.

	SNF	3DAH (LF)	10DAH (LF)	15 DAH (LF)	20DAH (LF)	25DAH (LF)	30DAH (LF)	35DAH (LF)
SAFA								
C12:0 Lauric acid	0.19	2.19	2.09	1.99	1.19	1.29	2.15	2.2
C13:0 tridecanoic acid	0.09	3.09	2.49	2.59	2.09	2.66	3.29	3.39
C14:0 Myristic acid	6.42	5.2	4.22	3.32	3.2	4.12	4.92	5.12
C15:0 Pentadecanoic acid	1.37	7.37	7.97	7.99	7.37	7.09	7.37	7.67
C16:0 Palmitoleic acid methyl ester	16.44	16.44	19.07	19.01	16.04	17.37	16.4	16.44
C17:0 Margaric acid methyl ester	0	12.91	13.15	12.01	12.01	11.41	12.11	12.91
C18:0 Stearic acid	8.81	8.81	8.01	8.62	8.21	7.91	8.71	8.79
C20:0Arachidic acid	0.4	0.4	0.37	0.47	0.28	0.71	0.33	0.4
MUFA								
C14:1c Myristoleic acid	0	3.12	2.92	3.02	3.12	2.12	3.92	3.12
C16:1c Palmitic acid methyl ester	12.47	6.01	4.51	4.01	6.11	5.01	6.81	6.01
C17:1 Heptadecanoic acid	1.41	2.32	2.12	2.72	2.12	1.32	2.72	2.32
C18:1c Oleic acid methyl ester	12.9	3.22	2.02	1.22	1.22	2.23	3.47	3.22
C18:1n9T Elaidic acid methyl ester	1.01	3	3	1.7	1.21	2.01	2.78	3.88
C20:1 Eicosanoic acid methyl ester	2.98	5.03	4.03	4.83	4.73	4.03	5.03	5.15
PUFA								
18:2n−6 Linoleic acid methyl ester	11.32	4.22	4.42	4.92	4.87	5.02	3.02	3.12
18:3n−6 Lineolic acid	0.01	2.32	2.32	2.39	2.98	2.32	2.02	2.3
20:2n−6 Eicosadienoic acid	0.04	0.44	1.04	0.99	0.44	0.46	0.44	0.24
20:3n−6-Eicosasatrienoic acid	0.06	0.03	0.03	0.98	2.67	1.53	0.73	0.33
20:4n−6 Arachidonic acid	0.88	0.54	1.3	0.54	1.54	1.54	1.04	1.54
18:3n−3 Linolenic acid	2	1.1	1.01	1.1	1.1	1.01	0.9	1.51
20:3n−3 Eicosatrienoic acid	0.16	1.7	1.17	1.74	1.9	2.7	1.3	1.1
20:5n−3 Eicosapentaenoic acid	8.61	3.21	4.11	4.51	5.21	5.41	2.21	2.21
22:5n−3 Docosapentaenoic acid	0	1.32	1.32	1.32	1.38	1.72	1.32	1.02
22:6n−3Docosahexaenoic acid	12.43	6.01	7.31	8.01	9.01	9.01	7.01	6.01

Each value represents % presence, where an average standard deviation of 0±0.07 to 0±0.38 for FA.

Fatty acid composition of rohu PLs

The comparative effect of live feed and SNF was significantly evident in the proximate and FA composition of the Rohu PL/fries body (Table 30 and 31). Table 31 clearly states a significant effect of LF and SNF on the proximate composition of rohu larvae. A significant decrease in moisture content was observed in contrast to the increasing trend of percent dry matter (DM) in both groups, however DM was significantly higher in SNF compared to LF through all the sampling points. Sampe pattern was observed for crude protein (%) and crude fats (%). The ash content was not significantly altered in both groups. Multiple comparison shown a significant

increase in SAFA in both experimental groups however, at each sampling point SAFA level was significantly higher in LF compared to SNF. Further, a relatively random trend was observed in MUFA % of rohu larvae, while at each sampling point the MUFA% was observed significantly higher in LF group compared to SNF however on 35DAH a reverse trend was observed i.e. significantly higher MUFA in LF.

Table 30. Changes in the fatty acid profile of *L. rohita* PLs while rearing on live feed and SNF during early ontogeny.

FA type	10 DAH		20 DAH		30DAH		35 DAH	
	LF	SNF	LF	SNF	LF	SNF	LF	SNF
∑SAFA	51±0.38^{f}	50±0.17^{g}	55±0.01^{de}	53±0.68^{e}	56±0.59^{c}	55±0.56^{d}	59±0.47^{a}	59±0.17^{b}
∑MUFA	15±0.38^{b}	12±0.32^{e}	14±0.19^{c}	13±0.28^{d}	14±0.21^{c}	13±0.26^{d}	15±0.52^{b}	15±0.84^{a}
∑n-6 PUFA	8±0.54^{cd}	10±0.76^{a}	8±0.35^{d}	9±0.08^{b}	8±0.67^{c}	8±0.87^{bc}	7±0.85^{f}	8±0.07^{e}
∑n-3 PUFA	24±0.69^{b}	26±0.75^{a}	22±0.42^{d}	23±0.25^{c}	19±0.79^{f}	21±0.5^{e}	16±0.22^{g}	16±0.91^{g}
∑PUFA	33±0.23^{b}	37±0.51^{a}	30±0.77^{d}	32±0.33^{c}	28±0.46^{e}	30±0.37^{d}	24±0.07^{f}	24±0.98^{f}
n3/n6	2±0.891^{a}	2±0.49^{d}	2±0.69^{b}	2±0.56^{c}	2±0.82^{a}	2±0.42^{de}	2±0.06^{e}	2±0.09^{e}
EPA	8±0.01^{c}	9±0.02^{a}	7±0.09^{e}	8±0.43^{b}	6±0.33^{f}	7±0.44^{d}	5±0.74^{g}	5±0.82^{g}
DHA	12±0.3^{ab}	13±011^{a}	11±0.08^{b}	11±0.07^{b}	10±0.44^{c}	10±0.07^{d}	8±0.03^{f}	8±0.55^{e}

One-way ANOVA was conducted, each value represent percent presence. superscripts describe significant variation in a row; where SAFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: poly unsaturated fatty acid, EPA: Eicosapentaenoic acid methyl ester, DHA: Docosahexaenoic acid methyl ester.

Table 31. Changes in the proximate composition and fatty acid profile of *L. rohita* PLs while rearing on live feed and SNF, during early ontogeny.

	10 DAH		20 DAH		30 DAH		35DAH	
	LF	SNF	LF	SNF	LF	SNF	LF	SNF
DM	9.8	9.8	15.9	16.8	21.2	22.7	23.45	25.85
CP	4.61	4.66	10.02	10.89	14.78	16.1	16.33	18.57
Fat	3.81	3.83	4.17	4.29	4.46	4.68	4.79	4.93
Ash	1.3	1.3	1.6	1.6	1.9	1.9	2.3	2.3
Moisture	90.1	90.2	84.01	83.02	78.78	76.99	76.5	73.95
Fatty acid								
SAFA								
C12:0 Lauric acid methyl ester	2.33	2.43	2.34	2.57	1.07	1.56	1.01	1.09
C13:0 tridecanoic acid methyl ester	1.11	1.32	1.91	1.87	2.01	1.76	2.09	1.98
C14:0 Myristic acid methyl ester	2.57	2.34	2.73	2.47	2.99	3.01	3.11	2.99
C15:0 Pentadecanoic acid	1.02	1.11	2.01	1.98	2.04	2.12	2.17	2.28
C16:0 Palmitoleic acid methyl ester	27.11	26.01	27.01	26.12	28.03	27.45	28.33	28.55
C17:0 Margaric acid methyl ester	0.12	0.11	0.77	0.68	1.01	1.09	1.87	1.76
C18:0 Stearic acid	17.01	16.4	17.22	17.01	18.12	17.34	19.33	18.44
C20:0Arachidic acid	0.11	0.43	1.02	0.98	1.32	1.22	1.56	2.08
MUFA								
C14:1c Myristoleic acid	0.56	0.61	0.52	0.54	0.62	0.61	1.21	1.73
C16:1c Palmitic acid methyl ester	0.53	0.62	0.23	0.27	1.14	0.18	0.54	0.61
C17:1 Heptadecanoic acid	1.87	0.99	1.93	1.94	1.99	1.97	2.76	2.64
C18:1c Oleic acid methyl ester	8.76	6.44	7.65	6.75	6.98	6.92	6.87	6.96
C18:1n9T Elaidic acid methyl ester	2.43	2.33	2.08	2.11	2.05	2.04	3.02	2.77
C20:1 Eicosanoic acid methyl ester	1.23	1.33	1.78	1.67	1.43	1.54	1.12	1.13
PUFA								
18:2n−6 Linoleic acid methyl ester	0.12	0.94	0.22	0.54	0.33	0.38	0.21	0.27
18:3n−6 Lineolic acid	1.11	1.21	0.98	1.09	1.33	1.32	1.12	1.17
20:2n−6 Eicosadienoic acid	2.23	2.54	1.97	2.04	1.88	1.92	1.66	1.72
20:3n−6-Eicosasatrienoic acid	1.31	1.37	1.09	1.07	1.09	1.12	0.89	0.92
20:4n−6 Arachidonic acid	3.77	4.7	4.09	4.34	4.04	4.13	3.97	3.99
18:3n−3 Linolenic acid	2.01	1.09	2.09	1.11	1.04	1.02	0.88	0.87
20:3n−3 Eicosatrienoic acid	1.23	2.32	1.07	1.42	1.01	1.99	0.94	0.96
20:5n−3 Eicosapentaenoic acid	8.01	9.02	7.09	8.43	6.33	7.44	5.74	5.82
22:5n−3 Docosapentaenoic acid	1.13	1.32	1.09	1.22	0.97	0.98	0.63	0.71
22:6n−3Docosahexaenoic acid	12.31	13	11.08	11.07	10.44	10.07	8.03	8.55

Each value represents % presence, where an average standard deviation for proximate composition was (0±0.11 to 0±0.65) and (0±0.07 to 0±0.3) for FA.

PUFA on the other hand, remained significantly higher in SNF group (compared to LF group) till the end of the trial. In addition, the trend of PUFA remains similar for both n3-PUFA and n6-PUFA.

Table 32. Growth performance of *L. rohita* PLs reared on live feed and SNF for 35 days LF.

Growth indices	Age (DAH)	Experimental Feeding Groups				
		LF	SNF	P-value	F-value	SEM
	03	0.0004	0.0004	0.777	0.086	0.0001
	10	0.11194	0.1772	<0.001	29.928	0.0041
	15	0.1736	0.25182	<0.001	203.09	0.0066
NWG	**20**	0.2416	0.40838	0.002	19.354	0.0036
	25	0.4512	0.84002	<0.001	190.44	0.0059
	30	0.6778	1.07624	<0.001	515.91	0.0158
	35	0.867	1.4761	<0.001	70.520	0.0314
	P-value	<0.001	<0.001			
	F-value	12244.4	2097.1			
	03	-	-	-	-	-
	10	6.986376	7.560633	<0.001	17.65	0.036
	15	7.535506	7.996559	<0.001	62.38	0.043
SGR	**20**	7.931918	8.596265	<0.001	99.66	0.020
	25	8.72088	9.491998	<0.001	320.9	0.013
	30	9.22583	9.799569	<0.001	1311	0.043
	35	9.531312	10.19102	<0.001	228.0	0.099
	P-value	<0.001	<0.001			
	F-value	7.094	6.464			

Results were analyzed via using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pair wise inter and intra group comparisons (n=5). Means (PLs weight values in g) LF= PLs reared on live feed, and SNF= PLs reared on Standardized Nano-particulate prepared feed.

Growth performance and survival

Growth regulation of L. rohita during early ontogeny while rearing the PLs on LF and replacing LF with nano particulate prepared feed has been shown in experiment 2 results. Prepared feed after standardization (SNF) for their lipid and protein levels as per *L. rohita* PLs requirements, the feed was supplemented to rohu PLs for 35 days after hatching. Formulation of SNF has been mentioned in table 31, and nano particulate confirmation/characterization in Table 15. Two way ANOVA revealed significant impact of feed type and age/DAH on rohu PLs (NWG, $F_{[1,56]}$=1653, *P<0.001*, η_p^2 =0.983; SGR, $F_{[1,40]}$=1029, *P<0.001*, η_p^2 =0.953) and (NWG, $F_{[6,56]}$=61032, *P<0.001*, η_p^2 =0.998; SGR, $F_{[5,40]}$=1759, *P<0.001*, η_p^2 =0.995). in addition these factors have significantly interacted to induce growth (NWG, $F_{[6,56]}$=242.3, *P<0.001*, η_p^2 =0.997; SGR, $F_{[5,40]}$=4.685, *P<0.001*, η_p^2 =0.330). The partial eta-squared (η_p^2) values of feed type and age DAH showed a similar and large effect on dependent variables, NWG and SGR while their interaction indicated a larger effect on NWG and a medium effect on SGR.

Multiple comparison analysis revealed a significantly high Growth (NWG &SGR) in SNF compared to LF at all the sampling points, while the survival% was also significantly higher (81.5%) in SNF compared to LF (63%) (Figure 28).

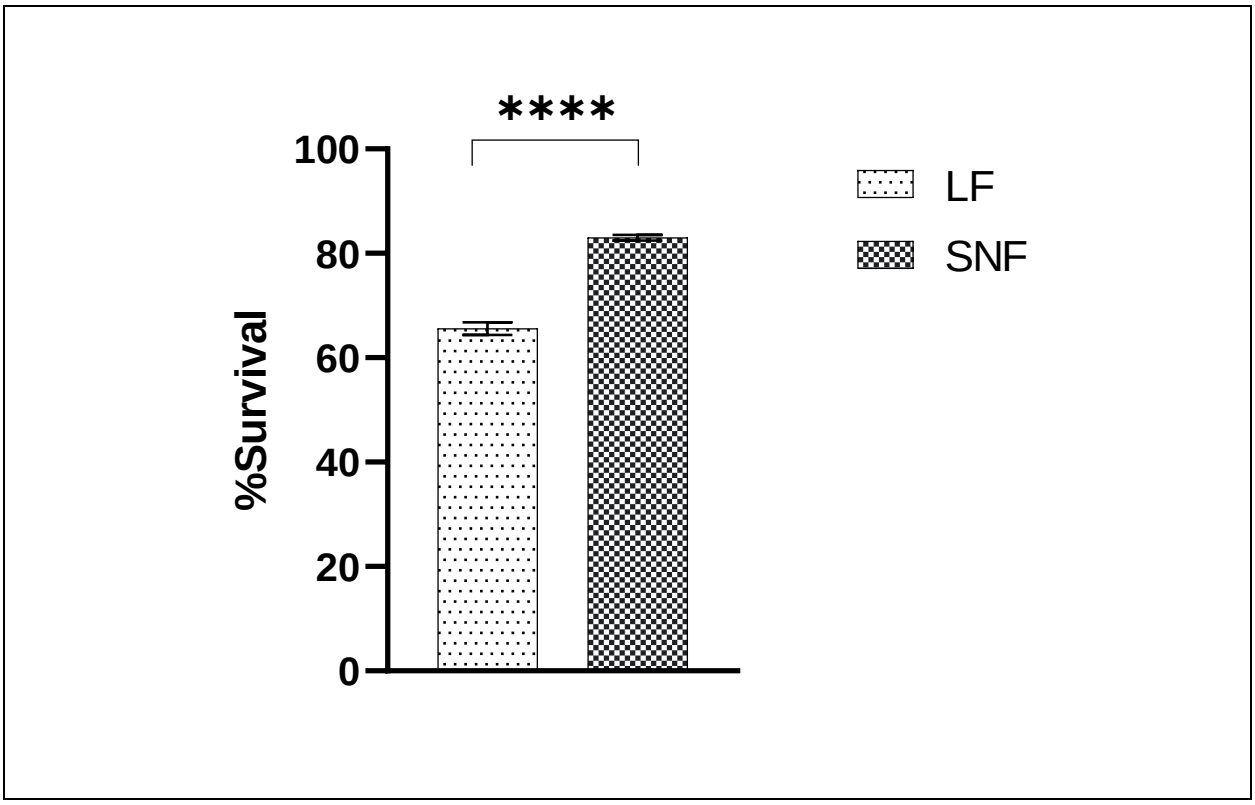

Figure 28. Survival rate of *L. rohita* PLs while rearing on LF and SNF for 35DAH.

Intestinal enzyme activity

Enzymatic activity regulated by live feed and nano particulate compound feed has been described in the 2nd experimental trial. However, after conducting the other two experiments for standardization of lipid and protein level in the larval diet of *L. rohita,* it was evident that digestive enzymes were significantly affected. Post standardization of the final formulated feed with best results in terms of growth and survival, SNF was observed to significantly enhance the release of guts enzymes.

Table 33. Enzymatic activity of the experimental groups fed on LF and SNF.

