https://doi.org/10.17221/129/2024-CJAS

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

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Citation: Lukić J., Vass N., Ardó L., Stanivuk J., Lengyel-Kónya É., Golíc N., Sándor Z.J., Ljubobratović U. (2024): Evaluation of weaning diets for sustainable indoor largemouth bass (*Micropterus salmoides*) larviculture. Czech J. Anim. Sci., 69: 471–483.

Abstract: This research evaluated the suitability of commercially available larval feeds, Otohime B2 (OB2), Aller Infa (AI), and Aqua Start (AS), and one Experimental Feed (EF), for the weaning of largemouth bass (*Micropterus salmoides*), LMB. Feeds were presented with various ω-3 fatty acid levels/bioavailability (high in OB2 and AI), fat percentage (high in OB2 and AS), free amino acid and short peptide (FAA + SP) levels (high in OB2), and various soluble protein (SPR) levels (high in AS and EF). Fish were co-fed *Artemia* plus OB2 from the 19th to 22nd day post-hatching (DPH), then *Artemia* in addition to one of the four above diets for seven days, with complete *Artemia* removal on the 30th DPH. Fish were sampled on the 32nd DPH. Morphometry, digestive enzyme activities, hormonal status, skeleton, muscle development, and potentially pathogenic *Flavobacterium* spp. levels were estimated. Survival was high (96% or more) in all the weaning regimes. Weaning to OB2 was linked to a fast fish growth rate (14.29%/day), while both OB2 and AI supported the skeleton development. Weight gain correlated with total fat, ash levels, free amino acids, and short peptide levels in the diet. Larvae weaned to soluble protein-rich AS and EF showed the lowest fish weight gain and skeleton development, and lower growth of potentially pathogenic *Flavobacterium* spp. This research suggests that the weaning diets for largemouth bass should have a balanced protein content and quality while allowing for the inclusion of fewer marine ingredients.

Keywords: fat; free amino acids; freshwater fish; intact proteins; marine ingredients; skeleton development

Supported by the European Union's Horizon 2020 research and innovation programme (Grant No. 871108 (AQUAEXCEL3.0), by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract No.: 451-03-47/2023-01/200042), and by the Research Excellence Programme and the Flagship Research Group Programme of the Hungarian University of Agriculture and Life Sciences. The corresponding author was granted with János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

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The development of sustainable feeds for aquaculture is the challenge posed to the aquafeed industry at the beginning of this century. Sustainable aquaculture encompasses both the rearing technology with maximal resource-sparing potential, e.g. recirculating aquaculture system (RAS), and the feed composition (FAO 2022). Using alternative sources of proteins and fatty acids (FA) to fishmeal and fish oil is the key to transitioning to sustainable diets. The extent to what this replacement is feasible depends on two factors. The first one is fish biology – namely, its natural diet and habitat, which affects the availability of highly unsaturated fatty acids (HUFA), including eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), which are more abundant in marine environments. The other factor is the level at what the endogenous synthesis of FA is possible, e.g. trout, which, due to its anadromous nature, has up to 25% capacity for EPA and DHA synthesis from lower-length precursor alpha-linolenic acid (ALA) (Colombo et al. 2018). As for the first criterion, trophic positioning additionally affects the availability of HUFA, since the top predatory fish have the highest demands for EPA and DHA (Parzanini et al. 2020).

Largemouth bass (*Micropterus salmoides*), LMB, is a warm-water fish species native to the USA, belonging to the sunfish (*Centrarchidae*) family. It is an opportunistic predator and has been introduced worldwide, mainly for recreational fishing purposes, though the introduction into non-native habitats may be detrimental to the local ichthyofauna. Largemouth bass flesh is attributed low fat percentage, it is rich in DHA and EPA, but also linoleic acid (LA), ALA, and oleic acid (OA), which reflects the diet featuring aquatic and terrestrial insects, aside from the vertebrate prey (Parmar et al. 2022). According to the data from 2020 (FAO 2022), worldwide largemouth bass production reached 0.7 mil t/year, with China being the largest producer. Introducing this species into farms in Europe could support aquaculture diversification and climate adaptation, which is a prerogative for aquaculture growth in the face of global warming and the narrowing of the possibilities for farming cold-water species, such as trout. Due to an increase in consumer quality standards, common carp farming is in decline (Hu et al. 2023) and largemouth bass could be an alternative species to boost sustainable growth, while, at the same time, ensuring the availability of nutritious and tasty meat. Compared to common carp, largemouth bass lacks intramuscular bones and its flesh is less smelly than the flesh of bottom-dwelling common carp, particularly the farmed one (Hu et al. 2023). Largemouth bass is currently reared in ponds from the nursing phase, but, given its predatory nature, acceptance of inert feed is more efficient when the feed training is performed in controlled conditions indoors (Sloane and Lovshin 1995). Nursing the fish indoors would eliminate the feed training step and prolong the grow-out period. It would also spare the land and water resources used for the nursing phase and present the biosecure material for stocking in RAS grow-out farms. Indeed, the potential of largemouth bass for grow-out in recirculation systems in Europe has recently been evaluated in several studies (Penka et al. 2021; Malinovskyi et al. 2022; Hematyar et al.2024), clarifying its potential and relevance for the diversification of European freshwater aquaculture.

A study dating back to 2013 evaluated the potential of different starter feeds for indoor larviculture of largemouth bass. The lowest mortality was seen when larvae were weaned to Otohime B, starting from the 14th day after the onset of exogenous feeding (Skudlarek et al. 2013). The first feed is important not only for the development of larvae but also it can affect the acceptance of different feeds in the later growth stages, regulating metabolism and gut microbiota, among other potential mechanisms. As nutritional programming has been documented in fish, the introduction of sustainable feeds during the early life of fish is gaining increasing attention in aquaculture (Hou and Fuiman 2020). This study aimed to evaluate the performance of largemouth bass larvae subjected to combinedweaning, starting with Otohime B2 (OB2) and ending with one of the four larval diets with various inclusion of marine ingredients, namely the three commercial feeds [OB2, Aller Infa (AI), and Aqua Start (AS)] with different inclusion levels of marine ingredients, and one experimental fish feed (EF). The three commercial feeds were selected according to the marine ingredient abundance, specifically, the presence of krill and squid meal, with OB2 having the highest and AS the lowest marine ingredient diversity, according to the manufacturer's declarations [Electronic Supplementary Material (ESM) S1)]. Our premise was that the opportunistic predatory nature of largemouth bass allows for including lower marine-ingredient diets during early life

https://doi.org/10.17221/129/2024-CJAS

stages. Eventually, acceptance of more sustainable diets during the weaning phase might provide more efficient adaptation to sustainable feeds during the later grow-out rearing phase.

