



Optimization of weaning strategy in the climbing perch (*Anabas testudineus*, Bloch 1792) larvae on growth, survival, digestive, metabolic and stress responses

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Abstract A 30-day experiment was carried out to know responses of different weaning approaches to the growth and survival of *Anabas testudineus* larvae. A total of 10800 larvae (Avg. weight 0.016 ± 0.03 mg; 3DPH) were randomly distributed in nine treatments (triplicates), including two controls. The strategies are as follows: C1 (Control I): feeding with live food (LF) for 30 days and C2 (Control II): feeding with micro-particulate diet (MPD) for 30 days; T1: LF for 5 days

and MPD for next 25 days; T2: LF for 10 days and MPD for next 20 days; T3: LF for 15 days and MPD for next 15 day; T4: LF for 20 days and MPD for next 10 days; T5: LF for 25 days and MPD for next 5 days; T6: LF for 5 days, then 25% LF replacement by MPD for next 5 days, 50% LF replacement by MPD for next 5 days, 75% LF replacement by MPD for next 5 days, and 100% LF replacement by MPD for last 10 days; and T7: LF for 10 days, then 25% LF replacement by MPD for next 5 days, 50% LF replacement by MPD for next 5 days, 75% LF replacement by MPD for next 5 days, and 100% LF replacement by MPD for last 5 days. Significantly ($p < 0.05$) higher WG and SGR were recorded in T2 (213.17 ± 0.32 , 23.98 ± 0.02) followed by T6, whereas the lowest was found in C2. Significantly higher ($p < 0.05$) percentage survival was manifested in the T7 (31.83 ± 0.22), followed by T2 (24.75 ± 0.13), and the lowest survival was observed in the C2. The digestive enzyme activities were found to be non-significant ($p > 0.05$) between different treatment groups. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) were reported to be significantly higher ($p < 0.05$) in C2 (68.52 ± 0.08 , 19.55 ± 0.10 , 21.79 ± 0.04 , and 0.044 ± 0.01) followed by T1; however, their reduced level was observed in C1. The activity of superoxide dismutase (SOD), catalase (CAT), glucose, and cortisol levels was observed significantly ($p < 0.05$) higher in C2 and lower in C1 and T2. As per the finding, it can be recommended that

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the appropriate weaning time for *A. testudineus* larvae is from 13 DPH onwards, in which larvae can be fed an initial ten days LF afterward MPD and the best weaning strategy can be adopted as in the T7 group for higher survival percentage.

Keywords *Anabas testudineus* · Weaning · Microparticulate diet (MPD) · Growth and survival · Digestive enzyme

Introduction

Species diversification is one of the important strategies in aquaculture to increase fish production. The selection of high-demand species is important to getting a higher return. *Anabas testudineus*, also known as koi or climbing perch, is one of the promising fish found in several countries, including Indian subcontinent (Tan and Lim 2004). It is well known for its superior taste and flavor and is a good source of iron and copper (Sarma et al. 2010; Bhuyan and Hussain 2018). It is highly resistant to unfavorable environments due to its air-breathing ability, which makes it a preferred species for aquaculture (Van Trieu and Long 2001). After fertilization, eggs will hatch in approximately 24h. The newly hatched larvae are 2–3-mm long and begin free swimming within two days (Wei et al. 2021). During larval development, the yolk sac is completely absorbed within 72h, indicating the first external feeding should be begun (Basak et al. 2014). The process of transforming the notochord into vertebrae through segmentation, as well as the development of the alimentary canal, takes place within a time frame of 144 to 192h. The final expansion of paired and median fins, mouth, and scale formation is completed by 336h after hatching. Pectoral, pelvic, caudal, and dorsal fins with fin rays fully form during this stage (Basak et al. 2014). The predominant digestive enzymes found in *A. testudineus* larvae are proteases and amylases (Chandan et al. 2023). Protease activity is higher than amylase activity, reflecting the carnivorous feeding habits (Singh et al. 2023). Protease activity within the larval gut is significantly higher in the hindgut region than in the foregut (Mondal et al. 2008).

Despite its cultural potential, species is not widely cultivated due to a lack of seed availability (Kohinoor et al. 2012; Jayasankar 2018). In this context,

successful larval culture for this species is one of the challenging tasks as the larval survival rate is very poor (Hamre et al. 2013; Singha et al. 2015, Singh et al. 2019; Alang et al. 2020), probably due to a lack of suitable live food (Rao 2003) and weaning diet and weaning strategy (Kolkovski 2001; Hauville et al. 2014; Jayasankar 2018).

Formulated microparticulate diet (MPD) is advantageous over live food because of the balanced nutrient supply, bulk preparation, easy storage, and lower cost of production (Holt 2011; Thompson et al. 2019). However, larvae are always very much fond of live prey like Rotifer, Moina, and *Artemia nauplii*, which are preferable live foods for koi larvae (Bell et al. 2003; Hamre et al. 2013). But the production cost of live food is high, and it is not nutritionally balanced (Cahu et al. 1998; Callan et al. 2003; Ma et al. 2015; Jamali et al. 2018) and can act as a vector for pathogens (Bonaldo et al. 2011). On the other hand, koi larvae are reluctant to accept artificial feed alone (Conceição et al. 2010; Rønnestad et al. 2013; Chen et al. 2020). Therefore, a suitable weaning strategy is required to be standardized for the large-scale culture of koi larvae with optimum survival rate (Kolkovski 2008), and the formulated feed should be nutritionally balanced with higher nutrient digestibility and well accepted by the larvae (Giri et al. 2002; Radhakrishnan et al. 2020).

Some research has been conducted on larval rearing of *A. testudineus* for higher seed production (Zalina et al. 2011; Singh et al. 2019) but failed to establish a suitable weaning strategy for successful larval culture (Ahilan and Thangarani 2020; Lazo et al. 2007). However, the majority of researchers suggest that koi fish larvae have a distinct and well-defined digestive physiology, and this information could be helpful in developing suitable MPD and optimal weaning protocol for higher survival and production of larvae (Lazo et al. 2000; Thompson 2013).

Weaning is a transition from one feed to another as well as a period of adjustment in which both types of feeds are supplied for an extended period, with a gradual reduction of live food and a gradual increase in prepared diet (Chèvre et al. 2011; Williot et al. 2011). This is especially required for carnivorous fish larvae, which have high protein and lipid requirements for both energy and body development (Andersen et al. 2016). A relatively successful weaning strategy involves mix feeding with live food and a formulated

diet for some period of time (Ljubobratović et al. 2015; Hung et al. 2022); however, co-feeding also plays a significant role in optimizing nutrient intake in the fish larval stage. Co-feeding results in significantly higher ($p < 0.05$) growth compared to just live feeds or microdiets alone during weaning (Djellata et al. 2021). Sudden weaning with a direct shift from live food to an artificial diet has poor success in larval survival (Herath and Atapaththu 2013). Live feeds provide exogenous enzymes and dietary compounds that stimulate larval digestive activity (Pradhan et al. 2014; Khoa et al. 2020). Co-feeding takes advantage of both live feed and artificial diet, enhancing digestion and growth (Djellata et al. 2021).