Enzyme Activity	Age (DAH)	Experimental groups				
		LF	SNF	P-value	F-value	SEM
	03	0.020^{G}	0.020^{G}	0.765	0.095	0.0003
	10	0.079^{F}	0.081^{F}	0.017	9.033	0.0005
	15	0.107^{E}	0.118^{E}	0.034	6.512	0.0025
Protease	**20**	0.173^{D}	0.202^{D}	<0.001	79.904	0.0048
	25	0.237^{C}	0.273^{C}	<0.001	117.299	0.0061
	30	0.304^{B}	0.422^{B}	<0.001	1201.57	0.0197
	35	0.348^{A}	0.392^{A}	<0.001	202.116	0.0074
	P-value	<0.001	<0.001			
	F-value	3424.186	5969.88			
	03	1.028^{G}	1.0432^{G}	0.472	0.570	0.0097
	10	6.146^{F}	6.118^{F}	0.610	0.282	0.0253
	15	9.354^{E}	9.322^{E}	0.670	0.196	0.0344
Lipase	**20**	11.058^{D}	10.99^{D}	0.617	0.271	0.0552
	25	14.71^{C}	14.77^{C}	0.452	0.625	0.0372
	30	15.284^{B}	15.33^{B}	0.564	0.363	0.0368
	35	16.562^{A}	16.544^{A}	0.877	0.026	0.0532
	P-value	<0.001	<0.001			
	F-value	9538.068	9550.75			
	03	0.485^{B}	0.485^{G}	0.481	0.574	0.0049
	10	0.29^{F}	0.280^{F}	0.029	7.033	0.0025
	15	0.313^{E}	0.332^{E}	<0.001	122.70	0.0034
Amylase	**20**	0.364^{D}	0.425^{D}	<0.001	129.133	0.0104
	25	0.58^{A}	0.653^{C}	<0.001	98.553	0.0126
	30	0.473^{B}	0.55^{B}	<0.001	1883.7	0.0137
	35	0.389^{C}	0.4684^{A}	<0.001	299.74	0.0133
	P-value	<0.001	<0.001			
	F-value	1858.81	874,396			
	03	-	-	-	-	-
	10	0.099^{F}	0.080^{F}	0.711	0.307	0.0007
	15	0.145^{E}	0.133^{E}	0.092	0.734	0.0056
Cellulase	**20**	0.217^{D}	0.216^{D}	<0.001	116.08	0.0071
	25	0.267^{C}	0.266^{C}	<0.001	301.79	0.0091
	30	0.309^{B}	0.309^{B}	<0.001	145.779	0.0090
	35	0.353^{A}	0.352^{A}	<0.001	404.75	0.0143
	P-value	<0.001	<0.001			
	F-value	2726.926	1827.690			

Results were analyzed by using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (Enzymes activity: U mg -1) LF= PLs reared on live feed, and SNF= PLs reared on standardized Nano-particulate prepared feed.

Table 34. Comparative effect of live feed and SNF on the ontogeny of development of protein digesting enzymes in the intestines of *L. rohita.*

Enzyme Activty	Age (DAH)	Experimental Groups				
		LF	SNF	P-value	F-value	SEM
	03	12.53^{G}	12.546^{G}	0.703	0.769	0.0001
	10	14.41^{F}	16.116^{F}	=0.001	30.278	0.3193
	15	22.63^{E}	41.678^{E}	<0.001	3951.58	3.1768
Trypsin	**20**	50.488^{D}	67.842^{D}	<0.001	2841.73	2.8964
	25	143.49^{C}	162.836^{C}	<0.001	633.871	3.2432
	30	222.48^{B}	289.738^{B}	<0.001	8977.8	11.2146
	35	310.458^{A}	406.696^{A}	<0.001	9940.56	7.5457
	P-value	<0.001	<0.001			
	F-value	85115.7	178523			
	03	13.998^{G}	13.496^{F}	0.543	0.262	0.0014
	10	27.782^{F}	33.632^{EF}	<0.001	548.104	0.9821
	15	43.222^{E}	54.814^{E}	<0.001	1015.37	1.9396
Chymotrypsin	**20**	212.94^{D}	252.52^{D}	<0.001	1042.99	6.6219
	25	531.42^{C}	692.66^{C}	<0.001	9512.74	26.8846
	30	678.008^{B}	882.96^{B}	<0.001	10397.7	34.1718
	35	1846.67^{A}	34007.3^{A}	<0.001	99675.6	66.1417
	P-value	<0.001	<0.001			
	F-value	23044.82	23384.8			
	03	2.83^{G}	2.83^{G}	0.321	0.0489	0.0012
	10	5.1^{F}	7.034^{F}	<0.001	2908.51	0.3227
	15	6.03^{E}	8.204^{E}	<0.001	5937.53	0.3625
Alp	**20**	9.046^{D}	11.296^{D}	<0.001	1826.29	0.3758
	25	11.028^{C}	14.428^{C}	<0.001	764.15	0.5696
	30	12.352^{B}	15.4^{B}	<0.001	294.71	0.5148
	35	14.632^{A}	16.65^{A}	<0.001	420.52	0.3395
	P-value	<0.001	<0.001			
	F-value	14275.9	3409.72			

Results were analyzed by using two-way ANOVA by taking two independent variables Feed type and Age followed by post hoc pairwise inter and intra group comparisons (n=5). Means (Enzymes activity: U mg -1), LF= PLs reared on live feed, and SNF= PLs reared on Standardized Nano-particulate prepared feed.

Two-way ANOVA revealed significant impact of feed nature on protein digesting enzymes after 35days feeding trial in *L. rohita* PLs, i.e. total protease activity ($F_{[1,56]}$=971, $P<0.001$, η_p^2=0.945), trypsin activity ($F_{[1,56]}$=89787, $P<0.001$, η_p^2=0.996) and chymotrypsin activity($F_{[1,56]}$= 28704, $P<0.001$, η_p^2=0.974). Further, feed type also regulated ALP ($F_{[1,56]}$=3984, $P<0.001$, η_p^2=0.986), amylase ($F_{[1,56]}$=744.8, $P<0.001$, η_p^2=0.930) and cellulase activity ($F_{[1,56]}$=16.46, $P<0.001$, η_p^2=0.946) significantly in the gut of rohu PLs. however, interestingly no significant alteration in lipase activity($F_{[1,56]}$=0.006, P=0.932, η_p^2=0.00) was observed in any experimental group after 35DAH. like feed type, significant change in enzymatic activity was observed with ontogeny/age in both experimental groups (total protease ($F_{[6,56]}$=9104, $P<0.001$, η_p^2=0.999),

trypsin ($F_{[6,56]}$=8325, $P<0.001$, η_p^2=1.00), chymotrypsin ($F_{[6,56]}$= 178215, $P<0.001$, η_p^2=1.00), ALP ($F_{[6,56]}$=10192, $P<0.001$, η_p^2=0.998), amylase ($F_{[6,56]}$=2478, $P<0.001$, η_p^2=0.996), and cellulase activity ($F_{[6,56]}$=6964, $P<0.001$, η_p^2=0.998) while not significant impact on lipase ($F_{[6,56]}$=19089, $P=0.9366$, η_p^2 = 1.00). However, the age*feed-type interaction was also significant in all the studied enzymes (total protease ($F_{[6,56]}$=199.9, $P<0.001$, η_p^2=0.955), trypsin ($F_{[6,56]}$=1468, $P<0.001$, η_p^2=0.996), chymotrypsin ($F_{[6,56]}$=16501, $P<0.001$, η_p^2=0.980), ALP ($F_{[6,56]}$=70.46, $P<0.001$, η_p^2=0.883), amylase ($F_{[6,56]}$=, $P<0.001$, η_p^2=0.865), lipase ($F_{[6,56]}$=0.2953, $P<0.001$, η_p^2 =0.031) and cellulase activity ($F_{[6,56]}$=5.927, $P<0.001$, η_p^2=0.869). The partial eta-squared (η_p^2) values indicated the large and almost similar effect size of both variables (feed type and age DAH) and their interaction on protease, trypsin, chymotrypsin, cellulase, ALP, and amylase. The partial eta-squared (η_p^2) value (0.00) show that Feed type although indicate no significant size effect on lipase activity but Age shows the maximum size effect (η_p^2=1.00) on lipase activity while a medium effect due to feed type × age (DAH) interaction.

Multiple comparison (Table 33) revealed significantly high protease, amylase, cellulase, ALP, trypsin, and chymotrypsin activity in Rohu PLs at 35 DAH, in SNF group compared to LF group. However, no significant change was observed in lipase activity, in any of the experimental groups. An important thing noted in the trial was the pattern of development of these enzymes, which was observed unique and different in all the studied enzymes. Figure 30, 27, 28 and 29 demonstrate the ontogenic pattern of enzymatic activity. Protease activity remained significantly unchanged in both LF and SNF groups till 10DAH, while less significant (Table 33) change from 10 to 15DAH. However, the difference in protease activity became more evident as the PLs development progressed, a significantly high protease activity was observed in SNF compared to LF. The pattern of ontogeny was same in the other protein digesting enzymes, however the notable thing was the point of sudden projection (Figure 30 and 32); uniform increase in protease activity, while a sudden upsurge in trypsin and chymotrypsin activity on 20DAH and 30DAH, respectively.

Amylase activity during the ontogeny remained the same as mentioned in experiment 2 results i.e. a high initial activity followed by a slower decline till 10 DAH and a resurge till 25DAH followed by a relatively less steep decline till 35DAH, in both LF and SNF. Like protease, amylase activity was also not significantly affected in both groups till 35 DAH, followed by a significant increase in SNF followed LF.

Lipase on the other hand increased with ontogeny uniformly, with no significant difference in LF and SNF. Cellulase activity followed a similar uniform increasing trend during the ontogeny, but here the LF has enhanced cellulase activity during the initial 15DAH and interestingly the change remains nonsignificant thereafter. ALP also remained significantly higher in SNF compared to LF at each sampling point while their ontogenic pattern of development was also uniform in both experimental groups.

Expression of genes regulating myogenesis

Post standardization of the prepared feed SNF was studied to determine the growth regulating factors, expression of genes which govern hypertrophy or hyperplasia, directly or indirectly. Two-way ANOVA of the studied independent factors e.g., age DAH and feed nature affecting the expression of the studied genes are stated in table (35 and 36). The present study indicated that feed nature has a significant potential to modulate the expression of MRFs including MYF5 ($F_{[1,56]}$=106.2, $P<0.001$, η_p^2=0.655), MEF2A ($F_{[1,56]}$=820.3, $P<0.001$, η_p^2=0.936), MyoD ($F_{[1,56]}$=322.7, $P<0.001$, η_p^2=0.835) and Myogenin ($F_{[1,56]}$=388, $P<0.001$, η_p^2=0.874). Further, the statistical analysis also reveals a significant effect of feed nature on GH ($F_{[1,56]}$=2266, $P<0.001$, η_p^2=0.976), *IGF-1*($F_{[1,56]}$=1317, $P<0.001$, , η_p^2=0.959) and Myostatin ($F_{[1,56]}$=302.8, $P<0.001$, η_p^2=0.844).

Like feed nature, time DAH also significantly regulates the expression of the studied genes positively i.e. in MEF2A ($F_{[6,56]}$=1677, $P<0.001$, η_p^2=0.994), GH ($F_{[6,56]}$=1981, $P<0.001$, η_p^2=0.995), *IGF-1* ($F_{[6,56]}$=176.675, $P<0.001$, η_p^2=990), myostatin($F_{[6,56]}$=179.6, $P<0.001$, η_p^2=0.951) or negatively i.e. in myf5 ($F_{[6,56]}$=168.5, $P<0.001$, η_p^2=0.948) and MyoD ($F_{[6,56]}$=463, $P<0.001$, η_p^2=0.985), while an irregular pattern of myogenin expression ($F_{[6,56]}$=1084, $P<0.001$, η_p^2=0.991).

Table 35. Relative expression of GH, *IGF-1* and myostatin in *L. rohita* PLs during rearing on LF and SNF.

	Age (DAH)	LF	SNF	P-value	F-value	SEM
	03	0.50^{F}	0.50^{E}	0.774	0.088	0.0045
	10	0.71^{E}	0.80^{D}	<0.001	159.69	0.0164
	15	0.90^{D}	1.03^{C}	<0.001	39.479	0.0226
GH	**20**	0.90^{D}	1.20^{B}	<0.001	1851.8	0.0499
	25	1.03^{C}	1.50^{B}	<0.001	254.29	0.0800
	30	1.11^{B}	1.60^{A}	<0.001	2829.2	0.0814
	35	1.20^{A}	1.60^{A}	<0.001	11477	0.0672
	P-value	<0.001	<0.001			
	F-value	371.66	2736.1			
	03	0.54^{G}	0.51^{G}	0.114	03.152	0.0092
	10	0.81^{F}	0.91^{F}	<0.001	240.17	0.0165
	15	1.01^{E}	1.07^{E}	<0.001	12.298	0.0144
IGF-1	**20**	1.41^{D}	1.59^{D}	<0.001	44.030	0.0325
	25	1.52^{C}	1.94^{C}	<0.001	927.52	0.0709
	30	2.42^{B}	2.91^{B}	<0.001	619.47	0.0825
	35	3.07^{A}	3.78^{A}	<0.001	586.34	0.1184
	P-value	<0.001	<0.001			
	F-value	7822.6	4542.6			
	03	0.90^{G}	0.90^{D}	0.763	0.097	0.0030
	10	1.03^{F}	1.02^{C}	0.808	0.063	0.0211
	15	1.11^{E}	$1.03B^{C}$	0.029	7.009	0.0188
Myostatin	**20**	1.20^{D}	1.04^{BC}	<0.001	46.486	0.0286
	25	1.30^{C}	1.11^{B}	<0.001	6034.4	0.0331
	30	1.40^{B}	1.10^{B}	<0.001	1029.91	0.0498
	35	1.50^{A}	1.20^{A}	<0.001	11401.8	0.0502
	P-value	<0.001	<0.001			
	F-value	303.96	23.967			

Two-way ANOVA for overall statistical analysis of two independent variables *Feed type* and *Age* and dependent variable *GH/IGF-1/Myostatin* expression followed by student T-test for intergroups comparison where n=5, significantly difference was checked at $p < 0.05$, LF: PLs fed live feed and SNF fed standardized nano feed.

Table 36. Comparative effect of live feed and SNF on MRF's expression in *L. rohita* during early ontogeny.

	Age (DAH)	LF	SNF	P-value	F-value	SEM
	03	1.20^{A}	1.20^{A}	0.978	0.001	0.0017
	10	1.10^{B}	1.20^{A}	0.001	458.51	0.0169
	15	1.10^{B}	1.11^{B}	0.840	0.0430	0.0166
MYF5	**20**	1.01^{C}	1.11^{B}	0.007	13.305	0.0195
	25	0.90^{D}	1.02^{C}	0.001	26.900	0.0227
	30	0.90^{D}	1.00^{C}	<0.001	75.697	0.0179
	35	0.80^{E}	0.90^{D}	<0.001	1222.1	0.0170
	P-value	<0.001	<0.001			
	F-value	80.328	102.81			
	03	1.39^{A}	1.40^{A}	0.348	0.994	0.0033
	10	1.20^{B}	1.30^{B}	<0.001	873.8	0.0171
	15	1.01^{C}	1.20^{C}	<0.001	34.52	0.3595
MyOD	**20**	0.90^{D}	1.10^{D}	<0.001	730.87	0.0335
	25	0.91^{D}	1.02^{E}	0.01	26.043	0.0212
	30	0.89^{D}	1.02^{E}	0.921	0.011	0.0019
	35	0.79^{E}	0.91^{F}	<0.001	79.501	0.0187
	P-value	<0.001	<0.001			
	F-value	259.07	213.72			
	03	1.10^{A}	1.09^{A}	0.436	0.671	0.0014
	10	0.79^{B}	0.81^{D}	0.068	4.435	0.0015
	15	0.79^{B}	0.80^{D}	0.495	0.512	0.0018
Myogenin	**20**	0.70^{C}	0.90^{C}	<0.001	1884.6	0.0340
	25	0.70^{C}	0.90^{C}	<0.001	3097.6	0.0331
	30	0.80^{B}	0.90^{C}	<0.001	2008.2	0.0167
	35	1.02^{B}	1.10^{A}	<0.002	19.257	0.0171
	P-value	<0.001	<0.001			
	F-value	4364.2	199.06			
	03	0.60^{E}	0.60^{G}	0.374	0.887	0.0014
	10	0.70^{D}	0.70^{F}	0.613	0.277	0.0011
	15	0.70^{D}	0.80^{E}	<0.001	628.63	0.0169
MEF2A	**20**	0.80^{C}	0.90^{D}	<0.001	2025.8	0.0170
	25	0.90^{B}	1.02^{C}	<0.001	31.656	0.0221
	30	0.98^{A}	1.11^{B}	<0.001	29.033	0.0061
	35	0.90^{B}	1.30^{A}	<0.001	31894	0.0669
	P-value	<0.001	<0.001			
	F-value	1089.5	889.78			

Two-way ANOVA for overall statistical analysis of two independent variables *Feed type* and *Age* and dependent variable *Myogenin/MYF5/MEF2A/MyoD* expression followed by student T-test for intergroups comparison where n=5, significantly difference was checked at $p < 0.05$, LF: PLs fed live feed and SNF fed standardized nano feed.

Interaction of the two studied independent variable age DAH and feed type was also significant in all the studied genes; MYF5 ($F_{[6,56]}$=6.579, $P<0.001$, η_p^2=0.413), MEF2A ($F_{[6,56]}$=215.9, $P<0.001$, η_p^2=0.959), MyoD ($F_{[6,56]}$=13.54, $P<0.001$, η_p^2=0.719), Myogenin ($F_{[6,56]}$=186.5, $P<0.001$, η_p^2=0.952), myostatin ($F_{[6,56]}$=30.76, $P<0.001$, η_p^2=0.767), GH ($F_{[6,56]}$=174.6, $P<0.001$, η_p^2=949), and *IGF-1* ($F_{[6,56]}$=176.7, $P<0.001$, η_p^2=950). The partial eta-

squared (η_p^2) values indicated the large and almost similar effect size of feed type and age DAH on the expression of GH, myogenin, *IGF-1* and MEF2A while medium effect of feed type and larger effect size of age DAH on MYF5, MyoD and myostatin. Moreover, the η_p^2 values indicates significant interaction between feed type and age DAH having large effect size on GH, myogenin, *IGF-1* and MEF2A gene expression while medium on MyoD and lower on MYF5 genes expression.