MATERIAL AND METHODS

Feeds

Four larval feeds were used in the study, and three commercial feeds: OB2 (Marubeni Nisshin Feed Co., Ltd, Tokyo, Japan), AI (Aller Aqua Group, Christiansfeld, Denmark), and AS (Aqua Garant, Austria), and locally produced EF. EF was made by a local company (H&H Carpio Halászati Kft, Ócsárd, Hungary) to reduce the costs of purchasing and importing commercial starter feeds. Proximate compositions of the diets were analysed by standard methods of the AOAC and are presented in Table 1 (Horwitz 2000). The moisture content was determined by drying the samples in an oven at 105 °C until a constant weight was achieved and then cooling in a desiccator. For crude ash determination, samples were weighed and placed in a muffle furnace at 550 °C for 8 hours. Crude protein was determined on the Kjeldahl digestion and distillation system VAPODEST 450 of Gerhardt Ltd, (Königswinter, Germany) crude fat by using SOXTHERM® Unit SOX416 from Gerhardt Ltd., crude fibre by using Fibretherm FT12 from Gerhardt Ltd. The concentration of fatty acids (FA) was done in an accredited laboratory. FAs were measured after esterification to fatty acid methyl esters (FAME) using a Shimadzu gas chromatograph (Type GC-2010; Shimadzu Corporation, Kyoto, Japan) and followed the Hungarian Standards (MSZ EN ISO 12966-2). The results of proximate feed analyses are expressed as weight percent to original feed matter. The results of FA analysis are expressed as weight percent of total esterified fat to original matter. Details on further feed biochemical analyses are provided in ESM S1.

Study design

Ethical permission for this study was provided by the Institutional Animal Welfare Committee of the Hungarian University of Agricultural and Life Sciences Szent István Campus (MATE-SZIC/416-1/2022). Animal experiments complied with the *A*nimal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines and were performed per EU Directive (2010/63/EU). Details on the fish origin are provided in ESM S1. On the 5th day post hatching (DPH), fish (30 000 larvae) were volumetrically counted and stocked into the common 1.4 m^3 cylindro-conical tank, with black walls and white conical bottom, supplied with up-welling flow with 50% of hourly tank exchange. The tank was within the larval rearing recirculation aquaculture system, with a drum filter of 32 µm mesh, moving bed bioreactor, trickling, UV, and ozone disinfection units. The temperature in the tanks was maintained at 24.9 ± 0.03 °C (SD). For the first 14 days larvae were fed enriched *Artemia* nauplii (SEP-ART GSL; INVE Aquaculture, Belgium). Next to the *Artemia* nauplii, fish were given OB2 feed as a transitional weaning feed for additional four days from the 19th to the 22nd DPH. In the previously set early weaning experiment, OB2 demonstrated the lowest mortality (ESM S1). Because of ethical reasons, the lack of previous data, and the limited year-round availability of LMB, OB2 was selected as a transitional diet for the current experiment.

Table 1. Proximate compositions of Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and experimental feed (EF), provided as mean values ± standard deviations (SD); *n*=2

	OB2	AI	AS	EF
Dry matter (%)	95.7 ± 0.03	95.5 ± 0.07	95.2 ± 0.02	95.3 ± 0.00
Crude protein (%)	56.4 ± 0.18	61.2 ± 0.12	60.68 ± 0.12	60.7 ± 0.17
Crude fat (%)	15.4 ± 0.16	10.1 ± 0.04	14.63 ± 0.18	8.58 ± 0.45
Crude ash (%)	12.5 ± 0.01	11.3 ± 0.05	8.61 ± 0.04	8.20 ± 0.01
Crude fiber (%)	2.5 ± 0.19	0.65 ± 0.18	0.80 ± 0.49	2.63 ± 0.11
[a]Nitrogen free extract (%)	8.9	12.2	10.5	15.1
[b]Gross energy (MJ/kg)	17.9	17.4	18.7	17.4

The nitrogen free extracts (NFE)[a] of diets and gross energy (GE)[b] values were calculated as NFE = 100 – (crude protein + crude fat + crude fibre + ash + moisture), and GE (MJ kg) = NFE × 23.6+ Protein*17.2 +Lipids* 39.5 (Halver and Hardy 2002)

https://doi.org/10.17221/129/2024-CJAS

Artemia nauplii were supplied manually five times a day, while the dry diet was added using an automatic mechanical feeder (LINN Gerätebau GmbH, Germany). The daily *Artemia* nauplii amount was 100 nauplii/larva; in five days, it was gradually increased to 500 nauplii/larva with 6 daily meals, while the feed amount was maintained constant at 30 g of feed per day supplied *via* automatic feeders every 15 min. The tank was cleaned daily. On the 23rd DPH, larvae were stocked again in the same tanks and under the same conditions as above, except for the number of larvae per tank which was 200 per 250 l tank. There were 12 tanks (3 tanks per feeding group) and four groups receiving OB2, AI, AS, and EF. Larvae were fed manually with enriched *Artemia* nauplii in the total amount of 500 nauplii/larva/day in 6 daily meals during the first two days, then gradual removal of one portion per day started on the 25th DPH of the experiment until complete removal on the 30th DPH. Dry feed was added in the amount of 3 g per tank per day for the first 3 days, then it was increased to 5 g from the 26th to the 29th DPH, then to 6 g on the 30th DPH, and, finally, 9 g on the 31st DPH. The study design is illustrated in Figure 1.

