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Technical feed quality influences health, digestion patterns, body mineralization and bone development in farming of the stomachless cleaner fish ballan wrasse (Labrus bergylta)

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Abstract:	Farmed ballan wrasse (Labrus berggylta) is an efficient cleaner fish used for non-medicinal delicing of Atlantic salmon in sea cages replacing to an increasing degree wild wrasse due to considerations for biodiversity and risk of overfishing local wrasse populations. Farming of ballan wrasse has been hampered by low growth rates, high prevalence of skeletal deformities and other welfare related pathologies. In this study we investigated how diets identical in composition but differing in their technical characteristics, by being prepared using different feed production technologies, affect fish performance, mineralization, bone development and gut health of the ballan wrasse larvae and juveniles. The different production technologies include the commonly used ‘high temperature’ extrusion, cold extrusion, and agglomeration, resulting in feed pellets with distinctive physicochemical properties. The results revealed that prolonged feeding periods with extruded pellets during ballan wrasse larvae weaning result in low body mineralization and the development of severe skeletal deformities. In juvenile ballan wrasse, the extruded pellet treatment resulted in higher mortality rates, fish with larger livers, indication for increased serum TAG and cholesterol in a similar manner, and increased activity of the digestive enzymes LAP and maltase, most probably as a compensatory mechanism to the assumed reduced availability of protein and carbohydrates of extruded pellets for this fish species. Smaller dietary effects were identified in terms of intestinal morphology and gene transcription rates.
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Response to Reviewers:	

Manuscript Number: ANIFEE-D-20-00062R1

Response to reviewer

The authors want to thank again the reviewer for her/his work in reviewing our manuscript.

Reviewer #1: Authors have addressed all the comments and provided logical explanation and rebuttals wherever required. However, I do not agree with the following comment with the authors- "After waiting for 5 months for the outcome of this review, we regret that the reviewer spent his/her time on that instead of focusing on the methods used, the obtained results and their discussion. We assume that correcting grammar and phrasing is optional and not the main duty of a reviewer." I can understand that it is very difficult to wait for such long period to get the reviewer's response. But, I have taken only 5 days to do the review (Review invitation received and accepted on August 27, 2020 and submitted the review report on September 01, 2020). If there is delay in the system, I cannot do anything about that. Authors are requested discuss about this issue directly with Editor and not with reviewer. However, these personal comments on the reviewer (me) will not affect my decision and observation on the paper.

Authors' response: Apologies for the comment, it was out of line. We do appreciate the meticulous work of the reviewer which helps us improve our manuscript.

Reviewer #1: I must appreciate that authors have clearly explained the hypothesis (effect of source of raw material and feed preparation methods) and provided sufficient data to address the hypothesis. But, authors need to work on the following minor issues-

Reviewer #1: L23: ballan wrasse (Labrus berggylta) or Labrus bergylta

Authors' response: Where mistaken, corrected to *Labrus bergylta*.

Reviewer #1: L78-81: Need to provide reference

Authors' response: The following missing reference was added:

Le, H.T.M.D., Shao, X., Krogdahl, Å., Kortner, T.K., Lein, I., Kousoulaki, K., Lie, K.K. and Sæle, Ø., 2019. Intestinal Function of the Stomachless Fish, Ballan Wrasse (*Labrus bergylta*). Front. Mar. Sci. 6:140. doi: 10.3389/fmars.2019.00140

Reviewer #1: L102-103: Make it simple without adding the 1, 2, 3

Authors' response: 1, 2 and 3 removed as suggested.

Reviewer #1: L129: Remove the "Treatments were"; for example- …….used: (1) WEx: fish were fed weaning and juvenile diets produced by extrusion; (2) CEx: juvenile fish were fed a …..and so on

Authors' response: Changed as suggested.

Reviewer #1: L261: Though the authors have mention that few primers were self-designed using Primer3web software and few obtained from existing literature, but did not mention which primers were self-designed and which were taken from literature. In the later case, authors need to provide references from where they have taken the primers.

Authors' response: All primers were designed by the researchers participating in the study presented in the current manuscript. Explanation was given at footnote in Table 4, and now also corrected in the text (removed: or taken from the literature).

Reviewer #1: L272: 55±62ºC (depending on the primers 273 used, 10 s; see Table 4)! I don't know is it my job to address this : 55-62oC. There are also few other grammatical issues and I believe authors will work on those issues.

Authors' response: Corrected. We thank the reviewer for noticing.

Reviewer #1: Tables and Figures: In the titles the fish name is mentioned as ballan wrasse and also B. wrasse; follow either one.

Authors' response: Changed to ballan wrasse.

Reviewer #1: Figures: The representation of XY-axis in the Figure 10 looks better and you may follow the same style for Figure 4, 9 and 11.

Authors' response:

Reviewer #1: Table 4: Footnote- Mention as- gapdh2, glyceraldehyde-3-phosphate de……..

Authors' response: Corrected

Reviewer #1: Tables: Authors must have to provide the actual P-values instead of ns (non-significant)

Authors' response: We believe that mentioning p values higher than the provided thresholds does not provide valuable information and is difficult to read. However, will have to provide all values if the magazine requires it.

Reviewer #1: Table-6: The components of proximate compositions need to be written as the following sequence and terms-

* Moisture
* Crude protein (no need of N X 6.25)
* Crude fat
* Total ash

Authors' response: Moisture is placed above Protein and Nx6.25 removed.

Reviewer #1: Table-8: Initial body weight and Final body weight; Mention the significance level ($P<0.05$) in the footnote as mentioned in case of other tables.

Authors' response: Corrected

Reviewer #1: Table-9: The dry matter row should come first as per the Title.

Authors' response: Corrected

Highlights

Use of harder, hot extruded weaning feeds results in skeletal deformities in Ballan wrasse larvae.

Softer feed particles allow higher mineral uptake in ballan wrasse larvae.

Extruded feeds lead to higher HSI and increased juvenile ballan wrasse mortality rates.

Technical feed quality influences health, digestion patterns, body mineralization and bone development in farming of the stomachless cleaner fish ballan wrasse (*Labrus bergylta*)

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Abstract

Farmed ballan wrasse (*Labrus bergylta*) is an efficient cleaner fish used for non-medicinal delicing of Atlantic salmon in sea cages replacing to an increasing degree wild wrasse due to considerations for biodiversity and risk of overfishing local wrasse populations. Farming of ballan wrasse has been hampered by low growth rates, high prevalence of skeletal

deformities and other welfare related pathologies. In this study we investigated how diets identical in composition but differing in their technical characteristics, by being prepared using different feed production technologies, affect fish performance, mineralization, bone development and gut health of the ballan wrasse larvae and juveniles. The different production technologies include the commonly used 'high temperature' extrusion, cold extrusion, and agglomeration, resulting in feed pellets with distinctive physicochemical properties. The results revealed that prolonged feeding periods with extruded pellets during ballan wrasse larvae weaning result in low body mineralization and the development of severe skeletal deformities. In juvenile ballan wrasse, the extruded pellet treatment resulted in higher mortality rates, fish with larger livers, indication for increased serum TAG and cholesterol in a similar manner, and increased activity of the digestive enzymes LAP and maltase, most probably as a compensatory mechanism to the assumed reduced availability of protein and carbohydrates of extruded pellets for this fish species. Smaller dietary effects were identified in terms of intestinal morphology and gene transcription rates.

Keywords: ballan wrasse; bone morphology; fish deformities; feed technology; mineralization.

Abbreviations: AA, amino acids; EGC, eosinophilic granular cells; IAA, indispensable amino acids; WB, whole body

1. Introduction

Over the last decade, both wild and farmed cleaner fish, including ballan wrasse (*Labrus bergylta*), have been used as a tool in combating infestations of farmed salmon with sea lice, a parasitic copepod (*Lepeophtheirus salmonis*). To assure cleaner fish welfare and avoid a decline in wild wrasse stocks, the industry needs to phase out using wild caught wrasses. To this end, appropriate diets and feeding regimes accommodating fish needs at all life stages is of outmost importance.

In praxis, during weaning from live to dry feed, ballan wrasse larvae are fed crustacean meal based formulated diets (Skiftesvik & Bjelland, 2003), often devoid of conventional fishmeal. Dietary inclusion of full fishmeal from the onset of weaning to artificial diets significantly reduces feed acceptance and weaning survival in this species (Kousoulaki et al., 2014b). Bogevik et al. (2016) suggested that ethoxyquin present in fishmeal and/or the secondary metabolites of fish oil oxidation may act as feeding repellents for the fussy ballan wrasse larvae, or mask the attractants deriving from the crustacean feed components. Nevertheless, though reaching up to 95% weaning survival, feeding ballan wrasse larvae a fishmeal-free common 'high temperature' extruded weaning diet crumbles over a longer period resulted in almost 100% head/jaw deformities (Kousoulaki et al., 2014b). This deformity may further hinder the ability of the fish to grow and become capable of removing salmon lice effectively. The mechanisms behind the development of these skeletal deformities are not yet understood. The feeding apparatus of marine fish larvae ossifies first (Sæle et al., 2004; Koumoundouros et al., 2009) and it can be expected that nutritional deficiencies during early development will first become evident in the mouthparts and related head structures. Additional sporadic observations have revealed that ballan wrasse larvae jaw-cranial deformities may not occur, or be less prominent, when agglomerated pellets are used instead of extruded crumbled pellets, or when fishmeal or the water soluble part of fish meal (stickwater) is included in the diet (Kousoulaki et al., 2014a;b). The water-soluble part of whole fish meal contains soluble phosphorus and free amino acids which are key micronutrients for the development of organisms as well as other metabolites stimulating feed intake and growth such as nucleotides, trimethylamine oxide (TMAO), creatine, organic acids (Carr et al., 1996; Wu and Bechtel, 2012). Moreover, it has also been shown that different fish meals and stickwater qualities affect the technical quality of extruded feeds considerably (Kousoulaki et al., 2014c; Samuelsen et al., 2014). Wild ballan wrasse consume only marginal amounts of fish in nature, while its diet was

found to mainly be composed of Echinodermata (45.1%), decapods (26.7%) and molluscs (11.1%) (Figueiredo et al., 2005). Thus, it is unlikely, that they have a specific requirement for fish derived components. On the other hand, as ballan wrasse have basic pH along their short digestive track (pH 7.7-8.2) they may lack the ability of efficient hydration and digestion of extruded feed pellets thus not being able to cover their nutritional needs during the fast-growing larvae stages (Le et al., 2019). The extrusion process involves relatively high temperature and pressure, which alters the physicochemical properties of the dietary nutrients. Ballan wrasse larvae are very sensitive to small dietary freshness differences which may also result from processing (drying, pelleting) on otherwise high quality marine raw materials (Kousoulaki & Opstad, 2012).

As in earlier stages, ballan wrasse juveniles do not accept well feeds without significant levels of full crustacean meals (Kousoulaki et al., 2014c), and still their growth rates are generally low at the same time as feed cost is very high, which threatens the economic viability of commercial ballan wrasse production. More economical ballan wrasse grow-out feeds need to be developed and the reason behind the slow growth of the fish fed commercial feeds requires investigation. Several efforts have been made to address this problem with little success so far. Feeds containing other marine raw materials as attractants, such as shrimp shell meal, blue mussel meal and squid meal seem clearly inferior because they give significantly higher mortality rates compared to a shrimp meal-based diet (Nordgreen et al., 2013). The answer may again lay thus on the negative effects of dietary fishmeal on feed intake in ballan wrasse. Moreover, slow growth may be also due to inefficient utilisation of feeds produced by common 'high temperature' extrusion which is the most common production technology due to a combination of the digestive physiology limitations of this species and the physical quality of the pellets.

The objective of this study was to elucidate the influence of feed production technology, and thus technical quality of feed crumbles and pellets on ballan wrasse larvae and juvenile general performance, skeletal development, mineralization, and digestive physiology. The technologies tested where: common 'high temperature' extrusion hereafter referred to as extrusion, cold extrusion, and agglomeration.

2. Materials and Methods

2.1. Feeding regime, feed formulation and preparation

Two trials with ballan wrasse were performed, firstly a larva weaning trial, and secondly a juvenile feeding trial. In both experiments, test diets were produced using similar raw material formulations but different processing technologies; extrusion (common 'high temperature' and cold) and agglomeration. In addition, an extruded weaning diet was produced exchanging major feed ingredients to test the hypotheses that raw materials and production technology can affect the physical quality of the diets which in turn is important for nutrient release, feed uptake, mineralization and general performance in ballan wrasse.

In the larval weaning trial, a commercial weaning protocol was used as positive control. This protocol includes brief co-feeding with Artemia, and extruded wrasse diet crumbles produced by Nofima (7-10 days), followed by introduction of a cold extruded commercial diet (OTOHIME, purchased by the supplier (PTAqua, Dublin, Ireland). The Nofima-wrasse diet contains cod muscle meal, shrimp meal, krill, and squid meals, no conventional fish meal or fish oil and has been tested in numerous trials in the past by the current manuscript's authors' groups securing sufficient feed intake in ballan wrasse weaning larvae. Moreover, the background knowledge of the current study is that feeding this extruded diet over prolonged weaning periods results in the development of severe fish head deformities (Kousoulaki et al., 2014b). Thus, the treatment of feeding this diet alone was used as negative control. A second larval diet was produced with identical formulation and raw materials as the extruded one, by

agglomeration. Last a third larval diet was produced by exchanging cod muscle meal with poultry byproduct meal (Table 1).

For the juvenile fish feeding experiment we produced three diets from the same raw material mix using different production technologies: 1) extrusion 2) cold extrusion and 3) agglomeration.

The following dietary treatment abbreviations are used: (1) WEx: fish were fed weaning and juvenile diets produced by extrusion. (2) CEx: juvenile fish were fed a diet produced by cold extrusion and identical formulation to the respective CEx juvenile diet; (3) Agg: fish were fed weaning and juvenile diets produced by agglomeration and identical formulation to the respective CEx diets; and (4) WExPB: fish were fed an extruded weaning diet containing poultry byproduct meal instead of cod muscle meal. Poultry byproduct meal is a more economic animal-based alternative to cod muscle meal used in the Nofima wrasse diet, with potential to affect the technical quality of the extruded pellets, as fishmeal does, but without causing feeding refusal in the fish, which is what farmers experience when attempting offering fishmeal based diets to ballan wrasse larvae. All diets contained shrimp meal as attractant.

