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# Marine raw material choice, quality and weaning performance of Ballan wrasse (*Labrus bergylta*) larvae

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## Abstract

Previous results show that weaning success of Ballan wrasse larvae greatly depends on the quality of the dietary marine raw materials. In the present study, six moist or agglomerated experimental weaning diets containing different combinations of high-quality marine raw materials, being fish meal (FM), cod muscle meal (CMM), shrimp meal (SM) and krill hydrolysate (KH), were tested in a 2-month weaning trial with Ballan wrasse larvae of 34.5 mg initial body weight. Larvae performance was good in all dietary treatments except those fed diet D1 containing only FM. The Ballan wrasse larvae fed weaning diets D4 and D5 containing FM and either SM or KH, respectively, had the highest final body weight (0.7 g) but also the highest mortality (50%). Best weaning survival (77%) was obtained using the dry agglomerated diet D3 containing CMM and SM. During the first month, fish survival correlated positively with dietary free amino acid and soluble protein levels and negatively with the combined levels of dietary lipid oxidation metabolites and ethoxyquin. During the second month, mortality rates were lower in all treatments. Fish larvae final body weight correlated negatively with total dietary fatty acids and positively with dietary cholesterol, phosphorous and DHA/EPA ratio.

KEY WORDS: Ballan wrasse, larvae weaning, marine protein quality

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## Introduction

Ectoparasitic copepod sea lice (*Lepeophtheirus salmonis*) infections have recently made a comeback as a major parasitic pest of farmed salmon, being a major economic, fish welfare and environmental threat concerning commercial Atlantic salmon (*Salmo salar* L.) culture in Europe and America (Costello 2009). One of the main reasons for the re-emergence of this parasite is the development of resistance to pharmaceuticals (SLICE™, hydrogen peroxide, AlphaMax™, Betamax™, Excis™) used for treating salmonid infestations over the last decade (Treasurer *et al.* 2000; Fallang *et al.* 2004; Sevatdal *et al.* 2005; Lees *et al.* 2008). Sea louse feed off the skin, mucus and blood of Atlantic salmon causing illness or death through secondary infection and osmoregulatory stress. The discovery that local wrasse displayed cleaning behaviour led to the commercial use of wrasse in salmon cages as an environmental-friendly control against sea louse during the early 1990s (Kvenseth 1996 and Skiftesvik *et al.* 2013). Today, there is a rapidly increasing interest in using farmed cleaner fish as primary and sustainable solution to combat sea louse infections in farmed Atlantic salmon (Skiftesvik *et al.* 1996; Stone 1996; D'Arcy *et al.* 2012). In particular, the largest species, Ballan wrasse (*Labrus bergylta*), has been shown to be tolerant of winter sea temperatures and more effective for delousing larger salmon (3–5 kg) in comparison with other wrasse species (Bjelland *et al.* 1996).

One of the main challenges in commercial production of new marine fish species for aquaculture is poor fish performance during the first life stages in larvae rearing, in terms of low survival, low growth rates and high incidences of deformities. Particularly, weaning, or else the transition from live feed to formulated artificial diets, represents probably the main bottleneck in sustainable hatchery operations (Tilseth 1990). Very little is known or published so far on this species, and currently, ongoing research is

focusing on determining its basic nutritional requirements and behaviour. Our previous experience shows that weaning is very challenging in this species, leading to high mortalities apparently due to starvation (Skiftesvik AB, Jensen PA & Opstad I, unpublished data). Moreover, besides survival, Ballan wrasse growth rates during and after weaning depend greatly upon the source and quality of protein in the feed (Kousoulaki *et al*. 2011). Unlike what is expected, most Ballan wrasse larvae do not accept weaning diets containing commercial fish meal but do respond very positively to the presence of shrimp meal in the diet in combination with cod muscle meal (Skiftesvik AB, Jensen PA & Opstad I, unpublished data). Likewise, the gut content of wild Ballan wrasse fish represented 0.09% of ingested food items, while Echinodermata, Decapods and Mollusca accounted for 45.1%, 26.7% and 11.1% of the food items, respectively (Figueiredo *et al*. 2005). Both shrimp meal and muscle meal are exceptionally expensive raw materials (approximately 25€ $kg^{-1}$) usually included in food preparations, but also commercially in rearing Ballan wrasse larvae and juveniles due to lack of alternatives that can sustain acceptable fish weaning performance in a predictable way (Berg-Jacobsen 2012). There is a need to formulate efficient Ballan wrasse weaning diets that are based on economic and available raw materials. In this paper, we present growth and survival results from a weaning study with Ballan wrasse larvae where different combinations of marine raw materials were used in the diets, namely fish meal (FM), cod muscle meal (CMM), whole shrimp meal (SM) and krill hydrolysate (KH).

