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Evaluation of weaning diets for sustainable indoor largemouth bass (*Micropterus salmoides*) larviculture

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Electronic Supplementary Material (ESM)

Supplementary material S1

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SUPPLEMENTARY MATERIAL S1

MATERIALS AND METHODS

Feeds –biochemical analysis

To assess the nutritive profile of feeds, levels of phospholipids (PL), free amino acids (FAA), soluble proteins (SP), and *in vitro* protein digestibility using LMB intestinal extract were estimated using biochemical assays (Lukic et al. 2019, 2021; Rungruangsak-Torrissen et al. 2002). All biochemical analyses of feeds were performed in four replicates per feed (except for FAA and SP for EF, which was done in triplicate). Before PL, FAA, and SP assays, feed samples (50 mg) were delipidated using 1 mL of the 2:1 chloroform: methanol mixture. Samples were incubated for 1 h at room temperature (RT) with shaking and centrifuged at maximal speed for 10 min at RT. Supernatants were transferred to new tubes and pellets were dried and used for FAA and SP analysis. Supernatants were desiccated to obtain semi-solid pellets for PL determination.

Phospholipid (PL) assay. Lipid pellets, obtained from desiccated supernatants after lipid extraction were suspended in chloroform. Ammonium thiocyanate reagent was prepared by dissolving 0.27 g of $FeCl_3$ x 6 H_2O and 0.3 g of NH_4SCN (ammonium thiocyanate) in 10 mL of distilled water. The reagent was mixed with lipid samples in 1:2 ratios, shaken in hand, and centrifuged at low speed for 1 min to separate lipid and water phases. The lower phase was transferred to new tubes and dried at RT until dryness. Pellets were suspended in 96% ethanol and solubilized by warming at 42°C. Absorbance was measured at 488 nm using a Multiskan Sky spectrophotometer (ThermoFisher Scientific, United States). Results are expressed as µg/mg of feed.

Free amino acid and short peptide (FAA+SP) assay. Pellets obtained after lipid extraction were dissolved in water and precipitated using 5% TCA. Supernatants obtained after precipitation (191 µL) were mixed directly in the wells of a 96-well microtiter plate with 9 µL of 0.1 M carbonate buffer with 0.005% trinitrobenzene sulfonate (TNBS). The plate was incubated at 37°C for 20 min, then 3 µL of concentrated HCl was added to each well to stop the reaction, and absorbance was measured at 420 nm. Results are expressed as mg/mg of feed.

Soluble protein (SPR) measurement. The same fraction of delipidated samples (pellets dissolved in water) that were used for FAA/SP analysis, was used for SPR measurement, using a commercial kit (Analyticon Biotechnologies GmbH, Germany). Samples (10 µL) were mixed with 190 µL of R1 reagent, incubated for 10 min at RT and the absorbance was measured at 546 nm. Results are expressed as mg/mg of feed.

In vitro protein digestibility assay. Intestines (~1.5 g, from the stomach-intestine junction to the posterior end of the intestine) from adult LMB (weight ~200 g, age: 1.5 y) were taken aseptically, longitudinally opened, and rinsed using saline. Afterward, 3 volumes of 50 mM Tris pH 8 + 200 mM NaCl were added, the intestine was homogenized using a KIMBLE Dounce tissue grinder set, pestle A only (Merck KGaA, Germany), and centrifuged at +4°C at a maximal speed for 15min. Supernatants were placed in Slide-A-Lyzer™ dialysis cassette, 10 kDa cut-off (ThermoFisher Scientific, United States), and left overnight at +4°C in 10mM Na-phosphate buffer pH 7.8. After extracting the homogenate from the dialysis tube, trypsin activity in the extract was determined as explained below (2.5) and the extract was diluted with 10 mM Na-phosphate buffer pH 8.2 and 5% chloramphenicol (0.5 µL/mL) up to 15 mL. Next, 1 mL of diluted extract was mixed with 50 mg of each feed and incubated at RT overnight (17 h) with gentle shaking. Samples before (0h) and after (17 h) simulated digestion were taken for FAA analysis, as explained in 2.3.2. Protein digestibility results are presented as released amino acids (mg)/mg of feed/U of trypsin activity.

Study design - fish origin and early weaning experiment

Larvae used for the trial were a product of semi-artificial reproduction of 4-year-old LMB broodstock. In the first week of May 2023, 30 pairs of breeders were harvested from a common pond, hormonally stimulated with salmon gonadotropin-releasing hormone analog (sGnRHa, [D-Arg6, Trp7, Leu8, Pro9-NEt]-GnRH, Bachem, Switzerland). Both males and females were treated with a 50 µg/kg dosage. Straight upon hormonal treatment, fish were transported to the 300 m^2 spawning pond with 30 nests of size 60 x 60 cm, made of artificial grass.

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Three days after hormonal treatment, 10 nests with fertilized eggs were transferred to the hatchery and further incubated at 23 ± 1 °C. Following hatching, larvae were reared in a common 1 m^3 water volume tank until the swim bladder inflation.