Fish sampling was performed on the 32nd DPH (10th day of the experiment). Fish were anesthetised using 0.4 ml/l of phenoxyethanol. Weight and length were measured after drying the fish with paper towel to remove remaining water. Twelve fish per tank were sampled for morphometric analysis (36 fish per group). For molecular analysis, 10–12 fish per tank were sampled, depending on the fish size, totally giving 30–36 fish per group. All samples for molecular analyses were stored at –80 °C until use.

In parallel to setting up indoor experiments, a portion of larvae (30 000 pieces) from the same batch was reared in the 700 m^2 earthen pond. Before filling up, the pond was fertilised with 2 t/ha of cow manure. The pond was filled up until half of its capacity five days before the stocking of the larvae. Upon the stocking of the larvae, the pond water level was increased gradually, about 15 cm per week, being at its full volume around four weeks after stocking the larvae when the sampling of the juveniles took place. Larvae were reared fully extensively on live zooplankton without additional feeding.

Homogenisation of fish RNA extraction, and quantitative PCR (qPCR)

Tissue homogenisation and RNA extraction were done as described in Ljubobratovic et al. (2017), with slight modifications. For enzyme assay and enzyme-linked immunosorbent assay (ELISA), whole fish were homogenised on ice by adding 10 volumes of homogenisation buffer to 300–400 mg of fish samples (phosphate buffered saline; PBS in the case of downstream ELISA assays or 50 mM Tris-HCl, pH 7 + 2 mM mannitol in the case of downstream enzyme assays). For RNA extraction, fish (3–4 whole fish, depending on the size, up to 200 mg total) were placed in the mortar, homogenised using liquid nitrogen, and 10 volumes of denaturing buffer [4 M guanidine thiocyanate, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, 0.5% (*w*/*v*) N-lauroylsarcosinate

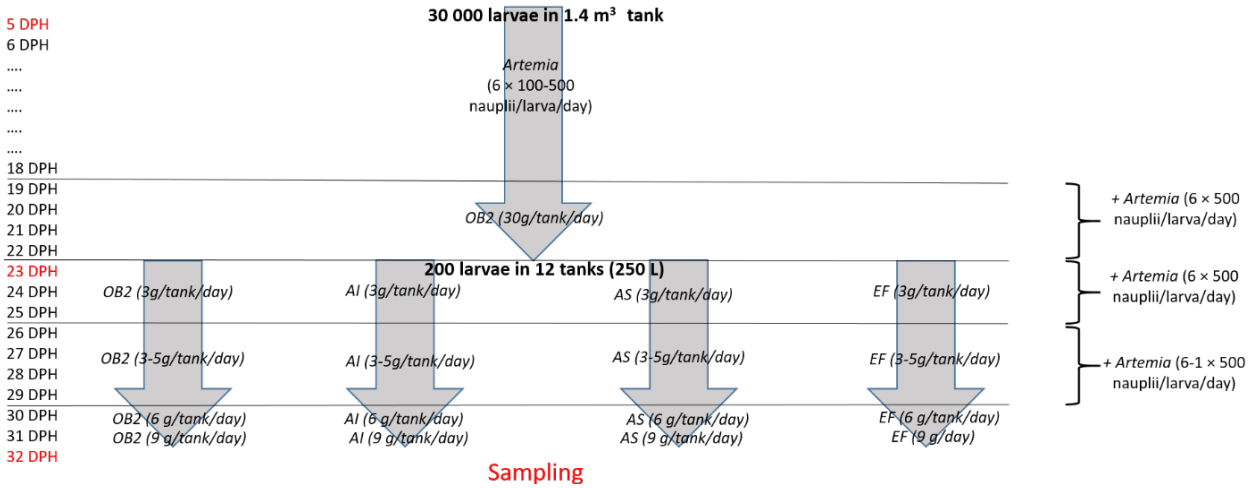

Figure 1. Study workflow

AI = Aller Infa; AS = Aqua Start; DPH = day post-hatching; EF = Experimental Feed; OB2 = Otohime B2

https://doi.org/10.17221/129/2024-CJAS

sodium salt] were added. More details on tissue homogenisation and RNA isolation are provided in ESM S1. Reverse transcription (RT) was performed using Xpert cDNA Synthesis Kit (Grisp, Lda., Porto, Portugal), while qPCR was performed using Xpert Fast SYBR 2X Mastermix (GRiSP Lda., Portugal). For qPCR, the template [complementary (c) DNA] was diluted at 1 : 5 before use. The reaction mixture consisted of 5 µL of Xpert Fast SYBR 2X Mastermix, 1 µl of each primer (100 mM concentration), and 1 µl of template cDNA (1/5 dilution) and PCR-grade water was added up to 20 µl. The following cycling conditions were used: 1 × 95 °C, 2 min; 40 × 95 °C, 5 s, and 60 °C, 30 seconds.

Primers (Table 2) for the specific genes were designed using mRNA sequencing data available from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Primers were designed using Primer-BLAST, NCBI. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Primers for *Flavobacterium* spp. were designed by Ljubobratovic et al. (2017).

Digestive enzyme assays

Assays for trypsin, chymotrypsin, lipase, alkaline phosphatase (AP), and amylase were done as detailed in ESM S1. Briefly, 10 µl of the sample was mixed with 190 µl of the reaction buffer containing the substrate [1 mM Nα-benzoyl-DL-arginine-ρ-nitroanilide hydrochloride (BAPNA) for trypsin, 0.5 mM succinyl-(ala)2-pro-phe-ρ-nitroanilide (SAPNA) for chymotrypsin, 1 mM ρ-nitrophenyl palmitate (ρ-NPP) for lipase, 5 mM p-nitrophenyl phosphate (p-NPP) for AP], as described in Ljubobratovic et al. (2017). The absorbance (410 nm in trypsin, chymotrypsin, and lipase assays and 407 nm in AP assay) was measured immediately (0 h) and every minute during the subsequent 10 min, maintaining the 37 °C incubation temperature. For the amylase assay, 50 µl of the sample was mixed with 50 µl of 1% starch solution. The mixtures were incubated for 15 min at room temperature (RT). Afterwards, 50 µl of stop solution was added and the mixture was boiled for 15 minutes. Finally, 50 µl of the reaction mixture was diluted with 150 µl of water and the absorbance was measured at 540 nm. Phospholipase A2 (PLA2) assay was done following the manufacturer's instructions, using the Abcam PLA2 Assay kit (Abcam, United Kingdom). Protein concentration in homogenates was analysed using the Analyticon Biotechnologies kit (Analyticon Biotechnologies AG, Lichtenfels, Germany). The results are expressed as µg/mg of protein for trypsin, chymotrypsin, lipase, and AP, as nmol/min activity/mg of protein for PLA2, and as mmol of maltose standard released per min per mg of protein.