The experimental diets were produced at the Feed Technology Center of Nofima in Bergen, Norway. The agglomerated feed was produced as described in Kousoulaki et al. (2014a). The extruded feeds were produced using a Wenger TX-52 co-rotating twin-screw extruder with 150 kg/h capacity. The settings of the extruder were "normal" *i.e.* the production can be up scaled to a feed factory. The considered extrusion conditions were: screw configuration (D), die opening (2 mm), knife speed (2908 rpm for the WEx and 3377 rpm for the WExPB), SME (6.8-7 kW for the WEx and 5.4 kW for the WExPBS), feed rate (125 kg/h for the WEx and 130 kg/h for the WExPB) and amount of steam (0 kg/h) and water (0.21-0.23 kg/min for the WEx and 0.14 kg/min for the CExPB). The cold extruded feeds were produced using the same equipment with some modifications in the production settings. Those were lower

feed mass temperature in the preconditioner, lower screw speed, cooling and less steam in the extruder (Table 2).

The measured feed mass temperatures along and exiting the extruder differed by 10-55^{o}C in different extrusion processes tested. The measured feed mass temperatures along and at the exit of the extruder were similar for the three 'high temperature' extruded test feeds and lower in the case of the production of the cold extruded control feed used in the juvenile trial (Figure 1). The feed masses contained all lipids according to the formulations and and no additional oils were coated on the pellets which had a diameter of 1.5-2 mm following extrusion. The moist pellets were dried in a tray dryer following extrusion. For the larval trial the pellets and agglomerates were ground and sieved to different crumble size fractions given to the fish according to the development of their mouth opening during the duration of the trial (pellet diameters used: 0.15-0.3 and 0.3-0.45 mm). The agglomerated feed particles produced for the juvenile fish feeding trial were dried using a fluid bed dryer and sieved to collect particle sizes between 1.2 and 2 mm.

2.2. Weaning trial

Ballan wrasse larvae feeding on Artemia were transferred from a single production tank at MOWI in Øygarden, Norway, to 15 200 l experimental tanks in the same facility. Approximately 200 fish were counted in each experimental tank and randomly attributed 1 of 4 feeding regimes. At experiment start the fish were 40 days old post hatching and weighed 34.5 mg. At first feeding and during weaning, fish were fed in excess. Until day 14 the fish were co-fed with 25,000 Artemia per tank and then onwards only with the experimental diets. The tank system was open flow through, with no aeration. Natural photoperiod was used with natural light from roof windows. The water flow rate was increased from 0 to 400 ml min^{-1} on day 20. The larvae were fed by hand three times a day in the beginning of the experiment and by automatic belt feeder after it was observed that they had started to eat the artificial diets. The

bottom of the tanks was cleaned every day and oxygen and temperature measurements were also taken daily. The water oxygen saturation levels in the experimental tanks ranged between 96% and 100%. The mean water temperature during the feeding trial was 16±0.5°C. The experimental diets were fed to the fish the same day as they were transferred to the experimental tanks, and the trial lasted for 34 days. No intermediate sampling was done. Fish growth rates (final body weight, SGR), survival, and deformity rates were calculated at the end of the trial. The larval weaning diets as well as whole fish at start and end of the trial were analysed for their content in a.o. protein, lipids, minerals, fatty acids and total and free amino acids (Supplementary tables 1 and 2, respectively).

2.2.1. Bone morphology evaluation by CT scanning

Micro-CT scanning was performed in fish from the larval trial by a SkyScan 1275 X-ray microtomograph (Bruker MicroCT, Kontich, Belgium). The scan parameters were adjusted for each sample to optimize the pictures. A typical scan was done with no filter, a source voltage of 26-40 keV and the source current maximized. The scans were high resolution, had a pixel size of 10-12 µm, a 360º rotation, a frame averaging of 2 and rotation steps of 0,2-0,4 degrees. The scans were reconstructed using NRecon (v 1.7.3.1 Bruker MicroCT, Kontich, Belgium). The smoothing was set to 0, the beam hardening correction was set to 36% and the ring artifact reduction was set to 3-6. The CT analyzer (CTAn 1.17.7.2+, Bruker MicroCT, Kontich, Belgium) was used to choose the fish bones as a volume of interest (VOI). The VOI was studied in a 3D visualization program (CTvox 3.3.0, Bruker MicroCT, Kontich, Belgium).

2.3. Juvenile trial

2.3.1. Physical properties of the feeds

The hardness of the agglomerated pellets, extruded and cold extruded pellets produced for the juvenile fish trial was measured using a texture analyzer (TA-HDi®, Stable Micro Systems Ltd, Surrey, UK) consisting of a load arm, equipped with a cylindrical flat-ended

aluminum probe (70 mm diameter). The pellets were broken individually between the probe and the bottom plate, and the major break of the pellet (the peak force) was measured and presented in Newton (N). Measurements were conducted for 20 pellets from each of the feed samples and reported as the average (Table 3).

2.3.2. Juvenile fish trial setup and measurements

Ballan wrasse used in the juvenile feeding trial were provided by MOWI, Norway. After transport the fish were acclimatized at the land-based trial facilities of Nofima at Sunndalsøra, Norway for two weeks before the start of the trial, and then distributed into 150 l flat-bottomed experimental tanks (150 fish per tank). The mean body weight of the fish was 11.4 ± 0.02 g at trial start. Each tank was equipped with a transparent lid and a small lamp above the lid. The photoperiod was 24 hours light. The mean water temperature was 15°C, and oxygen saturation was kept at 90 ± 6.6%. Small hides were placed in all tanks during the entire experimental period of 126 days to provide resting places as these fish like to rest for longer periods. The fish were fed continuously and in excess using automatic belt feeders. At start and end of the trial the weight of 25 fish per tank was recorded individually, thereafter the remaining fish in each tank were bulk weighed. Five fish per tank were sampled and frozen for whole-body analyses at trial end. Before sampling, fish were firstly anaesthetized, and then euthanized immediately by cervical dislocation. Blood samples were collected with heparinized vacutainers from caudal vein of fish for plasma preparation prior to tissue sampling. The abdominal cavity was opened to obtain the whole intestine which was then separated into four segments as defined in Lie et al. (2018), that were named IN1, IN2, IN3 and IN4, respectively. Samples for histology, RNA extraction and brush border membrane enzyme activity assessment were collected from each intestinal segment and the liver. Samples for histological evaluation were placed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing. Samples for RNA extraction were placed in RNAlater (Ambion,

Carlsbad, CA) at 4 °C for 24 h, and were stored at -20 °C. The remaining tissue of each segment was collected and snap-frozen in liquid nitrogen and then stored at -80 °C for brush border digestive enzyme activity assessment.

2.3.3. Blood chemistry

Photometric analyses were used to determine the content of lactate, glucose, magnesium, cholesterol and triacylglycerols in blood serum from 20 individuals per tank in the end of the juvenile trial using a Pentra C400 HORIBA, HORIBA Medical, Montpellier, France.

2.2.1.1. Gut mucosa enzyme activities

Intestinal tissues of fish from the end of the juvenile trial were homogenized in cold tris-mannitol buffer (1:20 w/v) containing the serine protease inhibitor (24 μg/ml), 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (Pefabloc® SC; Pentapharm Limited, Basel, Switzerland), using an Ultra Turrax® homogenizer (IKA, Staufen, Germany) followed by sonication at 4 °C for 15 s. The homogenates were frozen in liquid N_2 in aliquots and stored at −80 °C prior to analysis. The leucine aminopeptidase (LAP) and maltase activities were determined as described by Krogdahl and Bakke-McKellep (2005). The enzyme activities are expressed as specific activity, per mg protein in the homogenate, as well as total activity per unit of body weight of the fish. The protein concentration of homogenates was determined using the BioRad® Protein Assay kit based on the Bradford dye-binding method (BioRad Laboratories, Munich, Germany).

2.3.4. Gut mucosa gene expression

Expression profiling of a panel of 12 genes with key roles in intestinal immune and digestive function was conducted in fish tissues from the end of the juvenile trial using quantitative real-time PCR according to the MIQE guidelines (Bustin et al., 2009). Total RNA was extracted in a randomized order from 20-30 mg IN1 and IN4 tissue samples from 4 fish per tank replicate, *i.e.* 12 individual fish per diet by using Trizol reagent (Invitrogen™, Thermo Fisher Scientific,

Waltham, MA, USA) and purified with PureLink (Invitrogen™) including an on-column DNase treatment according to the manufacturer's protocol. RNA purity and concentration were measured using Take3 micro-volume plates and Epoch microplate spectrophotometer (BioTek Instruments). The integrity of the RNA samples was verified by the 2100 Bioanalyzer in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers (RIN) were >8 for all samples, with an average RIN of 8.9. Total RNA was stored at −80ºC until use. First-strand complementary DNA was synthesized from 0.5 µg total RNA from all samples using SuperScript1III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™). Individual RNA samples were pooled two and two within a tank replicate in the cDNA synthesis. Negative controls were performed in parallel by omitting RNA or enzyme. All qPCR primers used for amplification of gene-specific PCR products were designed for the current study using Primer3web software version 4.1.0 (http://primer3.ut.ee/). The primer details are shown in Table 4. All primer pairs were first used in gradient reactions to determine optimal annealing temperatures. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of the PCR products by agarose gel electrophoresis. PCR efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled cDNA. The expressions of individual gene targets were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland). Each 10 µl DNA amplification reaction contained 2 µl PCR grade water, 2 µl of 1:10 diluted cDNA template, 5 µl LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µl (10mM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95ºC (5 min), forty to forty-five cycles at 95ºC (10 s), 55-62ºC (depending on the primers used, 10 s; see Table 4) and 72ºC (15 s) and a melting curve step. Target gene expression was normalized to the geometric average of glyceraldehyde-3-phosphate dehydrogenase 2 (*gapdh2*), 14-3-3

protein epsilon (*ywhae*) and topoisomerase II alpha (*top2a*) expression after evaluation of their stability across and within the treatments as described by Kortner et al. (2011). Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification (Muller et al., 2002).

2.3.5. Histology liver/gut

Histology sections of the intestinal tract and the liver from 36 individuals from the end of the juvenile trial were prepared and stained with H&E stain. The sections consisted 36 each from 4 intestinal regions labelled as IN1 (proximal region), IN2, IN3, and IN4 (distal region). Histological sections were prepared following standard histological methods at the NMBU Faculty of Veterinary Medicine. The intestinal tissue sections were evaluated by light microscopy. The histological evaluation was focused on morphological changes associated with inflammatory reaction in the intestinal mucosa guided by our extensive experience of grading the severity of mucosal changes associated with soybean meal-induced enteritis (SBMIE) in the distal intestine of salmonids. Therefore, the morphological features that were evaluated included mucosal fold length, width and cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolization, as well as the frequency of intra-epithelial lymphocytes, mitotic figures and apoptotic bodies within the epithelial layer. Other morphological features unique to the ballan wrasse were also noted and graded during the histological evaluation. The degree of change for the different morphological characteristics evaluated were graded using a scoring system with a scale of 0-4 where 0 represented normal; 1, mild changes; 2, moderate changes; 3, marked changes, and 4, severe changes. The histological evaluation was conducted blind.

2.4. Chemical analyses

The larval trial diets and whole fish were analyzed for their chemical composition using standard methods: Kjeldahl protein (N x 6.25) (ISO 5983-1997), moisture (ISO 6496-1999),

ash (ISO 5984-2002), lipid (Bligh and Dyer, 1959), fatty acid profile (AOCS Ce 1b-89 FA), salt (AOAC 937.09), Ca, Mg, Na and K (Julshamn et al., 1999; ISO 6869:2000), P (ISO 6491), total amino acids (Cohen & Michaud, 1993) and free amino acids including taurine and anserine (Bidlingmeyer et al., 1987). The water-soluble protein fraction of the diets was extracted with boiling water, the extract was then filtered using paper filter and the crude protein content in the water phase was determined by the Kjeldahl method. Astaxanthin mono- and di- esters in whole fish were analysed using a method which determines the content of astaxanthin-esters in aquatic animals known to only contain carotenoids in the form of astaxanthin esters. The method is also used to determine any content of free trans-, 9cis- and 13cis-astaxanthin (Schüep and Schierle, 1995). Total starch and degree of starch gelatinisation were measured in diets using a modification of the glucoamylase methodology described by Chiang and Johnson (1977) and Samuelsen and Oterhals (2016). The juvenile trial diets were not analyzed as all three were produced from the same feed mix which was in turn similar to the recipe used in the larval weaning diets (WEx and Agg). All chemical measurements were based on averages of duplicate analyses.

2.5. Statistics

The biological performance and analytical data were subjected to one-way analysis of variance (ANOVA) using Microsoft Excel and SPSS 10.0 for Windows. When significant differences among groups were identified, multiple comparisons among means will be made using the Duncan's test. Treatment effects will be considered at a significance level of $P<0.05$, indication of difference was discussed at $P<0.1$. Differences in histological scores for the various evaluated morphological characteristics of the DI tissue were analysed for statistical significance using ordinal logistic regression run in the R statistical package (version 3.6.2; 2019) within the RStudio interphase (version 1.2.5033; 2019). Differences were examined based on odds ratios and confidence intervals of other diet groups being allocated higher

histology scores than samples from fish fed the agglomerated diet. For gene expression statistical data analysis Graphpad Prism version 8.1.2. was used. Diet and intestinal region (IN1 and IN4) were evaluated as class variables in a two-way ANOVA with interaction and further evaluated with a post-hoc Sidak's multiple comparisons test. All data were evaluated for homogeneity of variance and normality of residuals using the "residual by predicted" plot and histogram, respectively. When necessary, data were transformed to meet the statistical assumptions, and then refitted for a second evaluation. The level of significance was set at $P=0.05$, with $0.05<P<0.1$ reported as trends.

3. Results and Discussion

3.1.Weaning trial

3.1.1. General performance

The larval weaning performance results are presented in Table 5. There were no significant differences in final larval body weight between the feeding treatments. However, this result largely depends on the survival rates and presence of 'looser fish'. These fish were surviving but nearly not feeding and had a very low body weight at the time of sampling. Thus, they were not expected to survive further on. Fish grown under the commercial control regime (WEx 8 days + OTOHIME 26 days) where numerically the largest in body weight but also with the lowest survival rate among the four experimental treatments. As previously observed (Kousoulaki et al., 2018), a large percentage (>40%) of the fish fed continuously on the extruded cod muscle based feed (WEx) displayed obvious skeletal deformities after 34 days feeding with this extruded diet (Figure 3) whereas the fish weaned under the commercial protocol showed normal skull development (Figure 2). Fish weaned under the commercial feeding regime and fish fed the agglomerated diet (Agg) showed almost no skull deformities. Last, only 13.1% of the fish fed the extruded WExPB diet, displayed similar skull deformities as fish in the WEx treatment. The WExPB diet contained poultry byproduct meal instead of cod

muscle meal as major dietary protein source (Figure 2). Lower prevalence of skull deformities has previously been observed in ballan wrasse larvae fed extruded feeds containing full fishmeal compared to fishmeal free diets (Kousoulaki et al., 2018). Thus, it appears, that the dietary inclusion of poultry byproduct meal, as fishmeal, exerts positive effects on larvae skeletal development during weaning. This may be due to nutritional, but most probably due to the technical properties of the resulting extruded weaning feeds.