Multiple comparison of the studied gene shows significantly high expression of MyoD, MYF5, myogenin, GH, *IGF-1* and MEF2A in SNF compared to LF on 35DAH (Figure 34 and 35). On the other hand, myostatin expression was significantly increased at the same period. During the ontogeny, expression pattern of the studied genes was different from each other at each sampling point. Myf5 and MyoD pattern of development was similar, both significantly decreased at each sampling point uniformly, and at each point the significantly high expression in SNF compared to LF of those genes were constant. Unlike the above-mentioned genes, *IGF-1*, GH, Myostatin and MEF2A enhanced with ontogeny, except myogenin which decreased initially and increased subsequently. Further, during the ontogeny MyoD expression descends sharply till 20DAH and a less significant decline there after till 35DAH with LF but with SNF the decline curve was not sharp, and the decreasing curve became uniform after 25DAH (figure 34). *IGF-1*, myostatin and MEF2A expression initially remain same till 10DAH, afterward, the high comparative expression of gene in SNF was persistent at each sampling point. The GH expression followed the same trend during ontogeny, but GH expression was higher significantly in SNF group just after 3DAH till 35DAH.

Interesting pattern during the ontogeny was seen in myogenin where a sudden fall in expression level was noted after 3DAH which remain stable then till 15DAH in both groups, however after 15DAH the expression level of myogenin decreased in LF while increased in SNF.

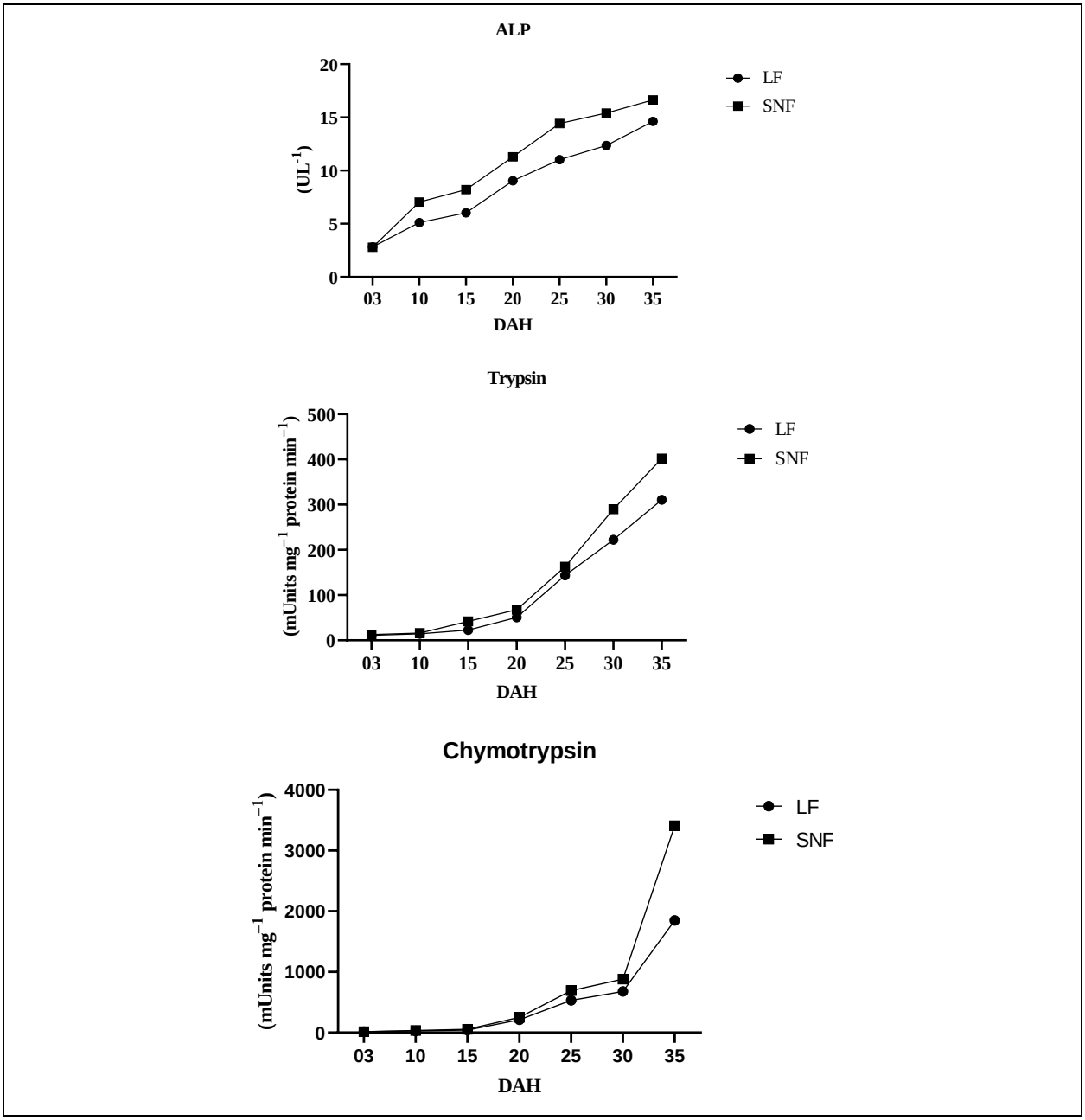

Figure 29. Pattern of activity of studied guts enzymes of *L. rohita* PLs in response to LF and SNF during early ontogeny.

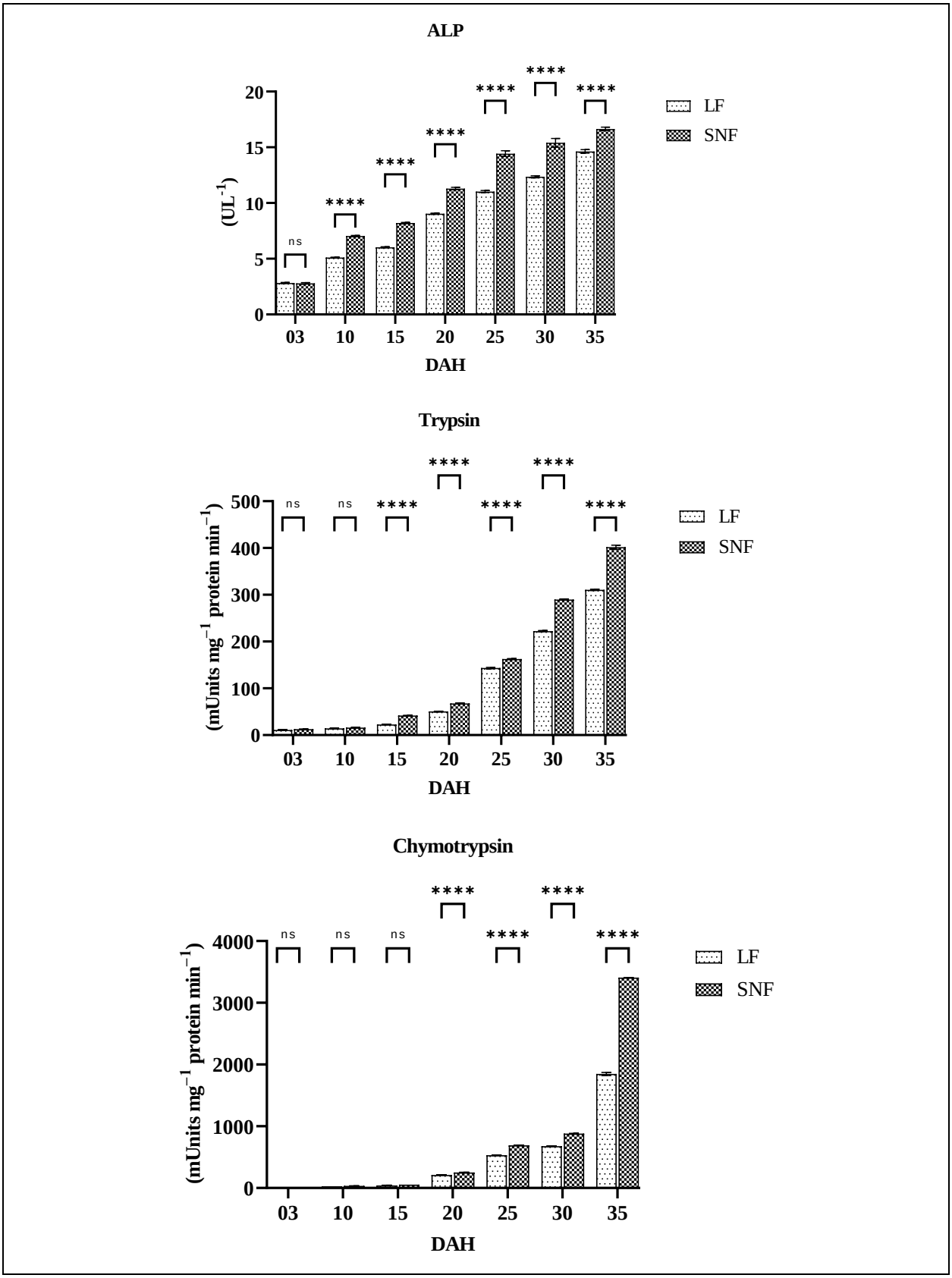

Figure 30. Bar graph showing inter group variation in ALP, trypsin, and Chymotrypsin activity during early ontogeny of *L. rohita* PLs.

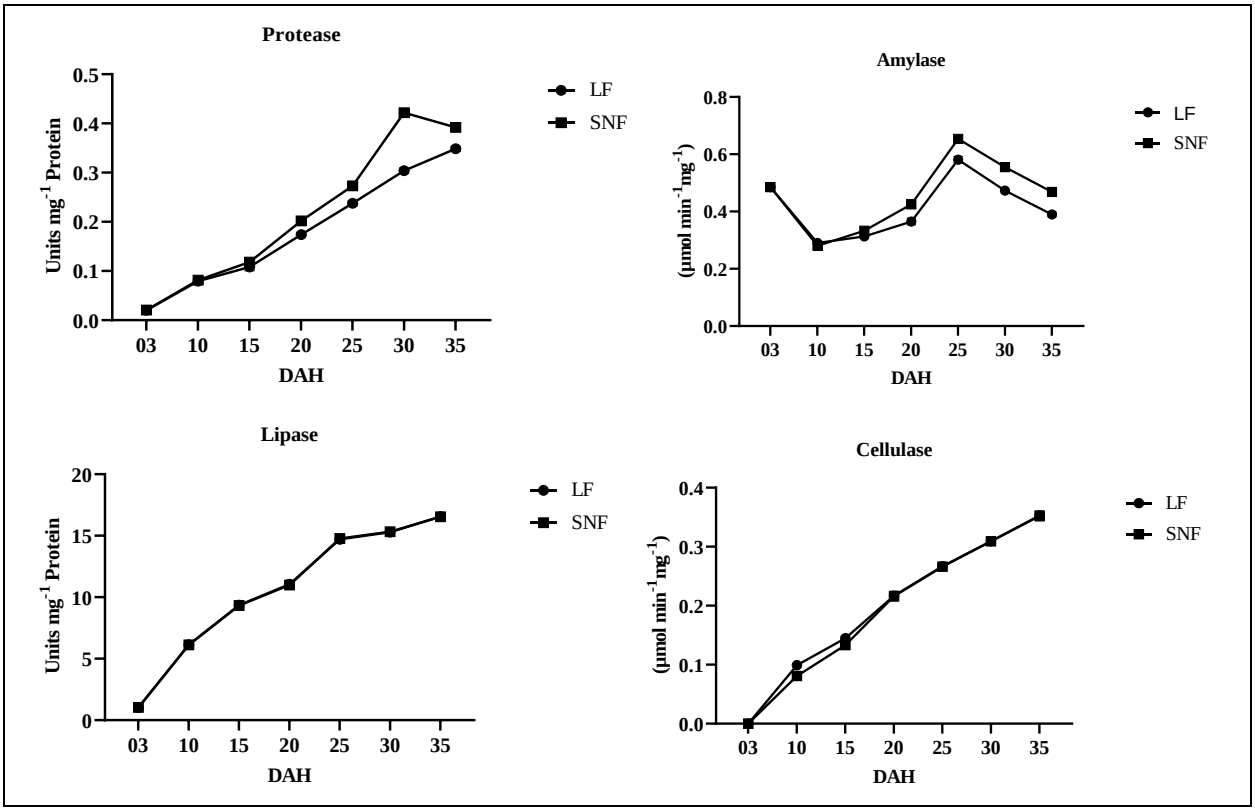

Figure 31. Pattern of development of gut enzyme release in *L. rohita* PLs in response to LF and SNF during early ontogeny.

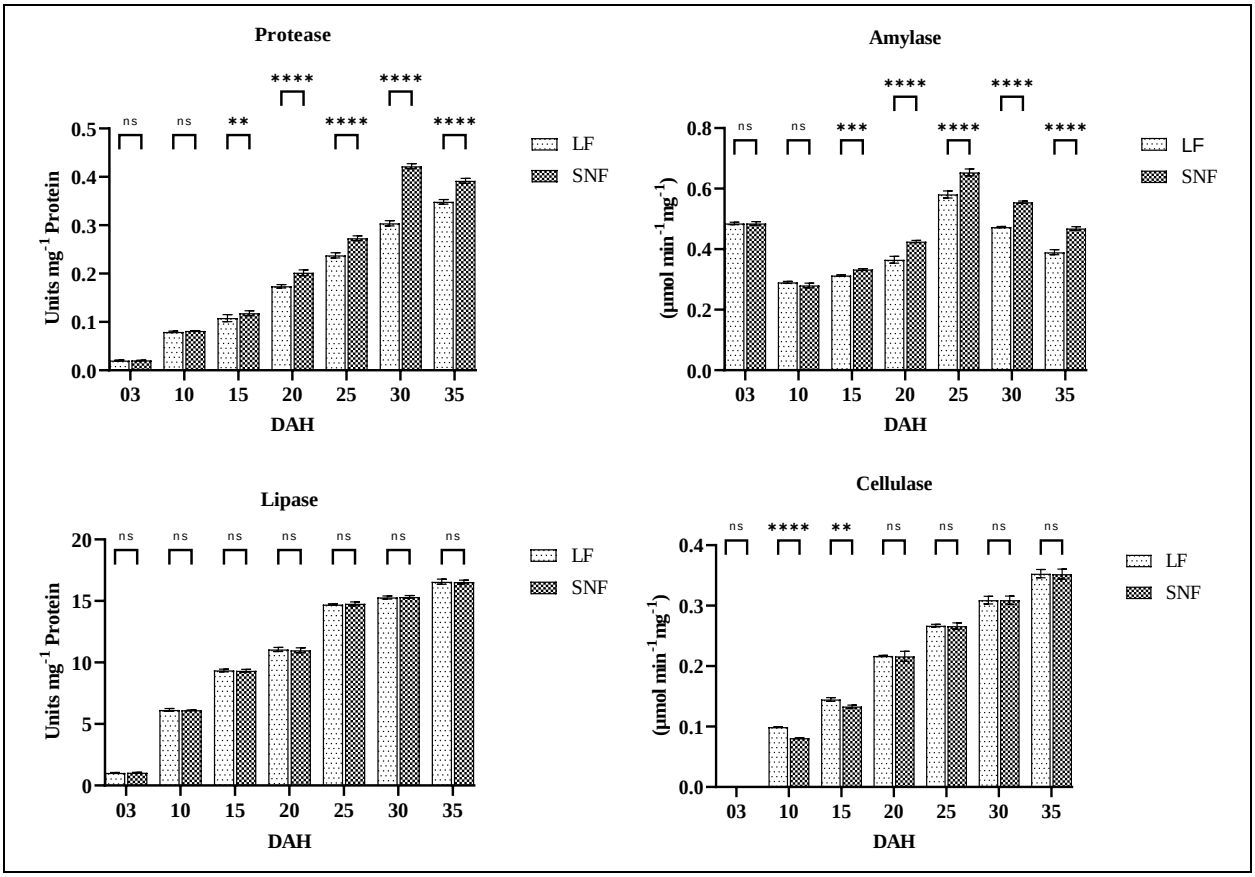

Figure 32. Bar graph showing inter group variation in Lipase, amylase, cellulase and protease activity during early ontogeny of *L. rohita* PLs.