ELISA

Since fish larvae were continuously fed, they were rapidly growing and difficult to assess for

Table 2. Primers used in the study

Primer abb.	Primer full name	Sequence	DNA strand complementarity
TrpT	Troponin T, fast skeletal muscle isoforms-like mRNA	TGCAATCGTCTCGGACTCAC	fw
		CCGCAAAGTAAACCTCAGCG	rev
TnnC	Troponin C, skeletal muscle mRNA	GAACCTTCCCTGATCGCCTT	fw
		GAGGAGGTGGGGCTTAAGTG	rev
Col1α2	Collagen, type I, α 2 mRNA	TCTGAGAGGACTGAACGGACT	fw
		AACAAGGTGTTTTCCCGCGT	rev
ColIα1	Collagen, type I, α 1a mRNA	GCGGTGAGAGGAATGAAAGGA	fw
		TGGCTGTCAGTTTCACCGTT	rev
PSN	Periostin, osteoblast specific factor b mRNA	ACCAAACCCAGCCGTTGTAA	fw
		TTTGTCAGTTATACCTATTGCAGGA	rev
Flavo	*Flavobacterium* spp. 16S rRNA	ATCCCCCACACTGGTACTGA	fw
		CCATAGGACCGTCATCCTGC	rev
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase mRNA	GCACTGTCACATCACCCACAT	fw
		TTCCTTCAGGCATCTAACAGGG	rev

fw = forward; rev = reverse

https://doi.org/10.17221/129/2024-CJAS

metamorphosis by morphological inspection. For this reason, molecular analysis of the levels of hormones regulating the morphogenesis process, thyroxine (T4) and triiodothyronine (T3), was performed. Furthermore, the levels of the main appetite and digestive function regulator, cholecystokinin (CCK), were estimated (Campinho 2019). Hormonal analysis was done using BioMatic (Canada) ELISA kits, based on the competitive reaction between the endogenous and the labelled target. Protein concentrations in the homogenates were estimated using the Analyticon Biotechnologies kit. Hormone concentrations are expressed as ng/mg of protein for T3 and T4 and as pg/mg of protein for CCK.

Statistical analysis

The results of the proximate, FA, and biochemical analysis of feeds are presented in the table as means ± standard deviation (SD) of replicated assays (n = 2, 2, and 3–4 per group, respectively). Data were analysed using the Kruskal-Wallis test with Dunn's post-hoc test, $P < 0.05$ (https://www.statskingdom.com/kruskal-wallis-calculator.html).

Morphometric evaluation results are presented as means ± SD of measurements of average tank values (n = 12 larvae per tank, 3 tanks per group). Specific growth rates (SGR) from the 3rd to 32nd DPH, allometric coefficients (b), and relative condition factors (Kn) were calculated as detailed previously (Peter et al. 2023). b was calculated as the length-weight relationship (LWR) slope:

$$LN(W) = a + b \log(L) \quad (1)$$

where:
W – fish weight (g);
L – fish length (cm);
a – the intercept of the regression line;
b – the slope of the regression line.

Kn was calculated using the Equation (2):

$$Kn = \frac{W_o}{W_c} \quad (2)$$

where:
Kn – relative condition factors
W_o – observed weight;
W_c – weight estimated using LWR.

Survival was expressed as % of live fish from the initial number of fish per tank. Comparisons for morphometric analyses were made using the Kruskal-Wallis test with Dunn's post-hoc test, $P < 0.05$.

The results of molecular analyses are presented in tables as means ± SD of the analysis performed on pooled samples (n = 9 pooled samples per group, 3 pooled samples per tank). The pooled sample comprised 3–4 fish, depending on the fish size. In case that normality and variance homogeneity, as assessed by Shapiro-Wilk and Levene's test ($P < 0.05$), could not be achieved by outlier removal or data transformation (logarithmic or reciprocal), Kruskal-Wallis test with Dunn's post hoc test ($P < 0.05$) was used for the analysis of unprocessed data (no outlier removal or data transformation). Otherwise, data were analysed using the analysis of variance (ANOVA) with Tukey's post hoc test ($P < 0.05$). Outliers were removed using Grubbs' test for a single outlier, $P < 0.05$ (up to one outlier per group) (https://contchart.com/outliers.aspx). The qPCR results are presented as relative mRNA expression, normalised to the housekeeping gene (GAPDH) mRNA expression, and calibrated to one arbitrarily selected sample from the OB2 group as the reference. The correlation was analyzed using the Pearson test, $P < 0.05$, between the average group values of all analysed variables and the feed nutritive profile (continuous variables only). Partial correlation (Pearson test, $P < 0.05$) was done when needed. ANOVA, Shapiro-Wilk and Levene's test, Pearson correlation analysis, and mean value ± SD calculations for all analyses were performed using IBM SPSS Statistics (v21). The Corrplot R package was used to visualize the correlation matrix (https://www.R-project.org).

RESULTS

Feed composition and biochemical profiles

Tables 1 and 3 outline the chemical and FA composition of the diets.