3.1.2. Whole body mineralization

We observed several significant differences in the chemical composition among the weaned larvae from the different experimental treatments, some of those as expected, were related to the chemical composition of the experimental feeds, as for instance their fatty acid profile. The experimental feeds were rather similar in composition, with some differences, mainly in total lipid levels (OTOHIME was 4-6% higher in lipids) and the higher levels of omega 6 fatty acids in the WExPB diet compared to the rest. The OTOHIME diet contained the highest total P levels among all test diets. Although WEx and Agg feeds were similar in formulation regarding total P, Ca and Mg, major minerals in bone structure, the fish fed these two diets were significantly different in terms of whole body P and Ca at the end of the weaning trial. The fish fed the WEx diet contained lower total whole body P levels as compared to the start of the feeding trial, whereas significatnly higher levels of both P and Ca where found in the whole bodies of fish fed the Agg diet ($P<0.05$) (Table 6; Supplementary table 2). Whole body levels of Mg, Zn and Fe followed the same patern as P and Ca, and that of Cu the reverse, but the differences were not allways statistically significant. The fish groups with in lower whole body mineral levels also displayed higher prevalence of skeletal deformities compared to fish groups with higher whole body mineral levels (Table 5), and there was a significant positive correlation between whole body P and fish population deformity rate (Figure 4).

Cephalic deformities have also been reported in common carp fed low phosphorus diets (Ogino and Takeda, 1976).

Fish take up Ca mainly from seawater through the gills, and the Ca dietary levels are thus considered as less important (Flik and Verbost 1993), except in periods with high demands, such as during reproduction and skeletal development, or when the water levels are low (Sundell and Björnsson 1988; Guerreiro et al. 2002). From our data it appears that the rate of Ca uptake is regulated by P uptake, as fish with significantly higher total whole body P levels had also significantly higher whole body Ca levels as well as whole body Ca/P ratio. Magnesium can also be taken up by drinking sea water and deposited by endocrine homeostasis regulation mechanisms (Bijvelds et al., 1996), apparently aiming at optimal tissue levels in relation to e.g. the P levels in bones and other fish body tissues. However, there is evidence that stomach is the primary region for magnesium absorption in fish (Bucking and Wood, 2007), and ballan wrasse is lacking that. Thus, in our study, as Ca and Mg could be supplied by sea water, the cephalic deformities observed were most probably caused by P deficiency due to lower uptake in the gut as a result of the differences in pellet technical quality and not due to the dietary P amounts or forms present, as these factors were constant in the test diets.

3.1.3. Bone morphology

The morphology of the head and spine deformities of the fish fed the extruded diets during the whole weaning trial were elucidated using CT-scanning. Fish from the commercial protocol (Figure 5 A and Figure 7 A-B), the Agg treatment (Figure 7 E-F) and most fish in the WExPB treatment (Figure 7 G-H), had long heads with the anterior part of the frontal bone descenting at low angle towards the ethmoid and the upper jaw (premaxillary and vomer). On the contrary, fish from the WEx treatment (Figure 5 B and Figure 7 C-D) had shorter heads, compressed mouth area, with decending frontal bone at sharper angle towards the mouth, lower apparent mineralization degree, and most characteristically deformed or even broken

parasphenoid. In induviduals with fractured parashhenoid, it appears that the fracture is along the fusion area between the parasphenoid and vomer. As this arise during ossification of the neurochranium, it is likeley that the fusion area represents a weak zone prone to break easily due to physical stress when mineralization is comprimized.

The parasphenoid is the median bone forming the ventral basis of the neurocranium and is connected with the vomer rostrally, a median bone forming the anterior part of the roof of the mouth of the fish (Figure 5), and the basioccipital caudally (not visible). The parsphenoid bone being the ventral basis of the neurocranium is supporting the viscerocranium, *i.e.* the upper and lower jaw and their supporting bones, which transfer forces from the jaws to the neurocranium. Obviously, the parasphenoid bone of fish with low mineralization status is weak, being deformed by the muscle adductor mandibulae forces appyied during jaw movement, e.g. during feeding and breathing. As in other teleosts, the ethmoid group is among the last neurocranial bones to ossify (Sæle et al., 2004, 2017). The ethmoid group of bones is not fully mineralized at this larval stage, further weakening the rostro-dorsal support of the neurocranium structure to the parasphenoid bone.

Moreover, fish in the WEx treatment had shorter and deformed neural and hemal spines with bending tips (Figure 6B) and very weakly mineralized pleural ribs and pterygiophores and invisible (non-mineralized) false ribs, as compared to the fish with better bone mineralization status (Figuer 6A), also signs of P defficiency in fish. The combination of low P availability in the extruded diets fed rapid growing larvae lead to the development of weaker bones and multible skeletal deformities in weaned ballan wrasse fish, as seen before in several farmed species (Baeverfjord et al., 1998; Ogino and Takeda, 1976, 1978; Ogino et al., 1979; Watanabe et al., 1980). A three dimentional view of the head and backbone area of well and low mineralized ballan wrasse larvae can be seen in the supplementary video materials.

3.1.4. Whole body amino acids and lipids (Table 6 & Supplementary Table 2)

Ballan wrasse larvae in the end of the weaning trial contained significantly higher protein ($P<0.001$) and numerically lower lipid ($P>0.05$) levels in whole body as compared to trial start, and the extrusion treatments WEx & CExPB resulted in fish with significantly higher whole body protein as compared to the commercial control and Agg treatments. Fish with higher levels of the analyzed minerals (except Cu) also had higher levels of total ash, and inversely related levels of protein. Irrespective of total whole body protein level, the concentrations of most essential amino acids in whole body of ballan wrasse larvae at the end of the trial did not differ betweem treatments, and increased during the experimental period. Whole body analysis showed signifciant differences between dietary treatments in whole body levels of non-essential amino acids e.g. hydroxyproline, glycine, alanine and proline, being at significantly higher amounts in the extrusion treatments CEx and CExPB indicating lower dietary essential amino acid and hence protein availability, and higher relative *de novo* production of non essential aminoacids which are present in the most abundant connective tissue body protein collagen.

Several treatment effects were observed in the whole body levels of free amino acids. For instance, fish fed according to the commercial weaning protocol (WEx+OTOHIME), had the highest final body weight (not significantly different from the other treatments) in the last period of weaning but also 2 or 3 times more free whole body free methionine, leucine, isoleucine and phenylalanine compared to start and end of the other feeding treatments. This can not be explained by the levels present in the diets, but may still demostrate better nutritional status of these fish. The same fish had also higher whole body levels of free lysine and total free amino acids but not significantly different compared to the other treatments.

3.1.5. Technical properties of experimental diets

Although WEx and Agg feeds had similar formulation, they performed very differently as weaning feeds. The difference between the these two feeds was the production process. WEx

resulted in mineral deficiency and consequently high level of ballan wrasse larvae skeletal deformities whereas Agg scored highest for all weaning performance parameters as compared to WEx. The two best performing feeding regimes were the commercial weaning protocol and Agg. OTOHIME, fed during the last ¾ of the weaning period in the commercial weaning treatment, and Agg are softer feeds produced at low temperatures with low levels of gelatinized starch and easily dissolved in water as compared to the feed used in the extruded weaning regimes WEx and WExPB (Table 7; Figure 8).

3.2. Juvenile fish trial

3.2.1. General performance

Rearing juvenile ballan wrasse diets with identical dietary formulations manufactured using different production technologies yielded some significant physiological effects (Table 8). Fish body weight tripled during the experimental period and there were no significant differences in growth among the dietary treatments. Fish fed the extruded diet performed poorer in terms of survival as compared to the fish fed agglomerated or cold extruded diets. Furthermore, fish fed the extruded diet had significantly higher HSI compared to the other two groups, which is a sign of suboptimal lipid metabolism. Relative fish liver weight is often seen to be affected by variation in diet composition, e.g. in ingredients, content of essential nutrients, antinutrients and other adventitious compounds (Caballero-Solares et al., 2018; Hansen et al., 2020). Overall, liver weight averaged 1.9% of body weight, a weight somewhat higher than often observed in healthy Atlantic salmon (Caballero-Solares et al., 2018; Hansen et al., 2020; Krogdahl et al., 2015).

3.2.2. Juvenile whole body composition

There were no significant differences in fish whole-body mineralization, although tendencies for differences between the dietary groups were present at end of the experimental. These findings contrast with our findings during the weaning stage, except for K, which was

lower in the WEx diet fed fish than in the CEx groups (Table 9). The same trend was observed for the other analyzed minerals, with higher mineral content in whole body of the CEx fed fish followed by intermediate content in the Agg fed fish, and lowest mineral content in WEx fed fish with (pair sample T test: $P<0.01$) (Table 10).

3.2.3. Serum chemistry

Blood serum analyses showed no significant differences among the dietary groups, but a tendency for higher triglyceride levels in the WEx diet fed groups ($P=0.1$) (Table 11). Moreover, there was a significant correlation between serum triglycerides and cholesterol ($R^2=0.668$) (Figure 9) which indicates that this fish group was using lipids for energy at higher proportion as compared to the other dietary groups. This result in combination with the suboptimal mineralization and lower relative essential to non-essential amino acid levels in fish whole body of the extruded dietary treatment suggests that both proteins and minerals are less available in extruded pellets for this fish species, whereas lipid availability may be higher. In extrusion, pellets expand more and generate more air and potential channels in the pellet that digestive enzymes may easier reach the lipids as compared to the proteins that may form less digestible molecules, including metalloproteins, when being processed at higher temperatures. The serum analyses results agree with the obtained higher HSI in fish fed the CEx diet indicating higher lipid accumulation in the liver of the fish in this treatment. Nevertheless, Hamre et al. (2013) found that ballan wrasse juveniles exhibit better growth performance on diets with relatively lower lipids to protein levels, and the present observation of higher HSI in the fish of the extrusion treatments may indicate suboptimal capacity of the fish to utilize dietary lipids.

3.2.4. Intestinal section weight

Figure 10 presents the results regarding relative weight of the four intestinal sections. The weight of the intestinal sections, as divided during sectioning, differed significantly,

decreasing from the proximal most to the distal most section, from 0.55 to 0.28%. In sum, the intestine comprised 1.6% of body weight, a number much lower than most often observed in Atlantic salmon (Kortner et al., 2016; Li et al., 2019). No significant diet effect was observed for the intestinal weights. In Atlantic salmon relative weighs of intestinal sections have been observed to vary with variation in level of nutrients and antinutrients in the diet and may serve as a useful biomarker for diet induced responses (Hansen et al., 2020; Krogdahl et al., 2020; Li et al., 2020).

3.2.5. Gut mucosa enzyme activities

As representatives of the digestive functions in the intestine, LAP and maltase capacity of the different sections were investigated (Figure 11). Overall, the activities and capacities in the fish of the present study were similar to those in our previous studies (Krogdahl et al., 2014) for all sections. A diet effect was seen for specific activity of LAP (U/mg protein) and maltase, being higher in fish fed the CEx diet. The results for the enzymes' capacity (U/kg fish) did not show significant diet effect. The intestine is a highly dynamic organ system which adapts to changes in diet composition, to optimize the digestives processes. Our present results may support our hypothesis that proteins in the extruded diet (CEx), but now also suggesting the same for the carbohydrate fraction, were less available to the fish which may have responded in a compensative manner by the production of higher proteolytic and maltose digestive enzyme amounts.

3.2.6. Histo-morphological observations

Inflammatory morphological changes were the most notable observations in the histological assessment of the ballan wrasse observed in the current histological assessment. Changes were observed predominantly in the IN-3 and IN-4 intestinal regions, but a few fish were also observed with mild to moderate inflammation in the proximal regions of IN-1 and IN-2. The inflammatory changes were characterized by increased cellular content of the

submucosal and lamina propria compartments as well increased infiltration of the intraepithelial space (Figure 12). The inflammatory responses appeared to involve eosinophilic granular cells (EGCs) and lymphocytic cells with both cell types observed to increase in numbers in the submucosa, lamina propria and the intra-epithelial space of the mucosal barrier.

Section IN-1: Results of the histological investigation are presented in Supplementary Figure 1. The morphology of the IN-1 sections was largely normal and healthy. Mild to moderate inflammatory responses characterized by an increased cellularity of the lamina propria was observed in a total of 4 fish (1 fish each from the Agg and WEx feeds, and 2 fish in the CEx; see Figure). Mild to moderate supranuclear vacuolization of enterocytes was observed in a few fish (Supplementary Figure 1 c). No statistically significant differences were observed between any of the feed groups in their influence on the occurrence and severity of the observed histological changes.

Section IN-2: Similar to the IN-1 sections, IN-2 sections were observed with predominantly normal and healthy mucosal appearance. The most changes observed were also inflammatory responses in the lamina propria compartment (Supplementary Figure 1 b) with a total of 8 of the 36 fish observed with mild to marked changes. Fish fed the CEx diet showed the most fish affected (4 fish) but differences between the groups were not statistically distinct.

Section IN-3: More fish were observed with inflammatory responses in the IN-3 intestinal segment. Mild to moderate changes were observed in the submucosa and lamina propria with the most changes (almost 50% of the fish assessed) observed in the lamina propria compartment (see Supplementary Figure 1 a and b). Infiltration of the epithelial layer was also observed in several fish especially from groups Agg and WEx while significantly fewer fish from the CEx group exhibited this response (Supplementary Figure 1 d).

Section IN-4: IN-4 sections were observed with the most fish showing changes for all the morphological features assessed. Most of the IN-4 sections showed mild to moderate

inflammation particularly in the lamina propria. Intraepithelial infiltration ranging from mild to marked was observed in 27 of the 36 fish evaluated. No significant dietary effects on the occurrence and severity of the morphological changes were discernible, however.

