

## RESEARCH ARTICLE

# New approach to the development of tailor-made feed for fish larvae using Zebrafish *Danio rerio* as a model

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## Abstract

Protein hydrolysates have been used extensively as dietary protein for larval fish. Typically, they are expensive, difficult to produce, and show varying results when utilized for different species. This study proposed a practical hydrolysis method that utilizes endogenous enzymes within the body to "auto-hydrolyze", or digest its tissue proteins with its own endogenous enzymes, and produce a fishmeal tailored to nutritional requirements and absorptive capacity of larval fish. The objectives for this experiment were to determine the: 1) effect of the proposed hydrolysis method on tissue protein breakdown level; and 2) effect of dietary inclusion of obtained hydrolysates on larval growth performance, using Zebrafish (*Danio rerio*). Whole-body adult Zebrafish were utilized to produce an unhydrolyzed fishmeal, and three fishmeals hydrolyzed for 1, 2, and 3 h, respectively. Three diets were formulated, defined by their dietary protein supply. The Unhydro diet contained unhydrolyzed Zebrafish meal. The 50% Hydro diet contained 50% Zebrafish meal hydrolysates and 50% unhydrolyzed Zebrafish meal. The 100% Hydro diet contained 100% Zebrafish meal hydrolysates. Five groups were utilized in this study, with three groups receiving one of the produced Zebrafish meal-based diets. Larvae fed a commercial starter diet and *Artemia*, respectively, were included as reference groups. Larval fish were randomly stocked into tanks (100 fish per tank) at 3 days-post-hatch (dph), and the trial was carried out until 18 dph. Electrophoretic analysis showed that the proposed hydrolysis method was able to efficiently hydrolyze the protein within Zebrafish body. The feeding trial results found no significant differences in weight, total length, or survival between the Unhydro, 50% Hydro, and 100% Hydro groups. The proposed hydrolysis method provides a practical and potentially cost-effective approach to producing species-specific fishmeal hydrolysates. Further research is necessary to determine whether the produced hydrolysates can improve the growth of larval fish in other species.

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## Introduction

To achieve more control over the nutritional input during the larval stage, and provide more optimal dietary protein, extensive research has centered on producing formulated diets to reduce the use of live feeds [1–7]. Generally, intact marine protein sources have been used in these formulated larval diets [1,8–10], with fishmeal (FM) being the most widely used [11,12]. Commercial FM has been traditionally produced from whole fish, typically small, oily species, or wastes originating from fish processing. Fishmeal is attractive as a protein source because of its high protein content, high digestibility, complete amino acid profile and high palatability [13–15]. Fishmeal also serves as a partial source of vitamins and minerals, and although the majority of lipids are extracted, the remaining lipids (5–10%) contain highly unsaturated essential fatty acids [11,16]. Positive results on the early introduction of FM-based diets have been observed in larval rearing, with most successes occurring in co-feeding protocols, where dry diets are fed along with live feed [17–19].

Still, there are limitations to the ability of FM-based diets to fully replace live feeds at first feeding [20–22]. For example, the complex proteins that are present in FM are not efficiently digested by larval fish, especially during the first feeding stage [20]. Many fish species raised in aquaculture are altricial, not possessing fully developed digestive tracts at the start of exogenous feeding [21]. The underdeveloped digestive tracts lead to reduced digestive capacity of altricial larval fish [21,22], and as a result, the utilization of protein hydrolysates has been extensively studied [7,21,23–25]. Providing hydrolyzed protein in a diet for larval fish helps account for the reduced activity of proteolytic enzymes in the underdeveloped digestive tract, by breaking down the protein to smaller peptides and free amino acids (FAA) prior to ingestion [26]. Altricial larvae have been found to have an increased assimilation efficiency of these peptides and FAA compared to intact protein [22,27]. While the inclusion of protein hydrolysates has led to improved utilization of formulated diets in some species [7,22,28–30], the full replacement of live feeds has remained elusive in the rearing of many commercial species.

In larval fish, amino acids are required at high levels for tissue synthesis and serve as the major source of energy [31–33]. The requirement levels for amino acids, the indispensable amino acids (IDAA) in particular, vary among species, and ages of fish within the same species [34]. While specific IDAA requirements are unknown for larval fish [35], the amino acid profile of same-species muscle has been proposed to represent the amino acid requirement for larvae [36–38]. Given this premise, and the known variations in amino acid profiles of FM produced from different species [16], perhaps the species-specific FM obtained from adult fish would provide the right amino acid profile for fish larvae. This would be beneficial for the advancement of formulated dry diets in larval rearing, with the species-specific FM potentially serving as a dietary protein source better suited to replace live feed, compared to current commercial starter diets. Additional species-specificity can be added to incorporate the previously mentioned benefits of hydrolyzed FM, using endogenous enzymes to mimic *in vivo* digestion [39,40].

An *in vitro* hydrolysis method utilizing same-species endogenous enzymes was developed in Kwasek et al. [41], and later tested in tandem with same-species muscle in Molinari et al. [42]. Specifically, the endogenous enzymes and muscle of adult Largemouth Bass (*Micropterus salmoides*) were used to produce a protein hydrolysate that served as a species-specific dietary protein source for first-feeding Largemouth Bass larvae [42]. Additionally, the use of species-specific enzymes and muscle was shown to increase growth in larval Walleye (*Sander vitreus*), compared to protein produced from non-species-specific sources [43]. The endogenous enzymes utilized in those studies were extracted from the harvested digestive tracts through homogenization and centrifugation, then mixed with isolated muscle. In this study, we proposed a modified and simplified method of protein hydrolysis that removes the individual extractions of muscle and digestive tracts and produces a species-specific FM from whole fish. This method has not previously been tested in any other studies as a means of producing dietary protein for larval fish.

The aim of this study was to develop a more optimal protein source for larval fish and to advance the production of formulated diets to replace live feeds. The objectives were to; 1) determine the effect of the proposed simplified *in vitro* hydrolysis method on tissue protein breakdown level and the production of a species-specific hydrolyzed FM; and 2) evaluate the effect of dietary inclusion of the obtained FM hydrolysate on larval fish. The performance of the hydrolysates was assessed based on growth and survival, the gene expression of the intestinal peptide transporter PepT1, and the postprandial muscle FAA pool, used as an indicator of dietary amino acid availability. This study utilized Zebrafish (*Danio rerio*), as they have been found to be an ideal model species for nutritional research [44].

## Materials and methods

### Experimental conditions

The feeding trial was conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University-Carbondale (SIUC), IL. The experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of SIUC. The SIUC Institutional Animal Care and Use approved all of the protocols performed (protocol #21-006). The experiment was carried out using a semi-recirculated aquaculture system (Iwaki Aquatic, Holliston, MA, USA). The system was equipped with two mechanical filters, a carbon filter, a UV light, and a biofilter. The photoperiod was maintained with overhead lights and the lights were on from 08:00–18:00 for 10 h of light and 14 h of darkness. The illumination provided was at 245 lux and the distance between the surface of the water and the light source was 10 cm. The inflow was set at 100 mL/min for each tank. During the study, the temperature was 27.37°C (± 0.62) and the pH was 7.25 (± 0.23). The salinity of the system was kept at 1–3 ppt to prolong the viability of the live feed [45].