Recent advancements in MPD composition have substantially shortened the early weaning period, enabling finfish culture to begin feeding on a specific larval diet as soon as the mouth opens (Cahu and Infante 2001). Weaning is most important stage in larval rearing because larvae need soluble proteins and fatty acids, which can be provided through a prepared diet (Rønnestad et al. 2013). A pre-digested protein source called fish protein hydrolysate (FPH) may be added as a dietary source of protein in the MPD. FPH has a simple form of proteins that includes peptides and amino acids, which serve as feed stimulants and attractants, which improve the diets flavor and acceptance (Chotikachinda et al. 2013; Siddaiah et al. 2022). In order to enhance larval growth performance as well as survival by incorporating FPH has been observed in some species, such as Atlantic salmon, *Salmo salar* (Berge and Storebakken 1996), Rainbow trout, *Onchorhynchus mykiss* (Dabrowski et al. 2003), and *Clarias magur* (Kumar et al. 2022). The FPH might aid in the digestion and absorption of artificial food (Zambonino-Infante et al. 1997; Carvalho et al. 1997; Cahu et al. 1999). Both abruptly weaned and gradually weaned larvae showed improved development and survival when FPH was added to a formulated diet (Kotzamanis et al. 2007; Srichanun et al. 2014). Anguas-Ve'lez et al. (2000) also noted that larvae fed with FPH had the highest overall rate of survival. Several papers discuss the advantages of an FPH-enriched larval diet, which raises the percentage of survival (Cahu et al. 1999; Gisbert et al. 2009; Kumar et al. 2022).

Considering above backgrounds, the current study was conducted to develop a suitable weaning strategy for *A. testudineus* larvae and its effects on survival,

growth, stress, and physio-metabolic and other enzyme responses.

Materials and methods

Experimental animal

The experiment was conducted at ICAR-Central Institute of Fisheries Education, Kolkata Center, West Bengal (India). *A. testudineus* larvae were produced through induced breeding by administering WOVA-FH™ (Biostadt India Limited, Kolkata) into females (23–38g; $n=3$) at 0.4 mlkg^{-1} and male (21–34g; $n=3$) at the rate of 0.2 mlkg^{-1} (Singh et al. 2019). After hormonal injection, male and female brooders were transferred into the fiber-reinforced plastic (FRP) tank at a 1:1 ratio. After 8–12h of latency period, eggs were released and temperature was maintained at $27 \pm 2 \text{ }^\circ\text{C}$ with mild aeration. Newly hatched larvae stocked in FRP container for the 3-day post hatch (DPH) period.

Microparticulate diet (MPD) preparation

The proximate analysis of ingredients and diet is mentioned in Table 1. The diet was prepared and as protein sources fish meal, groundnut oil cake, and FPH, whereas wheat flour was a carbohydrate source used. Butylated hydroxyl toluene was used as a preservative (antioxidant), and sodium alginate was used as a binder. A vitamin-mineral premix, vitamin C, and betaine hydrochloride were used as additives. Ground all the ingredients as suggested in the formulation, and mix them uniformly. Except for additives and oil, all other ingredients are mixed and the dough is made by mixing the water, then kept in a thick plastic bag and transferred for 25–30 minutes for steam cooking. After that, the mixture was crushed to allow it to cool. The other ingredients, namely, oil, BHT, soy lecithin, sodium alginate, betaine hydrochloride, mineral-vitamin mix, and FPH, were mixed properly. To make pellets, the mixture was placed in the pelletizer (SB-Panchal, Mumbai), then made in uniform spheroid size by using a spheronizer (die size: $200\mu\text{m}$) and dried using a mechanical dryer (UNIFLUID NANO, India). The dried feed (size approximately $200\text{--}250\mu\text{m}$) was kept in a closed container, marked properly, and kept in a cool place.

Table 1 Formulation and proximate composition of microparticulate diet used for feeding of *A. testudineus* larvae

Ingredients	Inclusion (%)
Fish meal	25.00
FPH ¹	15.00
Soybean meal	25.00
GNOC ²	17.00
Wheat flour	9.70
Cod liver oil	1.50
Sunflower oil	1.50
Vitamin mineral mix ³	1.50
BHT ⁴	0.02
Soy lecithin	2.00
Sodium alginate	1.00
Vitamin C	0.03
Betaine hydrochloride	0.75
Total	100.00
Proximate composition (dry matter basis)	
Moisture (%)	9.10
Crude protein (%)	48.09
Ether extract (%)	8.17
Crude fiber (%)	5.17
Total ash (%)	12.07
Nitrogen free extract (%)	26.50
Gross energy* (Kcal/100 g)	460.215

*Gross energy content was estimated as follows: (crude protein % × 5.65) + (ether extract % × 9.45) + (total carbohydrate % × 4.2)

¹FPH, fish protein hydrolysate

²GNOC, groundnut oil cake

³Composition of the vitamin-mineral mixture (quantity/kg): vitamin A, 55,00,000 IU; vitamin D₃, 11,00,000 IU; vitamin B₂, 2000 mg; vitamin E, 750 mg; vitamin K, 1000 mg; ascorbic acid, 2500 mg; vitamin B₆, 1000 mg; vitamin B₁₂, 6 mcg; calcium pantothenate, 2500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg; selenium, 125mg

⁴BHT, butylated hydroxytoluene

Experimental design, setup, and management

The ten thousand eight hundred 3 DPH larvae (2.7 ± 0.6 mm (length) and 0.016 ± 0.03 mg (wet body weight)) of *A. testudineus* were stocked in nine experimental groups in triplicates (Fig. 1) such as C1 (Control I): feeding with live food (LF) for 30 days, C2 (Control II): feeding with microparticulate diet (MPD) for 30 days; T1: LF for 5 days and MPD for next 25 days, T2: LF for 10 days and

MPD for next 20 days, T3: LF for 15 days and MPD for next 15 day, T4: LF for 20 days and MPD for next 10 days, T5: LF for 25 days and MPD for next 5 days, T6: LF for 5 days, then 25% LF replacement by MPD for next 5 days, 50% LF replacement by MPD for next 5 days, 75% LF replacement by MPD for next 5 days, and 100% LF replacement by MPD for last 10 days and T7: LF for 10 days, then 25% LF replacement by MPD for next 5 days, 50% LF replacement by MPD for next 5 days, 75% LF replacement by MPD for next 5 days, and 100% LF replacement by MPD for last 5 day. Accordingly, 27 glass aquaria ($45 \times 30 \times 30$ cm³; 40 l capacity and 20 l water volume; stocking density 20 larvae l⁻¹) were used. All aquaria were provided with continuous aeration facilities, and 30–35% of daily water was exchanged with freshwater. The temperature, dissolved oxygen (DO), and pH levels of water were continued at 27.7–29.6 °C, 6–8 mg/l, and 6.9–7.6, respectively, throughout the experimental period. The larvae were fed with LF (*Brachionus calyciflorus*) at the rate of 15–20 individual ml⁻¹ of water while MPD (Fig. 1) was fed five times per day based on visual inspection, approximately amounting to 10% of body weight respectively. Before the feeding at 8:00 a.m., feces were siphoned out every morning. The daily mortality of larvae was meticulously observed and recorded in every experimental aquarium over the entire duration of the experiment.