3.2.7. Gene expression

The intestinal gene expression results are presented in Supplementary Figures 2 and 3. In general, no apparent differences between dietary groups were found for expression levels of important intestinal immune and digestive related genes. The only exception was increased levels of the innate immune responder and antimicrobial agent lysozyme (*lyz*) in IN4 for fish fed the extruded diet (CEx), coinciding with the higher observed mortality in this treatment. As such, the gene expression results seem to be in accordance with the other gut related analytical endpoints, indicating few effects of diet on the general health and functional status of the gut. Expression levels of many of the profiled genes showed clear spatial differences between the proximal (IN1) and distal (IN4) gut segment, probably reflecting the different functions of the ballan wrasse intestine segments. Genes related to nutrient digestion, such as the vitamin C transporter (*slc23a1*), the membrane fatty acid transporter (*cd36*) and the cholesterol biosynthesis genes *sqle* and *cyp51a1* were expressed at higher levels in IN1 than IN4. These observations are in accordance with the study of Lie et al. (2018), which demonstrated that digestion-related genes were expressed more abundantly in the proximal segment with a gradient decrease along the intestine. Accordingly, Le et al reported that the ballan wrasse proximal intestine accounted for 74%, 86% and 50% absorption of protein, carbohydrate and total lipid, respectively (Le et al., 2019). On the contrary, the immune related *lyz*, the tissue remodelling related genes matrix metallopeptidase 13 (*mmp13*) and proliferating cell nuclear antigen (*pcna*), and, in particular, the water channel aquaporin 8 (*aqp8*) displayed higher transcript levels in IN4. The high IN4 *aqp8* expression levels are in agreement with the study

of Le et al (2019), and probably reflect an important role of this gut segment in both water absorption and ammonia excretion.

4. Conclusions

Extrusion, commonly used in aquaculture feed production technology involving high temperature processing of the feed raw materials, has a negative effect on the uptake rate of minerals in stomachless ballan wrasse larvae. The reduced uptake of minerals when fed extruded feeds had in turn negative effect on the skeletal development of the fish. Introduction of whole vertebrate meal, such as poultry by-product meal, alleviates this effect, resulting in lower incidences of skeletal deformities in weaned ballan wrasse larvae. Agglomeration and cold extrusion, *i.e.* feed production technologies using lower processing temperatures, resulted in feed particles that apparently allow higher uptake rate of minerals (e.g. P and Zn) in ballan wrasse larvae.

Juvenile ballan wrasse appears to become less robust demonstrated by higher mortality rates and develop larger and thus probably more fatty liver with concomitant higher TAG and cholesterol levels in the blood, when fed extruded as compared to cold extruded or agglomerated diets. Fish fed the extruded diet in our study demonstrated higher intestinal LAP and maltase activity. Comparing the larval *vs* the juvenile fish trials, this last observation, may be compensatory and related to the assumed reduced availability of proteins, minerals and carbohydrates in the extruded diets for the stomachless ballan wrasse with short intestine and lack of acid digestion intestinal process.

Based on the performance comparison of fish fed the test *vs* the commercial control, we recommend that ballan wrasse weaning diets should have higher levels of lipids and total P compared to the levels in the test diets of the present study. Moreover, diets prepared for farming ballan wrasse in all stages should be processed in relatively low temperatures, as for instance cold extrusion, using the same equipment as the one used for the commonly produced

extruded diets. It is though important in this case to bear in mind that the dietary starch used should be pre-gelatinizen in order to be digestible. Moreover, pellet endurance of cold extruded feeds may be inferior to commonly extruded diets and should thus be handled with more care.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization: KK, EG, GMB, ØS, IL, ÅK; Data curation: KK, EG, TMK, GMB, HN, ØS, IL; Formal analysis: KK, TMK, GH, NH, EMC; Funding acquisition: KK, EG, TMK, ÅK, ØS, IL; Investigation: KK, EG, TMK, GMB, ÅK, HN, ØS, IL; Methodology: KK, EG, TMK, GMB, GH, ÅK, HN, ØS, IL; Project administration: KK, EG, ÅK, ØS, IL; Resources: KK, EG, ÅK, ØS, IL, Software: KK, TMK, GH, EMC, Supervision: KK, EG, GMB, ÅK, ØS, IL, Validation: KK, EG, TMK, GMB, ÅK, ØS, IL, Visualization: KK, TMK, GH, HN, ØS, Roles/Writing - original draft: KK, Writing - review & editing: KK, EG, TMK, GMB, GH, ÅK, HN, ØS, IL.

Ethics statements

The feeding experiment followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th of June 2009.

The trial facilities were granted permission by the Norwegian Food Safety Authority to run the experiments. The decision was made on the basis of Regulations 18. June 2015 on the use of animals in experiments, §§ 6, 7, 9, 10 and 11.

Data availability statement

Generated Statement: This manuscript contains previously unpublished data.

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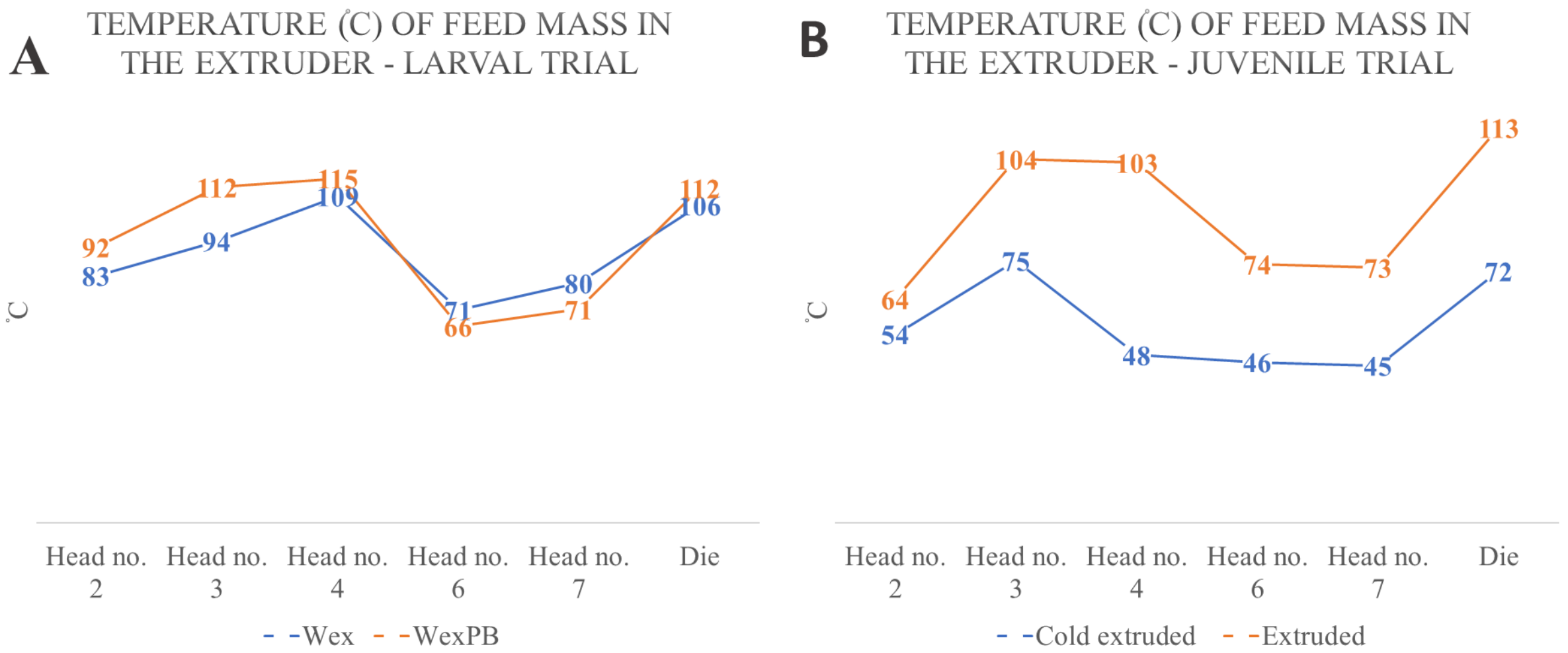


Fig 1. Temperature measurements of feed masses in the extruder during production of the two extruded larval diets (A) and (B) the extruded (orange line) and cold extruded (blue line) juvenile trial experimental diets.

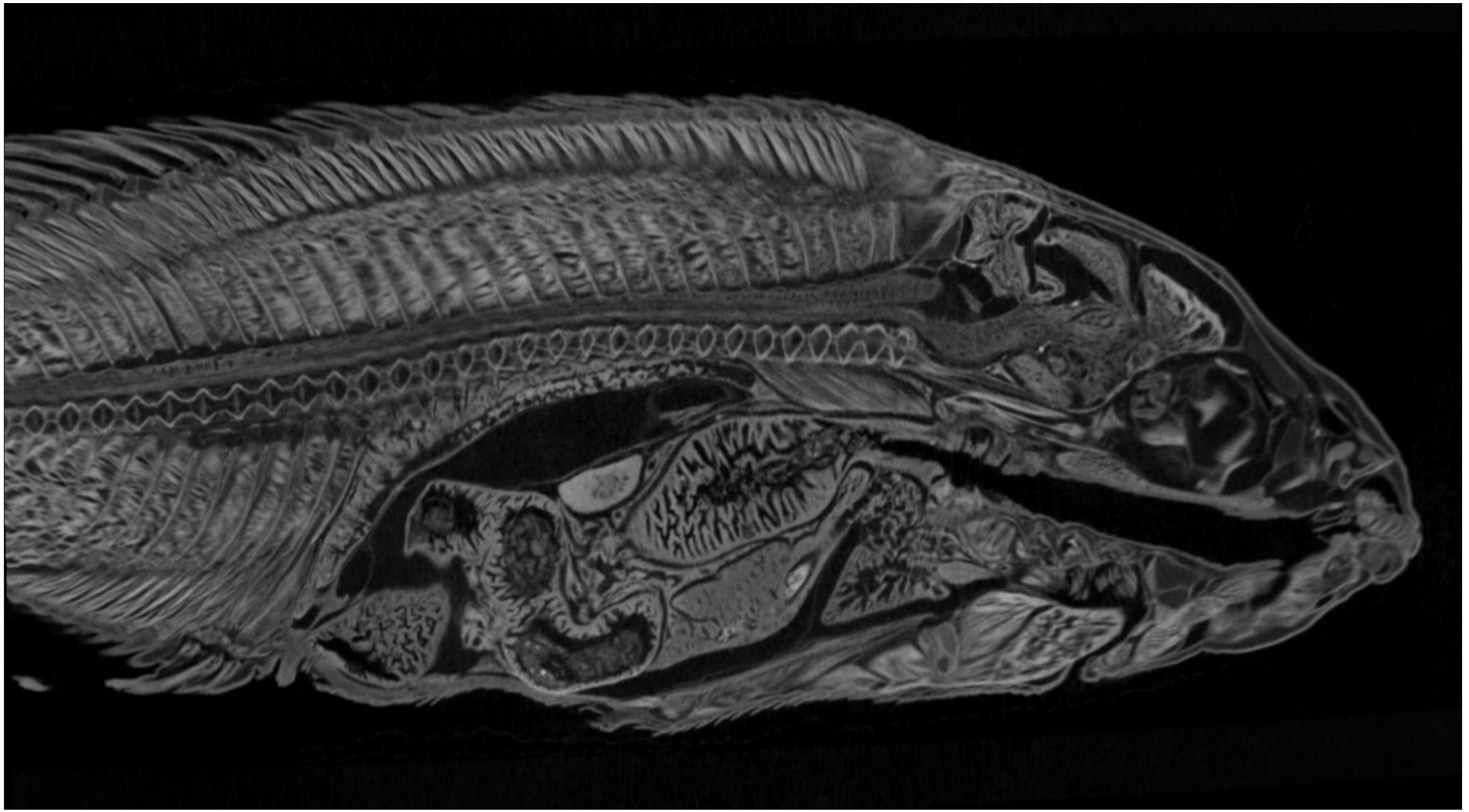


Fig. 2 CT scanner-X-ray picture using contrast liquid of ballan wrasse larvae with normal skeletal development.

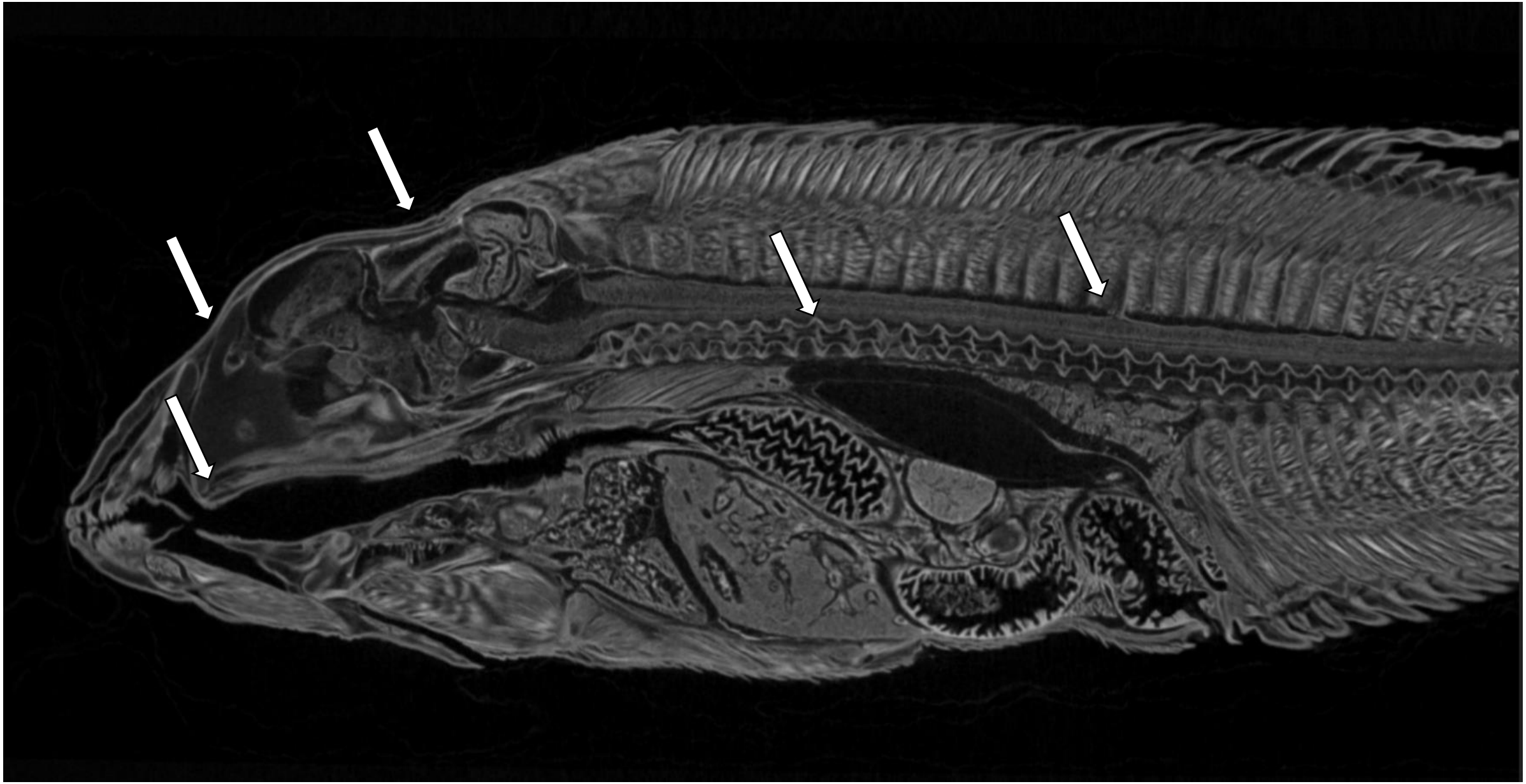


Fig. 3 CT scanner-X-ray picture using contrast liquid of deformed ballan wrasse larvae. Deformities are obvious both on the head and spinal cord areas (white arrows).