### Zebrafish hydrolysis

The hydrolysis method for this study was based on the *in vitro* method described in Kwasek et al. [41], with modification. Briefly, adult Zebrafish were kept at 27°C and fed to satiation one hour before harvesting. The fish were euthanized with an excess of tricaine methanesulfonate (MS-222) (Sigma-Aldrich, St. Louis, MO, USA) with a dose of 0.4 mg/mL. The whole bodies were ground three times with a meat grinder (General Food Service, Weston, FL, USA) and were stored on ice before and after each run through the grinder. After dilution with deionized water (1:2), the fish mince was homogenized with a PowerGen 1000 (Fisher Scientific, Waltham, MA, USA) tissue homogenizer on high speed for 10 min, at room temperature. The tissue to digestive tract ratio of the harvested Zebrafish was roughly 6:1. The homogenates were moved to containers (12 L), placed in a water bath at 27°C and stirred using an overhead stirrer (VWR VOS 16, VWR, Radnor, PA, USA) for the duration of the hydrolysis. The pH was adjusted to 7–9 using sodium bicarbonate for the entire hydrolysis to mimic the intestinal digestion of Zebrafish. Four Zebrafish meals were produced, three hydrolysates that were hydrolyzed for 1, 2, and 3 h, respectively, and an unhydrolyzed Zebrafish meal. After the hydrolysis, the solution was brought to

90°C for 15 min to stop any enzymatic activity. The unhydrolyzed Zebrafish meal was immediately brought to 90°C after homogenization to prevent any hydrolysis. While this meal is not a completely intact product, it is referred to as “unhydrolyzed” to clearly differentiate it from the products that underwent the controlled hydrolysis process. All products were frozen at -20°C and subsequently freeze-dried to remove moisture. After freeze-drying, lipids were extracted from each product using the Soxhlet method, with diethyl ether as the solvent [46]. Electrophoretic analysis of both unhydrolyzed and hydrolyzed products was conducted at The Ohio State University (Columbus, OH).

## Diets

Three diets were formulated to be isonitrogenous and isolipidic and meet the essential nutrient requirements of larval fish [9]. They were formulated to contain 51% crude protein, and 14% crude lipids (Table 1), and analyzed compositions were obtained after production (Table 2). The Unhydro diet did not contain any of the hydrolyzed Zebrafish meal and was solely based on the unhydrolyzed Zebrafish meal. The 50% Hydro diet was based on 50% Zebrafish hydrolysate mix and 50% unhydrolyzed Zebrafish meal. The 100% Hydro diet was 100% based on the Zebrafish meal hydrolysates for dietary protein. The hydrolysate mix contained equal parts of the 1, 2, and 3 h hydrolysates. This mix was used to provide a wide range of protein fragments in the diet.

## Experimental design

Larvae were produced from broodstock obtained from a local pet store (Petco, Carbondale, IL). The fish were separated by sex for two weeks prior to breeding. After this period, the broodstock were combined in a 2:1 ratio of females to males

**Table 1. Diet formulations for Zebrafish larvae feeding experiment.**

Ingredients (%)	Unhydro	50% Hydro	100% Hydro
Unhydrolyzed Zebrafish meal	74.00	37.00	—
Hydrolysate Mix <sup>a</sup>	—	38.75	78.00
Starch	4.85	3.10	0.85
Krill Meal <sup>b</sup>	5.00	5.00	5.00
Fish oil <sup>c</sup>	4.00	4.00	4.00
Lecithin <sup>d</sup>	4.00	4.00	4.00
Mineral mix <sup>e</sup>	3.00	3.00	3.00
Vitamin mix <sup>f</sup>	3.00	3.00	3.00
CaHPO <sub>4</sub>	1.00	1.00	1.00
Taurine	1.00	1.00	1.00
Choline chloride	0.10	0.10	0.10
Vitamin C <sup>g</sup>	0.05	0.05	0.05
Sum	100	100	100

<sup>a</sup>Equal mixture of 1, 2, and 3 h hydrolyzed Zebrafish meals

<sup>b</sup>Processed *Euphausia superba* (Florida Aqua Farms, Dade City, FL, USA).

<sup>c</sup>Cod liver oil (MP Biomedicals, Solon, OH, USA).

<sup>d</sup>Refined soy lecithin (MP Biomedicals, Solon, OH, USA).

<sup>e</sup>Bernhart-Tomarelli mineral mix with 5 ppm selenium in a form of sodium selenite (Dyets, Bethlehem, PA, USA).

<sup>f</sup>Custom Vitamin Mixture (mg/kg diet) Thiamin HCl, 6.84; Riboflavin, 7.2; Pyridoxine HCl, 10.29; Niacin, 16.35; D-Calcium Pantothenate, 75.84; Folic Acid, 1.89; D-Biotin, 0.24; Vitamin B12 (0.1%), 30; Vitamin A Palmitate (500,000 IU/g), 14.49; Vitamin D3 (400,000 IU/g), 12.39; Vitamin E Acetate (500 IU/g), 198; Menadione Sodium Bisulfite, 3.54; Inositol, 750 (Dyets, Bethlehem, PA, USA).

<sup>g</sup>L-Ascorbyl-2-Polyphosphate (Argent Aquaculture, Redmond, WA, USA).

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**Table 2. Analyzed composition of formulated diets.**

Analyzed Composition (g/100g) Dry Matter	Unhydro	50% Hydro	100% Hydro
Crude Protein (N x 6.25)	48.14 (± 0.18)	48.19 (± 0.09)	48.26 (± 0.17)
Crude Lipids	10.04 (± 0.08)	10.07 (± 0.14)	10.06 (± 0.12)
Ash	17.92 (± 0.04)	17.69 (± 0.05)	17.49 (± 0.03)
Alanine	2.94 (± 0.03)	2.88 (± 0.02)	2.90 (± 0.02)
Arginine	2.81 (± 0.01)	2.67 (± 0.04)	2.64 (± 0.02)
Aspartic Acid	4.32 (± 0.03)	4.26 (± 0.03)	4.24 (± 0.01)
Cysteine	0.46 (± 0.00)	0.46 (± 0.00)	0.48 (± 0.00)
Glutamic Acid	6.32 (± 0.15)	6.16 (± 0.06)	6.20 (± 0.08)
Glycine	2.92 (± 0.03)	2.80 (± 0.03)	2.93 (± 0.03)
Histidine	1.20 (± 0.00)	1.17 (± 0.01)	1.11 (± 0.02)
Hydroxyproline	0.58 (± 0.01)	0.55 (± 0.02)	0.61 (± 0.00)
Isoleucine	2.24 (± 0.01)	2.23 (± 0.02)	2.17 (± 0.02)
Leucine	3.56 (± 0.02)	3.52 (± 0.03)	3.45 (± 0.02)
Lysine	3.77 (± 0.03)	3.72 (± 0.03)	3.72 (± 0.01)
Methionine	1.20 (± 0.01)	1.17 (± 0.01)	1.19 (± 0.00)
Phenylalanine	2.01 (± 0.02)	1.99 (± 0.02)	1.94 (± 0.04)
Proline	2.17 (± 0.02)	2.12 (± 0.02)	2.18 (± 0.03)
Serine	1.86 (± 0.07)	1.79 (± 0.04)	1.83 (± 0.05)
Taurine	1.64 (± 0.25)	1.58 (± 0.03)	1.42 (± 0.05)
Threonine	1.97 (± 0.01)	1.94 (± 0.02)	1.93 (± 0.01)
Tryptophan	0.45 (± 0.01)	0.42 (± 0.01)	0.42 (± 0.00)
Tyrosine	1.52 (± 0.00)	1.44 (± 0.10)	1.50 (± 0.01)
Valine	2.42 (± 0.01)	2.40 (± 0.03)	2.35 (± 0.02)
Sum	46.37 (± 0.37)	45.27 (± 0.40)	45.21 (± 0.21)

Diets were analyzed in triplicates and the values are presented as mean (± S.D).

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and a wire mesh (1.5 mm) was added to the breeding tank with an artificial plant to induce spawning. The fish were allowed to breed for 24 h and then removed. The eggs hatched after approximately 48 h.