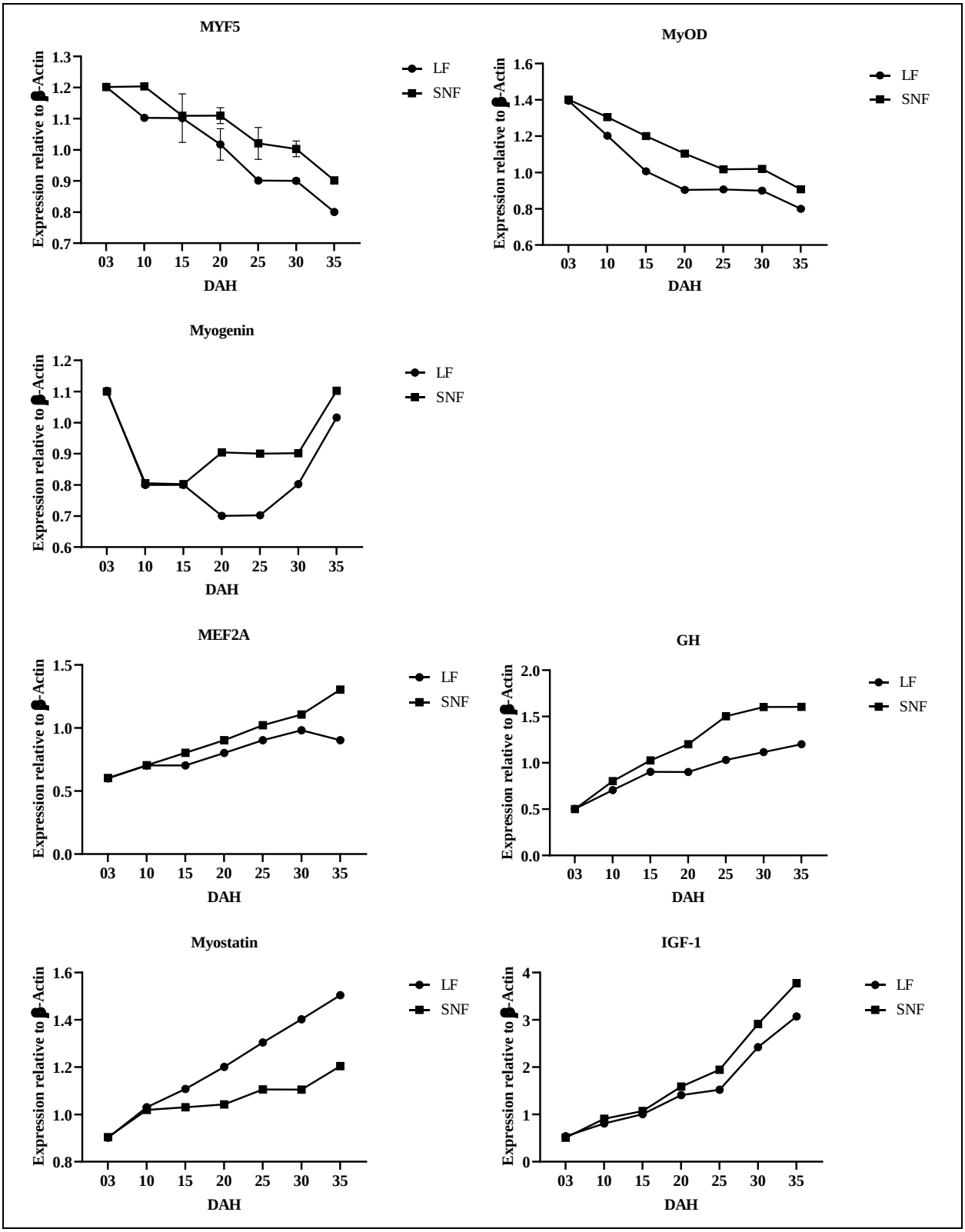

Figure 33. Pattern of expression of the studied genes of *L. rohita* PLs in response to LF and SNF during early ontogeny.

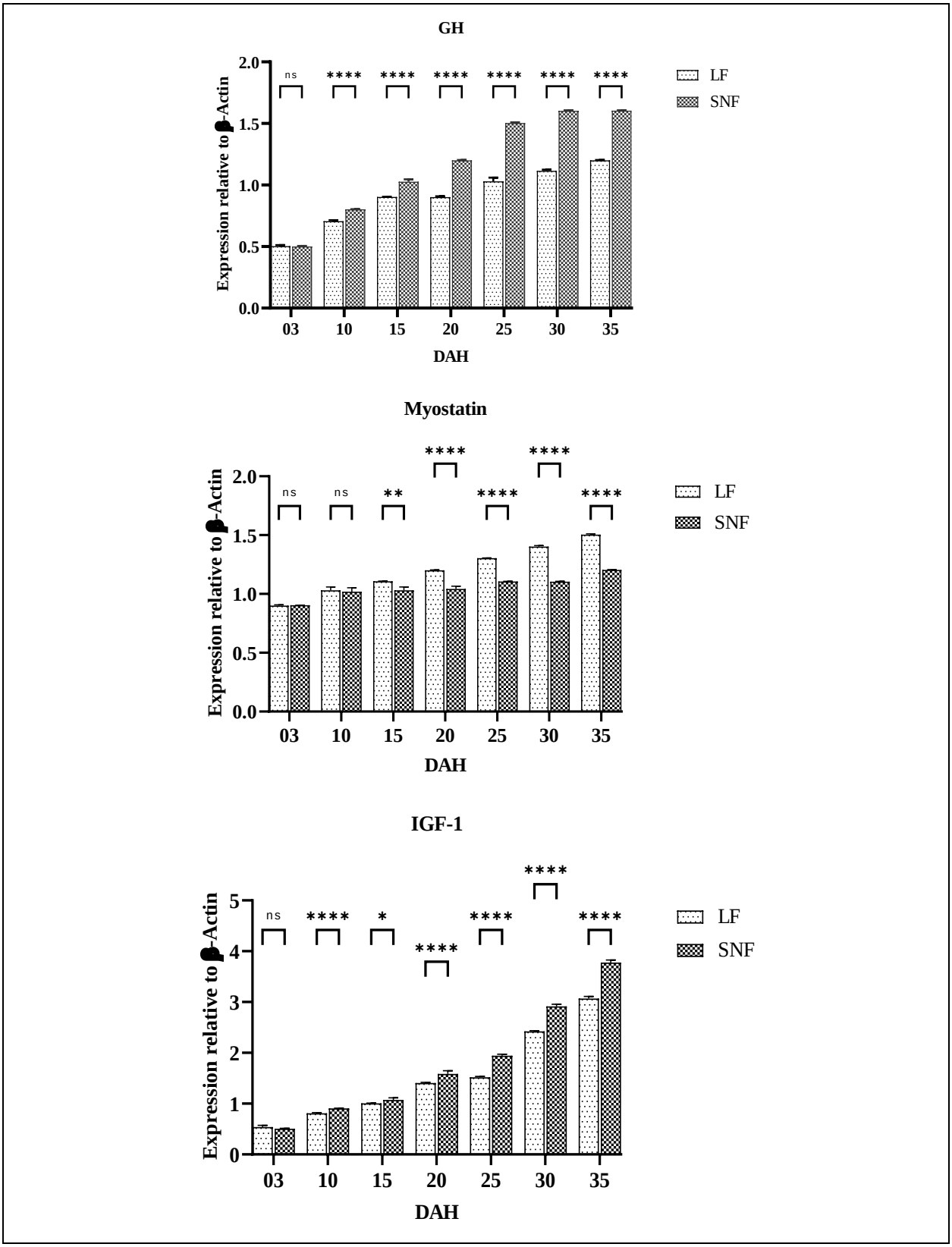

Figure 34. Bar graph showing inter groups variation in GH, *IGF-1* and myostatin expression during early ontogeny of *L. rohita* PLs.

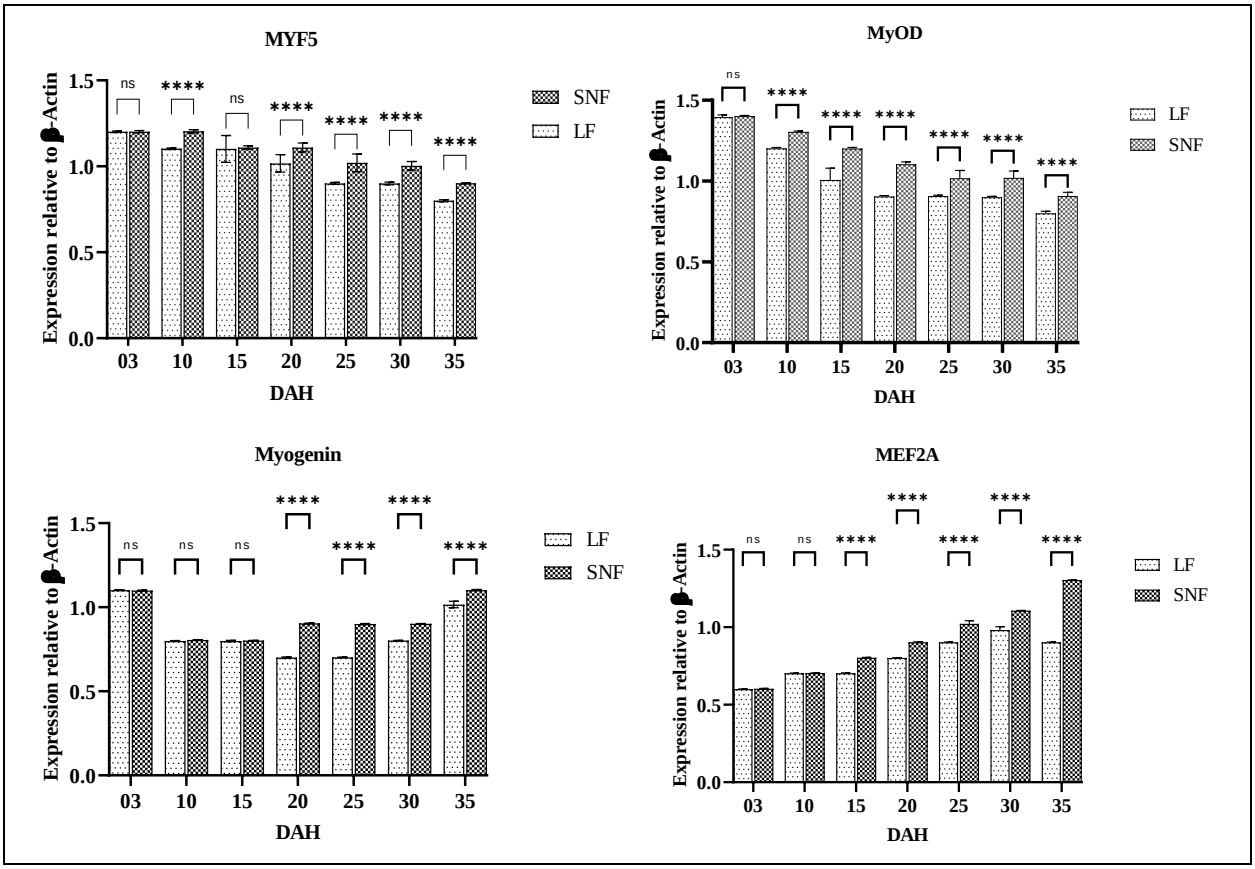

Figure 35. Bar graph showing inter groups variation in MRF's expression during early ontogeny of *L. rohita* PLs.

Table 37. Two-way ANOVA values of studied growth parameters and enzymes activity in response live feed and SNF for 35 days in *L. rohita* PLs.

Dependent factors	Independent factors	DF	F-Value	P-Value	% of total variation
Protease	Feed Type	1	971.0	<0.001	1.708
	Age (DAH)	6	9104	<0.001	96.08
	Feed type × Age (DAH)	6	199.9	<0.001	2.109
Amylase	Feed Type	1	744.8	<0.001	4.646
	Age (DAH)	6	2478	<0.001	92.76
	Feed type × Age (DAH)	6	59.94	<0.001	2.244
Cellulase	Feed Type	1	16.46	<0.001	0.0393
	Age (DAH)	6	6964	<0.001	99.74
	Feed type × Age (DAH)	6	5.927	<0.001	0.084
Lipase	Feed Type	1	0.006290	0.9371	5.489
	Age (DAH)	6	19089	<0.001	99.95
	Feed type × Age (DAH)	6	0.2953	0.9366	0.001546
ALP	Feed Type	1	3984	<0.001	6.072
	Age (DAH)	6	10192	<0.001	93.20
	Feed type × Age (DAH)	6	70.46	<0.001	0.6444
Trypsin	Feed Type	1	89787	<0.001	1.039
	Age (DAH)	6	8325	<0.001	98.11
	Feed type × Age (DAH)	6	1468	<0.001	0.8465
Chymotrypsin	Feed Type	1	28704	<0.001	2.397
	Age (DAH)	6	178215	<0.001	89.33
	Feed type × Age (DAH)	6	16501	<0.001	8.272
Growth	Feed Type	1	1653	<0.001	0.4476
	Age (DAH)	6	61032	<0.001	99.14
	Feed type × Age (DAH)	6	242.3	<0.001	0.3936
SGR	Feed Type	1	1029	<0.001	0.238
	Age (DAH)	5	1759	<0.001	89.52
	Feed type × Age (DAH)	5	4.684	<0.001	9.762

Table 38. Two-way ANOVA of the studied genes expression in response live feed and SNF for 35 days in *L. rohita* PLs.

Dependent factors	Independent factors	DF	F-Value	P-Value	% of total variation
MyoD	Feed Type	1	322.7	<0.001	9.967
	Age (DAH)	6	463.0	<0.001	85.80
	Feed type × Age (DAH)	6	13.54	<0.001	2.508
MYF5	Feed Type	1	106.2	<0.001	8.757
	Age (DAH)	6	168.5	<0.001	83.37
	Feed type × Age (DAH)	6	6.579	<0.001	3.256
MEF2A	Feed Type	1	820.3	<0.001	6.707
	Age (DAH)	6	1677	<0.001	82.24
	Feed type × Age (DAH)	6	215.9	<0.001	10.59
Myogenin	Feed Type	1	388.0	<0.001	4.811
	Age (DAH)	6	1084	<0.001	80.62
	Feed type × Age (DAH)	6	186.5	<0.001	13.88
GH	Feed Type	1	2266	<0.001	14.85
	Age (DAH)	6	1981	<0.001	77.91
	Feed type × Age (DAH)	6	174.6	<0.001	6.868
IGF-1	Feed Type	1	1317	<0.001	1.991
	Age (DAH)	6	10613	<0.001	96.32
	Feed type × Age (DAH)	6	176.7	<0.001	1.603
Myostatin	Feed Type	1	302.8	<0.001	18.68
	Age (DAH)	6	179.6	<0.001	66.48
	Feed type × Age (DAH)	6	30.76	<0.001	11.39

Table 39. Changes in intestinal morphology in response live feed and SNF for 35 days in *L. rohita* post-larvae.

Parameters	Anterior		Posterior	
	LF	SNF	LF	SNF
Villus height (µm)	500±3.5^{b}	570±2.4^{a}	471±4.1^{b}	492±2.5^{a}
Villus width (µm)	135±3.1^{b}	148±3.6^{a}	102±2.0^{b}	118±1.5^{a}
Crypt depth (µm)	21±1.6^{b}	26±1.50^{a}	14±2.35^{b}	17±3.31^{a}

LF: live feed, SNF: standardized nano-feed.

Histology

Significant alteration in gut morphology has been observed (Figure 36, 37,38). Changes in villi length was observed significantly changed in both anterior and posterior section of guts (Table 39). Further, our results indicated that in both sections, the SNF has been observed to enhance the villi length in comparison to LF group.

Unpaired T-test revealed a significant variation in both the width of Villi and crypt depth in both feeding groups. The increments in both villi width and crypt depth were significantly higher in SNF compared to LF, at both posterior and anterior section of gut (Figure 37).

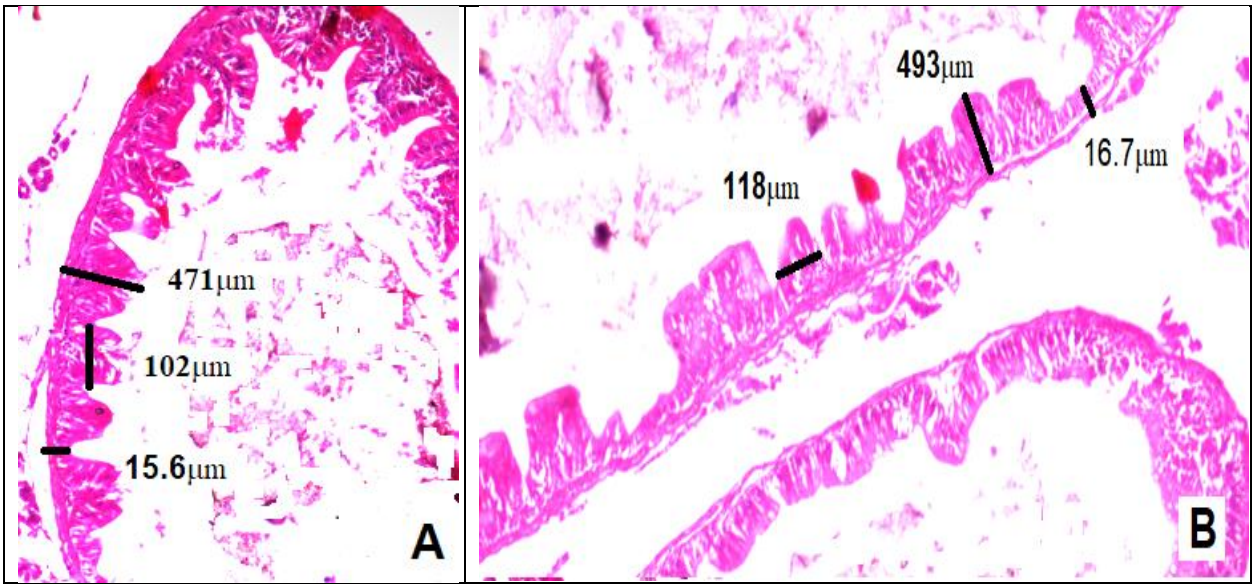

Figure 36. Histological observations of posterior section of intestine after 35DAH, A: feed with LF, B: feed with SNF.

Figure 36 show crypt thickness villi height and width in the anterior section of gut of rohu fry. Histology of gut clearly show differences in development of villi, while feeding on live feed and SNF. Cross section histological examination with 10X magnification of both anterior and posterior section of gut clearly demonstrate the increment in villi development with SNF compared to LF.