## Material and methods

### *Raw materials, diets and fish experiment*

Fresh frozen whole shrimps and cod fillets were freeze-dried (Christ Gamma 1-16 LSC, and Heto FD 8.0) at the pilot processing plant of Nofima AS in Bergen, Norway. The true protein digestibility in mink of the produced raw materials was determined according to Skrede (1979). Herring fish meal and stick water (SW) were produced in the Nofima AS pilot processing plant in Bergen, Norway, from iced herring. The herring were heated to 90 °C in water and pressed in a screw press (Stord Bartz P13, Bryne, Norway), and the press cake and soluble fraction were separated. An antioxidant (FEQ 500; Hillerød, Danmark) was added to the press cake and the finally produced fish meal. The soluble fraction was separated using a separator (Westfalia SA1, Oelde, Germany) into an oil fraction that was discarded and sludge which was mixed with the wet press cake and SW. The stick water was evaporated to be concentrated at 40–90 °C in a 4-step falling film vacuum evaporator (Anhydro APV, Soborg-Copenhagen, Denmark).

Five dry agglomerated and one moist experimental weaning diet were produced. The moist diet D2 was produced at the Institute of Marine Research in Austevoll, Norway, as practised in the latest years with successful Ballan wrasse weaning results. In our study, D2 functioned as an external control diet. Shortly, for the preparation of D2, the dry feed ingredients were blended well together, and the oil followed by cold water was added under mixing. The feed mix was passed through an extruder and was then introduced in a marumerizer or spheronizer to form feed particles which were then sieved to the required size fractions. The dry agglomerated diets were produced at Nofima AS, Bergen, Norway. For the production of the agglomerated diets, all dry ingredients were milled to particle size below 0.2 mm using a Retch mill. The dry ingredients were mixed well together, and stick water, water and oil were added under mixing during the agglomeration process. The agglomerates formed when dried at 60 °C for 8.7 min using a fluid bed dryer were then sieved to different particle sizes. The feed size fractions that were used from both the moist and dry agglomerated diets were in sequence 0.1–0.3, 0.3–0.6 and 0.6–0.8 mm.

The dry agglomerated diets were formulated to have a proximate composition resembling the one reported to give best performance results in farmed Ballan wrasse (Hamre *et al*. (2013a,b)). Diet 1 (dry agglomerated, D1, FM) contained only fish meal and herring stick water as marine protein sources. Diet 2 (moist, D2, CMM + SM) and diet 3 (dry agglomerated, D3, CMM + SM) contained cod muscle meal and shrimp meal, diet 4 (dry agglomerated, D4, FM + SM) contained fish meal and shrimp meal, diet 5 (dry agglomerated, D5, FM + KH) contained fish meal and krill hydrolysate (Sopropeche, Boulogne sur mer, France), and diet 6 (dry agglomerated, D6, CMM + KH) contained cod muscle meal and krill hydrolysate. In dry matter basis, the diets were similar in terms of crude energy. Diet 2 had higher crude protein (757 g $kg^{-1}$) and lower lipid level (151 g $kg^{-1}$) than the dry agglomerated diets that were among them similar to crude protein and lipid levels of approximately 700 and 170 g $kg^{-1}$, respectively. The marine raw material chemical composition and the formulation and chemical composition of the diets are presented in Tables 1–5. Due to cost, not all analyses were performed in all raw materials.

**Table 1** Freeze-dried shrimp meal (SM), freeze-dried cod muscle meal (CMM), spray-dried krill hydrolysate (KH),reference fish meal (FM) and reference fish meal stick water (SW) composition. Values are expressed in g $kg^{-1}$ sample in dry matter unless otherwise specified