At 3 days-post-hatch (dph), larval Zebrafish were randomly distributed into 15 (3 L) tanks, with 100 fish per tank. There were five treatment groups in this study, with three replicate tanks each. The first group, **LF**, was a live feed reference group, which received only live feed for the duration of the study. The LF group was fed rotifers (3–7 dph) and then switched to *Artemia nauplii* (7–18 dph), with a transition period from 7 to 11 dph where both types of live feed were provided. The next group, **Com**, was a dry feed reference group, which received a commercial starter diet (Otohime, Marubeni Nisshin Feed Co., Tokyo, Japan). The last three groups corresponded to each of the three diets produced utilizing the Zebrafish meal. These were the **Unhydro**, **50% Hydro**, and **100% Hydro** groups. The fish were fed in excess and, to ensure constant feed availability, fish received the feed every hour from 08:00–18:00. The size of the feeds added to the tanks were < 150 µm. The tanks were siphoned twice daily to prevent the degradation of water quality due to the build-up of waste and uneaten feed. The experiment was carried out until 18 dph.

### Sampling and measuring

At the conclusion of the study (18 dph), all fish from each tank were individually weighed and the total length measured. Samples for additional analysis were also taken at the conclusion of the study. Three fish from each tank were euthanized

with an overdose of MS-222 and stored in RNAlater (Invitrogen by Fisher Scientific, Waltham, WA, USA) for the analysis of PepT1 gene transcription in the gut. Samples for PepT1 expression were taken 2 and 24 h after feeding to analyze postprandial and fasting levels, respectively. Additionally, two sets of whole-body samples were taken from each tank, with five fish per sample. The sampled fish were euthanized in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further FAA analysis. These sets of samples were also taken 2 and 24 h after feeding and represented postprandial and basal FAA levels, respectively.

### PepT1 analysis

PepT1 analysis was conducted using the same method described in Molinari et al. [47]. The digestive tracts were removed from the sampled Zebrafish. The samples were processed using TRIzol Reagent (Ambion, Foster City, CA, USA) and RNA was extracted and purified using the On-Column PureLink DNase Treatment (PureLink™ RNA Mini Kit and PureLink DNase, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Once purified, the ng/ $\mu\text{L}$  of each RNA sample was obtained using a spectrophotometer (Nanodrop OneC, Thermo Fisher Scientific, Waltham, MA, USA). From this point, 2  $\mu\text{g}$  of RNA from each sample was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to obtain a 20  $\mu\text{L}$  cDNA solution. The cDNA solutions were then added to a tube with 380  $\mu\text{L}$  of water, to produce the cDNA sample for each tank. Gene expression of each cDNA sample was measured using a Bio-Rad (Hercules, CA, USA) CFX Opus 96 Real-Time PCR System. Each qPCR reaction mixture (20  $\mu\text{L}$ ) contained 9  $\mu\text{L}$  of cDNA sample, 10  $\mu\text{L}$  of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5  $\mu\text{L}$  of 800 nmol each of forward and reverse primers. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The primers are listed in [Table 3](#). Each qPCR reaction was run in technical duplicates. The qPCR cycle consisted of  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 20 seconds and  $60^{\circ}\text{C}$  for 35 seconds, followed by a melting curve to ensure the amplification of only a single product in each well. Relative gene expression was calculated using the  $2\Delta\Delta\text{Ct}$  method, normalizing the target gene expression to the expression of *ef1a* (reference gene).

### Free amino acid analysis

Free amino acid analysis of fish muscle was performed according to the method described in Kwasek et al. [41]. The muscle from the whole-body fish samples were obtained by removing the head, caudal fin, and viscera. Muscle samples of three fish from each tank were combined and homogenized together with 0.1 mol/L HCl in 1:9 (w/v) and spun at  $12,000 \times g$  ( $4^{\circ}\text{C}$ , 15 min). Supernatants were collected, filtered (Milipore, 10 kDa cutoff at  $15,000 \times g$ ,  $4^{\circ}\text{C}$ , 30 min), and later diluted with 0.1 mol/L HCl (1:19 v/v) containing norvaline and sarcosine (40  $\mu\text{mol/L}$ ) as internal standards. Blanks (0.1 mol/L HCl + 40  $\mu\text{mol/L}$  norvaline and sarcosine) and external standards (Sigma acid/neutral and basic amino acids) were prepared along with the sample preparation. The same concentration of glutamine in 0.1 mol/L HCl as an external standard was prepared and added to the basic amino acid standard. Free amino acids were quantified using Shimadzu Prominence Nexera-i LC-2040C Plus (Shimadzu, Japan) according to the Shimadzu protocol No. L529 with modifications.

**Table 3. Primers used for PepT1 analysis.**

Gene	GenBank Accension #	Primers	Function	Reference
PepT1/ <i>slc15a1</i>	AY300011	F: ATCATTGTGCTCATCGTGGC	Di- and tripeptide absorption	[48]
		R: GGCGAAGATGATGCTCACAG		
<i>ef1a</i>	NM_131263	F: TTGAGAAGAAAATCGGTGGTGCTG	Reference gene	[49]
		R: GAACGGTGTGATTGAGGGAAATTC		

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Free amino acid concentrations (expressed as  $\mu\text{mol/g}$  wet body weight) were calculated in LabSolutions software version 5.92 (Shimadzu, Japan) using internal and external standards.

### SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to visualize the products of tissue hydrolysis. Electrophoretic analysis of the samples was conducted on a discontinuous, reducing 12.5% T, SDS polyacrylamide gel with modifications of the method described by Updike et al. [50]. Briefly, samples were homogenized in dissociation buffer (8 mol urea, 2 mol thiourea, 60 mmol Tris buffer, pH 6.8, containing 3% SDS, 350 mmol DTT, and 0.002% bromophenol blue) overnight at 25°C with agitation and diluted with dissociation buffer as needed. Approximately 10  $\mu\text{L}$  of sample (10 mg/mL) dilutions were loaded onto each lane with a 3% stacking gel containing 1% SDS. The proteins were resolved at 150 V  $\text{cm}^{-1}$  until the dye front reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue dye (40% methanol, 5% acetic acid, 0.04% Coomassie Brilliant Blue G-250) overnight and destained with 10% acetic acid. The destained gel was scanned on an AZURE c600 scanner (Azure Biosystems, Dublin, CA, USA).

### Statistical analysis

Statistical analysis was run using R version 3.5.2 (Boston, MA, USA) [51]. The weight and total length measurements, along with survival data, were analyzed using one-way ANOVA and a Tukey test to detect significant differences between groups. The same statistical analysis was applied to the FAA data collected, with a one-way ANOVA and a Tukey test being used to detect significant differences in FAA levels between groups within each set of samples (2 and 24 h). Lastly, the data analyzed for PepT1 expression are presented as average fold change in relation to the LF reference group, however the one-way ANOVA and Tukey test were conducted on the dCt values within each set of samples (2 and 24 h). In the case of statistical analysis conducted on all data collected, differences between groups were considered significant at  $p < 0.05$ .