Growth performance and survival of larvae

At last of the trial, all larvae were collected from every experimental tank, anesthetized with ice, and rinsed in distilled water (DW), and average final body weight (FBW) was measured using an electronic balance. The weight gain (WG) and specific growth rate (SGR) were estimated as

$$\text{WG}(\text{mg}) = \text{Final wet weight}(\text{mg}) - \text{Initial wet weight}(\text{mg})$$

$$\text{SGR} = \frac{(\ln \text{ of final wet weight} - \ln \text{ of initial wet weight})}{\text{Experimental period}(\text{days})} \times 100$$

The survival percentage was calculated by

$$\text{Survival}(\%) = \frac{\text{Total number of larvae harvested}}{\text{Total larvae stocked}} \times 100$$

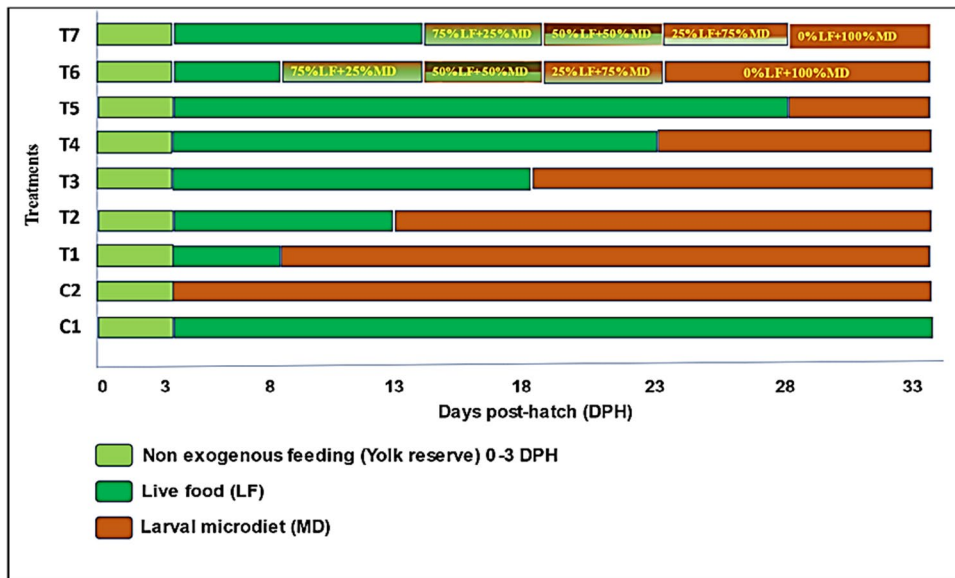


Fig. 1 Schematic representation of nine experimental groups of 3DPH to 33DPH *A. testudineus* larvae

Proximate composition of diet and carcass

The proximate composition of larvae and diet was examined according to the standard method of AOAC (AOAC 1995) on a dry matter basis. Moisture was estimated using a hot air oven (HTLP 28L, Memmert Type HTLP-013, IKON INSTRUMENTS, India), crude protein (CP) [total nitrogen percent (TN %) \times 6.25] was estimated using the MicroKjeldahl Semi-automate unit. The quantification of Ether extract (EE) or lipids was accomplished through the solvent extraction method, employing diethyl ether as the solvent (boiling point 55 ± 5 °C), and crude fiber (CF) content was determined using FibroTRON (FRB-8, Tulin Equipment, India), and total ash (TA) was estimated by muffle furnace at 550 °C for four hours. Nitrogen-free extract (NFE) of diet and total carbohydrate (TC) of the whole body calculated as follows:

$$\text{NFE}(\%) = 100 - (\text{crude protein}\% + \text{lipid}\% + \text{crude fiber}\% + \text{total ash}\%)$$

$$\text{TC}(\%) = 100 - (\text{crude protein}\% + \text{lipid}\% + \text{total ash}\%)$$

In samples, CP, lipid, TA, and TC contents were expressed on a wet-weight basis.

Assays of enzymes

Tissue collection and homogenate preparation

On completion of the experiment, 15–20 larvae from each replicate tank were pooled before the first feeding time, then anesthetized using ice, and instantly stored at -80 °C for biochemical examination. Each larva underwent a distilled water rinse and eliminated excess water using tissue paper before dissection. Whole fish larvae were dissected and anterior, and posterior sections were excised as described by Comabella et al. (2006) on the dry ice packet. Then, the larvae were homogenized while maintaining a cold temperature. A tissue homogenate comprising 0.05-M Tris hydrochloric acid and two percent sucrose was made. Centrifuging (REMI, India) at 5000 rpm for 10 min at 4 °C. The upper layer was collected in Eppendorf tubes and preserved (-80 °C) until analysis.

Total protein estimation from tissue homogenate

The estimated concentration of total protein of tissue homogenate was obtained by following Lowry et al.

(1951). The obtained results are used for enzymatic calculation.

Digestive enzymes

Acid protease

Anson method (1938) measured samples of acid protease (pepsin) activity. Hemoglobin substrate (0.25%) was mixed with sodium acetate buffer-HCl (0.1 M, pH 3). Sample homogenate (25 μ l) was added and incubated for 30 min. Trichloroacetic acid (TCA, 12%) was added to break the reaction. After centrifugation, supernatant absorbance was measured at 280 nm (Singh et al. 2023).

Alkaline protease

Alkaline protease activity was assessed by following a modified version of the Drapeau (1974) method. Tissue homogenate was incubated with a one percent casein solution in 0.05-M Tris phosphate buffer (pH 7.8) for 5 minutes. Then solution was then filtered after the addition of 10% TCA. Enzyme activity was quantified based on the acid-soluble fragment release, equal to $\Delta 0.001A_{280}$ per minute at 37 °C and pH 7.8.