Biometric indices

Individual body weights and SGR of larvae weaned onto AS were lower than in OB2 (P = 0.04

https://doi.org/10.17221/129/2024-CJAS

Table 3. Fatty acid (FA) composition of Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and Experimental Feed (EF), provided as percentage (%) of total FA in the respective feeds; *n* = 2

Composition	OB2	AI	AS	EF
l4:0	6.46	5.56	2.76	6.07
16:0	22.1	20.1	20.4	24.2
16:1	6.92	6.56	3.41	5.47
l8:0	4.27	2.27	5.23	4.85
l8:lω9	18.0	14.1	19.4	25.2
LA 18:2 ω6	5.66	5.71	15.8	10.4
y-18:3 ω6	0.17	0.11	0.14	0.14
ALA α-l8:3 ω3	1.10	0.94	1.89	1.26
20:0	0.18	0.15	0.40	0.24
20:1	3.71	11.1	2.67	2.99
20:2	0.20	0.24	0.32	0.24
20:3 ω6	0.10	0.07	0.09	0.12
ARA 20:4 ω6	0.72	0.52	1.18	0.71
20:3 ω3	0.13	0.10	0.16	0.14
EPA 20:5 ω3	13.3	11.4	5.62	7.68
22:l	1.35	1.90	0.53	0.82
22:5 ω3	1.39	1.00	1.01	0.67
DHA 22:6 ω3	12.2	15.0	16.0	7.18
24:1	1.05	2.46	1.00	0.58
SFA	34.0	28.8	30.8	36.3
MUFA	31.1	36.1	27.0	35.2
PUFA	35.0	35.1	42.2	28.6
PUFA ω6	6.86	6.65	17.6	11.6
PUFA ω3	28.1	28.4	24.6	16.9

ALA = α-linolenic acid; ARA = arachidonic acid; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; LA = linoleic acid; MUFA = monounsaturated FA; PUFA = polyunsaturated FA; SFA = saturated FA

Table 4. Biometric indices of fish weaned to Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and Experimental Feed (EF), provided as mean values ± SD; *n* = 3

Biometric indices	OB2	AI	AS	EF
Individual weight (mg)	82.9 ± 14.1^{a}	78.5 ± 15.3^{ab}	56.6 ± 19.0^{b}	58.6 ± 6.0^{ab}
Standard length (mm)	20.3 ± 0.96^{a}	21.0 ± 1.63^{a}	19.3 ± 1.85^{a}	19.1 ± 0.71^{a}
Sspecific growth rate, weight (%/day)	14.3 ± 0.52^{a}	14.0 ± 0.63^{ab}	13.0 ± 0.30^{b}	12.9 ± 0.90^{ab}
Relative condition factor (*Kn*)	1.01 ± 0.001^{a}	1.01 ± 0.000^{a}	1.01 ± 0.000^{a}	1.01 ± 0.000^{a}
Allometric coefficient (*b*)	1.93 ± 0.55^{a}	3.01 ± 0.70^{a}	2.43 ± 0.93^{a}	1.99 ± 1.13^{a}
Survival (%)	98.7 ± 1.04^{a}	96.0 ± 3.28^{a}	98.2 ± 1.89^{a}	97.5 ± 1.73^{a}

$^{a–b}$Letters in the superscript denote the absence of statistical significance

in both cases) (Table 4). No differences in other analysed parameters were observed between the treatments. Pond-reared fish presented the average length of 25.6 ± 3.4 mm and weight of 195.2 ± 96.8 mm.

mRNA gene expression

Expression of major organogenesis-related genes was assessed at the mRNA level, along with the expression of 16S rRNA from opportunistically

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Table 5. Relative gene (mRNA/rRNA) expression levels in the whole bodies of fish weaned to Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and Experimental Feed (EF), provided as mean values ± SD; n = 9

	OB2	AI	AS	EF
Collagen type I, α1 (ColIα1)^{KW}	1.19 ± 0.60^{a}	2.16 ± 2.07^{a}	0.37 ± 0.69^{b}	0.37 ± 0.53^{b}
Collagen type I, α2 (ColIα2)^{KW}	1.36 ± 0.70^{a}	1.42 ± 1.03^{a}	0.30 ± 0.42^{b}	0.48 ± 0.84^{b}
Troponin T (TrpT)^{AT}	1.73 ± 0.70^{a}	1.06 ± 0.56^{ab}	1.12 ± 0.66^{a}	0.47 ± 0.20$^{b\ (or)}$
Troponin C (TnnC)^{KW}	1.53 ± 0.58^{a}	2.13 ± 1.72^{a}	1.27 ± 0.68^{ab}	1.02 ± 0.59^{b}
Periostin (PSN)^{KW}	1.40 ± 0.63^{a}	1.86 ± 1.37^{a}	0.34 ± 0.40^{b}	0.61 ± 0.73^{b}
Flavobacterium spp. 16S rRNA^{KW}	0.94 ± 0.98^{a}	0.14 ± 0.22^{b}	0.13 ± 0.31^{b}	0.01 ± 0.01^{b}

$^{a–b}$Letters in the superscript denote the absence of statistical significance; sample 1 from the OB2 group was used as the calibrator; AT = ANOVA with data transformation; KW = Kruskall-Wallis test; OR = outlier removal

Table 6. Digestive enzyme activities in the whole bodies of larvae weaned to Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and Experimental Feed (EF), provided as mean values ± SD; n = 9

Enzyme*	OB2	AI	AS	EF
Alkaline phosphatase (μg/mg of protein)^{AN}	14.4 ± 3.69^{a}	7.93 ± 2.36^{b}	7.18 ± 2.24^{b}	7.06 ± 2.20^{b}
Trypsin (μg/mg of protein)^{AN}	1.87 ± 0.28^{a}	1.66 ± 0.33^{a}	1.91 ± 0.37^{a}	1.81 ± 0.54^{a}
Chymotrypsin (μg/mg of protein)^{AN}	2.25 ± 0.62^{a}	1.69 ± 0.45^{ab}	1.47 ± 0.49^{b}	1.15 ± 0.41^{b}
Trypsin to chymotrypsin activity (T/C) ratio^{AN}	0.91 ± 0.35^{a}	0.93 ± 0.2$^{a(or)}$	1.51 ± 0.40$^{ab(or)}$	1.25 ± 0.31$^{b(or)}$
Lipase (μg/mg of protein)^{AN}	0.29 ± 0.19^{a}	0.26 ± 0.10^{a}	0.32 ± 0.21^{a}	0.17 ± 0.20^{a}
Phospholipase A2 (PLA2) (nmol/min activity/mg of protein)^{KW}	3.57 ± 1.79^{a}	1.95 ± 0.86^{b}	2.44 ± 0.75^{ab}	2.08 ± 0.64^{ab}
Amylase (mmol of released maltose/min/mg of protein)^{KW}	0.087 ± 0.068^{a}	0.043 ± 0.039^{ab}	0.015 ± 0.025^{bc}	0.004 ± 0.012^{c}