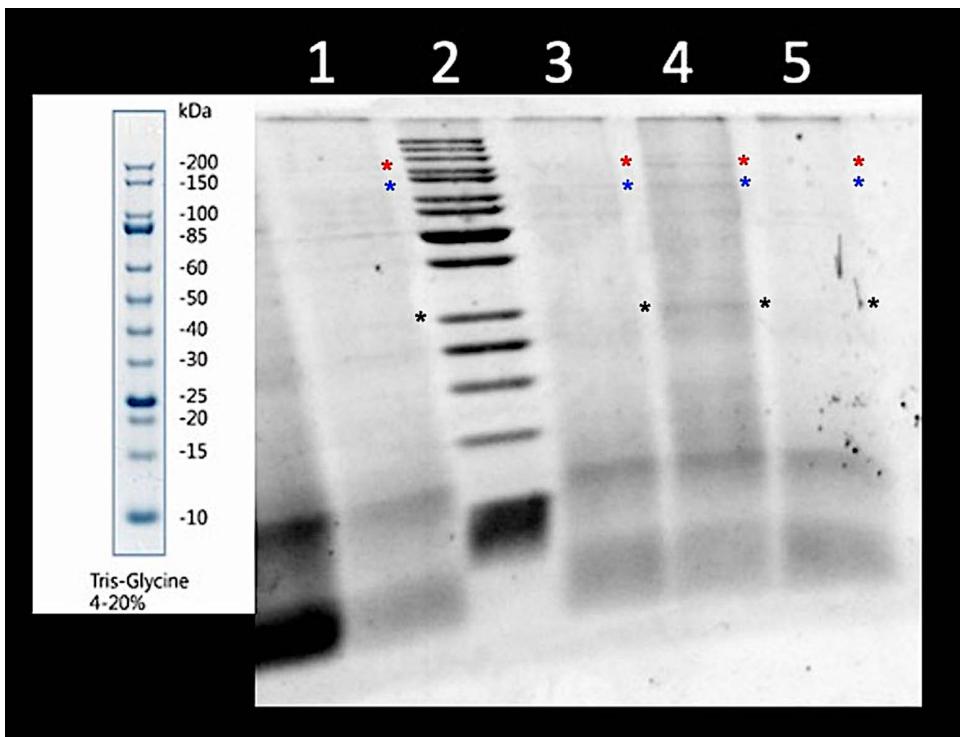
## Results

### Hydrolysis of zebrafish tissue

The results of the Zebrafish meal hydrolysis were obtained utilizing 12.5% T SDS-PAGE (Fig 1). The products added into each lane were: Lane (1) 2 h hydrolysate; (2) broad range molecular weight standard (200–10 kDa); (3) 1 h hydrolysate; (4) unhydrolyzed; (5) 3 h hydrolysate. The gel shows that the proposed method was able to efficiently hydrolyze the protein within the Zebrafish body and produce a hydrolyzed FM. The results show that as the hydrolysis progressed, from unhydrolyzed to the 1, 2, and 3 h hydrolysates, there appear to be decreasing levels of larger protein fragments ( $> 50$  kDa) and increasing levels of smaller proteins ( $< 25$  kDa), and small peptides lower than 10 kDa. Evidence of hydrolysis can be observed by the disappearance of protein bands at higher molecular weights in the hydrolysate samples, that are seen in the unhydrolyzed meal. The bands are seen at  $\sim 150$  kDa (red asterisk),  $\sim 100$  kDa (blue asterisk), and  $\sim 50$  kDa (black asterisk) in Lane 4, containing the unhydrolyzed Zebrafish meal. However, the bands only appear very faintly or not at all in lanes 1, 3, and 5, which contain the varying degrees of hydrolyzed Zebrafish meal.

### Growth and survival

The LF group showed significantly higher average weight ( $p < 0.001$ ) and total length ( $p < 0.001$ ) than the other groups (Table 4). Additionally, the Com group had a significantly higher average total length than the Unhydro, 50% Hydro, and 100% Hydro groups ( $p < 0.001$ ), and a significantly higher average weight than both the 50% and 100% Hydro groups ( $p < 0.001$ ). The 100% Hydro group had a significantly lower survival, compared to the LF group ( $p = 0.045$ ). There were no significant differences in growth or survival among the Unhydro, 50% Hydro, and 100% Hydro groups.



**Fig 1. Representative reducing 12.5% T SDS-PAGE of Zebrafish protein products.** Lanes (1) 2 h hydrolysate; (2) broad range molecular weight standard (200–10 kDa); (3) 1 h hydrolysate; (4) unhydrolyzed; (5) 3 h hydrolysate. Colored asterisks marked protein bands in the unhydrolyzed sample that are observed to disappear in the hydrolysate samples.

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**Table 4. Diet effect on growth and survival.**

Group	Avg. Weight (mg)	Avg. Total Length (mm)	Survival (%)
LF	8.53 <sup>c</sup> (± 0.57)	9.77 <sup>e</sup> (± 0.32)	90.67 <sup>b</sup> (± 5.03)
Com	1.98 <sup>b</sup> (± 0.21)	4.99 <sup>b</sup> (± 0.21)	80.67 <sup>ab</sup> (± 3.06)
Unhydro	1.18 <sup>ab</sup> (± 0.10)	4.16 <sup>a</sup> (± 0.18)	76.33 <sup>ab</sup> (± 1.16)
50% Hydro	0.98 <sup>a</sup> (± 0.02)	4.16 <sup>a</sup> (± 0.32)	76.67 <sup>ab</sup> (± 4.16)
100% Hydro	0.96 <sup>a</sup> (± 0.20)	3.84 <sup>a</sup> (± 0.41)	65.00 <sup>a</sup> (± 19.70)

Values are presented as means (± S.D.). Superscript letters indicate statistically significant differences between groups. The significance was determined using a One-Way ANOVA and a Tukey test with a p value < 0.05.

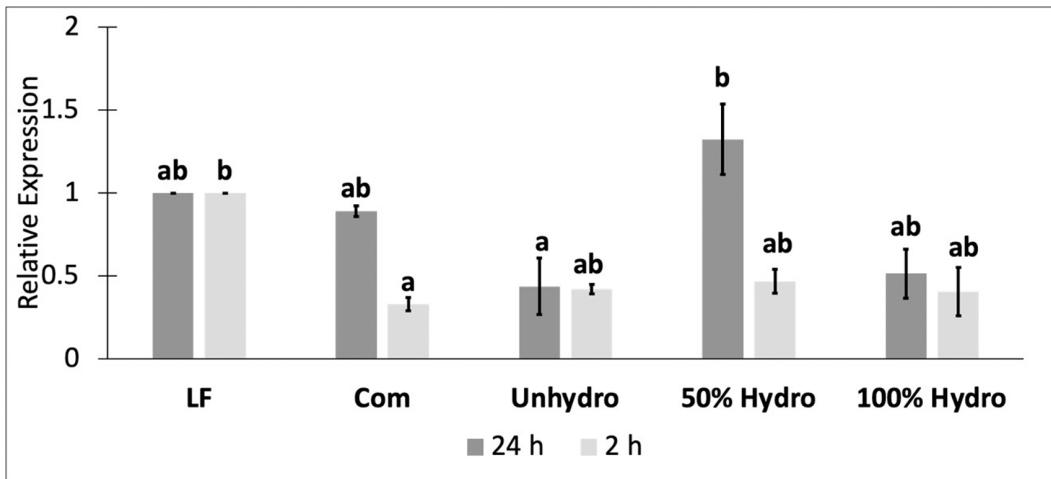
<https://doi.org/10.1371/journal.pone.0326665.t004>

### PepT1 expression

At 24 h after feeding, the 50% Hydro group had a significantly higher expression of PepT1, compared to the Unhydro group ( $p=0.038$ ) (Fig 2). No other significant differences were observed between the 24 h expressions of PepT1 of the remaining groups. At 2 h after feeding, the Com group expressed significantly lower levels of PepT1 than the LF group ( $p=0.049$ ). No other significant differences were observed between the 2 h expressions of PepT1 of the remaining groups.

### Muscle free amino acid composition

The Com group had significantly higher postprandial levels of total FAA and free IDAA in the muscle than all other groups ( $p<0.001$ ) (Table 5). The LF group had significantly higher postprandial levels of total FAA and free IDAA than the three



**Fig 2. PepT1 expression.** Values are presented as mean fold change in relation to LF group, and error bars represent standard error. The letters indicate significant statistical differences ( $p < 0.05$ ) among 24 h and 2 h expression. The significance was determined using a One-Way ANOVA and a Tukey test.

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groups that received the Zebrafish meal ( $p < 0.001$ ), and the 100% Hydro had significantly lower levels than all of the groups. Both the LF and Com groups had significantly higher postprandial levels of free dispensable amino acids (DAA) in the muscle than the other groups ( $p < 0.001$ ), and the 100% Hydro group had significantly lower levels of DAA in the muscle, compared to the other four groups.