Amylase

Activity of amylase was measured using the DNS technique with a starch solution, phosphate buffer and tissue homogenate (Rick and Stegbauer 1974). After incubation at 37 °C, absorbance was read at 540 nm following DNS addition. The activity was stated as maltose out from starch per minute at 37 °C (Singh et al. 2023).

Lipase

Activity of lipase was assessed via Cherry and Crandell's titrimetric method (1932), involving hydrolysis of triglycerides in olive oil emulsion. Fatty acids released were quantified by titration with standard NaOH. The assay comprised 1.5-ml emulsion, 1.5-ml (pH 8.0) Tris-HCl buffer, and 1.0-ml crude enzyme, incubated for 24 h. at 37 °C. Reaction stopped with 3-ml 95% ethyl alcohol, followed by titration with 0.01N NaOH and phenolphthalein indicator (Singh et al. 2023).

Protein metabolic enzymes

ALT (alanine aminotransferase) and AST (aspartate aminotransferase) activity was assessed in tissue homogenates using Wootton's method (1964). In AST, a substrate comprising 0.2M D, L-aspartic acid and 2-mM α -ketoglutarate in 0.05M (pH 7.4) phosphate buffer was added (0.5ml) to experimental and control tubes. Tissue homogenate (0.1 ml) initiated the reaction. After 60 minutes at 37 °C, 0.5ml of 1mM 2,4 dinitrophenyl hydrazine ended the reaction. In control, samples were added after DNPH and kept at room temperature, shaken occasionally. After twenty minutes, 5ml of NaOH was mixed and OD was recorded at 540 nm in contrast to blank (Washington and Hoosier 2012). ALT (alanine aminotransferase) activity was determined (Wootton 1964) using 0.2M D, L-alanine. Enzyme activity was expressed in nanomole pyruvate formed/min/mg protein at 37 °C.

Carbohydrate metabolic enzymes

Lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) activities in tissue homogenates were measured using established methods (Wróblewski and Ladue 1955; Ochoa 1955). For LDH, a reaction mixes with phosphate buffer, NADH, tissue homogenate, sodium pyruvate, and checked OD at 340 nm for 3 min. MDH activity was assessed similarly but with oxaloacetate. The activity was expressed in units/mg protein/min at 25 °C, with 1 unit equating $\Delta 0.01$ OD/min at 37 °C (Singh et al. 2023).

Oxidative stress enzymes

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was assessed by following Misra and Fridovich (1972) method. Samples were mixed with a buffer containing EDTA and epinephrine and the variation in OD at 480 nm for three minutes. SOD activity is determined by the protein amount needed for 50% inhibition of epinephrine oxidation (Karmakar et al. 2022).

Catalase (CAT)

Catalase activity was measured using Takahara et al. (1960) method. Tissue homogenate and H₂O₂ in

phosphate buffer were reacted; OD was checked at 240 nm in 15-second intervals for 3 minutes, with distilled water as the blank. The activity is shown as nanomoles of H_2O_2 decomposed/min/milligram of protein at 37 °C (Singh et al. 2023).

Glucose

Glucose estimation of larval tissue was done using an ELISA-based commercially available ERBA Glucose assay kit (Catalogue No. BLT00002, Cayman Chemical). The activity was expressed in mg/dl.

Cortisol

The cortisol levels in the sample were studied by using a Cortisol EIA kit (Catalogue No. 500360, Krishgen) as per the protocol given in the kit. OD was observed by using an ELISA reader at 412 nm. The activity is expressed in ng/ml.

Statistical analysis

The data study was done using a one-way analysis of variance (ANOVA) in SPSS version 22.0 Software. The normality assumption (using Shapiro-Wilk test) and homoscedasticity (Levene's test) were made. The results were presented in the format of mean \pm standard error. A one-way ANOVA and Duncan's test were employed to make comparisons between the mean values of the test concentrations and the control group. A significant change was observed at a 5% probability level ($p < 0.05$).

Results

Growth and survival

Different weaning strategies significantly ($p < 0.05$) affected the growth and survival of *A. testudineus* larvae reared under the experimental duration of 30 days (Tables 2 and 3). The highest WG and SGR were observed in T2 (213.17 \pm 0.32 mg; 23.98 \pm 0.02), followed by T6 (197.64 \pm 0.29 mg; 23.73 \pm 0.03, respectively), while the lowest WG and SGR (98.69 \pm 0.16 mg; 21.42 \pm 0.06, respectively) were found in C2. In the initial first 15 days of culture (3 DPH to 18 DPH), the highest survival was

Table 2 Effects of weaning strategy on growth performance of *Anabas testudines* larvae reared for the period of 30 days

Treatments	FBW ¹ (mg)	WG ² (mg)	SGR ³
C ₁	166.47 \pm 0.30 ^e	166.31 \pm 0.30 ^e	23.16 \pm 0.01 ^e
C ₂	98.85 \pm 0.16 ^a	98.69 \pm 0.16 ^a	21.42 \pm 0.06 ^a
T ₁	148.27 \pm 0.27 ^c	148.11 \pm 0.27 ^c	22.77 \pm 0.04 ^c
T ₂	213.33 \pm 0.32 ⁱ	213.17 \pm 0.32 ⁱ	23.98 \pm 0.02 ⁱ
T ₃	195.53 \pm 0.35 ^g	195.37 \pm 0.35 ^g	23.69 \pm 0.01 ^g
T ₄	159.03 \pm 0.30 ^d	158.87 \pm 0.30 ^d	23.01 \pm 0.05 ^d
T ₅	147.30 \pm 0.26 ^b	147.14 \pm 0.26 ^b	22.75 \pm 0.05 ^b
T ₆	197.80 \pm 0.29 ^h	197.64 \pm 0.29 ^h	23.73 \pm 0.03 ^h
T ₇	190.50 \pm 0.31 ^f	190.34 \pm 0.31 ^f	23.61 \pm 0.07 ^f
<i>p</i> -value	0.001	0.001	0.001

Data are expressed as mean \pm SE, $n=3$; mean values in the same column with different superscripts differ significantly ($p < 0.05$)

¹FBW final body weight

²WG weight gain

³SGR specific growth rate (% day⁻¹)

Table 3 Effects of weaning strategy on survival percentage of *Anabas testudines* larvae reared for the period of 30 days