| | FM | SW | SM | CMM | KH |
|---|---|---|---|---|---|
| Raw material composition in DM | | | | | |
| Crude protein | 777 | 242 | 681 | 956 | 700 |
| Ash | 118 | 75 | 160 | 59 | 200 |
| Crude Lipid | 120 | 91 | 142 | 37 | 14 |
| Water-soluble protein | 207 | | 465 | 178 | |
| True protein digestibility (mink) | 947 | | 891 | 984 | |
| P | 20 | | | | 21 |
| Zn, mg/kg | 82.1 | | | | |
| Salt | 35 | 42 | | | |
| Ethoxyquin, mg/kg | 23 | | | | |
| Putrecine | <0.10 | | | | |
| Cadaverine | <0.10 | | | | |
| Histamine | <0.10 | | | | |
| Total volatile N, mg/100 g | 43.8 | | | | |
| Trimethylamine-N, mg/100 g | 11 | | | | |
| Trimethylamine oxide-N, mg/100 g | 173.1 | | | | |
| Moisture (as used) | 87 | 282 | 48 | 50 | 50 |
| Total amino acid composition in DM | | | | | |
| Asparaginic acid | | | 57 | 89 | |
| Glutaminic acid | | | 82 | 141 | |
| Hydroxyproline | | | 0 | 0 | |
| Serine | | | 27 | 41 | |
| Glycine | | | 49 | 40 | |
| Histidine | | | 15 | 20 | |
| Arginine | | | 51 | 59 | |
| Threonine | | | 23 | 40 | |
| Alanine | | | 37 | 57 | |
| Proline | | | 40 | 29 | |
| Tyrosine | | | 22 | 34 | |
| Valine | | | 29 | 47 | |
| Methionine | | | 17 | 32 | |
| Isoleucine | | | 27 | 44 | |
| Leucine | | | 42 | 74 | |
| Phenylalanine | | | 25 | 41 | |
| Lysine | | | 46 | 89 | |
| Cystein | | | 26 | 12 | |
| Tryptophane | | | 6 | 11 | |
| Total | | | 484 | 670 | |
| Free amino acid composition in DM | | | | | |
| Creatinine | | | 0.0 | 1.2 | |
| Asparaginic acid | | | 0.3 | 0.1 | |
| Glutaminic acid | | | 3.4 | 2.2 | |
| Hydroxyproline | | | 0.1 | 0.0 | |
| Serine | | | 1.1 | 0.3 | |
| Asparagine | | | 0.5 | 0.0 | |
| Glycine | | | 24.8 | 1.0 | |
| Glutamine | | | 5.0 | 0.3 | |
| 3-amino-propionic acid | | | 0.2 | 0.5 | |
| Taurine | | | 10.5 | 6.9 | |
| Histidine | | | 0.4 | 0.0 | |
| 4-amino-butanic acid | | | 0.1 | 0.0 | |
| Citrullin | | | 0.0 | 0.0 | |
| Threonine | | | 0.6 | 0.2 | |
| Alanine | | | 4.7 | 1.0 | |
| Carnosine | | | 0.9 | 0.1 | |
| Arginine | | | 11.9 | 0.2 | |
| Proline | | | 24.1 | 0.3 | |
| Anserine | | | 0.0 | 7.3 | |
| Tyrosine | | | 1.3 | 0.1 | |
| Valine | | | 1.7 | 0.1 | |
| Methionine | | | 1.0 | 0.1 | |
| Cystine | | | 0.0 | 0.0 | |
| Isoleucine | | | 3.6 | 0.1 | |
| Leucine | | | 2.4 | 0.2 | |
| Phenylalanine | | | 1.1 | 0.1 | |
| Tryptophane | | | 0.4 | 0.0 | |
| Ornithine | | | 0.8 | 0.0 | |
| Lysine | | | 2.0 | 0.6 | |
| Total | | | 10.3 | 2.3 | |

The experimental feeds were given to triplicate 50-L round polyethylene tanks with 90 Ballan wrasse larvae in each over a period of 2 months. The fish were produced at the Research Station of the Institute of Marine Research at Austevoll on rotifers and Artemia. At the beginning of the experiment, the fish were 40 days old posthatching and weighed 34.5 mg wet weight. 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. During the first weeks, it became apparent that fish did not accept D1 (FM) and started dying. After 1 month from the beginning of the experiment, all fish from this treatment were counted, weighed and removed. A sample of fish in the other five dietary treatments was weighed, and the feeding trial continued for one more month. At the end of the weaning period, all fish in each tank were counted and weighed individually.