Significant differences were observed among the postprandial levels of individual FAA as well. The 50% Hydro group had a significantly higher level of free aspartic acid in the muscle, 2 h after feeding compared to the LF group ( $p = 0.017$ ). The postprandial levels of free glutamic acid and free arginine were significantly higher in the Com group compared to all other groups ( $p < 0.001$ ;  $p < 0.001$ ), and the LF group had significantly higher levels of both amino acids than the 3 groups that received the Zebrafish meal. The 50% Hydro group presented a significantly lower level of free asparagine than all other groups ( $p < 0.001$ ), and the Unhydro group had a level significantly lower than both the LF and Com groups. The postprandial levels of free serine and free alanine in the muscle were significantly lower in the three groups that received the Zebrafish meal ( $p < 0.001$ ;  $p < 0.001$ ), compared to the LF and Com groups. The measured levels of free glutamine were significantly higher in the LF and Com groups, compared to all other groups ( $p < 0.001$ ), and the Unhydro group had a significantly higher level than the 100% Hydro group. Significant differences between each group were observed for both free histidine and free glycine levels in the muscle ( $p < 0.001$ ;  $p < 0.001$ ). The highest levels were observed in the Com group, followed by the LF, Unhydro, 50% Hydro, and 100% groups, respectively. The 100% Hydro group had significantly lower levels of free threonine than all other groups ( $p < 0.001$ ), and the Com group had significantly higher levels than all other groups. The LF group had a significantly higher level of free tyrosine in the muscle 2 h after feeding than all other groups ( $p < 0.001$ ), and the Com group also had a significantly higher level than the three Zebrafish meal groups. The Unhydro group had a significantly higher level of free tyrosine than the 50% and 100% Hydro groups ( $p < 0.001$ ). The postprandial levels of free lysine, free methionine, and free valine were significantly higher in the Com group compared to all other groups ( $p < 0.001$ ;  $p < 0.001$ ;  $p < 0.001$ ), and the levels of all three amino acids were significantly higher in the LF group compared to the Unhydro, 50% Hydro, and 100% Hydro groups. For free lysine, the level was significantly lower in the 100% Hydro group compared to the Unhydro group ( $p < 0.001$ ). The level of free methionine was significantly higher in the Unhydro and 50% Hydro groups than the 100% Hydro group ( $p < 0.001$ ). The 50% Hydro group had a significantly higher level of free valine, compared to the 100% Hydro group ( $p < 0.001$ ). Both the LF and Unhydro groups

**Table 5. Postprandial FAA composition in muscle.**

Amino Acid (μmol/g)	LF	Com	Unhydro	50% Hydro	100% Hydro
<b>Aspartic Acid</b>	1.58 <sup>a</sup> (± 0.06)	2.21 <sup>ab</sup> (± 0.70)	1.97 <sup>ab</sup> (± 0.04)	2.91 <sup>b</sup> (± 0.38)	2.17 <sup>ab</sup> (± 0.26)
<b>Glutamic Acid</b>	3.54 <sup>b</sup> (± 0.20)	4.11 <sup>c</sup> (± 0.04)	2.17 <sup>a</sup> (± 0.26)	1.86 <sup>a</sup> (± 0.15)	1.74 <sup>a</sup> (± 0.16)
<b>Asparagine</b>	0.87 <sup>c</sup> (± 0.13)	0.89 <sup>c</sup> (± 0.04)	0.42 <sup>b</sup> (± 0.09)	0.21 <sup>a</sup> (± 0.01)	0.27 <sup>ab</sup> (± 0.01)
<b>Serine</b>	1.67 <sup>b</sup> (± 0.17)	1.85 <sup>b</sup> (± 0.01)	1.00 <sup>a</sup> (± 0.13)	0.89 <sup>a</sup> (± 0.02)	0.78 <sup>a</sup> (± 0.03)
<b>Glutamine</b>	3.56 <sup>c</sup> (± 0.13)	3.21 <sup>c</sup> (± 0.05)	1.73 <sup>b</sup> (± 0.20)	1.37 <sup>ab</sup> (± 0.15)	1.25 <sup>a</sup> (± 0.09)
<b>Histidine</b>	3.22 <sup>d</sup> (± 0.18)	4.72 <sup>e</sup> (± 0.10)	2.22 <sup>c</sup> (± 0.26)	1.56 <sup>b</sup> (± 0.08)	1.00 <sup>a</sup> (± 0.06)
<b>Glycine</b>	3.10 <sup>d</sup> (± 0.15)	3.75 <sup>e</sup> (± 0.12)	2.29 <sup>c</sup> (± 0.22)	1.62 <sup>b</sup> (± 0.05)	0.96 <sup>a</sup> (± 0.09)
<b>Threonine</b>	1.48 <sup>ab</sup> (± 0.11)	4.11 <sup>d</sup> (± 0.11)	1.91 <sup>c</sup> (± 0.20)	1.82 <sup>bc</sup> (± 0.16)	1.13 <sup>a</sup> (± 0.09)
<b>Arginine</b>	0.82 <sup>b</sup> (± 0.14)	1.05 <sup>e</sup> (± 0.04)	0.51 <sup>a</sup> (± 0.04)	0.59 <sup>a</sup> (± 0.07)	0.46 <sup>a</sup> (± 0.03)
<b>Alanine</b>	4.94 <sup>b</sup> (± 0.43)	4.72 <sup>b</sup> (± 0.02)	1.98 <sup>a</sup> (± 0.19)	1.74 <sup>a</sup> (± 0.05)	1.44 <sup>a</sup> (± 0.08)
<b>Tyrosine</b>	6.45 <sup>d</sup> (± 0.15)	4.96 <sup>e</sup> (± 0.34)	2.75 <sup>b</sup> (± 0.32)	1.42 <sup>a</sup> (± 0.53)	0.71 <sup>a</sup> (± 0.04)
<b>Lysine</b>	1.95 <sup>c</sup> (± 0.26)	2.91 <sup>e</sup> (± 0.07)	1.13 <sup>b</sup> (± 0.10)	1.06 <sup>ab</sup> (± 0.08)	0.74 <sup>a</sup> (± 0.04)
<b>Methionine</b>	3.01 <sup>c</sup> (± 0.06)	3.61 <sup>d</sup> (± 0.07)	1.13 <sup>b</sup> (± 0.12)	1.10 <sup>b</sup> (± 0.14)	0.79 <sup>a</sup> (± 0.05)
<b>Valine</b>	1.15 <sup>c</sup> (± 0.12)	1.37 <sup>d</sup> (± 0.03)	0.61 <sup>ab</sup> (± 0.04)	0.67 <sup>b</sup> (± 0.08)	0.46 <sup>a</sup> (± 0.03)
<b>Cysteine</b>	—	—	—	—	—
*Not detected					
<b>Tryptophan</b>	0.19 <sup>a</sup> (± 0.08)	0.34 <sup>e</sup> (± 0.01)	0.21 <sup>ab</sup> (± 0.01)	0.32 <sup>bc</sup> (± 0.04)	0.31 <sup>bc</sup> (± 0.02)
<b>Phenylalanine</b>	0.42 <sup>a</sup> (± 0.06)	0.67 <sup>b</sup> (± 0.02)	0.38 <sup>a</sup> (± 0.05)	0.41 <sup>a</sup> (± 0.03)	0.32 <sup>a</sup> (± 0.02)
<b>Isoleucine</b>	0.73 <sup>b</sup> (± 0.13)	1.33 <sup>e</sup> (± 0.03)	0.66 <sup>ab</sup> (± 0.04)	0.73 <sup>b</sup> (± 0.08)	0.52 <sup>a</sup> (± 0.04)
<b>Leucine</b>	0.89 <sup>ab</sup> (± 0.13)	2.18 <sup>e</sup> (± 0.04)	1.06 <sup>b</sup> (± 0.05)	1.13 <sup>b</sup> (± 0.12)	0.77 <sup>a</sup> (± 0.05)
<b>Proline</b>	2.51 <sup>d</sup> (± 0.33)	1.64 <sup>e</sup> (± 0.07)	0.94 <sup>b</sup> (± 0.12)	0.72 <sup>b</sup> (± 0.08)	0.20 <sup>a</sup> (± 0.01)
<b>IDAA</b>	13.84 <sup>e</sup> (± 1.03)	22.28 <sup>d</sup> (± 0.38)	9.84 <sup>b</sup> (± 0.90)	9.40 <sup>b</sup> (± 0.77)	6.51 <sup>a</sup> (± 0.36)
<b>DAA</b>	28.22 <sup>c</sup> (± 1.63)	27.35 <sup>c</sup> (± 1.10)	15.26 <sup>b</sup> (± 1.52)	12.75 <sup>b</sup> (± 0.19)	9.51 <sup>a</sup> (± 0.75)
<b>TFAA</b>	42.06 <sup>e</sup> (± 2.66)	49.63 <sup>d</sup> (± 1.47)	25.10 <sup>b</sup> (± 2.42)	22.15 <sup>b</sup> (± 0.89)	16.02 <sup>a</sup> (± 1.11)

The values presented are postprandial levels, 2h after feeding. Values are presented as means (± S.D.). Superscript letters indicate statistically significant differences between groups. The significance was determined using a One-way ANOVA and a Tukey test with a p value < 0.05. Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA=total free amino acids.