Treatments	3–18 DPH	18–33 DPH	3–33 DPH
C ₁	17.42 \pm 0.65 ^b	77.93 \pm 0.96 ^a	13.42 \pm 0.61 ^b
C ₂	10.00 \pm 0.14 ^a	93.33 \pm 0.67 ^b	9.20 \pm 0.08 ^a
T ₁	14.17 \pm 0.57 ^b	90.65 \pm 0.47 ^b	12.92 \pm 0.57 ^b
T ₂	27.83 \pm 0.96 ^{de}	88.36 \pm 0.98 ^b	24.75 \pm 0.13 ^e
T ₃	19.25 \pm 0.43 ^b	97.22 \pm 0.56 ^b	18.67 \pm 0.65 ^{bcd}
T ₄	18.08 \pm 0.21 ^b	96.76 \pm 0.46 ^b	17.50 \pm 0.01 ^{bc}
T ₅	19.83 \pm 0.26 ^{bc}	96.22 \pm 0.73 ^b	19.08 \pm 0.23 ^{cde}
T ₆	25.42 \pm 0.79 ^{cd}	93.68 \pm 0.19 ^b	23.83 \pm 0.18 ^d
T ₇	32.83 \pm 0.60 ^e	96.99 \pm 0.09 ^b	31.83 \pm 0.22 ^f
<i>p</i> -value	0.001	0.001	0.001

Data are expressed as mean \pm SE ($n=3$); mean values in the same column with different superscripts differ significantly ($p < 0.05$)

manifested by T7 (32.83 \pm 0.60%) followed by T2 (27.83 \pm 0.96%). The lowest survival was observed in C2 (10.00 \pm 0.14%). At the end of the next 15 days (18 DPH to 33 DPH) of culture, the larvae of T3 showed the highest survival, but insignificant results were found in the remaining treatments. Overall, at the end of the whole experimental duration (3 DPH to 33 DPH), the maximum survival was observed in T7 (31.83 \pm 0.22%), followed by T2 (24.75 \pm 0.13%),

and the lowest survival was found in the C2 (9.20±0.08%).

Carcass proximate composition

The proximate analysis of whole larvae varied significantly ($p < 0.05$) among the experimental groups (Table 4). The maximum crude protein content was found in T2 and T6, followed by T3 and T7, and the least crude protein was obtained in the C2 group. The higher moisture content was obtained in T2 and T6, and the lower levels were found in C2 and T5. On the other hand, elevated total lipid levels were recorded in C2, T5, and T7 and decreased in T2 and T6.

Effect of weaning strategy on digestive enzymes

The acid and alkaline digestive proteases, amylase, and lipase activities in *A. testudineus* larvae varied non-significantly ($p > 0.05$) between the treatments (Table 5).

Effect of weaning strategy on metabolic enzymes

The protein metabolic enzyme activities (AST and ALT) and carbohydrate metabolic enzymes (LDH and MDH) differed significantly ($p < 0.05$) (Table 6). The highest AST and ALT activities were observed in C2 (19.55±0.10 and 68.52±0.08, respectively), whereas the lowest values were observed in C1 (8.25±0.04

Table 4 Effects of weaning strategy on whole body proximate (% wet weight basis) of *A. testudines* larvae reared for the period of 30 days

Treatments	Moisture	Crude Protein	Lipid	Total Ash	Total Carbohydrate
C ₁	83.26±0.05 ^{de}	11.90±0.05 ^e	1.56±0.02 ^{cd}	2.37±0.07 ^{abcd}	0.90±0.06 ^c
C ₂	82.20±0.09 ^a	10.45±0.07 ^a	2.12±0.01 ^g	3.41±0.03 ^f	1.83±0.03 ^{de}
T ₁	82.98±0.05 ^{cd}	11.47±0.04 ^c	1.58±0.01 ^d	2.48±0.13 ^{bcd}	1.49±0.12 ^d
T ₂	83.72±0.27 ^f	12.40±0.08 ^f	1.48±0.03 ^a	2.15±0.05 ^a	0.24±0.11 ^a
T ₃	83.33±0.04 ^{de}	12.03±0.04 ^e	1.54±0.01 ^{bc}	2.25±0.12 ^{abc}	0.84±0.11 ^c
T ₄	83.15±0.08 ^{de}	11.68±0.04 ^d	1.58±0.01 ^{cd}	2.66±0.04 ^{de}	0.94±0.04 ^c
T ₅	82.51±0.05 ^{ab}	10.74±0.03 ^b	2.02±0.01 ^f	2.82±0.05 ^e	1.92±0.06 ^e
T ₆	83.40±0.11 ^e	12.37±0.04 ^f	1.51±0.01 ^b	2.19±0.17 ^{ab}	0.53±0.13 ^b
T ₇	82.67±0.02 ^{bc}	11.96±0.01 ^e	1.94±0.02 ^e	2.53±0.03 ^{cd}	0.89±0.04 ^c
<i>p</i> -value	0.001	0.001	0.001	0.001	0.001

Data are expressed as mean±SE ($n = 3$); mean values in the same column with different superscripts differ significantly ($p < 0.05$)

Table 5 Effects of weaning strategy on activities of digestive enzyme on *Anabas testudines* larvae reared for the period of 30 days

Treatment	Acid protease ¹	Alkali protease ²	Amylase ³	Lipase ⁴
C ₁	1.49±0.04	1.16±0.03	1.38±0.03	0.87±0.03
C ₂	1.44±0.02	1.09±0.00	1.33±0.02	0.83±0.02
T ₁	1.46±0.06	1.12±0.02	1.36±0.02	0.85±0.04
T ₂	1.53±0.02	1.20±0.03	1.44±0.02	0.93±0.02
T ₃	1.50±0.04	1.18±0.02	1.41±0.02	0.89±0.03
T ₄	1.47±0.05	1.15±0.04	1.37±0.03	0.86±0.02
T ₅	1.45±0.03	1.11±0.02	1.34±0.02	0.84±0.03
T ₆	1.52±0.02	1.19±0.03	1.42±0.02	0.90±0.03
T ₇	1.49±0.03	1.17±0.02	1.39±0.02	0.88±0.03
<i>p</i> -value	0.537	0.095	0.060	0.355

Data are expressed as mean±SE ($n = 3$)

¹Acid protease activity is expressed in U/mg protein/min

²Alkali protease activity is expressed in millimole of tyrosine released/mg protein/min

³Amylase activity is expressed in micromole of maltose released/mg protein/min

⁴Lipase activity is expressed in U/mg protein/min

Table 6 Effects of weaning strategy on protein and carbohydrate metabolic enzymes activities in *Anabas testudines* larvae reared for the period of 30 days