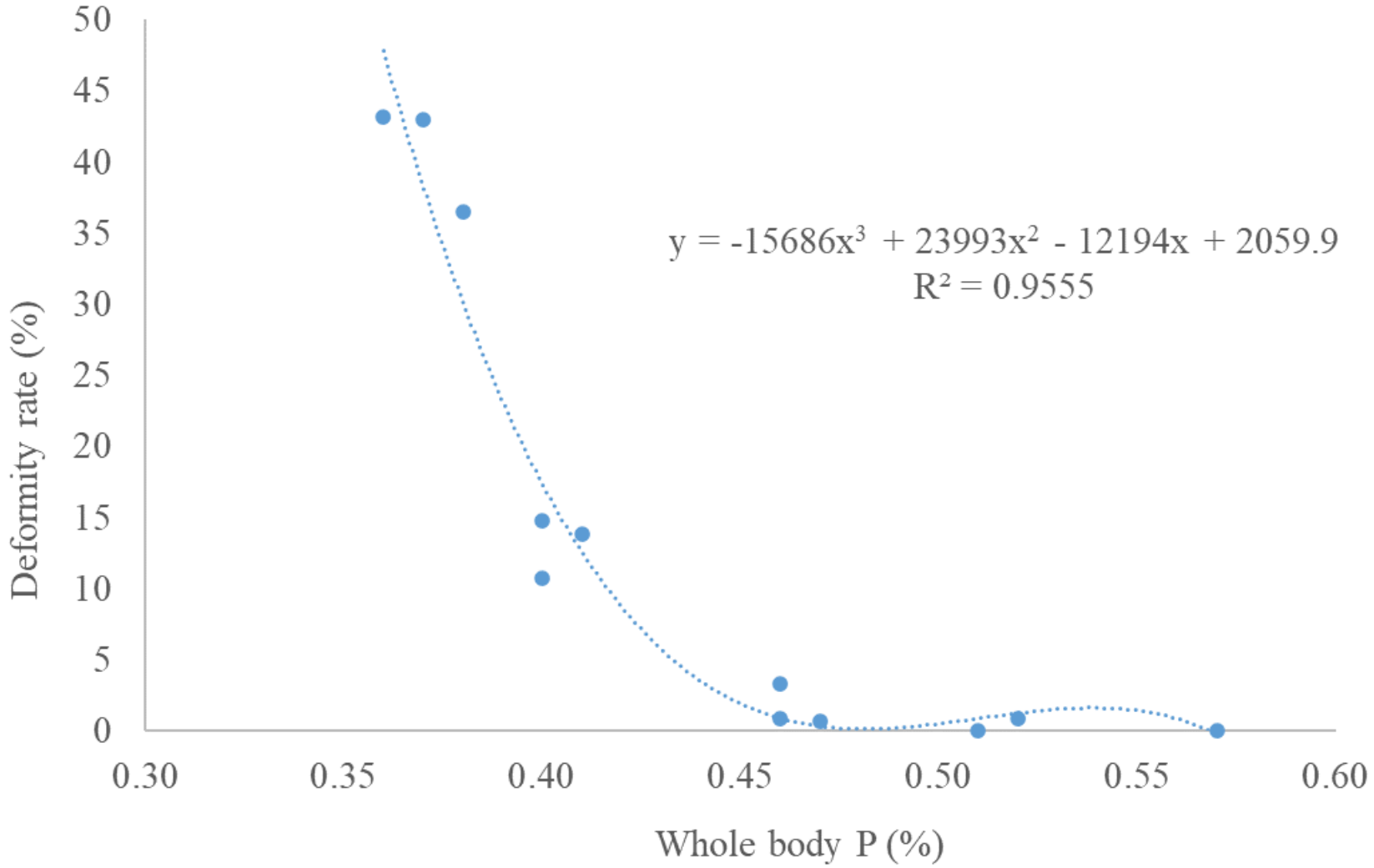


Fig. 4 Relationship between total phosphorous levels in the whole body (WB) of weaned ballan wrasse larvae and occurrence of skeletal deformities to the skull.

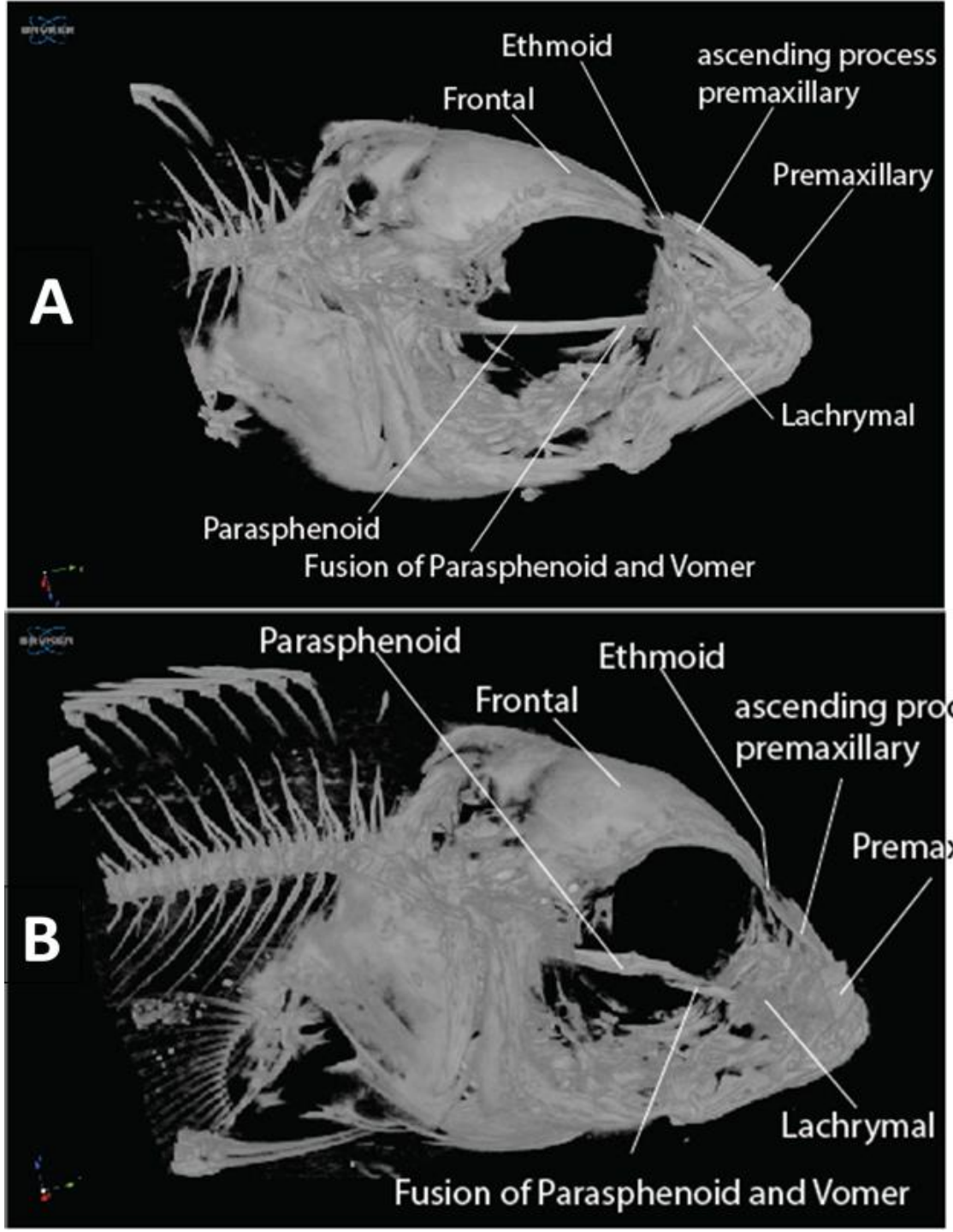


Fig. 5 CT-scan pictures of the ballan wrasse larvae cranium skeleton from A: well mineralized fish and B: poorly mineralized fish. A: Healthy frontal, ethmoid, premaxillary, lachrymal and parasphenoid bones marked. B: Curved frontal, compressed mouth area and fractured parasphenoid bone.

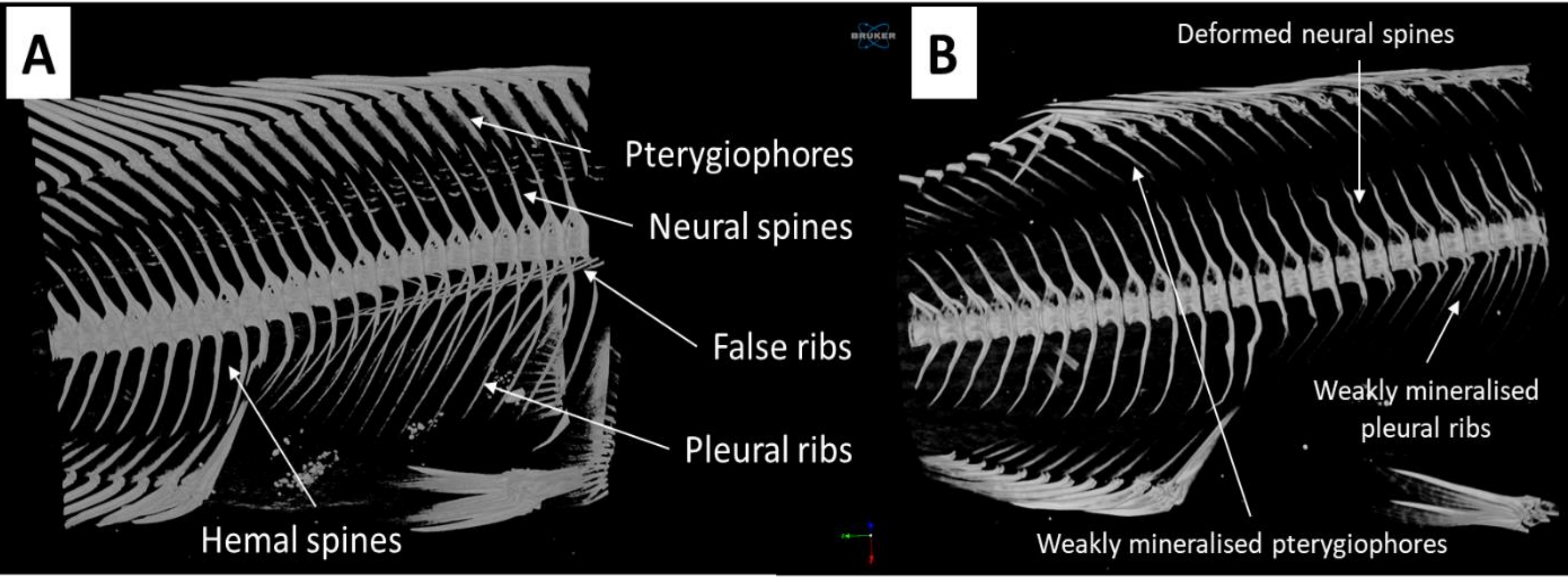


Fig. 6 CT-scan pictures of the ballan wrasse larvae main body skeleton area from A: well mineralized fish and B: poorly mineralized fish. A: long healthy well mineralized pterygiophores, neural and hemal spines and pleural and false ribs. B: short, poorly mineralized pterygiophores, short, deformed and poorly mineralized pleural ribs and neural spines and invisible false ribs.

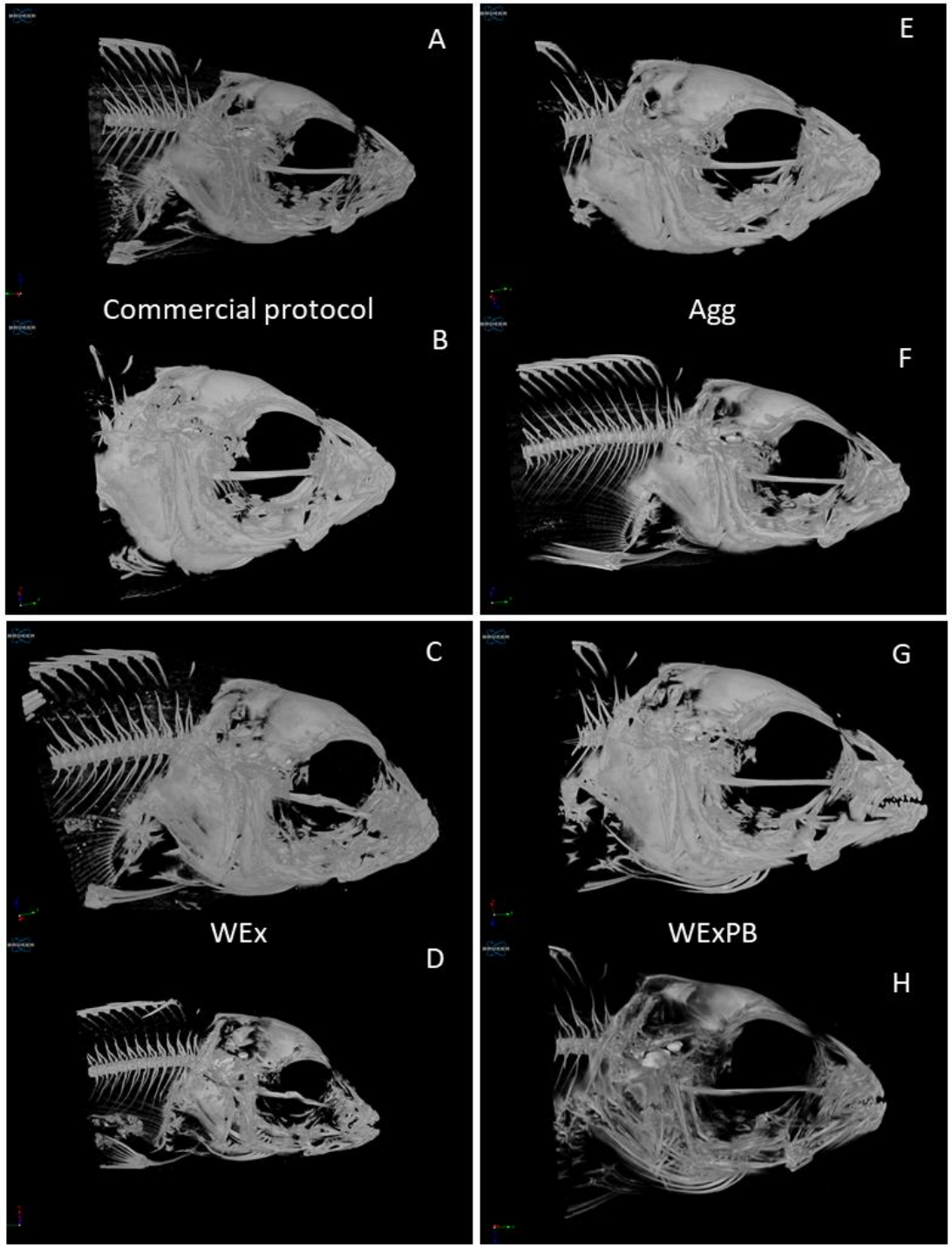
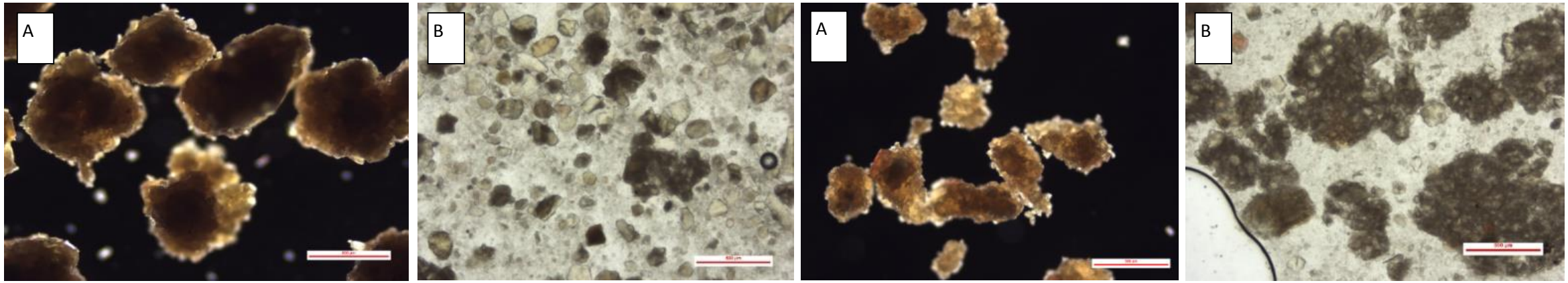