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had significantly lower levels of free tryptophan in the muscle, compared to the Com group ( $p=0.003$ ). The level of free phenylalanine in the muscle 2h after feeding was significantly higher in the Com group than all other groups ( $p<0.001$ ). The Com group also had significantly higher levels of free isoleucine and free leucine, compared to all other groups ( $p<0.001$ ;  $p<0.001$ ). Both the LF and 50% Hydro groups had significantly higher levels of free isoleucine than the 100% Hydro group ( $p<0.001$ ). The 100% Hydro group showed a significantly lower level of free leucine, compared to the Unhydro and 50% Hydro group ( $p<0.001$ ). Lastly, the LF group had a significantly higher level of free proline in the muscle than all other groups ( $p<0.001$ ), and the Com group had a significantly higher level than the three groups that received the Zebrafish meals. The 100% Hydro group had a significantly lower level of free proline than all other groups ( $p<0.001$ ). The results for all basal levels of FAA analyzed are presented in [Table 6](#).

## Discussion

Given the difficulties in quantifying amino acid requirements for larval fish [34,35], the approach utilized in this study served as an attempt at a practical proof of concept for the long-held notion that a species' amino acid requirements match the amino acid profile of the same-species tissue [36–38,52]. Both ethical and biosecurity considerations were taken into account when applying this approach, which involved feeding species-specific protein to larvae. Natural

**Table 6. Basal FAA composition in muscle.**

Amino Acid (μmol/g)	LF	Com	Unhydro	50% Hydro	100% Hydro
<b>Aspartic Acid</b>	1.08 (± 0.22)	1.64 (± 0.07)	1.94 (± 0.03)	2.37 (± 0.03)	2.44 (± 0.21)
<b>Glutamic Acid</b>	2.28 (± 0.60)	2.11 (± 0.13)	1.29 (± 0.14)	1.82 (± 0.04)	2.07 (± 0.07)
<b>Asparagine</b>	0.52 (± 0.05)	0.40 (± 0.04)	0.15 (± 0.02)	0.11 (± 0.01)	0.29 (± 0.00)
<b>Serine</b>	0.43 (± 0.09)	0.59 (± 0.01)	0.35 (± 0.04)	0.67 (± 0.03)	0.64 (± 0.04)
<b>Glutamine</b>	2.00 (± 0.57)	1.30 (± 0.08)	0.81 (± 0.09)	1.02 (± 0.01)	1.18 (± 0.05)
<b>Histidine</b>	2.46 (± 0.29)	3.17 (± 0.15)	1.10 (± 0.13)	1.49 (± 0.02)	1.48 (± 0.07)
<b>Glycine</b>	3.11 (± 0.21)	2.61 (± 0.14)	1.27 (± 0.17)	1.37 (± 0.02)	1.52 (± 0.07)
<b>Threonine</b>	0.26 (± 0.09)	2.06 (± 0.08)	0.88 (± 0.07)	1.15 (± 0.04)	0.93 (± 0.07)
<b>Arginine</b>	0.12 (± 0.05)	0.27 (± 0.01)	0.22 (± 0.02)	0.52 (± 0.03)	0.40 (± 0.02)
<b>Alanine</b>	2.51 (± 0.38)	1.93 (± 0.09)	1.07 (± 0.11)	2.03 (± 0.05)	1.75 (± 0.10)
<b>Tyrosine</b>	5.17 (± 1.27)	4.10 (± 0.37)	0.67 (± 0.08)	1.77 (± 0.45)	2.50 (± 0.14)
<b>Lysine</b>	0.35 (± 0.12)	0.74 (± 0.03)	0.39 (± 0.04)	0.82 (± 0.05)	0.63 (± 0.04)
<b>Methionine</b>	2.25 (± 0.53)	2.61 (± 0.08)	1.03 (± 0.11)	0.92 (± 0.02)	0.92 (± 0.01)
<b>Valine</b>	0.46 (± 0.08)	0.59 (± 0.04)	0.33 (± 0.03)	0.39 (± 0.01)	0.40 (± 0.02)
<b>Cysteine</b>	—	—	—	—	—
*Not detected					
<b>Tryptophan</b>	0.07 (± 0.04)	0.28 (± 0.01)	0.24 (± 0.03)	0.14 (± 0.00)	0.33 (± 0.01)
<b>Phenylalanine</b>	0.09 (± 0.02)	0.25 (± 0.01)	0.18 (± 0.03)	0.27 (± 0.02)	0.26 (± 0.02)
<b>Isoleucine</b>	0.11 (± 0.02)	0.23 (± 0.01)	0.19 (± 0.02)	0.33 (± 0.02)	0.30 (± 0.02)
<b>Leucine</b>	0.20 (± 0.05)	0.40 (± 0.02)	0.34 (± 0.03)	0.63 (± 0.04)	0.48 (± 0.03)
<b>Proline</b>	0.16 (± 0.21)	0.17 (± 0.02)	0.01 (± 0.00)	0.16 (± 0.00)	0.06 (± 0.01)
<b>IDAA</b>	6.39 (± 1.25)	10.60 (± 0.41)	4.90 (± 0.49)	6.66 (± 0.10)	6.14 (± 0.29)
<b>DAA</b>	17.25 (± 3.41)	14.84 (± 0.74)	7.56 (± 0.66)	11.32 (± 0.53)	12.45 (± 0.65)
<b>TFAA</b>	23.63 (± 4.63)	25.44 (± 1.15)	12.46 (± 1.15)	17.98 (± 0.63)	18.59 (± 0.93)

The values presented are basal levels, 24 h after feeding. Values are presented as means (± S.D.). Indispensable amino acids (IDAA) = Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. Dispensable amino acids (DAA) = Ala, Asp, Asn, Glu, Gln, Gly, Pro, Ser, and Tyr. TFAA = total free amino acids.

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cannibalistic behavior observed in both farmed and wild populations of many fish species [53,54] provides support that research utilizing this method falls within ethical boundaries. Additionally, the heat treatment applied for both the unhydrolyzed and hydrolyzed Zebrafish meal produced not only deactivated the enzymatic activity, but served to eradicate any potential infectious agents present in the adult Zebrafish tissue. This method adhered to guidelines for production of protein products from farmed fish [55,56] and prevented disease transfer from the adult Zebrafish to the larvae. Research based on this concept is an important step towards understanding larval fish nutritional requirements, particularly as it relates to their amino acid requirements that can ultimately lead to development of optimal species-specific diets.