Treatments	AST ¹	ALT ²	LDH ³	MDH ⁴
C ₁	8.25 ± 0.04 ^a	59.80 ± 0.08 ^a	10.76 ± 0.14 ^a	0.022 ± 0.03 ^a
C ₂	19.55 ± 0.10 ⁱ	68.52 ± 0.08 ⁱ	21.79 ± 0.04 ^g	0.044 ± 0.01 ^f
T ₁	17.14 ± 0.05 ^h	54.75 ± 0.12 ^h	19.38 ± 0.05 ^f	0.041 ± 0.05 ^e
T ₂	10.44 ± 0.07 ^b	47.62 ± 0.15 ^b	14.48 ± 0.05 ^b	0.026 ± 0.01 ^b
T ₃	16.92 ± 0.04 ^g	64.44 ± 0.16 ^g	18.79 ± 0.41 ^e	0.036 ± 0.04 ^d
T ₄	15.76 ± 0.05 ^f	52.60 ± 0.11 ^f	17.66 ± 0.08 ^d	0.033 ± 0.09 ^c
T ₅	14.21 ± 0.05 ^e	57.71 ± 0.08 ^e	16.83 ± 0.11 ^c	0.031 ± 0.06 ^c
T ₆	11.61 ± 0.10 ^c	53.37 ± 0.30 ^c	14.73 ± 0.11 ^b	0.027 ± 0.02 ^b
T ₇	12.17 ± 0.05 ^d	57.59 ± 0.01 ^d	16.65 ± 0.24 ^c	0.031 ± 0.01 ^c
<i>p</i> -value	0.001	0.001	0.001	0.001

Data are expressed as mean ± SE (*n* = 3); mean values in the same column with different superscripts differ significantly (*p* < 0.05)

¹AST aspartate aminotransferase; the activity is expressed as nanomoles of oxaloacetate released / mg protein/min

²ALT alanine aminotransferase; the activity is expressed as nanomoles of sodium pyruvate formed/mg protein/min

³LDH lactate dehydrogenase; the activity is expressed in U/mg protein/min

⁴MDH malate dehydrogenase; the activity is expressed in U/mg protein/min

and 59.80 ± 0.08, respectively). Similarly, the highest LDH and MDH activities were observed in C2 (21.79 ± 0.04 and 0.044 ± 0.01), followed by T1 and T3 and lower activity was found in C1 (10.76 ± 0.14; 0.022 ± 0.03) followed by the T2 group.

Effect of weaning strategy on oxidative stress or antioxidant enzymes

The antioxidant enzymes viz., SOD (50% inhibition of epinephrine auto-oxidation/mg protein/min) and CAT (nanomoles H₂O₂ decomposed / mg protein/min) significantly varied (*p* < 0.05) between treatments (Fig. 2). Significantly (*p* < 0.05) higher SOD value was obtained in C2 followed by T1 and T3, whereas the least activity was found in C1 and T2. Similarly, the CAT value was significantly (*p* < 0.05) highest in C2, followed by T1, and a lower value was found in C1, followed by T2 and T6.

Tissue glucose and cortisol level

The glucose (mg/dl) and cortisol (ng/ml) levels significantly differed (*p* < 0.05) among treatments (Fig. 3). The significantly lowest (*p* < 0.05) glucose levels found in larvae of C1, T2, T6, and T7, and the highest level was found in the C2 followed by T1. Significantly (*p* < 0.05) lower cortisol levels found

in T2 followed by C1 and reported higher in C2 followed by T1, although the larvae of T3 and T4 exhibited comparable cortisol levels (*p* > 0.05).

Discussion

The primary objective of this investigation was an optimization of the weaning strategy with a formulated microparticulate diet (MPD) and live food (LF) for a culture of *A. testudines* larvae. At onset of exogenous feeding, the larval digestive system is functional but simple, lacking a true stomach and pyloric caeca (Kolkovski 2001). Digestive enzyme activity is low or undetectable at an early stage (Mozanzadeh et al. 2021). Major changes in digestive physiology occur during metamorphosis from larvae to adults in the gut. However, significant functional changes happen during the larval phase ((Kolkovski 2001). Assessing enzyme activity levels in relation to growth and survival data obtained from co-feeding provides insights into the most favorable weaning period (Yufera et al. 2012). In some fish species, gradual replacement of live food with MPD has shown to be an effective weaning approach (Hamre et al. 2013; Biswas et al. 2022). It is well known that adopting larvae on MPD needs a strategy or protocol to adjust them to significant morphological, physiological and

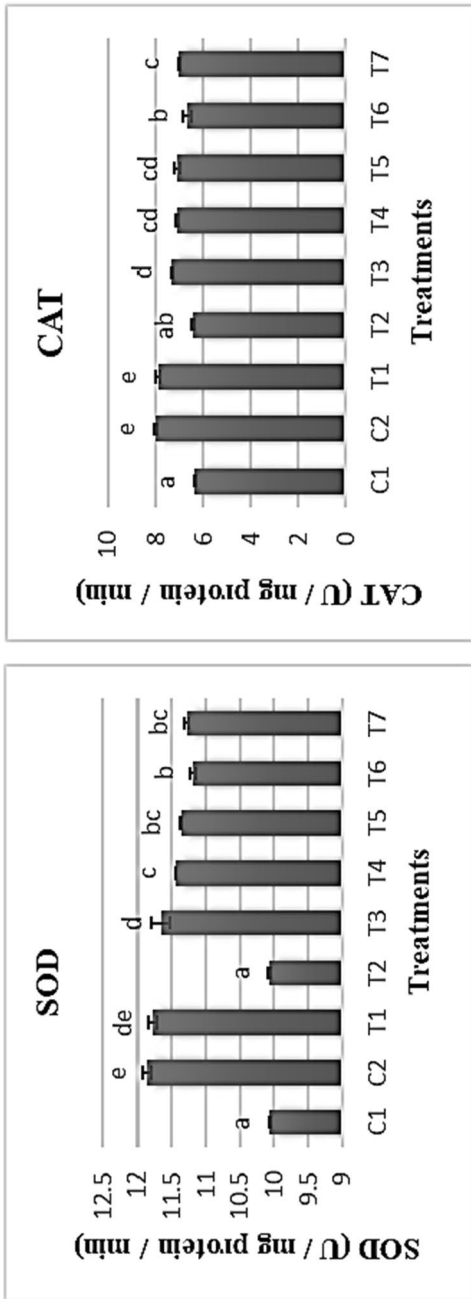


Fig. 2 Effects of weaning strategy on the activities of tissue oxidative stress enzymes such as SOD and CAT of *A. testudines* larvae reared for the period of 30 days