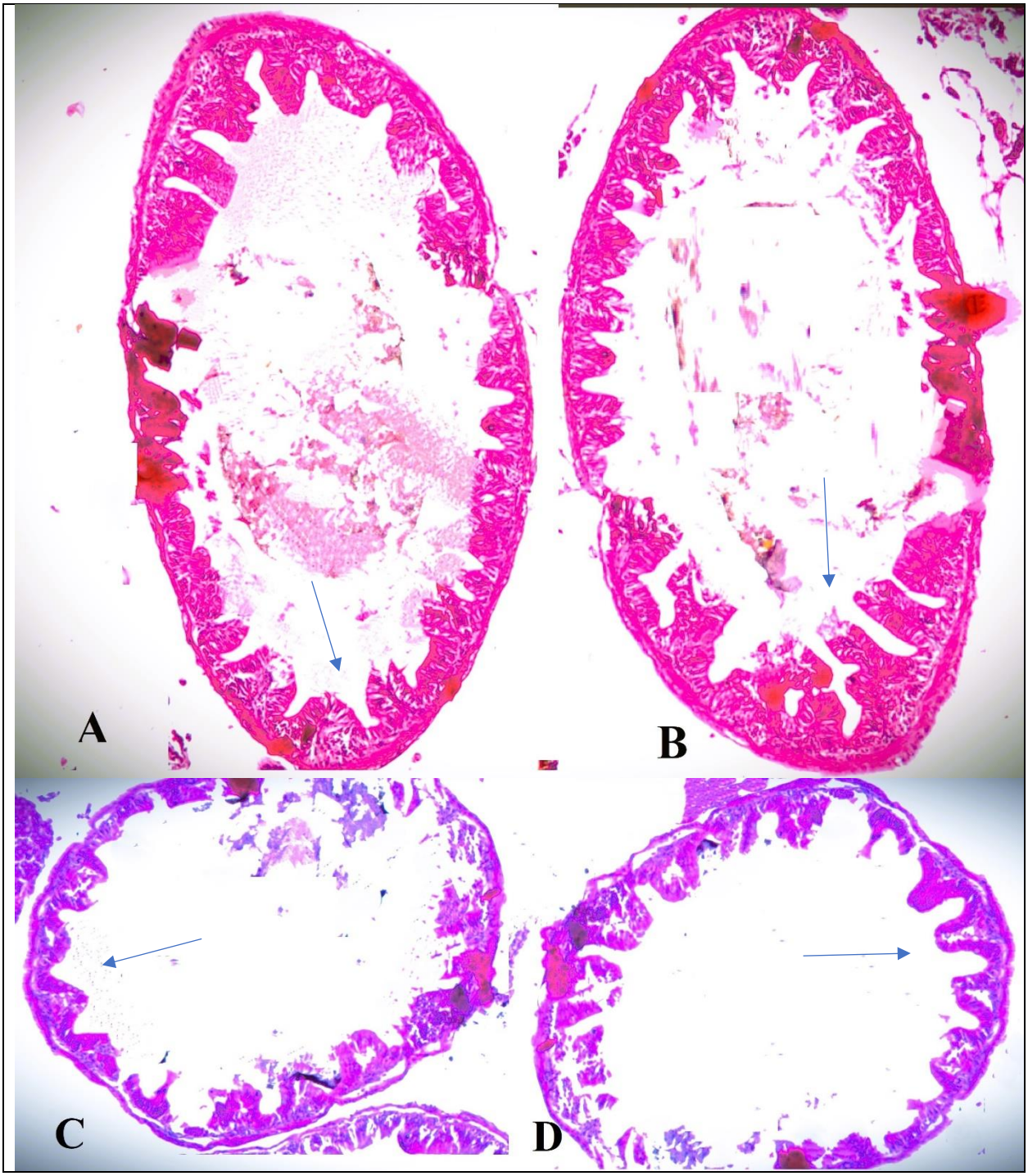

Figure 37. Cross section histological observations of Anterior (A,B) and posterior section(C,D) of intestine after 35DAH; A & C: fed with LF, B &D: feed with SNF.

The individual villi observation with 40X magnification although show the difference in width (more in SNF group), however the difference in villi length cannot be clearly seen with 40X. The difference in these parameters can be seen with 10X examination (37) evident in figure 39.

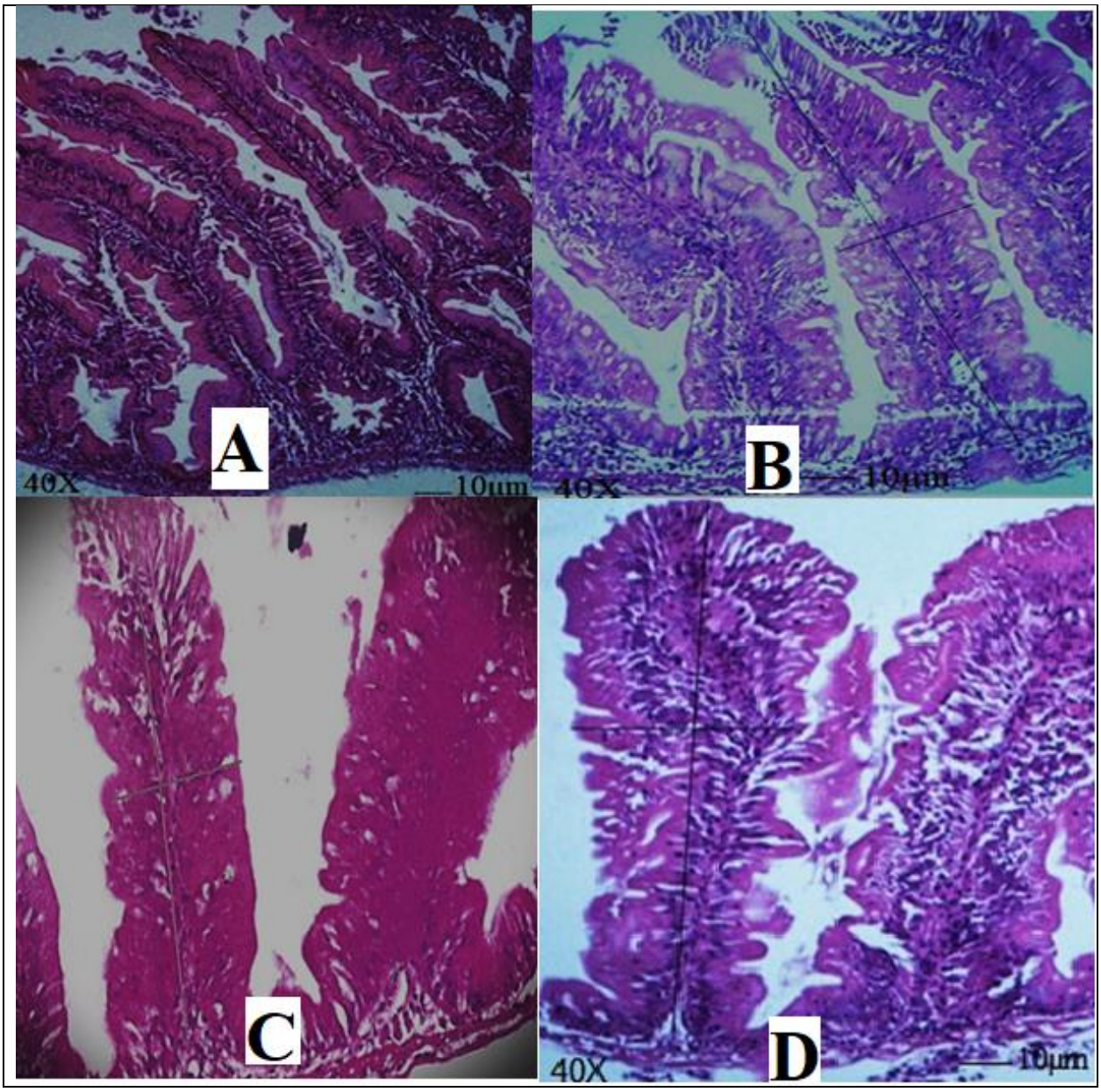

Figure 38. histological observations of villi with 40X of Anterior (A,B) and posterior section(C,D) of intestine after 35DAH; A & C: fed with LF, B &D: feed with SNF.

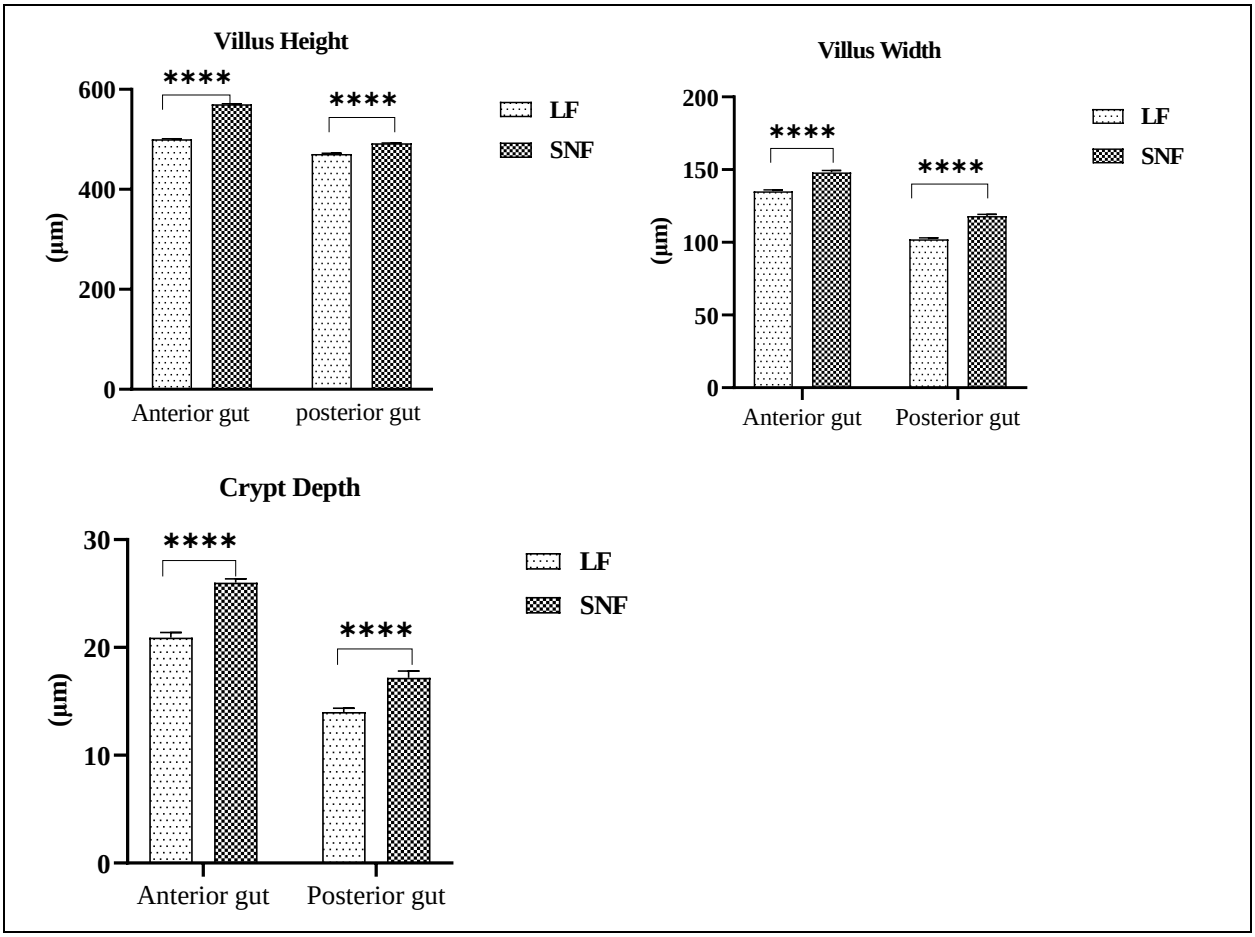

Figure 39. Variation in height and width of villi and crypt depth in anterior and posterior section of gut of rohu fry (35DAH).

Discussion

In fish culture, Larval rearing is one of the challenging phases, requiring special care and attention. It is well documented that by controlling mortality and retarded growth, the most critical problems during early rearing, this venture could be profitable (Das et al., 2012). During the development of fishes, larval phases are sensitive periods which are responsive to both extrinsic and intrinsic factors. Among extrinsic factors, feed/diet is a crucial one that affects the growth and survivorship during early rearing as well as the provision of quality fish seed. Therefore, insightful knowledge regarding the role of feed in the regulation of feed intake, release of digestive enzymes and early growth is a prerequisite for improving the survival and growth of fish. In Pakistan, Rohu (*L. rohita*) is the abundantly cultured Indian major carp. However, higher mortality (>70%) and retarded growth during early rearing and low-quality seed are the major challenges for the sustainable culture of this species. These problems could be due to the unavailability of larval-prepared feed and the rearing of PLs in nursery earthen ponds on live feed. It is well documented that an imbalanced nutritional profile, missing essential nutrients and the presence of parasites in live feed affect larval performance (Menden-Deuer and Lessard, 2000; Cahu et al., 2003; Marañón, 2008 & 2013). These inherent problems associated with live feed highlight the importance/ requirement of larval prepared diet.

The literature review declares certain lacunae in preparation of larval feed, like insufficient /lack of knowledge about nutritional requirements of larvae, physiological changes in the digestive system during ontogeny, formulation, particle size and texture of larval diet etc. (Teshima et al., 2000; Langdon, 2003; Pepin, 2023). To address these issues the present comprehensive study was designed, and efforts were made to understand the impact of feed nature/texture on the regulation of feed intake, temporal release of digestive enzymes and growth of 3DAH larvae for 35 days rearing period. *L. rohita* is a warm-water fish that shows growth potential and breeding activity from March to October. It's all phases of development are generally cultured in earthen ponds in extensive to semi-intensive culture conditions. Like most fish species, the larvae of *L. rohita* are small and mostly cultured on live feed. Thus, for the determination of nutritive requirements during early rearing, an initial study was focused on culturing of live feed in earthen ponds by the conventional and commonly used method, i.e., fertilization of pond and variation in plankton density and their nutritive value, abundance and composition were determined during March to October.

Earthen ponds are examples of freshwater ecosystems supporting communities of various aquatic organisms, including plankton. The nutrient loading in earthen ponds is generally related to management practice and is mainly dependent on fertilizers, feed input and excreta of cultured animals (Boyd et al., 2010). Fertilization enhances natural food production, i.e., promotes the growth and production of phytoplankton (primary producers), and zooplankton, the primary consumers, and secondary producers (Boyd, 2018). Generally, phytoplankton, an integral component of the pond ecosystem contributes to the progression of zooplankton (Li et al., 2019), a food source for cultured fishes especially their larvae (Shao et al., 2019). To produce live feed, earthen ponds were prepared with the addition of both organic and inorganic fertilizers and lime to maintain the pH of the bottom soil. The productivity of the ponds was checked with Sacchi disc and maintained with the addition or restriction of fertilizers.

Many investigators reported the temporal changes in the plankton composition, species-wise relative abundance, variation in body composition and nutritive value and suggested the role of environmental fluctuations (light, temperature, and salinity) in altering the dynamic of plankton (Ndebele-Murisa et al., 2010). The observed month-wise (temporal) variation in the plankton community, i.e., relatively higher phytoplankton (%) in March to May while higher Zooplankton (%) in June to September was in accord to literature and confirmed the role of environmental factors in the succession of the planktonic community. Among environmental factors water temperature is the main factor that regulates the plankton abundance and their community structure (Tulsankar et al., 2021; Sun et al., 2022). It is well documented that the total plankton abundance was higher during warmer months (Elakkanai et al., 2017; Cole et al., 2019; Tulsankar et al., 2021) compared to the colder months. The observed higher abundance of plankton during warmer months or increase in relative abundance with an increase in water temperature or vice versa may be due to increased daylight hours and water temperatures that trigger the abundance of phytoplankton (Sommer et al., 2012). These phytoplankton in turn become the source of zooplankton nutrition (Coman et al., 2003).

Water temperature not only affects the species richness but also influences the plankton community structure, the relative abundance, and the biomass of dominant species (Sun et al., 2022). Generally, among phytoplankton, green algae (Cyanophyceae) dominated during the warmer months (temperature >20 °C) (Kratina et al., 2012), while Chlorophyceae along with

Zygnematophyceae and Trebouxiophyceae dominated during the colder months (Tulsankar et al., 2020). In the present study, Blue-green algae also showed an increasing trend with an increase in temperature, i.e., from March to August and then showed a declining trend. In contrast to cyanophycean, Chlorophyceae showed a decreasing trend with an increase in temperature, i.e., from March to August and then increased with a lowering of temperature from September to October. It seems prokaryotic phytoplankton flourish at a higher temperature compared to eukaryotic phytoplankton which thrives at somewhat low temperatures (Kosten et al., 2012). The Increasing water temperature causes a decrease in the viscosity of surface water, which in turn favor the sinking of non-motile phytoplankton and adjusting of Cyanophyceae to dominate plankton communities (O'Neil et al., 2012).

Zooplankton are the diverse group of heterotrophic organisms that are responsible for the transferable of energy to higher trophic levels (Steinberg and condon, 2009). These are the major sources of natural food for fish, especially for larvae (Alam et al., 1987). The distribution and diversity of major groups of zooplankton (Rotifers, Copepods, Cladocerans and Ostracods) depend on the physiochemical properties of water especially water temperature (Saba and Sadhu, 2015). In the present study, Rotifers Cladcerans and Copepods are abundant compared to other zooplankton. Many investigators reported a similar abundance of these among zooplankton community (Tulsankar et al., 2021; Singh et al., 2021). Like the results of many investigators, Rotifers abundance (March to September) was dominated by Brachionus spp. followed by Keratella spp. The abundance of Brachionus spp. here is an indicator of organic load and indicated the alkaline nature of water (Ezz et al., 1967). In food web of pond ecosystem, Rotifers are the important Zooplankton group that provide food to adult copepod (Lokko et al., 2017), while phytoplankton abundance support the growth of copepod nauplii (Sharip, 2019). Though in earthen ponds environmental factors influenced the relative abundance of plankton and their community structures, but the effects of the farming practices, i.e., use of fertilizers, ratio of organic and inorganic fertilizer, amount of fertilizer /ha and periodicity of application, stocked animal, feed type (type and composition) etc. cannot be ruled out (Casé et al., 2008; Boyd, 2009). In the present study, initially, organic fertilizer (cow dung) and nitrogen-phosphorus-potassium (inorganic fertilizer) at the rate of 3333.33kg/ha and 125 kg/ha were used and lime at the rate of 250kg/ha was applied to maintain the pH of bottom soil. Afterwards, the productivity of ponds was checked periodically and maintained with the addition or restriction of organic or inorganic fertilizer. It

seems, this strategy also contributed to the fluctuation of plankton abundance and their community structure.