The first main objective of this study was to assess the efficacy of the proposed hydrolysis method on tissue protein breakdown level and the production of a species-specific hydrolyzed FM. The efficacy of the hydrolysis process was visualized using electrophoretic analysis (SDS-PAGE), showing the disappearance of larger protein fragments in hydrolyzed meals. The electrophoretic results suggest the proposed method was able to hydrolyze the tissue proteins in the Zebrafish body and produce protein/peptide products with a higher proportion of low molecular weight proteins than the unhydrolyzed product. The increased proportion of these low molecular weight proteins/peptides was intended to provide dietary protein that would be more efficiently absorbed by the larval fish. Studies suggest that small peptides (5–75 kDa) are more efficiently absorbed by larval fish than larger, intact proteins [6,22]. Based on electrophoretic analysis, there appears to be

more of these smaller peptides in the hydrolyzed Zebrafish meals, compared to the unhydrolyzed meal, with increasing amounts of peptides < 10 kDa appearing as the time of hydrolysis progressed.

Given the previous success observed with the use of species-specific hydrolysates as a protein source for larval fish [41–43], the hydrolysis method in this study was proposed to create a more practical and applicable approach for the production of these hydrolysates. The major benefits of the proposed hydrolysis method are rooted in its simplicity, reproducibility, and species-specificity. The autolytic basis of this hydrolysis method is similar to that utilized in the production of fish silage, where fish by-products or whole fish are minced, and the tissue is allowed to be autolyzed by proteolytic enzymes contained in both the guts and muscle [57–60]. During the silage process, the endogenous enzymes break down the tissue proteins to produce a liquid with high levels of water-soluble peptides and FAA [61]. Silage production represents a cost-effective and simple way to produce hydrolyzed fish protein. However, this process can be time consuming and presents significant variations in protein recovery and degree of hydrolysis [57,62]. As a result, recent research has focused on enzymatic hydrolysis as a method of producing fish protein hydrolysates. Enzymatic hydrolysis involves the addition of separately obtained proteolytic enzymes to hydrolyze the protein in an incubated system [61,63]. This method provides more control over the peptide profile of the hydrolysate, with the selection of enzymes being based on their cleavage-specificity [64]. There is also greater control over the degree of hydrolysis as the pH and temperature of the method can be adjusted throughout the process to maintain an optimal environment for the activity of the selected enzymes [63]. This ultimately results in a higher degree of hydrolysis in a shorter amount of time, and a more consistent and reproducible protein profile than silage production [63,64]. A major pitfall to the use of enzymatic hydrolysis is the difficulty in selecting the most ideal enzyme(s) and their high cost [60,65].

The hydrolysis method in this study provides a middle ground between these two production methods, with added benefits of species-specific protein and species-specific enzymes. In our method, whole fish were minced, and the proteins were autolyzed using only the endogenous enzymes. While the digestive enzymes play the most significant role in the autolysis process in fish, other enzymes contained within the body have proteolytic capabilities and contribute to the breakdown of body proteins [61,66]. The autolysis was continuously monitored and controlled to maintain a constant temperature and pH, mimicking the *in vivo* digestion of the Zebrafish digestive tract. The *in vitro* hydrolysis method of mimicking *in vivo* digestion with endogenous enzymes has been proven to be effective in this and other studies [41,67,68]. This mimicked *in vivo* digestion produces a dietary protein that is hydrolyzed with the species-specific composition of proteolytic enzymes, representing a molecular weight profile that likely matches that of protein digested within the fish body. It also produces a FM that provides the optimal amino acid profile for the larvae of the same species [52,69]. The continuous control over pH and temperature of this method allows it to be applicable to other species as a means of producing species-specific FM. Although in the present study the pH was maintained to be constantly alkaline to mimic the intestinal digestion of Zebrafish, an agastric species, the same general incubation method was utilized in a gastric species [41]. That study switched the pH of the hydrolysis solution from acidic to alkaline to mimic the movement from stomach (acidic digestion) to intestine (alkaline digestion), and found that the activity of the endogenous enzymes, and thus hydrolysis of the protein, continued after the pH switch [41]. The proposed method provides a practical way to produce hydrolyzed, species-specific FM, where the cost-effectiveness, simplicity, and autolysis aspects of silage production are maintained, and the efficiency and control are improved through monitored incubation.

Another benefit of the presented hydrolysis method is that it produces a hydrolyzed FM out of whole-body fish, instead of just isolated muscle. Not only does this contribute to the simplicity of the method by removing the dissection and isolation of muscle and digestive tracts, it also provides a more sustainable and complete dietary nutrient source. The harvesting of muscle from fish, or filleting fish, leaves behind 50–70% as waste [63,70]. The fillet from fish contains only 15–25% of the total protein in the body [64,71] and leaves behind a fish frame that contains the head, tail, scales, bones, and viscera [72]. These often-discarded parts of the fish contain ~58% protein [72] and can provide additional nutrients including minerals, vitamins, and essential fatty acids [73–75]. Although the levels and effects of these components were

not directly assessed in this study, further investigation and modifications to this method could present the potential of the hydrolyzed FM to be a species-specific source of these nutritional components as well. In an industry where future growth hinges on increased sustainability, a novel method that discards over 50% of its materials as waste would be short-lived. Thus, the use of whole fish as a source of highly digestible protein/peptides in FM as described in this study presents a more viable and environmentally conscious approach to produce a species-specific diet for larval fish that has shown positive effects in previous studies [41–43].

The second main objective of this study was to assess the effect of dietary inclusion of the obtained species-specific FM hydrolysate on larval Zebrafish. The LF and Com groups served as benchmarks for growth and physiological responses of Zebrafish under typical laboratory rearing methods [76]. Only the 100% Hydro group exhibited a significant difference in survival, significantly lower than the LF group. The significant reductions in all growth parameters in the dry feed groups relative to the LF group highlight the need for the development of suitable dry diets for larval rearing. Among the dry feed groups, all three of the Zebrafish meal groups showed a significantly lower total length compared to the Com group, and the 50% Hydro and 100% Hydro groups exhibited significant reductions in average weight. While the Com group provides a reference for larval rearing on available commercial starter diets, important variables like dietary composition, ingredients, and physical structure are not controlled for, which limits the ability to make strong conclusions on the dietary effects behind the differences observed. Thus, the most informative comparisons in this study come from the three groups fed with the formulated Zebrafish meal-based diets.

The growth and survival results from this study showed no significant differences in weight, total length, or survival between the Unhydro, 50% Hydro, and 100% Hydro groups, therefore the inclusion of the hydrolyzed FM failed to improve the growth of the larvae. These results are opposite of those seen previously in studies that tested the use of species-specific protein for larval diets and observed a significant increase in growth [41,43]. Additionally, the lack of improvement through the inclusion of hydrolyzed protein in general contradicts results observed in many other larval studies [7,24,28,42]. As mentioned previously, the inclusion of the hydrolyzed Zebrafish meal in the diet was intended to promote increased growth by providing protein in the form of small peptides and FAA. The dietary provision of these small peptides and FAA has been found to increase absorption of dietary protein by larval Walleye [27], and Striped Bass (*Morone saxatilis*) [27], as well as larval Atlantic Halibut (*Hippoglossus hippoglossus*) [77], and Senegalese Sole (*Solea senegalensis*) [6]. The absorption of the di-/tripeptides from the diets was assessed based on the expression of PepT1, an intestinal peptide transporter [78,79]. PepT1 expression has been found to be responsive to different molecular weight profiles of dietary protein [80–82].