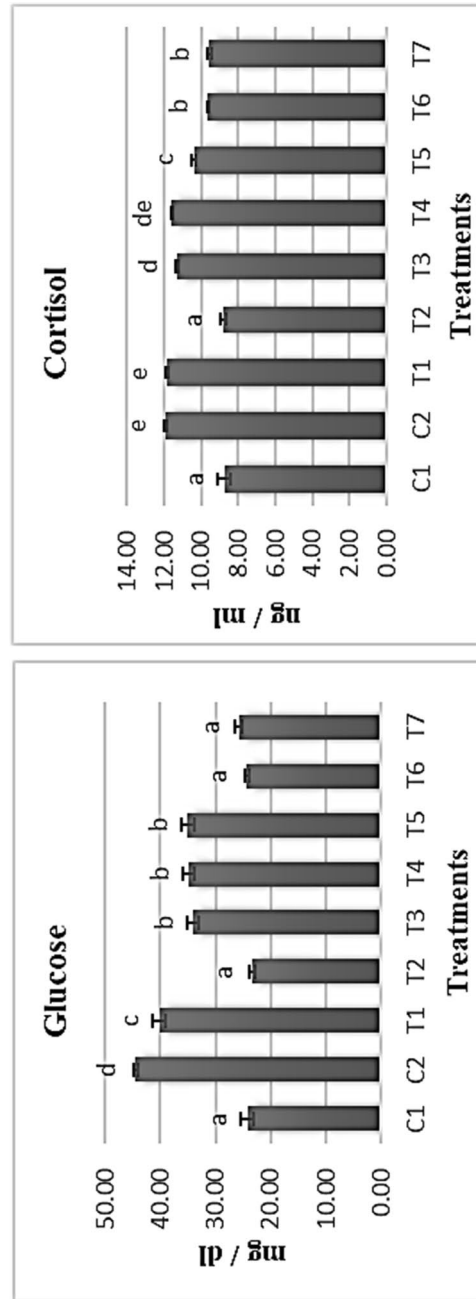


Fig. 3 Effects of weaning strategy on the tissue glucose and cortisol level of *A. testudines* larvae reared for the period of 30 days

behavioral changes (Rao 2003). In contrast, the developmental stage was a significantly non-dependent factor in determining larval development and dietary status (Andrade et al. 2012). In the current investigation, different weaning strategies have shown a substantial impact on the growing performance and survivability of *A. testudines*. Variations in larval size are inevitable and can be corrected over the rising phase; however, weaning is critical to larval growth and survival during the rearing phase (Ali et al. 2003; Curnow et al. 2006; Valente et al. 2013; Hung et al. 2022).

Growth performance and survival

Several studies showed a good effect of live prey on the growth performance of various species of aquaculture (Conceição et al. 2010; Singh et al. 2019; Chen et al. 2020). A lack suitable weaning diet during initial larval rearing and cannibalism are the main constraints for successful larviculture of *A. testudineus* (Morioka et al. 2009; Singha et al. 2015; Singh et al. 2019). In existing investigation, significantly ($p < 0.05$) higher WG and SGR of the larvae were found in T2, which might be due to the better acceptability of live food at the early stage. The larvae lack a well-developed digestive system at their initial development stage to digest a formulated diet; hence, live food could be considered a better one as it probably could supply exogenous digestive enzymes for synergistic effects with endogenous enzymes (Singh et al. 2019). The findings corroborated the observations in larvae of African catfish, *Clarias gariepinus* (Verreth and Tongeren 1989; Verreth et al. 1992) and Redtail catfish, *Mystus nemurus* (Kamarudin et al. 2011), where feeding fish of appropriate weaning age with artificial diet could result in better weight gain and SGR. Mir et al. (2018) also reported that *Clarias magur* larvae could exhibit higher SGR and survival after weaning with an artificial diet. Kolkovski et al. (1997) also demonstrated that the co-feeding of European seabass larvae with formulated feed and live food increased growth and nutrient metabolism. In the current study, poor growth of larvae in C2 might be due to feeding of larvae with artificial feed from the first day of an experiment, which probably could result in lower feed intake due to low palatability with poor nutrient digestibility at early stage of larvae. As MPD lacks the endogenous enzyme, which results in

low feed digestion, it consequently results in lower growth of larvae (Cahu and Infante 2001). Similar findings were observed in Sand bass *Paralabrax maculatofasciatus* (Civera-Cerecedo et al. 2008) and *Senegalese sole* (Engrola et al. 2007), where larvae exhibited poor growth in C1, probably due to limited availability of dietary nutrients resulting mobilization of nutrient from body reserve for energy production and satiation leading to poor growth of larvae. Therefore, an inadequate weaning strategy may cause poor survival as well as growth of larvae (Ma et al. 2012).

The main challenge in *A. testudineus* larviculture is poor survival, lack of proper weaning strategy, and cannibalism among larvae (Singha et al. 2015). Previous outcomes of *A. testudineus* larval rearing showed only 4–16% survival solely feeding on formulated diet (Trieu and Long 2001) and 16–28% feeding on live food (Morioka et al. 2009; Singh et al. 2019) during a 15-day period. Furthermore, cannibalistic behavior can be developed and it leads to heterogeneous growth, leading to reduced survivability of larvae (Thanh et al. 1999). However, *Dicentrarchus labrax* larvae showed low cannibalism after the feeding of the artificial feed after the appropriate age (Infante and Cahu 1994a, b). Therefore, a weaning strategy was optimized in the present study to enhance larval survivability and growth. In comparison to control groups (C1 and C2), higher survival reported in T7 and T6 probably could prove that instead of continuous feeding with live food certain co-feeding patterns may enhance larval survivability, as reported by Brown et al. (1997). Lower survival in larvae of C1 might be due to a deficiency of certain essential nutrients in live food (Segner and Witt 1990; Mejri et al. 2021; Grayson and Dabrowski 2022). Moreover, the lowest survival in C2 also suggests that feeding artificial feed alone can't sustain larval survival, probably owing to lower feed intake and nutrient digestibility and, eventually, starvation (Salhi and Bessonart 2012).

Proximate composition of carcass

Food availability, fish growth and anabolic or catabolic response are all aspects that affect the chemical composition of the fish body (Abdel-Tawwab et al. 2006). A significant difference ($p < 0.05$) in carcass proximate analysis found in present study and contradicts the findings of earlier findings may be due to

variations in feeding regimens, fish stage, type, nutrient digestibility, and research environment. However, according to Jafari et al. (2011), there was a considerable change in the whole-body composition of *Rutilus frisii kutum* larvae on different feeding regimens.

Effect of weaning strategy on digestive enzymes

There were no significant ($p > 0.05$) changes detected in digestive enzyme response between the treatments. In fish larvae, acid protease activity normally emerges after the development of a functioning stomach (Cahu and Infante 2001; Kolkovski 2001). The current research found insignificant acid protease activity, representing that stomach and acid protease action were functional at the end of the experiment. Accordingly, functional intestinal track and pepsin activity were found in the sea bass, *Lates calcarifer* larvae at 17 days (Walford and Lam 1993) and in red porgy *Pagrus pagrus* at 30 days (Darias et al. 2005). In larvae, protein assimilations began through alkaline proteolytic enzymes at the initial phase (Darias et al. 2007). The alkali protease activity in larvae was also not affected significantly by MPD or live food (Grendell and Rothman 1981; Tseng et al. 1982; Infante and Cahu 1994a, b). The evolution from the early to the juvenile stage is assumed to have ended when the gut and proteolytic enzyme secretion appear (Govoni et al. 1986). Similarly, amylase and lipase activities were non-significant ($p > 0.05$) in the present work; most digestive activity, such as acid protease, alkaline protease, amylase, and lipase, shows comparable patterns (Ribeiro et al. 1999). A similar pattern has also been reported in European sea bass, gilthead sea-bream, red drum, and black spot sea bream (Moyano et al. 1996; Ribeiro et al. 2008). It indicates that once their digestive systems are completely formed and the larvae have acclimated to the formulated diet, larvae are very flexible to various dietary regimes (Pittman et al. 2013; Rønnestad et al. 2013).