$^{a–b}$Letters in the superscript denote the absence of statistical significance

AN = ANOVA; KW = Kruskall-Wallis test; or = outlier removal; *(amount of converted substrate/(time unit)/amount of proteins in the sample)

pathogenic *Flavobacterium* spp. (Table 5). EF group had the lower *TrpT* mRNA expression in comparison with OB2 (P < 0.000 1) and AS (P = 0.042). Similarly, *Tnnc* expression was significantly lower in EF-weaned fish than in the OB2 group (P = 0.03) and AI group (P = 0.02). EF and AS-fed larvae showed lower *ColIα2* expression than OB2- (P = 0.01 and 0.005, respectively) and AI-fed fish (P = 0.02 and 0.008, respectively). Likewise, PSN expression was lower in EF and AS groups again than in OB2- (P = 0.04 and 0.003, respectively) and AI-weaned fish (P = 0.03 and 0.002, respectively). *ColIα1* expression was higher in OB2- and AI-weaned larvae than in EF (P = 0.006 and 0.003, respectively) and AS (P = 0.008 and 0.005, respectively) groups. *Flavobacterium* spp. 16S rRNA levels were significantly higher in OB2 in comparison with AI (P = 0.02), EF (P = 0.000 4) and AS (P = 0.02).

Digestive enzyme activities

Activities of major digestive enzymes relevant to digestion in fish larvae were evaluated using biochemical assays. AP activity was significantly higher in OB2-weaned fish in comparison with the other three diets (P < 0.000 1) (Table 6). PLA2 activity was also higher in larvae weaned onto OB2, but only in comparison with fish weaned onto AI (P = 0.000). OB2 groups also showed the significantly higher chymotrypsin activity than EF (P < 0.000 1) and AS (P = 0.012) groups. The trypsin to chymotrypsin (T/C) activity ratio was lower in OB2 and AI groups than in EF-fed larvae (P = 0.003 and 0.006, respectively). Amylase activity was again significantly higher in the OB2 group compared to EF (P = 0.000 1) and AS (P = 0.002) fish, but also in the AI-weaned group in comparison with the EF group (P = 0.01). No differences in trypsin and lipase activities were seen between the treatments.

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Table 7. Concentrations of T3, T4, and CCK in the whole bodies of fish weaned to Otohime B2 (OB2), Aller Infa (AI), Aqua Start (AS), and Experimental Feed (EF), provided as mean values ± SD n = 9

????	OB2	AI	AS	EF
Tri-iodothyronine (T3) (ng/mg of protein)^{KW}	0.187 ± 0.059^{ab}	0.164 ± 0.058^{a}	0.196 ± 0.051^{ab}	0.248 ± 0.088^{b}
Thyroxine (T4) (ng/mg of protein)^{AN}	2.02 ± 0.70^{a}	1.90 ± 0.62 $^{a(or)}$	2.42 ± 0.77 ^{a}	2.87 ± 1.06^{a}
Cholecystokinin (CCK) (pg/mg of protein)^{AN}	0.053 ± 0.025^{a}	0.032 ± 0.014^{a}	0.054 ± 0.024^{a}	0.058 ± 0.023^{a}

$^{a–b}$Letters in the superscript denote the absence of statistical significance