At the 2 h sampling time, there was a numerical trend of lower PepT1 expression in the Unhydro, 50% Hydro, and 100% Hydro groups, and a significant downregulation in the Com group compared to the LF group. This highlights a major benefit of live feeds. Live feeds contain high levels of water-soluble protein in low molecular weight form, which makes the dietary protein more bioavailable than that in formulated dry diets [34,83]. This may have resulted in a faster absorption of di-/tripeptides in the LF group and a delayed expression of PepT1 in the dry feed groups. This delay in PepT1 expression has been observed in Rainbow Trout (*Oncorhynchus mykiss*), where the expression of PepT1 was significantly higher 12 h postprandially, compared to 2 h in response to dry diets [84]. Consequently, the 2 h postprandial results showed no significant differences in PepT1 expression between the Unhydro, 50% Hydro, and 100% Hydro groups. However, 24 h after feeding, there was a significant upregulation in PepT1 expression in the 50% Hydro group over the Unhydro group. This might suggest that the 50% inclusion of the hydrolyzed Zebrafish meal significantly increased the absorption of dietary protein in di-/tripeptide form. Previous studies that have shown increases in PepT1 expression in response to dietary peptides also observed these differences at 24 h post-feeding [82,85,86]. In larval Snakehead (*Channa argus*), the 50% inclusion of a protein hydrolysate led to a significant increase in the 24 h PepT1 expression over a diet containing no protein hydrolysates and a diet containing 100% of its dietary protein in hydrolyzed form [82]. These results are similar to the 24 h PepT1 results in the present study, although there was only a numerical decrease in PepT1 expression in our 100% Hydro

group compared to the 50% Hydro group. In the larval Snakehead, the significant increase in PepT1 correlated with a significantly higher growth due to the increased absorption of di-/tripeptides [82], however in the present study, there were no significant differences in growth between the Unhydro, 50% Hydro, and 100% Hydro groups. The lack of significant differences in growth between these three groups, and the higher growth on an intact protein-based commercial diet, could suggest that Zebrafish are adapted to dry feeds at first feeding and are able to utilize dietary protein in different molecular forms efficiently for growth. The adaptation to dry feeds may stem from a high level of domestication in Zebrafish.

Domestication in fish has been found to influence the digestive abilities of larvae [87–89]. Research has found that upon introduction of compound diets, domesticated larvae started showing significantly higher growth rates compared to the wild-type larvae [88]. This increased growth rate corresponded with an overall decrease in the activity of most digestive enzymes, but an increase in proteolytic enzymes in the domesticated larvae [88]. This difference in digestive enzyme activity was suggested to be a result of “nutritional monotony”, where domesticated fish had been fed high-protein diets for generations, leading to a potential shift towards higher proteolytic activity within the gut [88]. This phenomenon has also been observed in Gilthead Seabream [89]. These studies provide evidence that domestication in fish species can affect the abilities of larvae to digest and utilize dietary components from formulated feeds over generations.

Additional evidence of the effect of possible Zebrafish domestication on the results in this study and other larval studies may be found in the high survival rates achieved with full live feed replacement. The Unhydro and 50% Hydro diets were able to fully replace live feed in this study without significant reductions in survival, supporting survival rates ~76%. This full replacement of live feed has previously been achieved in the rearing of larval Zebrafish with similar survival levels [23,90–92]. Compared to the low survival levels of complete live feed replacement in commercial species like Largemouth Bass [42], Barramundi (*Lates calcarifer*) [93], Sea Bass (*Dicentrarchus labrax*) [1], and Senegalese Sole [94], the observed success of dry feed-only rearing for Zebrafish presents a stark contrast. The significant difference in survival between Zebrafish and those other commercial species could be due to the varying levels of domestication. Domesticated fish have exhibited higher feed intake and reduced stress in intensive settings, contributing to improved survival over their wild-type counterparts during intensive rearing [87,95–97]. Zebrafish are a popular ornamental and lab-raised model species and are classified at the highest level of domestication [98,99]. Common Carp (*Cyprinus Carpio*) is another cyprinid species within this same level of domestication that has shown high survival of larvae reared on dry diets only [100]. Meanwhile, most commercial species in aquaculture exist at lower levels of domestication [101]. For Carp, this increased domestication is a result of a longer history of culture compared to other commercial species. In contrast, Zebrafish have been commercially cultured for much less time, but still reached the highest domestication level. This is a result of very short generation times in Zebrafish (~2–4 months) [102], which has allowed for genetic changes related to domestication to occur much more rapidly compared to other commercialized species [103]. Thus, the varying degrees of domestication among species may be a factor behind the differences in survival between the live feed replacement studies.

Another plausible reason behind the lack of differences in growth across the Unhydro, 50% Hydro, and 100% Hydro groups, is that the dietary protein profiles of each diet were more similar than they were formulated to be. The Unhydro diet was formulated to provide a dietary protein profile with larger, intact fragments, while the 100% Hydro diet was formulated to provide dietary protein in the form of low molecular weight peptides and FAA from the hydrolyzed Zebrafish meal. The 50% Hydro diet was formulated to be a middle ground between those diets, with half of its dietary protein supplied from the unhydrolyzed Zebrafish meal, and the other half from the mix of hydrolyzed Zebrafish meal. Looking at the results from the SDS-PAGE, it is feasible that our “unhydrolyzed” Zebrafish meal may have undergone unintended and unincubated partial hydrolysis. While the unhydrolyzed Zebrafish meal does contain a higher level of large protein fragments >25 kDa compared to the hydrolyzed products, there seems to be a fair amount of smaller proteins <25 kDa present. These small fragments may be a result of unintended autolysis occurring during storage prior

to and during the homogenization of whole fish. While the fish were stored on ice during harvest, reducing the post-harvest hydrolysis rate [104], the water used during homogenization was room-temperature. This could have allowed for the enzymes contained within the body to partially hydrolyze the body protein during the 10 min homogenization at room temperature. A partial hydrolysis of our ‘unhydrolyzed’ Zebrafish meal could have reduced the variations in dietary molecular weight profiles among our formulated experimental diets. This likely contributed to reduced differences in growth from occurring among the Unhydro, 50% Hydro, and 100% Hydro diets. This poses a limitation to this method, and further research to prevent unintended hydrolysis from occurring prior to incubation would improve control over the final degree of hydrolysis.

The postprandial levels of FAA in the muscle were significantly lower in the 100% Hydro group, compared to the other groups. While this reduction in postprandial FAA has typically been attributed to excess leaching from hydrolysate-based diets [8,77,105], it is unlikely that this led to the reduced FAA levels in the 100% Hydro group. Precautions like smaller meals in shorter time intervals and 2x daily cleanings were taken to ensure pellets consumed by the larvae had not experienced prolonged exposure in the water column. Additionally, no significant differences in growth or survival were seen among the Zebrafish-meal fed groups, suggesting any potential leaching differences among diets did not significantly affect larval performance. However, the 100% Hydro group was the only group to show a significant reduction in survival relative to the LF group. Previous studies have found that high inclusion levels of hydrolysates have hindered the utilization of dietary protein in larval fish, leading to a rapid absorption and excretion of amino acids, or an oversaturation of FAA transporters that limited absorption [30,106,107]. The supply of amino acids from the diet is critical to support the high levels of tissue synthesis required during larval metamorphosis [34]. Given the significantly reduced survival and postprandial FAA levels, it is likely that the full supply of dietary protein in hydrolyzed form reduced dietary amino acid absorption and utilization to a point that hindered the proper development of the larval Zebrafish and led to a significant decrease in survival of the 100% Hydro group.

## Conclusion

Overall, the results from this study showed that the proposed hydrolysis method was able to efficiently hydrolyze the tissue protein within Zebrafish body. This provides a practical, cost-effective, and sustainable method for producing hydrolyzed species-specific FM, that is applicable to a wide range of species. Due to observed partial hydrolysis of the protein that did not undergo the incubated hydrolysis procedure, further research is recommended to prevent this and provide more control over the hydrolysis level of protein produced with this method. Results from the feeding trial show that the inclusion of the species-specific hydrolysates did not improve the growth of the larvae compared to the unhydrolyzed Zebrafish meal. The growth results paired with PepT1 gene expression potentially indicate Zebrafish larvae as being highly adapted to dry feeds at first feeding and able to utilize dietary protein in different molecular forms efficiently for growth. Future research should explore this novel concept on other, less domesticated, fish species.

## Supporting information

**S1 File. Analyzed parameters.** This file contains the raw data for growth, PepT1 expression, and FAA analysis. (XLSX)

**S2 File. Raw SDS-PAGE gel.** (TIF)

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