Effect of weaning strategy on protein metabolism

The protein metabolic activities are indicators of nutritional status, growth condition, and stress of animals, including fish (Pan et al. 2003; Haridas et al. 2017). Increased AST and ALT activities may be explained in two ways in relation to growth. Accordingly, increased activities of these enzymes with

improved growth indicate that apparently produced non-essential amino acids participate in cellular metabolism in the body, leading to growth, in contrast with increased enzyme activity with reduced growth indicates oxidation for energy generation may restrict growth and at the same time releasing ammonia in the environment might create stress in fish. Thus, the highest AST and ALT values in the C2 group with reduced growth may be because less food ingestion and nutrient utilization and leads to energy satiation met through oxidation of body protein-derived amino acids leading to growth retardation. Similarly, higher AST and ALT values follow dietary stress due to low acceptability of feed with reduced feed intake in rats (Stadler et al. 2005). The higher AST and ALT levels with higher growth in larvae of certain weaning groups than C1 might be due to the participation of synthesized amino acids leading to better development of larvae. Moreover, the live food-fed group probably had a deficiency of some essential amino acids, leading to lower growth of larvae (Grayson and Dabrowski 2022).

Effect of weaning strategy on carbohydrate metabolic enzymes

The LDH and MDH activities also followed a similar pattern and were found significantly increasing in C2; this might be due to anaerobic oxidation leading to large amounts of lactate being released since lactate is the preferred substrate for gluconeogenesis to cope with stress and produce glucose and therefore LDH activity is raised (Chatterjee et al. 2006). However, the C1 group has low LDH and MDH, indicating that the live food-fed group had low stress due to better acceptability of live food in this group. Also, MDH is essential for gluconeogenesis as well as lipogenesis, which catalyzes the reversible malate oxidation to oxaloacetate in the cytosol (Panepucci et al. 2000). The increased MDH activity in fish suggests that there is a rising incidence of Krebs's cycle as a result of rising energy demand to overcome stress (Kumar et al. 2011). However, lower LDH and MDH activities in T2 and T6 suggest they adopted a weaning protocol. Kumar et al. (2015) showed that MDH activity was increased in *L. rohita* fingerlings in response to temperature shock than control, implying a higher energy requirement during the stress condition.

Effect of weaning strategy on oxidative stress enzymes

The SOD catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide and CAT converts hydrogen peroxide to oxygen and water (Morales et al. 2004; Pérez-Jiménez et al. 2007). These enzymatic pathways reduce stress and have the potential to balance proper oxidation-reduction equilibrium, ameliorate reactive oxygen species (ROS) equilibrium, and save cells from oxidation (Abdel-Tawwab and Wafeek 2017; Hoseinifar et al. 2020). SOD and CAT activities were significantly ($p < 0.05$) varied between the experimental groups, with C2 indicating the highest SOD and CAT might be due to the higher stress in the larvae that were fed with MPD. As a CAT, SOD speeds up the dismutation of superoxide anion (Kehrer et al. 2010). The C1 had considerably decreased SOD and CAT compared to others due to the exclusive live food fed in this group, demonstrating relatively reduced stress. Lim et al. (2002) also reported that the live food-fed fish group had much less stress than commercial diet-fed fish.

Glucose and cortisol

Glucose and cortisol are another two major stress indicators in fish, and their elevated levels indicate stress in fish. As a useful tool for evaluating the health status of fish larvae, measuring glucose levels is also a good biomarker for research on secondary stress reactions (Wedemeyer 1981). The primary glucocorticoid released by interregional muscle in the kidney of teleost fish is cortisol (Iwama et al. 1999). The value of glucose and cortisol found to rise in fish is an indicator of various stressors (Luz et al. 2008; Fiúza et al. 2015). Elevated levels of cortisol and glucose levels were observed in both C2 and T1 groups, indicating that the larvae fed with an artificial diet for 30 days and 25 days encountered starvation stress. Similarly, feeding a microdiet to Chinese longsnout catfish larvae significantly increases body glucose content (Liu et al. 2012). A considerable increase in cortisol levels in the C2 group fed with MPD indicates they were under nutritional stress as nutritional requirements have not yet fulfilled, which is also indicates reduced growth in this group (Yu et al. 2019).

Conclusion

The initial phase of larval rearing is a major aspect impeding the growth and survival of *Anabas testudineus* farming because live food production is both costly and nutritionally deficient and it also needs specialized production facilities. As a result, optimizing the weaning protocol to minimize dependency on live food is of both technical and economic importance. The research outcomes suggest that different weaning approaches substantially influenced growth and survival. Also, proximate composition and enzymatic activities associated with digestion, protein, and carbohydrate metabolism exhibited significant differences among the treatments. Furthermore, antioxidant enzyme activities also displayed variations with the control group. The glucose and cortisol levels demonstrated substantial differences among the experimental groups. Major findings of the experiment reveal *A. testudineus* larvae can be weaned after 13 DPH on formulated feed. The present study also concludes that feeding *A. testudineus* larvae with LF for 10 days, then 25% LF replacement by MPD for next 5 days, 50% LF replacement by MPD for next 5 days, 75% LF replacement by MPD for next 5 days, and 100% LF replacement by MPD for last 5 days can be the best weaning strategy as far as survival (31%) is concerned. The implementation of this weaning protocol may ensure seed availability for a widespread culture of *A. testudineus*. Additional research is advised to explore the comprehensive understanding of larval nutrition, like molecular level examination is essential for precise dietary interventions, along with the implementation of innovative methodologies for investigating the nutritional requirements of *A. testudineus* larvae.

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Vijayakumar Mannur: Formal analysis, Software implementation. **Anusha Patel:** Data curation and editing of the manuscript.

Data availability The necessary data has been included in the article as a table and figures in the main article.

Declarations

Ethics approval This study was carried out in accordance with the recommendations made by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of government of India. These recommendations ensure the welfare and maintenance of fish used in laboratory studies for biomedical research and product testing.

Consent for publication I, Dr. Sikendra Kumar, give my consent for information about a research paper to be published in Fish Physiology and Biochemistry journal.

Competing interests The authors affirm that they have no known financial or interpersonal conflicts that would have seemed to have an impact on the research presented in this study.

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