AN = ANOVA; KW = Kruskall-Wallis test; OR = outlier removal

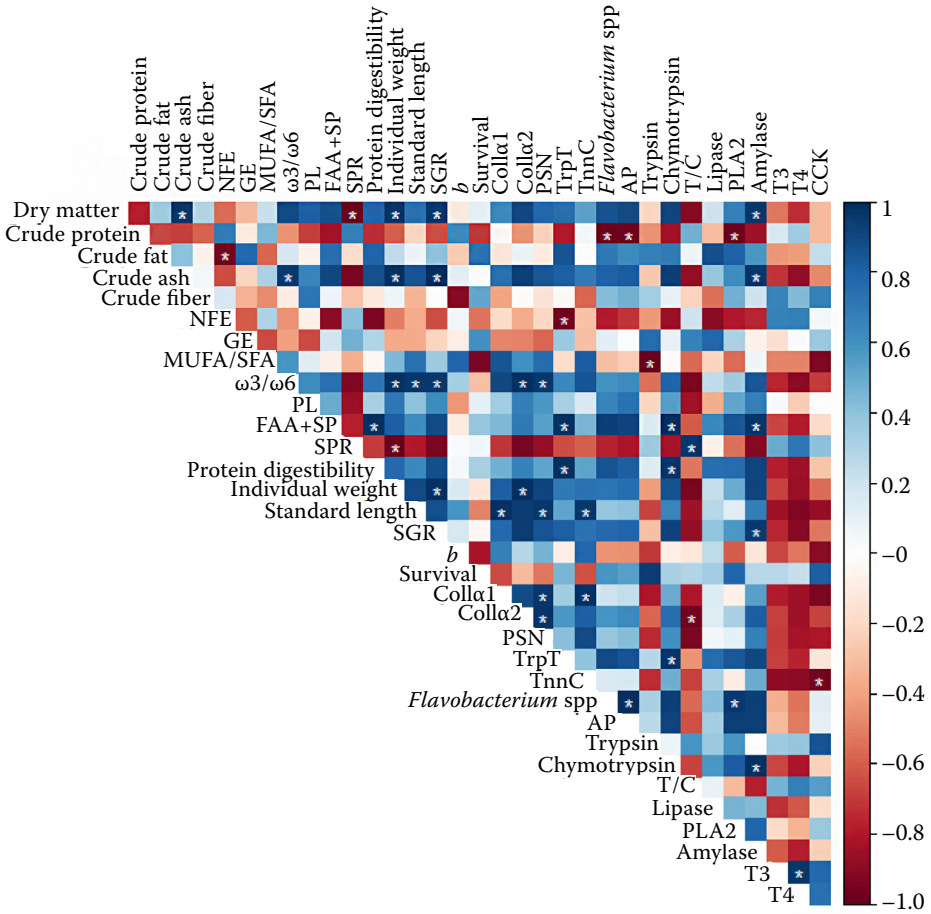

Figure 2. Correlation plot showing correlations between the analysed parameters

*Indicates a significant (P < 0.05) correlation between two variables; AP = alkaline phosphatase; b = allometric coefficient; FAA+SP = free amino acids + short peptides; GE = gross energy; MUFA = monounsaturated fatty acids; NFE = nitrogen-free extract; PL = phospholipids; PLA2 = phospholipase A2; SFA = saturated fatty acids; SGR = specific growth ratio; SPR = soluble proteins; T/C = trypsin to chymotrypsin activity ratio; ω3/ω6 = ratio between ω3 and ω6 polyunsaturated fatty acids (PUFA)

Hormonal status

Concentrations of metamorphosis and gut activity regulators were assessed through ELISA. AI group showed a significantly lower level of T3 in comparison with EF-weaned fish (P = 0.03) (Table 7). No differences in concentrations of other hormones were observed.

https://doi.org/10.17221/129/2024-CJAS

Correlation analysis

Details of correlation analysis are given in ESM S1. A visual representation of the correlation matrix is provided in Figure 2, while a correlation matrix with *P*-values is provided in ESM S2. Results of partial correlation analysis have been incorporated into the discussion when needed.

DISCUSSION

This research compared the efficacy of weaning the indoor-reared largemouth bass, using three commercial diets (OB2, AI, AS) and one experimental feed (EF). According to the fatty acid profile analysis of the diets, OB2 and AI had the highest DHA and EPA percentage, reflecting the higher content of marine ingredients (Table 3), and the highest ω3 to ω6 PUFA ratio. Nevertheless, the overall fat level in AI was low and the mono-to-saturated fatty acid (MUFA/SFA) ratio was the highest in the AI diet, indicating a high level of vegetable ingredients in this feed (Zong et al. 2018).

Contrary to OB2 and AI, AS and EF diets were very inferior in ω3 fatty acid content and bioavailability. Namely, AS was very rich in LA, in addition to low levels of DHA and EPA. LA can reduce the endogenous DHA and EPA synthesis by competing at the binding sites of biosynthetic enzymes with ALA (Peter et al. 2023). Furthermore, given the differences in the bioavailability of fatty acids when supplemented in the form of phospholipids and neutral lipids in fish larvae (Tocher et al. 2008), AS is expected to be very inferior in terms of PUFA supplementation, since its fatty acids appear to be mainly bound to neutral lipids, as the PL level in this feed was quite low. EF was developed by incorporating a diverse range of marine ingredients. However, DHA levels were at the lowest in the tested diets, and EPA levels were also similarly low compared to AI and OB2 diets (Table 2).

The highest weight gain was observed in fish weaned using OB2 and AI. Despite similar weight gain, it appears that AI outperformed OB2 in terms of morphogenesis progress. Namely, T3 levels were significantly lower in AI-weaned larvae than in EF-weaned fish, while T4 showed a statistical trend ($P < 0.1$) towards the decrease in AI *vs* EF. This indicates the most advanced morphogenesis since levels of T3 and T4 drop after the end of metamorphosis, which is supposed to be completed on the 32nd day post-hatching of largemouth bass (Campinho 2019). Inferior overall maturation of B2-fed larvae may appear contradictory, given the highest alkaline phosphatase activity in this group, which is indicative of the most advanced gut maturation, presumably as the result of high phospholipid levels in OB2 (Wang et al. 2022). A closer look at the expression levels of cholecystokinin may aid the interpretation of observed results. Cholecystokinin is the key regulator of fish digestion, affecting the levels of pancreatic secretions. It furthermore acts as an appetite suppressor, slowing the stomach emptying (Ronnestad et al. 2013). The statistical trend of lower cholecystokinin concentration was seen only in AI compared to EF-fed fish. This could be attributed to the differences in saturated fatty acid levels between the four diets (lowest in AI), given the previously reported inverse association of the fatty acid saturation degree with cholecystokinin expression in humans (Robertson et al. 2022). Three dietary factors seemed to stimulate the fish weight gain of OB2 and AI-fed fish in the present research: free amino acids and short peptides, fat and ash percentage, which is indicative of the mineral content of the diet. OB2 and AI-fed fish also showed greater amylase and chymotrypsin specific activities. This is also supported by the correlation analysis showing positive links between free amino acid and short peptide levels on the one hand, and amylase and chymotrypsin specific activities on the other hand. In support of this, in Nile tilapia (*Oreochromis niloticus*) culture, amylase activity and fish growth were stimulated by free amino acids (Wangkahart et al. 2023), while studies in rats showed that free amino acids can activate pancreatic enzyme secretion (Hashimoto and Hara 2003).

In contrast to the above-mentioned three nutrients, the balanced fat composition (ω3 : ω6) in OB2 and AI supported the skeleton growth, correlating positively with the mRNA expression levels of collagen 1 alpha 1 (*Col1a1*), collagen 1 alpha 2 (*Col1a2*), and periostin (PSN), but also with the levels of slow muscle-specific *troponin C* (*TnnC*) (Darias et al. 2008). Slow muscle fibres support long-term slow contractions which may be critical for the proper skeleton development (Luna et al. 2015). Mutations in *Col1a1* have also been linked with skeleton malformations in zebrafish, suggesting that OB2 and AI-fed fish might be less prone to skeletal deformities in subsequent rearing stages

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(Huang et al. 2021). Although the ω3 : ω6 in the feed supports the skeleton growth, the lower total fat percentage in the diet in AI did not compromise the larval development according to the expression of ossification markers and fish length. In this line, the standard length indicates maturation progress in fish larvae, which correlated positively with ossification marker expression in the present study (Singleman & Holtzman 2014). The above implies that largemouth bass can be weaned to more sustainable feeds, containing lower fat percentage and more vegetable ingredients, as long as the ω3 : ω6 meets the nutritional demands of the species.