Live feed organisms like rotifers and copepods, are often the feed of larval and juvenile stages of many fishes and crustaceans (Tulsankar et al., 2021). Thus nutritional quality of live feeds is vital for the successful culture of any fish species. Seasonal variation in the plankton community alters the proximate and biochemical composition of both phytoplankton and zooplankton (Jo et al., 2017). The term "proximate composition" describes the proportions of key organic substances in the biomass of these creatures, including proteins, lipids, carbohydrates, and ash. The seasonal water temperature fluctuation considerably impacted the protein content of phytoplankton and zooplankton. It is well documented that among phytoplankton, diatom-dominated communities with higher protein content enhance (Hillebrand et al., 1999) while the lipid content tends to decline (Zhang et al., 2019), with an increase in water temperature. These changes have consequences for energy transmission in aquatic food webs. This temperature-driven phytoplankton composition showed a positive impact on the amount of protein of copepod prey (Bonnet et al., 2009). The present results are also in accord with the literature and showed fluctuation in protein and crude fat contents with temperature. The highest values of both protein and lipid contents were observing during warmer months, especially in July and August, the period when *L. rohita* larvae require energy and protein for survival and growth.

Live feed mainly constitutes C14:0, C15:0, C16:0, 20:4n−6, 20:5n−3 EPA (Eicosapentaenoic acid), 22:5n−3, 22:6n−3 DHA (Docosahexaenoic acid) during the summer season (Jiménez-Prada et al., 2018). In the present study, the prominent zooplankton species found in nursery earthen ponds were cladocera, copepods, moina, rotifers and artemia. Copepods usually remain advantageous due to abundance of unsaturated FA, essential for fish growth and development (Bell et al., 2003; Olivotto et al., 2006; Conceica et al., 2010) and presence of PUFA/HUFA in the phospholipid fraction which make it more bioavailable, compare to Artemia (where PUFA occur in neutral lipid fraction) (Conceica et al., 2010). It seems the dominance of PUFA during June and July favors the development of *L.rohita* larvae. However, rotifers and artemia are still the most abundant live food utilized in feeding fish larvae (Sorgeloos et al., 2001; Kang et al., 2006). *Moina macrocopa***,** rich in nutrients, contains more superior protein profile than artemia (Loh et al., 2012) and is widely used in finfish and shellfish culture (Poynton et al., 2013; Ingram, 2009; Aguado et al., 2009; He et al., 2001). Cladocera mostly accumulates EPA and

Arachidonic acid (ARA) while copepods predominately accumulate DHA (Farkas 1979; Persson and Vrede, 2006; Smyntek et al., 2008; Burns et al., 2011; Mariash et al., 2011). The seasonal changes in dietary ARA concentrations may be counteracted by converting linolenic acid or 22:5n-6 into ARA (Brett et al., 2006; Taipale et al., 2011) with an increased ARA accumulation in Daphnia in late summer and autumn. The present results clearly indicate these seasonal changes in the FA profile of live feed and species composition. These variations and inconsistent nutrient supply of nutrients in live feed indicate the crucial need for a formulated standard diet for the successful culturing and weaning of fishes during early rearing.

After getting a rough idea about the nutrient profile of live feed during the rearing period of *L. rohita* larvae, efforts were made to formulate and prepare a larval diet. It is well documented that besides nutrient profile, the texture, and particle size of larval feed also affect the pelletability and palatability and overall absorption and bioavailability of nutrients for growth (Baskerville-Bridges and Kling, 2000). These issues were also taken into consideration and based on the proximate composition of live feed, 60% CP (Table 12) nano-particulate feed having a particle size of <80 nm with a smooth surface area was prepared ((Table 12) with the aid of Nanotechnology. It is well documented that Nano-scale feed particles due to distinctive properties, i.e., small size, shape, and high surface area to volume ratio improve the absorption and bioavailability of nutrients and affect the growth of animals (Chau et al., 2007).

Generally, prepared feed possesses relatively stable nutrients (Baskerville-Bridges and Kling, 2000) however literature revealed variable results. For instance, many investigators observed lower growth and survival rate in silver sea bream larvae (*Rhabdosargus sarba* (Forsskål, 1775) =*Sparus sarba* Forsskål, 1775) (Kelly et al., 2000), gilthead seabream (*Sparus aurata* Linnaeus, 1758) (Fera et al., 2000) and red drum larvae (*Sciaenops ocellatus* (Linnaeus, 1766)) (Lazo et al., 2000) along with skeletal deformities when live feed was replaced with prepared diet and suggested that it could be due to the poor texture of prepared feed and delayed adjustment of the larvae to the exogenous feeding, thus leading to malnutrition (Cahu and Infante, 2001; Little et al., 2002). However, some scientists reported nutrient anomalies in extending the live feeding regime during early life stages in European sea bass (*Dicentrarchus labrax* (Linnaeus, 1758)) (Hamza et al., 2016) and common sole (*Solea solea* (Linnaeus, 1758)) (Parma et al., 2013), which may be due to an unstable nutrient profile, since live feed does not have all the essential ingredients required for growth (Hertrampf and Piedad-Pascual, 2003).

In the present study, for the evaluation of the efficiency of Nano-particulate prepared feed (ND) compared to Life feed (LF), 35 days feeding trial in a indoor facility was conducted and larvae were divided into three groups, T1 and T2 reared exclusively on LF and ND respectively while T3 co-fed with both LF and ND (1:1). For oncogenic changes, sampling was done at 3,10,15,20,25,30 and 35DAH from the experimental groups. The results of feed-dependent increase in growth and the expression of genes involved in feed intake and growth with age DAH were in accord with literature and indicated the role of feed during early rearing of *L. rohita*. Moreover, improved growth performance and survival (%) of the T2 group compared to T1 could be due to improved texture, nutritional profile, higher absorption, and assimilation of ND. It seems small size feed particles with smooth surface texture facilitate larvae to adjust to exogenous feeding, improve digestion, and absorption of ND and reduce nutrient loss in fecal matter (Pieszka et al., 2019).

To overcome the limitations of live feed and prepared diet several scientists adopted the Co-feeding (LF+ND) strategy for the early rearing of larvae and reported improved growth performance in both marine; totoaba (*Totoaba macdonaldi* (Gilbert, 1890)(Mata-Sotres et al., 2015), Atlantic cod (*Gadus morhua,* Linnaeus, 1758) (Baskerville-Bridges and Kling, 2000), silverfish (*Trachiotus ovatus* (Linnaeus 1758)) (Ma et al., 2015), and freshwater species; common carp (*C. carpio* (Linnaeus, 1758)) (Szlamińska and Przyby, 1986), barramundi (*Lates calcarifer* (Bloch, 1790)(Curnow et al., 2006), butter catfish (*O. bimaculatus* (Bloch, 1794) (Pradhan et al., 2014), Chinese longsnout catfish (*Tachysurus dumerili* (Bleeker, 1864)= *Leiocassis longirostris* Bleeker, 1864) (Liu et al., 2012), and *L. rohita* (Mitra and Mukhopadhyay, 2002). The improved growth performance in response to co-feeding could be due to the early weaning of larvae on formulated feed due to the presence of chemical attractants like free amino acids and metabolites in the live feed (Cahu and Infante, 2001; Kolkovski, 2004), availability of the deficient nutrients (Conceição et al., 2010) and improvement in the assimilation and the utilization of nutrients (Parma et al., 2013) due to the presence of exogenous enzymes in the live feed (Sorgeloos et al., 2001). Here the higher growth rate in the T3 group (reared on LF+ND) was in accord with the literature.

Digestive enzyme activities directly affect the absorption and digestion of nutrients which ultimately results in animal growth (Xia et al., 2018). Their regulation is essential for studying the

effect of nutrient digestion on growth performance (Wang 2007). The ability of fish larvae to digest nutrients from the diet is chiefly dependent on the availability of appropriate digestive enzymes which are modulated by the physical and chemical nature of the feed (Cahu and Infante, 2001). Many studies have reported a relationship between feed type and digestive enzyme profile in different fish species e.g., Atlantic cod (Wold et al., 2007); red porgy (*Pagrus pagrus* (Linnaeus, 1758)) (Suzer et al., 2007), and white bream (*Blicca bjoerkna* (Linnaeus, 1758) (Cara et al., 2003). Among digestive enzymes, amylase plays an important role in the breakdown of carbohydrates into simple sugar to make energy sources available for the body. The observed significantly higher amylase activities in response to ND could be due to the high carbohydrate level in prepared feed (ND) compared to 6-10% carbohydrates in the live feed (Ma et al., 2005) or may be related to size and texture of ND as amylase level effected by efficient consumption/utilization of feed (Hoehne-Reitan et al., 2001). A similar result has been reported by Bhilave et al. (2014) where formulated feed resulted in the highest amylase activities in *L. rohita*. Yet, Shan et al. (2009) observed variation in amylase activity in miiuy croaker (*M. miiuy* (Basilewsky, 1855)) larvae with changing zooplankton species composition.

Protease activity is closely associated with the feed intake, protein nature and level in feed and age of fish (Córdova-Murueta and García-Carreño, 2002). Like present results, many investigators reported significantly high protease activity with co-feeding or during weaning from live feed to a prepared diet. For instance, Meetei et al. (2014) and Lazo et al. (2000) observed higher protease activity in *catla*, (*Gibelion catla* (Hamilton, 1822) =*Labeo catla* Hamilton, 1822) and *S. ocellatus* larvae respectively in response to co-feeding while Engrola et al. (2007) and Kolkovski (2001) observed this higher activity in *S. senegalensis* and *S. ocellatus* respectively during weaning. Similarly, Nguyen et al. (2011) reported the modulation of the expression levels of tryptophan-pathway genes (trp), ctr 1 and pep in opah, (*Lampris guttatus* (Brünnich, 1788)) larvae during the transition from live prey feed to the micro-diet at 28 DAH. The higher protease activity observed here and reported by others in response to co-feeding may indicate the maturation of the digestive tract (Vega-Orellana et al., 2006) or a higher need for an enzyme for digesting protein-rich micro-diet with variable molecular weight peptide distribution (Skalli et al., 2014). It seems that in co-feeding, live feed augmented the secretion of protease. The improvement of protease compared to amylase in response to co-feeding in turbot (*Scophtalmus maximus* (Linnaeus, 1758)) has been reported by Munilla-Moran et al. (1990) and suggested that it could be

related to the differential role of live feed in stimulating digestive enzymes, proteases 43–60%; esterase 89–94%; exonuclease 79–88%; amylase 15–27%. Moreover, Engrola et al. (2007) reported the delayed maturation of the digestive tract in Senegalese sole in response to prepared feed.

Early exposure of larvae to different microorganisms modulates their larval intestinal microbiota community which in turn affects the growth and digestive enzymes activity of the host (Deng et al., 2021). Herein the comparatively higher cellulase activity in the T1 followed by the T3 compared to the T2 might indicate higher activities of intestinal bacteria in response to live feed. Similarly, Fagbenro (1990) also observed high cellulase activity in the catfish (*Clarias agboyiensis* (Sydenham, 1980) =*Clarias isheriensis* Sydenham, 1980) reared on plankton. This may be due to the establishment of gut microbiota in the presence of live feed. The lower cellulase activity in the T2 group may be due to the limited number of microbiotas.

Here the initial higher amylase (5DPH) and lower protease and cellulase activities reveal the role of amylase in the transition period of rohu larvae (Siebenaller, 1984). However, a sudden drop after 3DAH to 10 DAH and a resurge after 10 DAH indicated larvae adaptation to carbohydrate utilization (Chakrabarti et al., 2006) with a peak at 25DAH and then again, a sluggish descent after 25DAH to 35DAH. Such decline after 21DAH was reported by Chakrabarti et al. (2006) in rohu larvae and attributed to the developmental changes in the digestive system of rohu larvae. Like our results, Kousoulaki (2015) also reported multi-peaks of amylase activity in *D. labrax*. On the other hand, the regular increasing pattern of protease and cellulase indicates the structural transition of the gastrointestinal tract irrespective of feed composition (Dement'eva, 1976).

For a better understanding of the role of nature and texture of diet on the early development of *L. rohita* larvae, the present study also analyzed the mRNA levels of genes related to appetite and growth. Generally, *Ghrelin* an orexigenic hormone, regulates growth, feed intake and energy metabolism (Kang et al., 2011). Growth is regulated with the interlinking of the *Ghrelin-GHS-R* axis with the growth hormone (GH)-insulin-like growth factor-1 (*IGF-1*) axis (Zhang et al., 2016). Here, the higher expression of *Ghrelin* and GHSR and lower expression of *leptin* in the T1 compared to the T2 may indicate the stimulation of appetite and demand for increased energy

intake due to the unbalanced nutrient profile of live feed (Ma et al., 2005). No literature is available to compare the results, however, the lower expression of IGF in the T1 supports this view.

Several studies indicated the significant effect of nutrient profile on the secretion and mRNA levels of appetite and growth-regulating hormones (Bertucci et al., 2019). For instance, Navarro-Guillén et al. (2017) reported high *ghrelin* expression and higher feed intake in response to live feed in Senegalese sole. Moreover, the higher expression of IGF and *leptin* in the T2 group may indicate GH-induced IGF production and somatic growth (Picha et al., 2008) due to improved nutritional profile, higher absorption, and assimilation of ND. It is well documented that *leptin* reflects the endogenous energy status and responds differently to the nutritional state in the fish (Kling et al., 2012). The relationship between a positive energy state to elevated plasma *leptin* levels decreased energy intake, and increased energy utilization has been reported by Anubhuti and Arora (2008). Like present results, many investigators reported significantly higher expression of *leptin* in various fish species in the absence of live feed, e.g., rainbow trout (Johansson and Björnsson, 2015) and fine flounder (Fuentes et al., 2012). Furthermore, the highest expression of *ghrelin* and the lowest expression of *leptin* in the T3 group could be due to increased consumption of feed to fulfilthe increasing demand for nutrients /energy for a higher growth rate. No parallel study indicates the expression of genes related to feed intake and growth in response to co-feeding. Yet, the observed highest expression of GHRS and IGF in the T3 as compared to other groups are in accord with the observed growth performance data and may indicate the stimulation of GH-GHSR and GH-IGF systems in the presence of a balanced nutrient profile (Giorgioni et al., 2022). Furthermore, the higher expression of *Ghrelin* and GHSR in the T1 and the T3 groups may also indicate the contribution of chemical attractants like free amino acids and metabolites in the live feed for the stimulation of appetite (Cahu and Infante, 2001; Kolkovski et al., 2004). Concluding the inference, it is quite clear that co-feeding is the best feeding strategy during early rearing as the combined effect of both overcomes the deficiency/ lacunae of each other. However, this strategy still shows the dependence on live feed. The other option is to standardize nutrients (especially macronutrients; protein, fats) in the prepared larval feed (in accordance with the nutritional demand of rohu larvae) and reduce the lacunae in formulated feed.

Knowledge regarding the nutritional requirement of fish larvae is still a challenge for the fish nutritionist. Generally, nutritional requirements are defined as the ‘requirement of nutrients

for maximal survival and growth. According to Izquierdo and Lall (2004), the relationship between fish-diet-feeding contributes most significantly to the determination of the quantitative needs of the fish. Feed formulation according to the nutritious requirement of larvae can support optimum growth (Craig and Helfrich, 2002). Therefore, assessment of dietary requirements is vital for providing the right amount of nutrients to cultured species (Khan and Maqbool, 2017). Among micronutrients, Protein lipids/fats constitute an integral part of the fish diet. A good knowledge of the larval nutritional requirements throughout development would contribute to optimizing diets and feeding protocols, and thereby improve larval and juvenile quality (Hamre et al., 2013). Nevertheless, considering the vulnerability of fish larvae, it is always difficult to identify and meet nutritional requirements when several physiological and metabolic constraints are linked and each of them may prevent growth or an appropriate development. A good understanding of the different factors and events interacting in food acquirement and processing is prime important for designing larval diets that can fulfil the larval requirements for optimal ingestion, digestion, and absorption of these diets. The requirement for a particular nutrient can be defined from a physiological point of view as the nutrient intake needed to fulfil a physiological role (Izquierdo and Lall, 2004).

Protein is one of the micronutrients that is required for optimum growth, survival, and reproduction (Wilson and Halver, 1986; Berlinsky et al., 2020). The dietary protein provides amino acids for the synthesis of new protein for growth (Lovell, 1989; NRC, 2011). The low amount of protein in diets can result in retarded growth and impairment of the immune system, on the other hand, if it is present in excess, then additional protein is converted into energy by an oxidation reaction and results in extra production cost and nitrogenous stress on culture system (Webb and Galtin, 2003; Wu and Galtin, 2014). The other significant point that needs consideration about dietary protein is the digestion capacity of fish larvae, as most fish lack stomach during early stages (Radhakrishnan et al., 2020) and have a low specific protease activity (Gawlicka et al., 2000; Rojas-Garcı´a & Rønnestad 2002). The present study revealed a significant protein digestion potential of *L. rohita* PLs with 55%CP in their diet. Rohu larvae are carnivores in nature during the early stages of development (Khan and Siddiqui, 1973) thus showing the best performance on a high CP diet (P55) compared to P45 and P50). The observed higher activity of trypsin, chymotrypsin, and total protease activity in response to 55% CP was also parallel to the growth performance data group.