Early weaning experiment. 1) One share of larvae was transferred to the 12 cylindro-conical tanks of 250 L water volume of the same features as the previous tank and within the same recirculation loop. In total, 200 randomly harvested larvae were stocked in each tank. Different feed treatments (OB2, AI, EF, and AS) were randomly assigned to each tank, with three tanks per treatment. Larvae were fed manually with enriched *Artemia* nauplii (500 nauplii/larva/day) in 6 daily meals. Dry feed was introduced on the 11th DPH and was added in the amount of 6 g per tank per day for the first 3 days, then it was increased to 5 g from the 14th to 18th DPH. Feed was supplied via an automatic belt feeder in 5-minute intervals. Throughout the experiment, the feed was over the daily requirement. This is based on previous studies in fish larviculture when the larvae were fed *ad libitum* (Malzahn et al., 2022). Tanks were cleaned once per day and mortalities were counted. The feeding cycle matched the light duration in the photoperiod set at 16:8 light/day (LD) both during the experimental period and the period before. Dissolved oxygen concentrations were 8.8 ± 0.7 mg/L. On the 19th DPH, the experiment was discontinued due to very high mortality of larvae, reaching an average of 36.3% in the AI group and being the lowest in the OB2 group (22.3%), then AS (26.7%) and EF group (27.3%).

Homogenization of fish, RNA extraction, and Quantitative PCR (qPCR) – protocol details

Tissue homogenisation for enzymatic assays and ELISA was done using a glass Dounce grinder. After 20-30 strokes with pestle A, samples were filtered through Falcon® 70 µm Cell Strainer (Corning Inc., United States), and the filtrate was further homogenized using pestle B. After one cycle of freeze-thawing at -20 to assist the lysis of the cells, samples were centrifuged at +4°C, 15 500 g (maximal speed) for 15 min. Supernatants were stored at –20 °C until use.

For RNA extraction, the liquid nitrogen homogenized mixture in Denaturation Buffer was transferred to 2 mL tubes, mixed with one volume of acidic phenol, pH 4, 1/1- volume of 2 M sodium acetate, pH 4, and 1/5 volume of chloroform, manually mixed and centrifuged at maximal speed, +4°C, 15 min. The upper phase was transferred to a new tube and the acidic phenol extraction was repeated. After the second phenol extraction, one volume of isopropanol was added and the RNA was precipitated at -20°C for 30 min. Samples were centrifuged for 15 min, +4°C at maximal speed and the pellets were washed using 70% ethanol. Samples were centrifuged again, the ethanol was removed and the pellets were dried at RT for 15 min. Pellets were dissolved in PCR-clean distilled water, RNA concentration was measured using Nanodrop 2000 (Thermo Fischer Scientific, United States), and the RNAs were stored at -80°C until use.

For cDNA synthesis, an RNA template (1 µg) was mixed with 4 µL of 5 x Reaction Buffer, 1 µL of dNTP mix, 1 µL of Random Hexamer, and 1 µL of reverse transcriptase and filled with distilled water up to 20 µL. In RT minus controls, reverse transcriptase was heat-inactivated at 85°C for 10 min. Mixtures were incubated at 25°C for 10 min, then at 55°C for 15 min, and then at 85°C for 5 min in SuperCycler Thermal Cycler SC3005 (Kyratec, Australia). Samples were stored at -20°C until use.

Digestive enzyme analysis – protocol details

Digestive enzyme assays were done using protocols from Ljubobratovic et al. (2017). Reaction buffers for trypsin, chymotrypsin, alkaline phosphatase, and lipase assays were as follows:

1 mM Nα-benzoyl-DL-arginine-ρ-nitroanilide hydrochloride (BAPNA) dissolved in 50 mM Tris-HCl, pH 8.2 + 20 mM CaCl2 + 1% dimethyl sulfoxide (DMSO), in the case of trypsin assays

0.5 mM succinyl-(ala)2-pro-phe-ρ-nitroanilide (SAPNA) dissolved in 50 mM Tris-HCl, pH 8.2 + 20 mM CaCl2 + 1% DMSO, in the case of chymotrypsin assay;

5 mM p-nitrophenyl phosphate (p-NPP) in 30 mM carbonate buffer, pH 9.8, 1 mM $MgCl_2$, in the case of AP assay; and

1 mM ρ-nitrophenyl palmitate (ρ-NPP) in 50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.3% Triton X 100, in the case of lipase assay.

Differences between the two points at the linear part of the curve were calculated and used in the following formula:

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Enzyme activity (mU/mg of protein) = (((ΔAhomogenate – ΔAblank)/time (min)) x 1000 x total volume of the reaction mixture (mL))/(ε x volume of the homogenate (mL) x prot conc (mg/mL))

- ΔA is the difference in the absorbance between the two time points

- ε is the extinction coefficient of the reaction product (8 800 for the trypsin and chymotrypsin assays, 18 300 for the AP assay, and 15 000 $M^{-1}cm^{-1}$ for the lipase assay)

Stop reagent for the amylase assay was made by mixing distilled water, Component A (96 mM 3,5- dinitro salicylic acid (DNSA) dissolved in 20 mM sodium phosphate buffer, pH 7), and Component B (sodium-potassium tartarate dissolved in 2 M NaOH in a ratio of 1.5:1 (weight: volume)) in a ratio of 1:1.7:1.7. In blank samples, samples were added after the addition of the stop reagent, before the boiling step.