Contrary to free amino acids and short peptides, soluble proteins in AS and EF negatively affected fish weight gain. This was presumably the result of suppressing the amylase specific activity, as observed by a partial correlation controlling for nitrogen-free extracts (statistical trend, $P = 0.076$), as carbohydrates are well-known inhibitors of amylase activity (Frias-Gomez et al. 2023). Proteins originating from plant material and microorganisms have been attributed amylase-suppressing activity (Svensson et al. 2004), presumably indicating the presence of plant-derived proteins in AS and EF, which were rich in soluble proteins in the present research.

Crude proteins in AI, AS, and EF seemed to downregulate phospholipase A2 and alkaline phosphatase activity. Alkaline phosphatase is associated with gut maturation in fish larvae (Infante and Cahu 2001). Dietary proteins in excess might be detrimental to fish due to the high energy used to catabolize proteins and the potential accumulation of toxic nitrogen waste molecules resulting from amino acid degradation (Jang et al. 2022). This might have caused an 'adaptive' suppression of alkaline phosphatase activity to reduce the uptake of amino acids from the intestinal lumen. Notwithstanding, the suppression of alkaline phosphatase and phospholipase A2 activity in this fish species did not affect the growth, but it reduced the amount of potentially pathogenic *Flavobacterium* spp., presumably by reducing the amount of available amino acid monomers and hydrolysed phospholipid products in the intestinal lumen. *Flavobacterium* spp. is among the most important freshwater fish pathogens, inhabiting digestive and external fish surfaces, including the mucus and gills (Loch and Faisal 2015). Feed training of largemouth bass has been associated with a greater risk of *Flavobacterium* spp. infection (Bebak et al. 2009). As observed here, the OB2 group exhibited higher levels of *Flavobacterium* spp. compared to larvae fed AI, AS, and EF, presumably due to the low crude protein level. However, the possibility that the food affected the immune system by possessing different amounts of immunostimulants, i.e.

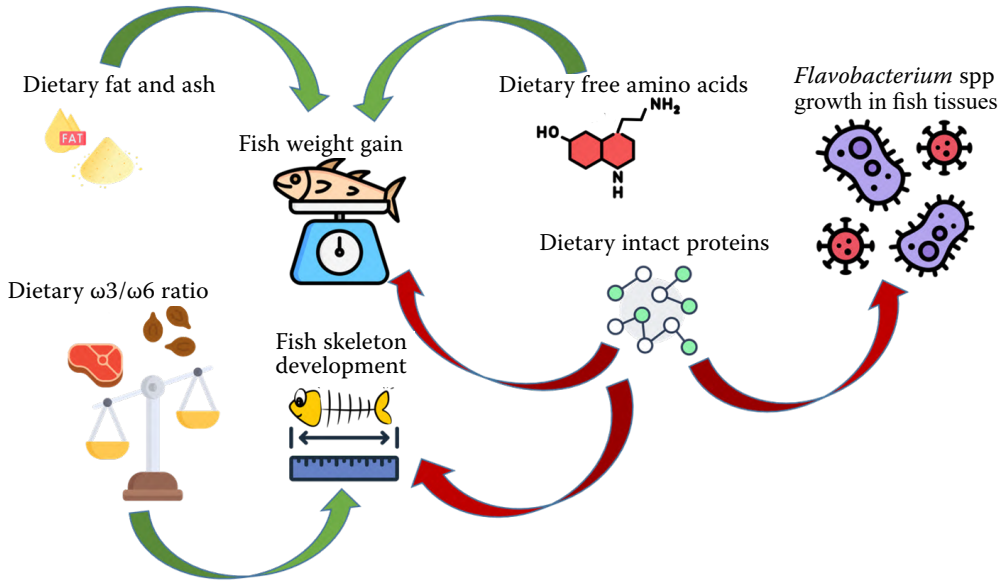

Figure 3. Illustration showing the presumed interactions between feed ingredients and fish performance
Green arrows show positive correlations, while red arrows show negative correlations
Source of the icons for the illustration: www.flaticon.com (accesed Apr 30, 2024) under the free license and are used in this manuscript as per the Terms of Use provided at https://www.freepikcompany.com/legal#nav-flaticon (authors of the icons: Iconjam, Freepik, Backwoods, Sir.Vector, smashingstocks, juicy_fish, Paul J., cah nggunung, and andinur)

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vitamins, essential FAs and amino acids, minerals, cannot be excluded (Vijayaram et al. 2024).

To summarise, this study has revealed previously unknown potentially omnivorous feeding habits of larval largemouth bass, including the association of amylase activity with fish weight gain. Moreover, the study has shown that increasing the dietary ω3 : ω6 and hydrolysed protein levels might optimally support the skeleton development of the indoor weaned LMB (Figure 3). Although ω3 fatty acids have unequivocal benefits for the skeleton development, the total fat percentage in the doses applied in this research does not appear to affect the larval growth parameters. This allows for the design of sustainable and lower-cost first feeds with the inclusion of a lower amount of marine ingredients. Finally, hydrolysed and intact proteins are critical in controlling infection risks. The study demonstrated the technical feasibility of rearing largemouth bass larvae in the intensive system with negligible fish mortality, despite the lower growth than in pond-reared larvae. Moreover, given the several-times lower price of AI than that of OB2, the above-outlined findings have a wide exploitation potential in aquaculture. Further nutritive and technological optimisations, applying the findings of this research, and, in particular, seeking to eliminate the transitional OB2 weaning stage, would upscale the largemouth bass farming in Europe.

Contlict of interest

The authors declare no conflict of interest.

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Received: August 3, 2024
Accepted: October 29, 2024
Published online: December 23, 2024