Alkaline proteases mainly trypsin play a key role in the digestion of protein in the intestine (Zambonino-Infante and Cahu, 2001). Like the present results, many investigators observed an increase in enzymatic activity, protease (Xiong et al., 2012), and trypsin (Martinez et al., 2018) with dietary protein level in feed and suggest a link between the protein content of the diet and proteolytic enzymes. However, beyond the optimum dietary CP level, a negative impact on the performance was reported, which may be due to stress caused by the deterioration of water quality because of the accumulation of nitrogenous metabolites (Mohanta et al., 2007). The present results are also in accord with the literature and showed a positive trend up to 55% CP while beyond that (at 60% CP) negative impact on growth performance and enzymatic activity started appearing.

Similarly, beyond optimum level a depression in growth was observed in *C. auratus* (Fiogbe and Kestemont, 1995); *O. niloticus* (Kaushik et al., 1995); *P. fluviatilis* (Fiogbe, 1996); hybrid catfish (*H. bidorsalis x C. anguillaris*) (Diyaware et al., 2009); *H. fossilis* (Siddiqui and Khan, 2009); *H. niloticus* (Monentcham et al., 2010); *C. gariepinus* (Farhat and Khan, 2011).

Literature indicated variation in the optimum dietary requirement of protein during early development of different fish species, e.g., 48- 53% CP for *Salmo trutta* larvae (Arzel et al., 1995); 45-53% (*C. auratus*, Fiogbe and Kestemont, 1995); 48.5-49.4 (*P. fluviatis*, Fiogbé, 1996); 42-56% (*C. gariepinus* and *H. longifilis*, Kerdchuen, 1992); 50-55% (hybrid catfish *H. bidorsalis x C. anguillaris*, Diyaware et al., 2009); 28-35% (*O. niloticus*, El Sayed and Teshima, 1992); 28-30% (*O. niloticus,* De Silva et al., 1989); 30-36% (*O. niloticus,* Shiau, 2002) and 35- 42% (*C. carpio*, Tacon, 1987). The comparatively high CP demand in rohu PLs compared to other carp's larvae), observed in the present study might be due to physiological and behavioral differences. Generally, L. *rohita* is relatively carnivorous in nature during early life stages and preferred zooplankton compared to phytoplankton. This selective feeding behavior indicated a higher protein requirement because their natural diet consists of zooplankton, which are protein rich.

Successful ontogeny ensures enhancement in organogenesis and hence effective myogenesis (Riskin et al., 2011). Growth is addressed in biological terms as hyperplasia and hypertrophy through which myogenesis in larvae and adult take place (Vo et al., 2016) The expression of genes in skeletal, cardiac, and smooth muscle cells could be controlled by a shared myogenic regulatory pathway. Muscle growth by hypertrophy and hyperplasia of muscle fibers is regulated by several genetic factors such as myogenic regulatory factors (MRFs: including MyoD

and MYF5), structural genes, and other growth factors (Rescan, 2005). After fertilization, differentiation of skeletal muscle is initiated by MyoD, which binds directly to the regulatory regions of a wide number of genes and regulates their expression during differentiation. Both MyoD and MYF5 are necessary for the initiation of myogenesis in vertebrates (Francetic and Li, 2011). play a key role in regulating muscle development and growth (Shi et al., 2022). Though no comparative study was found to compare with present results, decreasing trends in the expression of MyoD and MYF5 with age DAH indicated the normal and expected pattern of muscle cell specification and proliferation to differentiation and maturation. Generally, during development, MyoD and MYF5 play a role in initiating the formation of myoblasts (muscle precursor cells), thus showing higher expression during early development. However, as muscle development proceeds, myoblasts exit the cell cycle and begin to differentiate into mature muscle fibers. Thus, there is a downregulation of MyoD and MYF5 factors and an up-regulation of other myogenic factors for the promotion of muscle tissue differentiation and maturation. Moreover, comparatively higher expression of these two myogenic factors in response to P55 compared to other protein levels may indicate active muscle cell specification and commitment (Wu et al., 2021).

In the present study, a positive correlation between GH and dietary protein level up to 55% CP indicates the role of GH in the regulation of protein synthesis and cellular processes like growth and development. Generally, GH is the main regulator that controls somatic growth (Björnsson et al., 2002) by affecting the target tissue liver, and influencing other tissues like muscles etc. and promoting overall body growth (Herrington and Carter-Su, 2001). The increased GH level in parallel to the proteolytic enzymes, trypsin, chymotrypsin and protease and higher levels of both GH and enzymes in response to 55% crude protein may indicate the higher digestion and conversion of dietary protein into muscle tissue, leading to improved growth performance of *L.rohita* larvae. Though optimum protein is crucial to growth, the dietary lipid level also plays an important role in growth, and energy supply, however, excess lipids may lead to poor feed intake (Mohanta et al., 2008).

Dietary lipids play a major role in providing a good source of concentrated energy, essential fatty acids and fat-soluble vitamins as the fishes have a limited ability to utilize carbohydrates as an energy source (Izquierdo et al., 2000; Tocher 2008; National Research Council (NRC), 2011; Stickney et al., 1989). Lipids involve the provision of high energy values, promotion of the

absorption of fat-soluble vitamins, inhibition of dust, improvement of palatability, etc., which are important for the feed industry (Yang et al., 2019). Many intrinsic and extrinsic factors including water temperature, species, development stage, culture conditions, the types of feed and the ingredients sources affect the dietary requirement of lipids for fish (Miller et al., 2005; Menoyo, 2004, 2006). The present result indicates successful ontogeny of the digestive system of *L. rohita* PLs and comparatively higher growth performance over 8% inclusion of fats, somewhat comparable to 9% inclusion for the best growth performance of juvenile rohu (Gandotra et al., 2017). The optimal level of lipid observed in this study was comparable to recommended by BIS (2014a) for the spawn and fry of carps, fingerlings of *L. rohita,* and a small carp*, Barbonymus gonionotus* (Mishra and Samantaray, 2004). However, it was somewhat higher than reported by other investigators, like 5 to 7% for *C. mrigala* (Gumus & Ikiz, 2009), 6.5% for the fry of *Mystus montanus* (Raj *et al.,* 2007), 7% for *Ompok pabda* fry (Paul *et al.,* 2011), and 7.5% for *Ctenopharyngodon idella* fry (Choi *et al.,* 2015), Moreover, larvae of *Clarias batrachus* and *Silurus asotus* showed best performance at somewhat higher inclusion level 10% and 17% respectively of dietary fat(Kim *et al.,* 2012; BIS 2014b). This difference in optimum dietary lipid level may indicate the need for the optimization of this level in feed according to age and fish species.

The best growth performance at an 8% inclusion level of lipids may be due to protein protein-sparing effect. Generally, at optimum dietary inclusion level, lipids fulfil the energy demand and spare the dietary protein for growth and development (Li *et al*., 2012), the high lipase activity in CF8 groups suggests an efficient utilization of lipids as an energy source. Such protein-sparing effects have been demonstrated in many fish species like salmon *S. salar* (Johnsen et al., 1991), rainbow trout *O. mykiss* (Garcia et al., 1981; Beamish and Medland 1986), carp *C. carpio* (Watanabe et al., 1987), hybrid striped bass, *M. saxatilis*×*M. chrysops* (Nematipour et al., 1992). The observed comparatively poor growth and survival (%) of *L. rohita* larvae at suboptimal levels of dietary lipid (4%) may be due to metabolic disturbance along with a decrease in the efficiency of feed protein and the lack of fat-soluble vitamins and essential fatty acids (Bonvini et al., 2015). Moreover, poor growth performance, survival (%) and low GH level at higher dietary lipid content (16%) may indicate unsatisfactory rearing results (Brown et al., 1996; Kestemont et al., 2003). It is well documented that dietary lipids beyond the optimum level adversely affect the growth rate, normal functioning of the digestive system, feed conversion efficiency and immunity

of the fish (Ai et al., 2008). Many studies reported the suppressed growth rate in different fish species, including Chinese long-nosed catfish, *Leiocassis longirostris* (Pei *et al.,* 2004), mrigal carp, *C. mrigala* (Jafri et al., 1995), small carp, *Carassius gibelio* (Bloch, 1782), tilapia, *Oreochromis aureus* (Stickney and McGeachin, 1983), hybrid tilapia, *O. niloticus* × *O. aureus* (Chou and Shiau, 1996) due to the excessive intake of dietary lipids. The previously reported results are in accordance with the currently observed suppressed growth in response to the 16% inclusion of dietary lipids. The retarded growth observed here and reported by other investigators may be due to inhibition of de novo fatty acid synthesis, and reduced digestion and absorption of nutrients (Sargent et al., 1989). It seems that excessive level of dietary lipid reduces the digestibility and utilization of dietary proteins, thus limiting growth performance (Ling et al., 2006), causing oxidative stress and negatively affecting survival, disease resistance, stress response, and lipid metabolism (Kjær et al., 2008).

Dietary lipid level beyond the optimum requirement also negatively affects the digestive enzymes, especially proteases, lipase and amylase, leading to impaired energy metabolism and suboptimal growth may be due to poor carbohydrate and protein digestion and utilization (Deng *et al.,* 2021). The observed decrease in the activity of protease and amylase and poor growth at 16% inclusion level is in accord with the literature which indicated the reduced activity of these enzymes in response to excessive dietary lipid in a variety of fish species, including the Asian red-tailed catfish, *H. wyckioides*, the orange-spotted grouper, *Epinephelus coioides*, the Munchurian trout, *B. lenok*, the rainbow trout, and the Japanese sea bass, *L. japonicus* (Deng *et al.,* 2021). Intestinal ALP is an important enzyme that is mostly affected by dietary lipid levels. It is involved in the digestion and absorption of nutrients, mainly lipids and phosphate compounds, maintenance of intestinal health and bone metabolism (Santos et al., 2022). The comparatively higher level of ALP in the CF8 group was parallel to growth performance and may indicate the improved hydrolysis of dietary lipids into fatty acids and glycerol and their absorption through the intestine.

The ontogeny of fish larvae may directly be associated with myogenesis. Lipids inclusion rate did not regulate MYF5 unlike MyoD expression in the guts, which suggests that lipids inclusion by 8% enhances the organogenesis, as high MyoD to MYF5 expression may lead the cells to exit their cycle into differentiation (Kitzmann and Fernandez, 2001). The growth pattern also justifies the significantly high

expression of GH in *L. rohita* PLs in CF8. These two trials indicate the optimum dietary requirement of crude protein and crude fat in the larval feed of *L. rohita.*

After understanding the ontogenetic variation in the digestive enzymes trend in response to LF and ND and various protein and lipid levels a standardized nanoparticulate feed (SNF) with 55% CP and 8% crude fat was formulated and prepared (Table 7) and 35 days feeding trial was conducted for the comparative evaluation of efficiency SNF with LF. During the experiment, Lf was collected from earthen ponds, therefore proximate composition as well as fatty acid profile was analyzed periodically. It is well-documented that environmental factors, especially water temperature affect the relative abundance of plankton and their proximate and biochemical composition (Bonnet et al., 2009; Tulsankar et al., 2021; Sun et al., 2022). During feeding trial, the higher level of SAFA and low level of PUFA in LF compared to SNF may be associated with the abundance of lipid-deficient cyanobacteria (Müller-Navarra et al., 2000; Wacker et al., 2001)., According to the literature, SAFA predominantly meets the need for energy during the early stages (Mourente and Tocher, 1992; Farhoudi et al., 2011). Though SNF was significantly lower in SAFA compared to LF, it assumed that a high carbohydrate level in SNF compensates for the energy provision of the larvae. Additionally, high MUFA in SNF may also compensate for the lower SAFA as fish larvae use MUFAs mostly as energy sources (Tocher 2003; De Roos and Persson 2013).

The observed improved growth performance in response to SNF may be due to the presence of significantly higher levels of unsaturated FA (MUFA and PUFA: both n3 and n6) in SNF, which are crucially required for the successful ontogeny in fish (growth and survival). For instance, omega-3 fatty acids in salmonid feed enhanced pigment uptake and resistance to stress and diseases (Sargent et al., 2002; Glencross, 2009). Many investigators reported the important role of PUFAs like EPA, ARA and DHA for growth and reproduction (Heckmann et al., 2008; Brett et al., 2009; Schlotz et al., 2012; Twining et al., 2016) like DHA is an integral constituent of neural membranes (Feller, 2008; Wassell and Stillwell, 2008; Tocher, 2010) and visual cell membranes (Brett and Muller-Navarra, 1997). The absence of DHA in a larval feed of herring impairs visual performance (Bell et al., 1995; Shields et al., 1999) while more DHA was correlated with enhanced growth via enhanced vision to take feed in cyclpoid larvae. Fish larvae usually take feed by sight (Conceica et al., 2010).

The proximate body composition of fish commonly includes moisture, protein, lipid, and ash (Ahmed et al., 2022). It is influenced by their diet, life stage, body size and habitat of fishes (Breck, 2014). The present study also indicated the change in proximate composition and fatty acid profile of *L. rohita* while rearing on LF and SNF. Initially, both groups of PLs did not show any difference in their whole-body proximate composition, however from 20 DAH larvae differences started appearing and remained in fries (35 DAH) collected at the end of the feeding trial. The somewhat higher CP content and lower moisture in larvae reared on SNF may be due to size/body difference, The decrease in moisture content and increase in protein content with an increase in the size of fish is well documented (Ahmed et al., 2022)

The nutrient composition of the diet can influence the fatty acid profile of fish (Taşbozan and Gökçe, 2017). It seems variation in the fatty acid profile of larvae reared on SNF and LF could be due to types and amounts of fatty acids in the fish's diets (Tables 28& 29). It is known that fish bioaccumulate and incorporate fatty acids into their own tissues from their food. Many investigators reported a decrease in SAFA with age in different fish species while feeding *Schizochytrium limacinum* based feed, *Salmo salar* (Kousoulaki et al., 2015, 2020), giant grouper (*Epinephelus lanceolatus*) (Garcia-Ortega et al., 2016), longfin yellowtail (*Seriola rivoliana*) (Kissinger et al., 2016), *Pagrus major* (Seong et al., 2019) and olive flounder (*Paralichthys olivaceus*), (Qiao et al., 2014) and suggested that these fatty acids are catabolized as an energy source, improving deposition of long-chain polyunsaturated fatty acids (LC-PUFA) in the fish flesh (Sargent et al., 2002; Turchini et al., 2009). However, contrary to the literature, the present study observed a slight increase in SAFA in the whole-body composition of both groups of larvae/fries. It could be due to enough supply and availability of SAF in diets. The high carbohydrate level in SNF might also be the reason for increasing SAFA in the body composition of *L. rohita* fries. Moreover, a slightly higher level of PUFA in larvae reared on SNF could be the higher PUFA content in feed. The PUFA retention in the body indicated growth and development (Sargent et al., 2002; Glencross, 2009; Qiao et al., 2014; Eryalçin et al., 2015; Kissinger et al., 2016; Cardinaletti et al., 2018; He et al., 2018; Kousoulaki et al., 2020). The retention of EPA and DHA in fish tissues is important for both fish growth and fish health (Glencross, 2009; Bou et al., 2017) as DHA is used in the membrane of body tissues while EPA is selectively used for β-oxidation (Sargent et al., 2002; Bou et al., 2017). It seems comparatively better levels of both PUFA in SNF improved the growth of larvae.

Digestive enzyme activities reflect the ability of fish to digest and absorb nutrients (Xia et al., 2018). The regulation of enzymes in response to diet ultimately effect the growth performance (Wang, 2007). During early rearing feed both in terms of composition and availability directly induces the development and maturation of the digestive tract (Ma et al., 2005). In the present study, the higher activity of all enzymes except amylase (similar in both groups) and cellulase in response to SNF may indicate improved digestion of nutrients as compared to nutrients in LF. This could be related to the balanced nutrient profile in SNF, as it was prepared after the determination of major nutrient (protein and lipid) requirements or may be due to the size and texture of feed particle size. It is well documented that prepared feed possesses a relatively balanced nutrient profile (Baskerville-Bridges and Kling, 2000), while Nanoscale ingredients have distinctive physical or chemical properties due to their small size, shape, high surface area to volume ratio (Chau et al., 2007) and have higher absorption and assimilation of ingredients. The positive effect of Nanoscale ingredients on fish growth, immunity and the antioxidant system is well-reported (Carriquiriborde et al., 2004; Faiz et al., 2015; Kanwal, 2012; Jamil 2013; Khan et al., 2017; Kumar et al., 2023 It seems relatively stable nutrient profile of SNF, while small size feed particles with smooth surface texture facilitate the larvae to adjust to exogenous feeding, improved digestion, and absorption and assimilation and reduce nutrient loss in fecal matter (Pieszka et al., 2019). The digestive enzyme activity during the rearing of larvae is parallel to growth performance data and supports the higher efficiency of SNF compared to LF.

Moreover, comparatively low activity of digestive enzymes and growth performance in response to LF may be due to an imbalanced nutrient profile since live feed does not have all the essential ingredients required for growth (Hertrampf and Piedad-Pascual, 2003). Literature reveals nutrient anomalies in *Dicentrarchus labrax* (Linnaeus, 1758) and *Solea solea* (Linnaeus, 1758 (Hamza et al., 2016) in response to live feed during early rearing. The relationship between feed composition and digestive enzymes profile is well illustrated in different fish species e.g., *Blicca bjoerkna,* Linnaeus, 1758 (Cara et al., 2003), *Pagrus pagrus,* Linnaeus, 1758 (Suzer et al., 2007), *Gadus morhua* (Wold et al., 2007) etc.