Fig. 7 CT-scan pictures of the ballan wrasse larvae cranium skeleton area from representative fish of each dietary treatment. A-B: +Commercial protocol including use of the warm extruded feed WEx for 8 days and 24 days OTOHIME (cold extruded); C-D: WEx; E-F: Agg (agglomerated); G-H: WExPB warm extruded feed where cod muscle meal in the wrasse feed formulation is substituted with poultry byproduct meal.



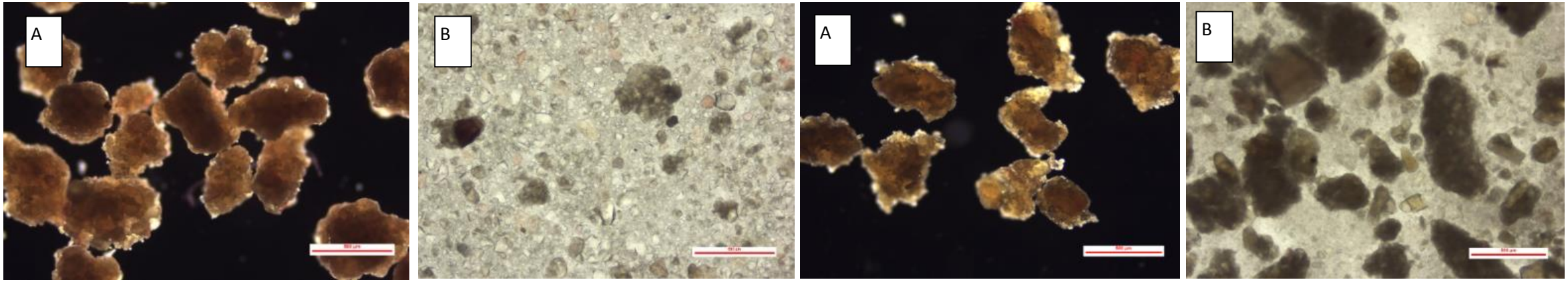


Fig. 8 Experimental diets in dry form (A) and wet form (B) following a solubilization test. Pictures taken under the microscope. Solubilization test: feed samples (0.5 g in 5.0 ml water) after 1 hour in the water, with vigorous shaking every 20 minutes (at 20, 40 and 60 min in the water).

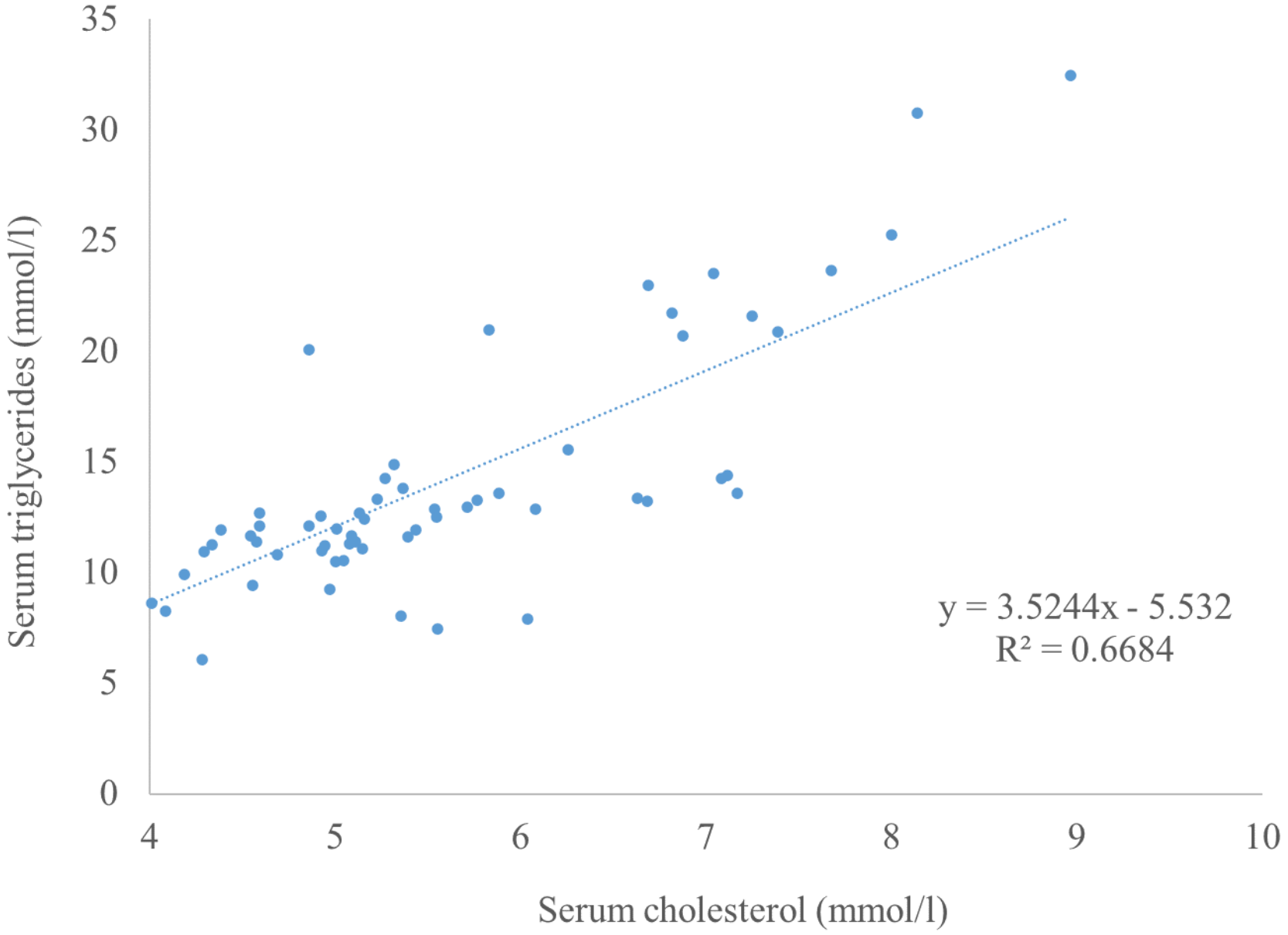


Fig. 9 Significant positive correlation between levels of serum triglycerides and serum cholesterol in ballan wrasse fed diets from different production technologies, i.e. agglomeration, cold and warm extrusion.

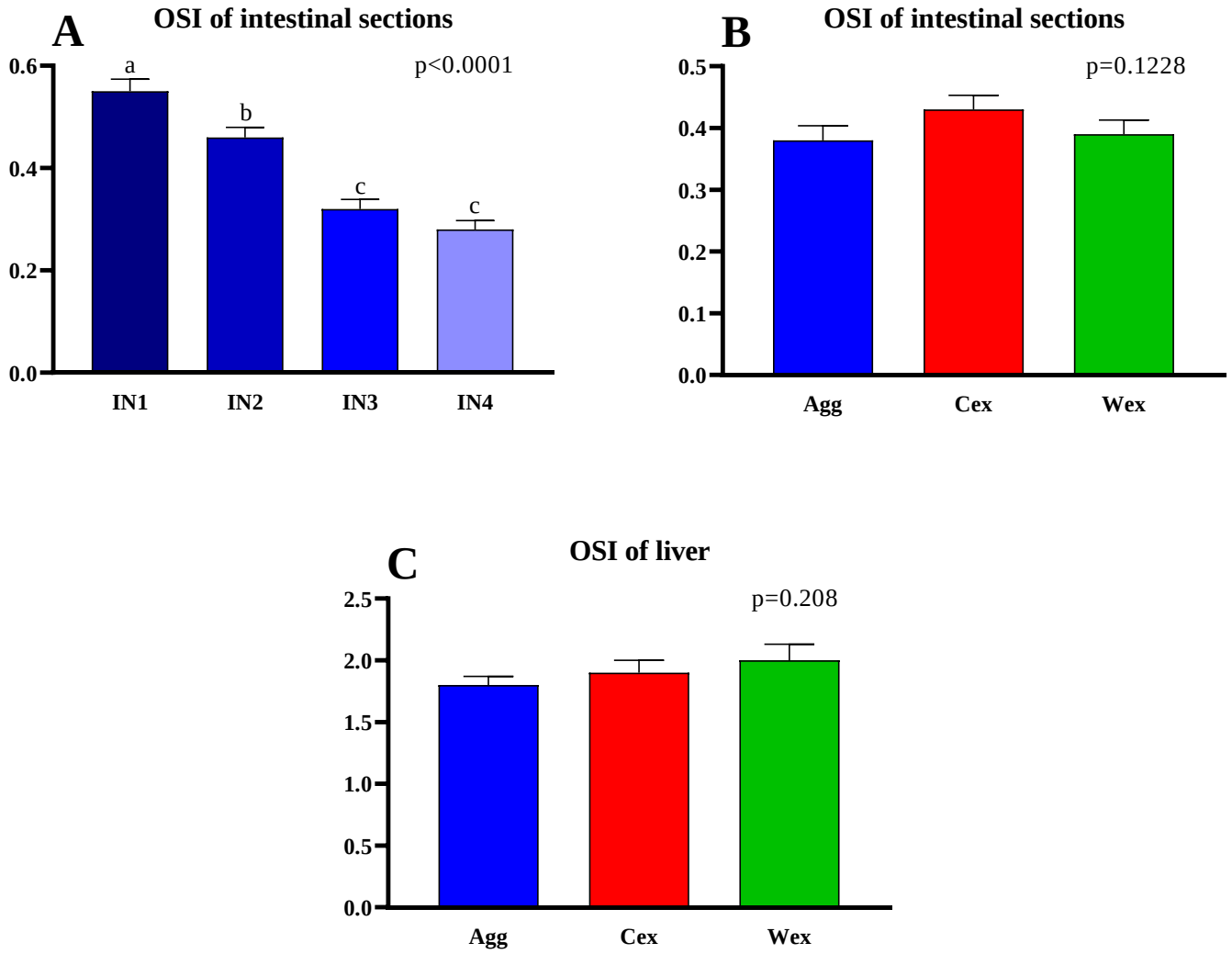


Fig. 10 Relative weight (organosomatic index, OSI) of the four sampled intestinal sections (IN1-4) shown in the figure section A, and effects of diet (Agg=agglomerated, CEx=cold extruded, WEx=warm extruded) on organosomatic index of the intestinal sections (figures section B) and liver (figure section C).

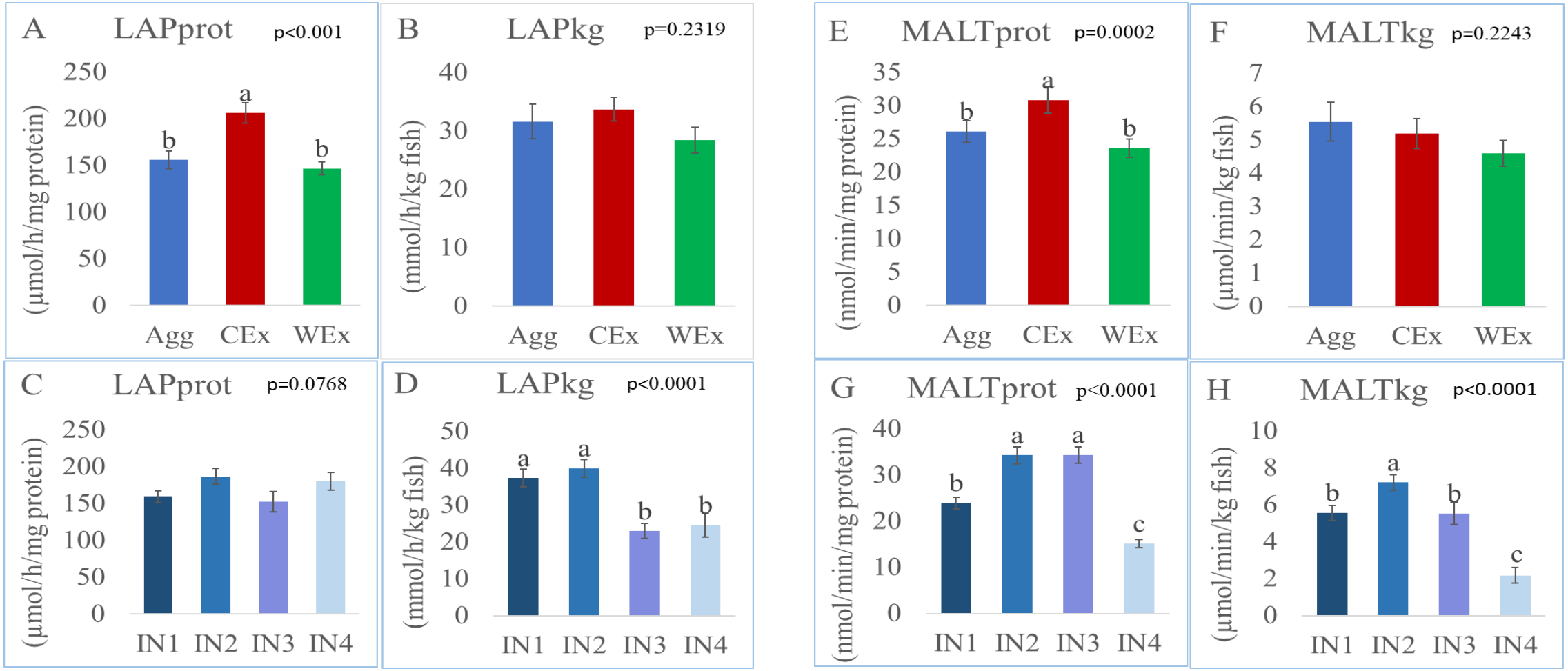


Fig. 11 Effects of diet (Agg=agglomerated, CEx=cold extruded, WEx=warm extruded) on activity of leucine amino peptidase (LAP, figure A-D) and maltase (MALT, figure E-H) in tissue sampled along the intestinal tract (IN, section 1 - 4). The activity is expressed per mg protein (prot, figure A, C, E, G)) and capacity in the fish (kg, figure B, D, F, H).