RESULTS

Correlation analysis – details of statistical analysis

Pearson's test revealed a positive correlation of individual weight and SGR with dry matter and crude ash percent, as well as ω3/ω6 ratio, but a negative correlation with SPR level in the diet. At the same time, individual weight correlated positively with SGR and *ColI*α2 mRNA expression in fish larvae, while SGR correlated positively with amylase-specific activity. Standard fish length correlated positively with ω3/ω6 ratio in the diet and *ColI*α1, *PSN,* and *TnnC*. *ColI*α1 mRNAs. It also correlated positively with *PSN* and *TnnC* mRNA expression levels. In addition, a positive correlation between *PSN* and *ColI*α2 mRNA expression was observed and both were associated positively with ω3/6 ratio in the diet. At the same time, *TnnC* negatively correlated with CCK concentration, while *ColI*α2 correlated negatively with T/C activity ratio. The levels of *Flavobacterium* spp. 16S rRNA correlated positively with AP and PLA2 specific activities, while all three parameters correlated negatively with dietary crude protein level. Trypsin specific activity was negatively associated with MUFA/SFA ratio in the diet. Chymotrypsin specific activity correlated positively with feed FAA+SP and protein digestibility, and fish *TrpT* mRNA expression and amylase specific activity. Amylase activity correlated positively with dry matter, crude ash, FAA+SP levels in the diet, and the fish SGR. Aside from a negative correlation with *ColI*α2 mRNA expression, the T/C activity ratio correlated positively with dietary SPR level. T3 and T4 concentration levels showed a positive inter-correlation.

DISCUSSION

Chymotrypsin specific activity was positively associated with the mRNA expression level of fast muscle fiber-specific TrpT, indicating the role of chymotrypsin in fast swimming during the larval period. Although no effects of higher TrpT mRNA expression on larval growth in this study were observed, fast swimming might be critical for protection from predators or predatory activity in ponds and natural habitats.

Considering the SPR level, and in alignment with the preference of trypsin and chymotrypsin for larger and shorter peptides, respectively, the **T/C activity ratio** correlated positively with intact SPR level in the diet, which was the highest in AS and EF. Interestingly, intact SPR and high T/C ratio correlated inversely with the expression of *ColI*α2 mRNA (the statistical trend for SPR, p=0.052). Whether this resulted from increased T/C activity ratio and the release of specific peptides and amino acids which, individually or collectively, might have affected collagen gene transcription, remains to be investigated. An interesting phenomenon related to LMB, also observed in previous research (Lukic et al. 2023) is the ability to trade off the expression of ossification-related markers for fast growth in the post-larval stage. This research supports the results of the above studies, as the T/C ratio is a common predictor of future fish growth (Rungruangsak-Torrissen et al. 2006).

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Table 1. Raw materials in Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS) and experimental feed (EF)

Feed	Feed composition
OB2, particle size 360-650 µm https://www.mn-feed.com	Krill meal, fish meal, squid meal, potato starch, wheat flour, fish oil, brewer's yeast, calcium phosphate, guar gum, soy lecithin, betaine, licorice plant, apple extract, wheat germ
AI, particle size 400 µm https://www.aller-aqua.com/	Fish meal, fish-oil, grain products, krill meal, vegetable proteins, vitamins and minerals, undisclosed functional ingredients
AS, particle size 300-500 µm www.aqua-garant.com	Fish meal, peas, fish oil, soy lecithin, fish gelatin
EF, particle size 300 µm This study	Fish-meal, pre-digested fishmeal, krill-meal, squid-meal, egg powder, chicken protein concentrate, brewer's yeast, wheat flour, egg protein, liver-meal

The particle size and the list of ingredients for OB2, AI, and AS have been downloaded from manufacturers' websites

Table 2. Phospholipid (PL), free amino acid and short peptides (FAA+SP), soluble protein (SPR) levels and protein digestibility of Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS) and Experimental Feed (EF), provided as means ±standard deviations (SD)

Nutrient	OB2	AI	AS	EF
PL(µg/mg of feed)	42.5 ± 3.6	25.9 ± 7.2	*5.4 ± 3.0	29.7 ± 7.1
FAA+SP (mg/mg of feed)	0.027 ± 0.013	0.018 ± 0.004	0.016 ± 0.006	*0.01 ± 0.007
SPR (mg/mg of feed)	0.042 ± 0.012	0.079 ± 0.011	*0.173 ± 0.012	*0.139 ± 0.039
Protein digestibility (released amino acids (mg)/mg of feed/U of trypsin activity)	3622 ± 792	2258 ± 208	2015 ± 1817	*341 ± 532

*Statistically significant difference in comparison to OB2