Here, the higher activity of cellulase in response to LF may indicate the higher colonization of bacteria/microorganisms. Generally, most vertebrates, including fish don't produce cellulase endogenously but derive this from bacteria and other microorganisms that harbour the gut. According to Deng et al. (2021), early exposure of larvae to different microorganisms modulates their larval intestinal microbiota community which in turn affects the growth and digestive

enzymes activity of the host. Like the present observation, Fagbenro (1990) also observed high cellulase activity in the catfish (*Clarias agboyiensis* (Sydenham, 1980) =*Clarias isheriensis* Sydenham, 1980) reared on plankton and suggested the establishment of gut microbiota in the presence of live feed. Moreover, the lower cellulase activity in a group reared on SNF may be due to the limited number of microbiotas.

Furthermore, the gradual increase in the activity of protease, lipase, trypsin, chymotrypsin, and ALP was in accord with the literature (Kolkovski, 2001) and indicated the maturation and successful transformation of the digestive system (Peňa et al., 2016). However, higher activity of these enzymes in a group reared on SNF than LF may signify higher efficacy of SNF. Ontogeny of digestive system comprises stepwise development of pancreatic enzymes (lipase, trypsin, chymotrypsin, protease, amylase) followed by secretion of intestinal enzymes (ALP) and lastly stomach formation (Gisbert et al., 2009) with exception of no stomach formation in carps (Chakrabarti et al., 2006); loaches (Cobitidae), killifish (Cypridontidae), clingfish (Gobiesocidae) and gobies (Gobioidae) (Harder, 1975). The digestibility of nutrients reflects the growth of fish larvae (Wang et al., 2006). The availability of nutrients in feed modulates metabolism by altering the major digestive enzymes (Goswami et al., 2020) and morphological transformation of the digestive tract (Moraes et al., 2020). The observed morphological change in villus height, villus width, and crypy depth in response to SNF and LF (Table 30, Fig. 32) also indicated the role of feed in the development of digestive system.

Among the most crucial intrinsic factors that govern digestion, growth and development during early ontogeny include the gut-brain axis (Goldstein et al., 2021), GH-IGF system, Myogenic regulatory factors (MRF's) system and other crosslinking hormones and enzymes (discussed earlier). GH and *IGF-1* pathway seem to be significantly induced by SNF as high expression of these genes were observed in the SNF group. *IGF-1* regulates body growth and induces differentiation and proliferation (Wood et al., 2005). However, GH and its receptors (mainly hepatic GHR) govern the expression of *IGF-1* (Picha et al., 2008). Like present results, high expression of *IGF-1* mRNA in the muscles was reported to enhance growth in *O. niloticus* (Eppler et al., 2007). The lower myostatin expression in SNF also justifies the upward growth curve. Myostatin acts as a negative growth regulator that prevents satellite cell proliferation during muscle growth development (Garikipati and Rodgers, 2012). myostatin acts as a growth suppressor that inhibits the proliferation and differentiation of satellite cells during muscle growth

development (Prabu et al., 2020; Prabu et al., 2021). In fish species, dietary changes alter the relative expressions of myostatin (Sudha et al., 2022).

The MRFs are a family of highly conserved sequences that play different functions in the myogenesis process of fish growth (Rescan, 2005). Myogenic regulatory factors (MRFs), a family of basic helix-loop-helix (bHLH) transcription factors, play a pivotal role in the determination and differentiation of skeletal muscle and they have the property of converting a variety of cells into myoblasts and myotubes. Members of this gene family like MyoD, MYF5, and myogenin have been identified in many fish species and found to be expressed in developing somites and skeletal muscles though no report is available in any Indian carp species. A delay/retention of MyoD and MYF5 expression in SNF along with high MEF2A expression suggest the continuation of tissue differentiation till the fry' stage as MyoD and MYF5 play a common role in establishing myoblast identity, whereas myogenin is involved in terminal differentiation by regulating the expression of different muscle proteins and enzymes like myosin, troponin, and creatine kinase (Du et al., 2003). High myogenin expression in SNF till 30DAH may be related to an intense differentiation and fusion of myoblasts to existing myofibers during hypertrophy, as reported by Johansen and Overturf (2005), these shifts induce the development of digestive structure as seen in Figure 36 and Table 39. The villi length and width significantly enhanced with SNF may be due to the above-mentioned pattern of MRF's expression in response to live and prepared feed. However, the sudden surge in myogenin expression on 35DAH may suggest compensatory weight gain or the nutrient in the live feed might be available by that period (as variation in species composition has been shown in experiment 1). MyoD and MYF5 synergistically regulate the expression levels of myogenic genes genome-wide by binding their consensus E-box sequences (Kim & Shendure, 2019). MyoD has been well-established as a master transcription factor in myogenic cell-lineage specification during development (Rudnicki et al., 1993; Davis et al., 1987; Weintraub et al., 1987). The early high expression of MYF5 and MyoD suggests their potential role in tissue differentiation. Similar high expression was reported in common carp at 30 h after fertilization (Cole et al., 2004). In the present study, the high expression of MyoD and MYF5 in the embryonic stages of *L. rohita* may indicate a consistent postnatal muscle growth involving hypertrophy of muscle fibers which require additional nuclei to maintain a relatively constant nuclear to cytoplasmic ratio. These nuclei are provided by activated myogenic stem cells which also express myogenic proteins. Similarly, in general, MYF5 and MyoD expressions in skeletal muscles are followed by upregulation of myogenin and of MEF2 family factors. These results also confer the

early ALP activity in the SNF which indicates an early brush border development, also justified in the histological observation of guts.

Conclusion

The classical conventional method of rearing fish larvae over live feed is critical both spatially and temporally. Our study shows that Live feed including zooplankton and phytoplankton significantly alter during the different season in accordance with temperature and other physical properties of water. Such change alters the nutrient value of the available feed for fish/rohu larvae. Replacing live feed with a balanced formulated nano particulate feed partially and completely has met with success up to some level. Briefly, the inclusion of formulated prepared diet has enhanced growth (both alone and in the co-feeding group) however, high mortality was seen with formulated feed alone. The reason was possibly the nutrient profile of formulated nano feed (as the nutritional requirements of rohu larvae were not known). Secondly, the lower expression of *Ghrelin* and GHSR and high expression of *leptin* in the guts (in response to formulated nano feed) suggest poor compatibility with the digestive system, while high *IGF-1* expression indicated high growth. The optimization trials for macro-feed ingredients indicated that for successful ontogeny of rohu larvae, 50-55% protein and 8-12% lipids are required. Feeding formulated one standard diet with optimized protein and lipid concentration, our inference indicated very lower mortality and higher growth with SNF. Molecular study has revealed a relatively higher expression of growth factors with SNF compared to Live feed which possibly resulted in higher net weight gain. Further, the expression of Myogenic Regulating Factors (MRFs) including MYF5, MyoD, myogenin, and MEF2A, which govern the differentiation and maturation of somite cells into myocytes, was also regulated significantly by SNF. The early weaning with SNF implicates successful ontogeny which also resulted in higher survival %. Overall, this study confirms the role of the extrinsic factor, larval feed on the regulation of ontogenetic changes in the release of digestive enzymes and their effect on survival and growth and indicates the most significant effect of SNF compared to live feed. Thus, the replacement of life feed with SNF could be recommended for improving the survival and growth during early rearing and to produce quality seed of *L. rohita*.

Future perspective

To improve the formulation, standardization of vitamins and minerals is highly suggested. Secondly, understanding the digestibility of protein, fats, and carbohydrates by rohu larvae will open a window for trying other feed ingredients alternatives like the inclusion of hydrolyzed protein, the inclusion of simple carbohydrates. Finally, a very significant study recommended related to rohu ontogeny is the profile set up of gut microbiota over ontogeny, while rearing on live feed and prepared feed.

SUMMARY

Good quality fish seed and feed according to nutritional needs are the integral components required for the economic viability of any aquaculture system. The quality/health of the seed directly depends on the feed/nutrients provided during ontogeny. During the development of fishes, there are sensitive periods which are responsive to extrinsic factors including feed/feeding and affect the growth and survival during early rearing. Therefore, insightful knowledge regarding the role of feed in the regulation of feed intake, release of digestive enzymes and early growth is a prerequisite for improving the survival and growth of fish seeds. Rohu (*Labeo rohita*) is the abundantly cultured Indian major carp in most Asian countries including Pakistan. It is popular in polyculture systems and mostly reared in earthen ponds. However, higher mortality and retarded growth during early rearing and low-quality seed are the major challenges for the sustainable culture of this species. Due to the unavailability of larval-prepared feed, postlarvae (PL) are generally cultured in nursery earthen ponds on live feed. The present study was designed to study the impact of live feed and prepared larval feed on the ontogeny of the digestive system of *L. rohita* and addresses the major limitations with live feed, challenges in preparation and formulation of larval feed of *L. rohita*, standardization of macronutrients and preparation of feed that could be intake and digest easily and positively affect the growth and survival during early rearing. The study was conducted in four phases. In the first phase nursery ponds were prepared by conventional methods, i.e., by liming and fertilization (animal manure and NPK fertilizer) and live feed was produced. The fertility of the ponds was checked with Sacchi disc and maintained from March to October by supplementary fertilization. The density, diversity, fluctuation, and nutritive value, i.e., proximate composition and fatty acid profile of plankton were assessed during the studied period. Results indicated month-wise variation in primary and secondary productivity of ponds, with a significantly high phytoplankton biomass ratio compared to zooplankton mass during March, April, and May, while zooplankton mass dominated from May onward. Furthermore, among phytoplankton mass, green algae dominated during March, April, and May while Diatoms dominated the rest of the months. Similarly, rotifers, cladocerans, and copepods dominated the overall zooplankton mass. Species-wise occurrence indicated *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella variabilis*, *Aphanothe ceconferta*, *Cryptococcum Brasiliense*, *Oscillatoria sancta*, *Navicula bottnica*, *Eudorina elegance*, *Pediastrum tetras* as the predominant phytoplankton species across all the months (Fig 5), while *Asplanchna* spp., *Brachionus* spp.,

Daphnia spp. and *Moinea* Sp. dominated the zooplankton community. The proximate composition of both phytoplankton and zooplankton indicated an increasing trend in CP (%), CF (%) and dry matter (%) from March to August. The fatty acid profile indicated a gradual increase in saturated fatty acid (SAFA) from March to August with a peak value in August. Unlike SAFA, MUFA decreased during July and August, while their peak values were observed in March and April. Among all n-3 and n-6 polyunsaturated fatty acids, Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) indicated an increasing trend from March to July and subsequently showed a decreasing trend. In the second phase of the study, based on the proximate composition of live feed, a 60% CP nanoparticulate larval diet (ND) was prepared and a 35-day feeding trial in a replicate of five was conducted to evaluate the impact of partial and total replacement of live feed (LF) with ND on rohu (*L. rohita*) during early rearing. Larvae, 3 days after hatch (3 DAH) were evenly distributed into 3 groups; T1 reared exclusively on LF, T2 on ND and T3 co-fed with both LF and ND (1:1). For ontogenetic changes, sampling was done at 3,10,15,20,25,30 and 35DAH from the experimental groups. All groups showed feed dependent increase in growth and the expression of genes involved in feed intake and growth with age DAH. The T3 group showed significantly higher WG, SGR, and expression of *IGF-1* followed by the T2 group while the highest expression of *ghrelin* and *GHS-R* were observed in a T3 followed by a T1 group. Furthermore, *leptin* showed the highest expression in a T2 followed by the T1 group. The intestinal enzymes showed variable trends with the highest activity of cellulase, amylase and protease in the T1, T2 and T3 groups respectively. Moreover, in all groups, cellulase increased continuously with age DAH, while amylase and protease showed a positive correlation up to 30 DAH and then declined. The overall result of this trial showed the most significant effect of co-feeding followed by feeding ND, thus indicating the need for standardization of the prepared diet by determining the optimum requirement of CP (%) and crude fat (%). Thus, in the third phase of the study, two independent experimental trials were conducted to standardize protein and fat levels in the larval feed of *L. rohita*. To optimize the dietary protein requirement, four experimental larval feeds having 45, 50, 55 & 60 % CP were prepared and a 35-day feeding trial in replicate of five was conducted. The 3DAH larvae were evenly distributed in 20 small circular tanks, randomly divided into 4 groups and fed on their respective diet. The sampling was again conducted at 3,10,15,20,25,30 and 35DAH. Results of this trial indicated a significant impact of protein concentration on the growth, ontogeny of the digestive system and expression of growth-regulating hormones/factors (GH, MYF5 and MyoD). Among all diets, P55 followed P60 showed the most

significant positive effect on all studied indices except amylase activity which did not show any significant difference among groups. Moreover, all groups showed a significant age-dependent increase in trypsin and chymotrypsin while protease and amylase showed a curvilinear relationship with age DAH, i.e., increasing trend (positive correlation) up to 30DAH and 25 DAH respectively and then decreasing trend. Moreover, in all groups, GH showed a positive correlation, while MyoD and MYF5 showed a negative correlation with age DAH. To optimize the dietary lipid level, another experiment was conducted. Experimental design and rearing conditions were the same as reported for the evaluation of optimum dietary protein requirement except that 50% CP was fixed in experimental diets while fat (fish oil) was added at graded levels (4, 8, 12, 16 %), using fish oil as the major source. Here again, both independent variables, i.e., lipid inclusion level and age DAH showed a significant effect on survival (%), growth, activity of intestinal enzymes, and expression of genes regulating growth /myogenesis. Lipid at 8% followed by 12% inclusion level showed the most significant effect on all studied indices except on the activity of lipase which did not show any significant difference among groups at any sampling time. The growth indices showed a positive correlation with age DAH while ontogenetic changes in the release of digestive enzymes pattern indicated a uniform increase of alkaline phosphatase (ALP) and lipase with age DAH, in all groups, while amylase trend was unique; an initial high amylase activity followed a decline then a steep surge at 25DAH and again decrease after 25DAH in all the experimental groups. The ontogeny variation of protease activity showed a uniform increase up to 25DAH, followed by a surge at 30DAH and then slight but non-significant decrease in activity. The ontogenetic changes in GH gene expression showed a positive correlation with age DAH, i.e., expression uniformly increased with age DAH while MyoD and MYF5 expression showed a negative relation with DAH, i.e., uniformly decreased with age DAH. After understanding the ontogenetic variation in the digestive enzymes trend in response to live feed, ND and various protein and lipid levels a standardized nanoparticulate feed (SNF) with 55% CP and 8% fish oil were formulated and prepared and 35 days feeding trial in a replicate of 5 was conducted to compare the efficiency of SNF as compared live feed (LF). The 3DAH larvae were equally distributed in 10 small circular experimental tanks (capacity: 8.0L), at the rate of 500 larvae /tank. The tanks were randomly distributed in two groups: LF and SNF and PLs reared on their respective feed, i.e., live feed and SNF respectively. The sampling was again done at 10, 15, 20, 25, 30 and 35 DAH. The fatty acid profile of both LF and SNF showed significantly higher levels of MUFA and PUFA in the SNF compared to LF. Results indicated a significant effect of both independent variables (feed and age

DAH) on all studied indices. The comparative effect of LF and SNF indicated significantly higher survival (%), growth rate, activity of digestive enzymes except lipase and expression of MyoD, MYF5, GH, *IGF-1* and MEF2A in a group reared on SNF while, myostatin expression was significantly higher in a LF group compared to SNF group. The ontogenetic pattern of intestinal enzymes indicated a slight but significant change in protease activity in both LF and SNF groups from 03 to 15 DAH. However, the difference in protease activity became more evident as the larvae development progressed. The other proteolytic enzymes also showed a positive correlation with age DAH, however, both trypsin and chymotrypsin uniformly increased up to 20 DAH and 15 DAH respectively and then showed a sudden surge and afterwards also showed an increasing trend with a higher level of activity. The ontogenetic trend of amylase activity indicated initial high activity followed by a slower decline till 10 DAH, then gradually increase and a resurge at 25 DAH followed by a relatively less steep decline to 35 DAH, in both LF and SNF groups. In contrast to amylase, cellulase, lipase, and ALP activity showed a positive correlation and increased uniformly with age DAH. During ontogeny, the expression pattern of the studied genes showed variation, like the expression of both MYF5 and MyoD significantly decreased with age DAH uniformly, while, unlike these genes, *IGF-1*, GH, Myostatin and MEF2A showed a positive correlation with age DAH. However, myogenin showed sudden down-regulation after 3DAH which remained stable till 15DAH in both groups, however after 15DAH, the expression level of myogenin decreased in a LF group up to 25DAH and then increased while in SNF group showed a continuously increasing trend. Both lipase and proteases were seen to be enhanced with optimum lipid and protein level in the diet (experiment 3 and 4). Discussing this increase in proteases and lipase as the possible outcome of efficient utilization (in term of early maturation of digestive tissues/organ) of optimum protein and lipids, in experiment 5 although a high protein % was available in LF but the trypsin and chymotrypsin activity was significantly higher in SNF group. This inference may conclude a slight instantaneous impact of diet over direct release of certain digestive enzymes, however the major change come from the maturation of that tissue. Further, the only slight change in proteas activity in experiment 5 (compared to experiment 3, P55) may be due to the early shift of larvae to gut peptidases (high trypsin and chymotrypsin activity with SNF). Overall, this study confirms the role of the extrinsic factor, larval feed on the regulation of ontogenetic changes in the release of digestive enzymes and their effect on survival and growth and indicates the most significant effect of SNF compared to live feed. Thus, the replacement of

life feed with SNF could be recommended for improving the survival and growth during early rearing and to produce quality seed of *L. rohita*.

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