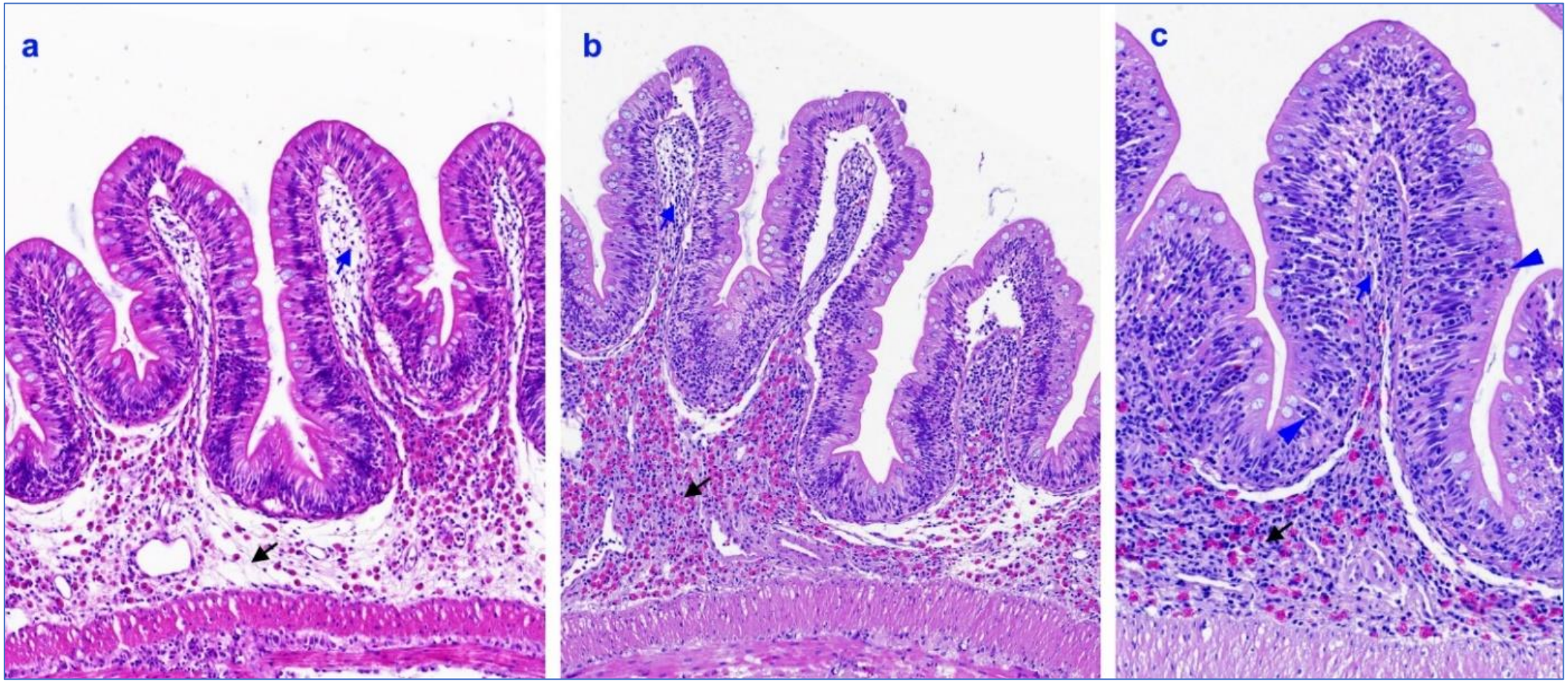
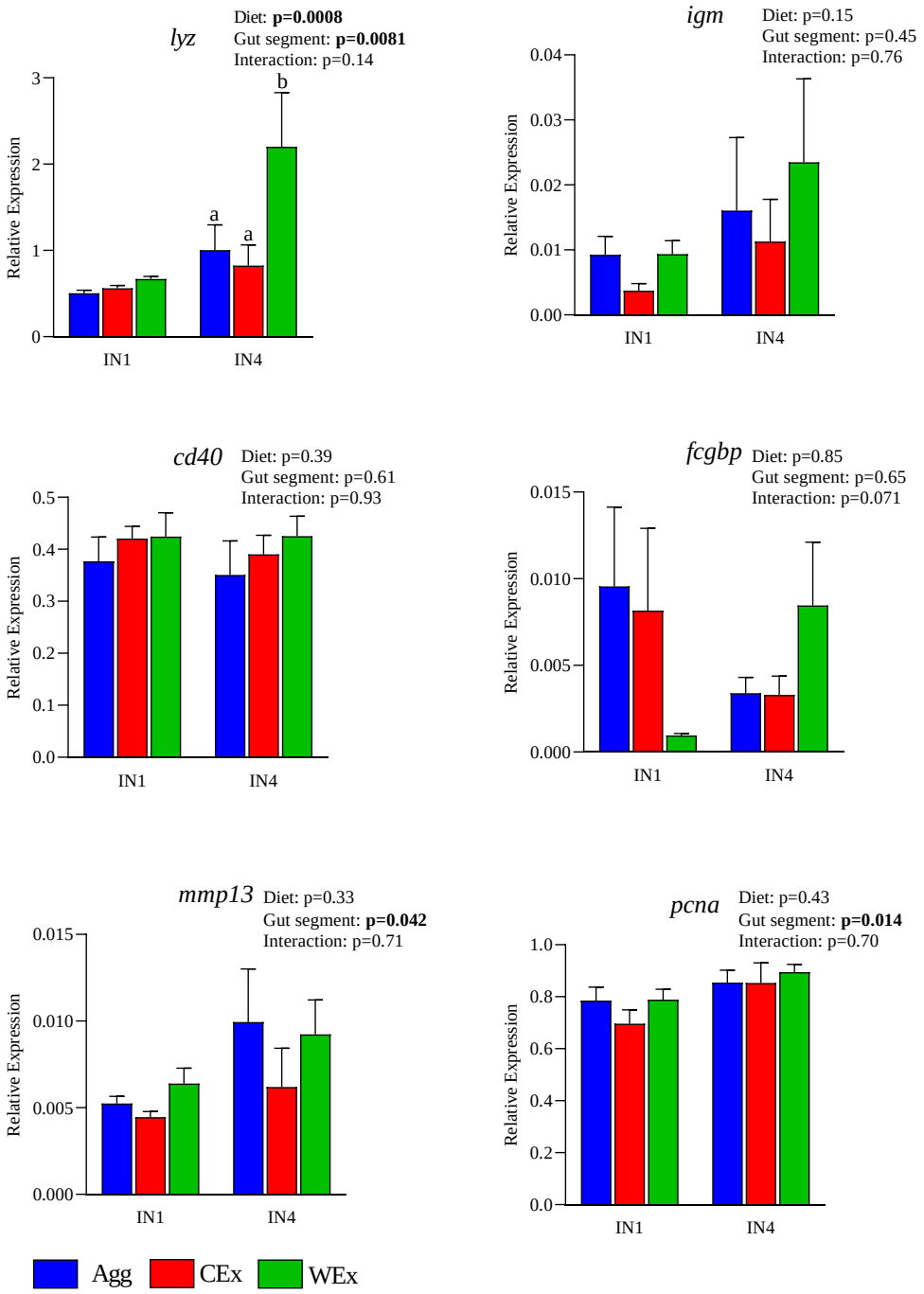
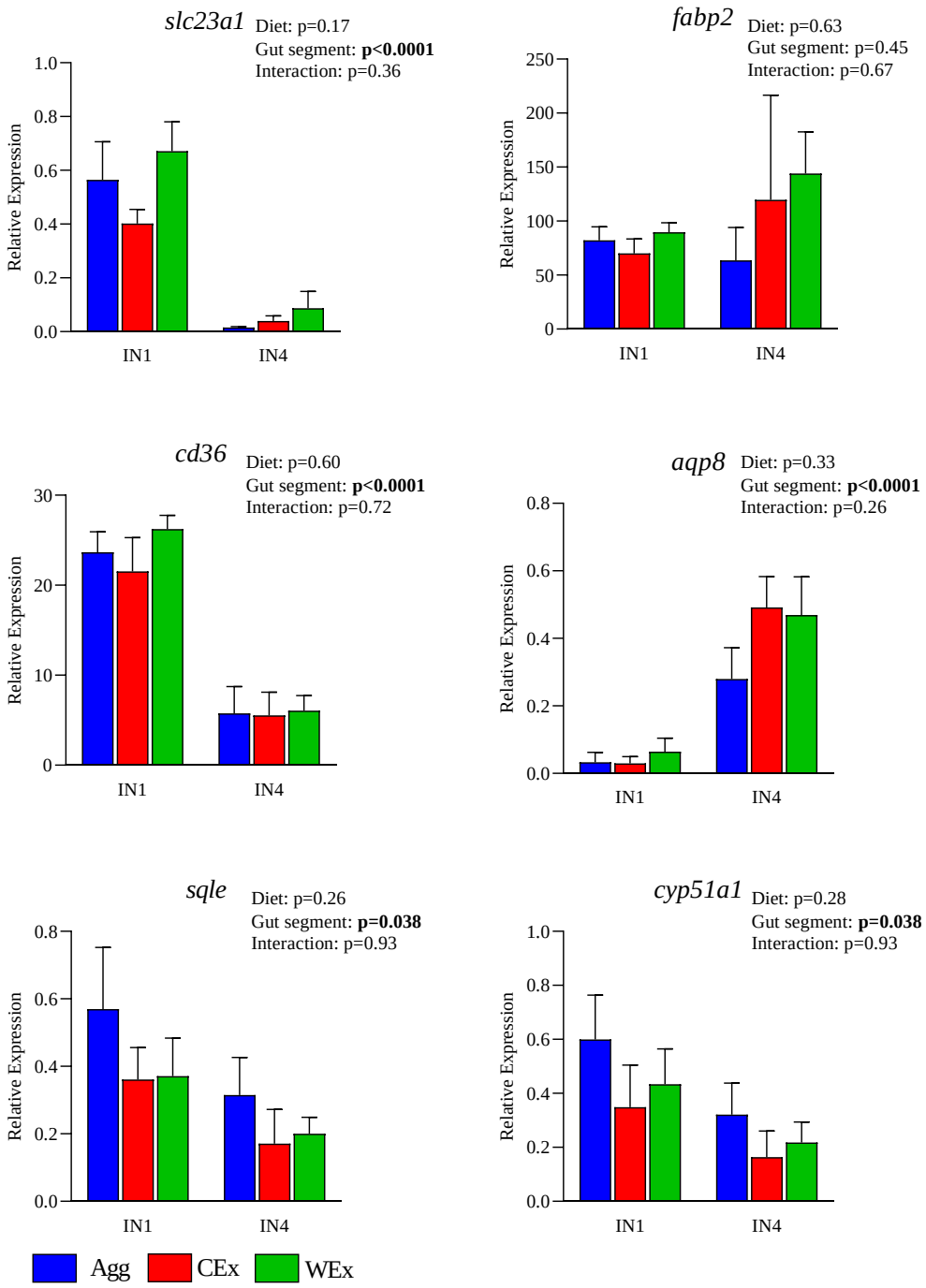


Fig. 12 Representative images of the morphological features observed in the histological assessment of the ballan wrasse intestinal sections from the juvenile fish trial. All images are of the IN-3 intestinal region. Changes observed included the increased cellularity of the submucosa (black arrows) and the lamina propria (blue arrows) as well increased intraepithelial infiltration by lymphocyte-like cells and eosinophilic granular cells (EGCs). Picture a shows normal morphological appearance of sparse cellularity of the submucosal and lamina propria compartments dominated by EGCs. Picture b illustrates increased cellularity of the submucosa and lamina propria by a mixed population of cells including lymphocytic cells and EGCs. Picture c shows marked infiltration of the intestinal epithelial layer by EGCs and lymphocyte-like cells.

Supplementary Fig. 1 Number of Ballan wrasse intestinal sections (numbers in the stacked columns) evaluated that were scored as 'normal' or 'healthy', 'mild', 'moderate', 'marked', or 'severe' for selected histo-morphological characteristics of increase in width and cellular content of (a) submucosa and (b) lamina propria; (c) presence of supranuclear vacuolization in enterocytes, and (d) increase in infiltration of the epithelial layer of the intestinal mucosa. Diet groups with different superscript letters are statistically different. Panel a-d: Results from intestinal section N1; Panel e-h: Results from intestinal section N2; Panel i-l: Results from intestinal section N3; Panel n-p: Results from intestinal section N4.



Supplementary Fig. 2 Immune-related gene expression in proximal (IN1) and distal (IN4) intestine. Data are mean relative expression levels of n = 12 fish per diet. Bars are standard error. The P values for the 2-way ANOVA are given. Statistical differences between dietary treatments within one intestine segment are denoted with different lettes. See Table 5 for explanation of gene symbols.



Supplementary Fig. 3 Digestion-related gene expression in proximal (IN1) and distal (IN4) intestine. Data are mean relative expression levels of n = 12 fish per diet. Bars are standard error. The P values for the 2-way ANOVA are given. Statistical differences between dietary treatments within one intestine segment are denoted with different letters. See Table 5 for explanation of gene symbols.

Table 1
Formulations of the experimental diets used in the larvae and juvenile trials of the current study.

Trial	Larvae weaning		Juvenile
Number of diets	2	1	3
Abbreviation	WEx and Agg	WExPB	WEx, CEx and Agg respectively
Production technology	Extrusion and agglomeration, respectively	Extrusion	Extrusion, cold extrusion and agglomeration
Unit	%	%	%
Shrimp powder[1]	28.50	28.50	28.50
Cod muscle powder[1]	31.27	-	45.15
Poultry by-product meal[2]	-	29.07	
Squid meal[1]	12.00	12.00	
Pre-gelatinized starch			7.50
Krill hydrolysate[3]	6.60	6.60	6.60
Whole wheat	6.06	6.06	3.50
Krill oil[4]	4.00	4.00	3.50
Soya lecithin	2.00	3.00	
Cholesterol	-	0.70	
Alltech SP1[5]	0.60	0.60	
Choline chloride	0.50	0.50	0.50
Aquate[5]	0.40	0.40	0.40
Minerals[5,6,7]	3.69	3.69	1.85
Vitamins[6]	2.88	2.88	2.27
Tau, Met, Lys	1.45	1.95	0.20
Astaxanthin (10%)	0.05	0.05	0.03
Total	**100.00**	**100.00**	**100.00**

[1]Seagarden, Stavanger, Norway
[2]GePro Geflügel-Protein Vertriebsgesellschaft mbH & Co. KG, Diepholz, Germany
[3]Olympic, Ålesund, Norway
[4]Aker Biomarine, Oslo, Norway
[5]Alltech Inc, Kentucky, USA. Products included: SP1, Aquate, Bioplex Zn, Bioplex Cu, Sel-Plex 2300, Bioplex Mn and Bioplex Fe
[6]Norsk Mineralnæring, Norway.
[7]Monosodium phosphate

Table 2
Extrusion conditions during experimental feed production.

	Cold extrusion	Extrusion (juvenile diet production)
Pre-conditioner		
Speed (rpm)	220	220
Steam (kg/h)	4-6	7-8
Water (kg/min)	0.150 - Cold	0.150 - Warm
Temperature (°C)	55-60	85
Extruder		
Dice opening (mm)	1.25	1.25
Knife speed (rpm)	1354	1441
Speed (rpm)	160	400
Engine shear (kW)	3.5	5.7
Motor load/SME (kg/h)	0	0
Water (kg/min)	0.350	0.330
Pressure at Head no. 5 (bar)	14	7.8
Pressure at Head no. 7 (bar)	16	11.7

Table 3
Pellet hardness of the experimental juvenile trial feeds.

	Agglomerated	Cold extruded	Extruded	ANOVA (P-value)
Pellet hardness (N)	3.2^{a} ±0.59	7.7^{b} ±2.33	10.1^{c} ±2.20	0.000

Table 4

Primer pairs and related information for real-time PCR assays.

	5' -3' primer sequence					
Gene symbol*	Forward	Reverse	Amplicon size (bp)	Annealing temperature (℃)	Efficiency	Gene bank accession no.
gapdh2	TATTTGTGTCCGTGTGCCCC	GCCTCCGTCCACTGATGAAT	129	62	1.99	XM_020633887.1
lyzg	CTTGGGACAGCGAGGAACAC	TCCATCGCCCATGTTGTAGG	140	62	1.96	XM_020660641
cd40	AGCAGTAAACCCGACTGAGG	GCTTTGGTCGTCCTCGTTCT	85	60	1.99	XM_020651338.1
mmp13	TCTCGACGCCGCTTATGAAA	CACGCACGGGTTTATAGCCA	95	60	1.90	XM_020631204.1
fcgbp	CAACTCTCCCTGTCTCTCCAG	GCTTCACAGAGGCAATTCTCC	126	62	2.04	XM_020655516.2
cd36	ACGGAGGGATAAAACGCACA	TATGCTGTGGTTCCAGGCTC	181	62	2.01	XM_020649455.1
aqp8	TTGGCTCCTTTCCTTGTGGG	CCGAGAATGAGCCTGAGCAA	197	60	1.95	XM_020642545.1
slc23a1	CCCACTGAACACCTCACACA	AGACCAATCAGCAGCTCCAC	93	60	1.83	XM_020655303
sqle	ACGAGAGATCAGCGACCAAC	CAGGTTCTGGAGCCACTGTT	117	62	1.94	XM_020635029
cyp51a1	AAGGACTGCTGTTCCGATGG	CCTCTCCACAAAACCACCGA	113	60	1.79	XM_020648620
fabp2	TACAGCCTTGCGGATGGAAC	ATCCTCTTAGCCTCCACACCT	173	60	1.95	XM_020643842.1
pcna	GCCAACAACACACAAAGGCT	TCGTCTTTCTGCGTCACTCC	106	62	1.88	XM_020647462.1
igm	ATCTCTTGTGGAACAGGGCAC	CCTTGAAGTCAGCAAAACGCT	101	55	1.89	XM_020660315.1

* Full gene names: gapdh2, glyceraldehyde-3-phosphate dehydrogenase 2; lyzg, lysozyme g; cd40, cluster of differentiation 40; mmp13, Collagenase 3; fcgbp, IgGFc-binding protein; cd36, cluster of differentiation 36; aqp8, aquaporin 8; slc23a1, solute carrier family 23 member 1 ; sqle, squalene monooxygenase; cyp51a1, Lanosterol 14-alpha demethylase; fabp2, fatty acid binding protein 2; pcna, proliferating cell nuclear antigen; igm, immunoglobulin m. All primers were designed in-house.

Table 5

Growth performance, mortality rates, percentage of fish with skeletal deformities and fin status of weaned ballan wrasse. Values are mean ± standard variation (n=3).

Weaning protocol	WEx 8 days + OTOHIME 26 days	WEx 34 days	Agg 34 days	WExPB 34 days	1-WAY ANOVA (P-value)
Feed processing	Extruded + Cold extruded	Extruded	Agglomerated	Extruded	
Start fish number	200	200	200	200	-
End fish number	119	126	139	121	ns
Initial weight (g)	0.035	0.035	0.035	0.035	-
Final weight (g)	0.40±0.01	0.36±0.03	0.37±0.08	0.34±0.02	ns
SGR	7.18±0.11	6.81±0.22	6.86±0.59	6.65±0.17	ns
Survival – looser fish (%)	48±9.22	60±9.67	64±13.71	54±6.83	ns
Deformities (%)	0.28^{a}±0.49	40.86^{c}±3.79	1.61^{a}±1.46	13.09^{b}±2.	0.000
Normal fish (%)	48.0^{ab}±9.2	34.3^{a}±8.4	62.7^{b}±13.2	46.3	0.047
Damaged fins (%)	33	0	33	^{ab} 0	-

*Numbers in the same line with different superscript letter are significantly different following Duncan post-hoc test (P<0.05)

ns: non-significant

Table 6

Chemical composition of ballan wrasse larvae before and after weaning with different diets.

Feeding regime		Start	WEx+ OTOHIME	WEx	Agg	WExPB	Anova* P value
Feed technology			Extrusion + Cold extrusion	Extrusion	Agglomeration	Extrusion	
Moisture	%	81.5^{ab}±0.2	81.2^{a}±0.6	81.8^{b}±0.1	82.0^{b}±0.1	81.8^{b}±0.2	0.050
Protein	%	12.6^{a}±0.2	13.9^{b}±0.4	14.6^{c}±0.1	14.0^{b}±0.2	14.4^{c}±0.2	0.000
Fat	%	3.0±0.1	2.4±0.4	2.3±0.6	2.3±0.7	2.2±0.2	ns
Ash	%	2.33^{ab}±0.21	2.90^{c}±0.10	2.20^{a}±0.10	2.57^{b}±0.06	2.33^{ab}±0.21	0.001
Ca	%	0.39^{b}±0.02	0.61^{d}±0.01	0.35^{a}±0.02	0.53^{c}±0.02	0.39^{b}±0.01	0.000
P	%	0.43^{c}±0.01	0.53^{e}±0.03	0.37^{a}±0.01	0.46^{d}±0.01	0.40^{b}±0.01	0.000
Ca/P		0.89^{a}±0.027	1.17^{c}±0.002	0.95^{b}±0.027	1.14^{c}±0.031	0.97^{b}±0.029	0.000
Mg	%	0.042±0.001	0.044±0.001	0.034±0.006	0.037±0.001	0.035±0.004	0.093
Zn	ppm	22.0^{b}±1.00	20.5^{ab}±0.71	19.0^{a}±1.00	21.0^{b}±1.00	20.3^{ab}±0.58	0.029
Fe	ppm	10.3±3.56	10.5±0.71	6.5±1.01	9.9±6.56	7.0±0.72	ns
Cu	ppm	2.77±1.61	1.65±0.07	2.20±0.62	1.63±0.15	1.90±0.44	ns
Sum FAA[1]	%	1.06^{AB}±0.05	1.15^{B}±0.24	0.91^{AB}±0.14	0.83^{A}±0.08	0.89^{AB}±0.09	0.082
Sum total AA[2]	%	11.53^{a}±0.46	13.18^{b}±0.24	13.72^{bc}±0.56	13.30^{b}±0.18	14.09^{c}±0.30	0.000
sum total IAA[3]	%	5.68^{a}±0.26	6.78^{b}±0.15	7.12^{bc}±0.29	6.90^{b}±0.08	7.41^{c}±0.09	0.000

* Numbers in the same line with different small or capital superscript letter are significantly different or have indication for difference, respectively, following pairwise comparisons with Duncan post-hoc test ($P<0.05$); ns: non-significant; [1]Free amino acids; [2]Amino acids; [3]Indispensible amino acids.

Table 7
Starch content and gelatinization as well as observation on feed technical qualities before and after water treatment.

	Hardness	Stability for 1 hour in water	Starch	Degree of starch gelatinisation
	Observation		%	% of total starch
OTOHIME	Soft	Dissolved	2.7	67.0
WEx	Hard	Partly intact	4.9	94.7
Agg	Soft	Dissolved	5.7	20.0
WExPB	Hard	Partly intact	5.3	90.0

Table 8
Juvenile ballan wrasse performance when fed diets of different physical properties.

	Agg	CEx	WEx	ANOVA (P*)
Mean body weight START (g)	11.4±0.1	11.4±0.0	11.4±0.1	ns
Mean body weight END (g)	37.2±5.8	36.2±2.3	33.5±4.0	ns
Fork length (cm)	11.8±0.3	12.2±0.1	11.5±0.6	ns
SGR	0.93±0.12	0.92±0.05	0.85±0.09	ns
Mortality (%)	37.3±6.1[a]	41.3±5.3[a]	57.3±2.9[b]	0.006
Mean sample fish body weight (g)	51.9±2.24	55.5±2.21	48.3±6.66	ns
HSI (in sample fish)	1.74±0.03[a]	1.88±0.10[a]	2.06±0.09[b]	0.009
Condition factor (in sample fish)	3.10±0.25	3.06±0.04	3.11±0.16	ns

* Numbers in the same line with different small or capital superscript letter are significantly different, following pairwise comparisons with Duncan post-hoc test ($P<0.05$); ns: non-significant.

Table 9
Whole body dry matter and mineral composition of ballan wrasse juveniles fed diets of different physical properties.

	Agg	CEx	WEx	ANOVA (P*)
Dry matter % (g/100g)	30.6±1.2	31.4±1.1	30.9±0.7	ns
Ca (mg/kg)	10300±2402	10800±1311	9967±929	ns
Na (mg/kg)	1400±0	1500±173	1333±58	ns
K (mg/kg)	3667 ± 58^{ab}	3900 ± 200^{b}	3567 ± 58^{a}	0.042
Mg (mg/kg)	423±25	443±31	417±31	ns
P (mg/kg)	6800±1153	7333±802	6500±436	ns

* Numbers in the same line with different small or capital superscript letter are significantly different, following pairwise comparisons with Duncan post-hoc test ($P<0.05$); ns: non-significant.

Table 10
Paired samples T test comparison of mineral levels and dry matter of ballan wrasse juveniles fed diets of different physical properties (CE: cold extruded; A: agglomerated; WE: extruded, P-value).

	Agg	CEx
CEx	CEx>Agg (0.062)	
WEx	Agg>WEx (0.075)	CEx>WEx (0.066)

Table 11
Juvenile ballan wrasse serum chemistry.

Treatment	Agg	CEx	WEx	ANOVA (P value)
Sample fish body weight (g)	52.5±1.3	55.5±2.2	48.3±6.7	ns*
Cortisol (nmol/l)	1166±514	980±663	1014±666	ns
Lactate (mmol/l)	1.16±0.04	1.31±0.38	1.10±0.10	ns
Glucose (mmol/l)	2.04±0.19	2.28±0.29	1.84±0.47	ns
Magnesium (mmol/l)	0.98±0.13	0.90±0.05	0.91±0.07	ns
Cholesterol (mmol/l)	5.12±0.14	5.17±0.88	5.93±0.28	ns
Triglycerides (mmol/l)	11.93±0.20	12.58±2.57	15.16±1.00	0.1

* non-significant

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Author Contributions

Conceptualization: KK, EG, GMB, ØS, IL, ÅK; Data curation: KK, EG, TMK, GMB, HN, ØS, IL; Formal analysis: KK, TMK, GH, NH, EMC; Funding acquisition: KK, EG, TMK, ÅK, ØS, IL; Investigation: KK, EG, TMK, GMB, ÅK, HN, ØS, IL; Methodology: KK, EG, TMK, GMB, GH, ÅK, HN, ØS, IL; Project administration: KK, EG, ÅK, ØS, IL; Resources: KK, EG, ÅK, ØS, IL, Software: KK, TMK, GH, EMC, Supervision: KK, EG, GMB, ÅK, ØS, IL, Validation: KK, EG, TMK, GMB, ÅK, ØS, IL, Visualization: KK, TMK, GH, HN, ØS, Roles/Writing - original draft: KK, Writing - review & editing: KK, EG, TMK, GMB, GH, ÅK, HN, ØS, IL.

Ethics statements

The feeding experiment followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th of June 2009. The trial facilities were granted permission by the Norwegian Food Safety Authority to run the experiments. The decision was made on the basis of Regulations 18. June 2015 on the use of animals in experiments, §§ 6, 7, 9, 10 and 11.

Data availability statement

Generated Statement: This manuscript contains previously unpublished data.

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CT scanner video of low mineralized Ballan wrasse larvae head area

Supplementary table 1

Supplementary table 2