**Table 2** Weaning diets' formulation and chemical composition (in g $kg^{-1}$ unless otherwise specified)

| Feed processing | AG | CPW | AG | AG | AG | AG |
|---|---|---|---|---|---|---|
| Feed name | FM | CMM + SM | CMM + SM | FM + SM | FM + KH | CMM + KH |
| Diet number | D1 | D2 | D3 | D4 | D5 | D6 |
| Herring meal[1] | | | | 589.4 | 716.9 | 107.0 |
| ECO LT fish meal[2] | 755.7 | | | | | |
| Herring stick water[3] | 161.7 | | 120.0 | 100.0 | 100.0 | 100.0 |
| Cod muscle meal-FD[4] | | 613.5 | 468.9 | | | 497.6 |
| Shrimp meal - FD[5] | | 306.7 | 234.5 | 234.5 | | |
| Kill hydrolysate[6] | | | | | 102.5 | 102.5 |
| Möller's Cod liver oil[7] | | 61.4 | | | | |
| Fish oil[8] | | | 85.7 | 30.1 | 31.7 | 90.3 |
| Vit Min Mix (NRC)[9] | | 18.4 | | | | |
| Wheat[10] | | | 40.0 | | | 49.0 |
| Vitamin & Mineral mix[11] | 25.5 | | 25.5 | 25.5 | 25.5 | 25.5 |
| Monosodium phosphate[12] | 5.0 | | 10.0 | 10.0 | 10.0 | 10.0 |
| Soya lecithin[13] | 46 | | 9.3 | 4.4 | 7.3 | 12.0 |
| Stay C[14] | 3.7 | | 3.7 | 3.7 | 3.7 | 3.7 |
| Vit D3[15] | 1.9 | | 1.9 | 1.9 | 1.9 | 1.9 |
| Carophyll Pink (10%)[16] | 0.5 | | 0.5 | 0.5 | 0.5 | 0.5 |
| Dietary moisture (g $kg^{-1}$ as fed) | 77 | 391 | 73 | 77 | 85 | 87 |
| Analysed composition (in dry matter) | | | | | | |
| Crude Protein | 674 | 757 | 681 | 683 | 701 | 698 |
| Total lipid | 168 | 151 | 163 | 174 | 169 | 171 |
| Ash | 158 | 94 | 101 | 143 | 137 | 92 |
| Total water-soluble protein (g kg $protein^{-1}$) | 251 | 398 | 323 | 305 | 319 | 310 |
| Total P | 16 | 11 | 9 | 14 | 14 | 10 |
| Soluble P | 11 | 4 | 7 | 9 | 12 | 11 |
| Crude energy (KJ $g^{-1)}$ | 21.5 | 23.5 | 22.9 | 22.3 | 22.2 | 23.4 |
| Ethoxyquin (mg $kg^{-1}$) | 0 | 0 | <1 | 7 | 8 | 2 |
| Anisidin number (meq kg $lipid^{-1}$) | 29 | 3 | 16 | 40 | 32 | 6.1 |
| Peroxide number (meq kg $lipid^{-1}$) | 4.3 | 0 | 0.8 | <0.1 | 1.6 | <0.1 |

[1] Reference fishmeal produced by Nofima AS, Bergen, Norway.
[2] ECO LT fish meal provided by Norsildmel AS, Fyllingsdalen, Norway.
[3] Produced from fresh frozen herring by Nofima AS, Bergen, Norway.
[4] Produced from fresh frozen bone- and skin-less cod fillet by Nofima AS, Bergen, Norway; FD = freeze-dried.
[5] Produced from fresh frozen shrimps by Nofima AS, Bergen, Norway; FD = freeze-dried.
[6] Provided by Sopropeche (Boulogne sur mer, France).
[7] Provided by Axellus AS, Oslo, Norway.
[8] Provided by Norsildmel AS, Fyllingsdalen, Norway.
[9] Supplied by Normin, Hønefoss, Norway, and mixed at Nofima AS providing in the final diet 3000 IU Vit $D_3$,160 mg $kg^{-1}$ Vit E, 20 mg $kg^{-1}$ Vit $K_3$, 200 mg $kg^{-1}$ Vit C, 20 mg $kg^{-1}$ Vit $B_1$, 30 mg $kg^{-1}$ Vit $B_2$, 25 mg $kg^{-1}$ Vit $B_6$, 0.05 mg $kg^{-1}$ Vit $B_{12}$, 60 mg $kg^{-1}$ Vit $B_5$, 10 mg $kg^{-1}$ folic acid, 200 mg $kg^{-1}$ Vit $B_3$, 1 mg $kg^{-1}$ Vit $B_7$, 92.31 mg $kg^{-1}$ $MnSO_4 + H_20$, 3125 mg $kg^{-1}$ $MgHPO_4 + 3H_2O$, 182.4 mg $kg^{-1}$ $FeSO_4 + H_20$, 352.9 mg $kg^{-1}$ $ZnSO_4 + H_20$, 23.62 mg $kg^{-1}$ $CuSO_4 + H_20$, 1413 mg $kg^{-1}$ $K_2CO_3$, and 6.67 mg $kg^{-1}$ Se.
[10] Supplied by Tereos Syral, Europe (www.syral.com).
[11] Provided by Normin, Hønefoss, Norway.
[12] Provided by Agrokorn, Videbaek, Denmark.
[13] Provided by Normin, Hønefoss, Norway.
[14] Provided by DSM, Europe (www.dsm.com).
[15] Provided by Normin, Hønefoss, Norway.
[16] G.O. Johnsen AS, Oslo, Norway

### *Chemical analysis*

In the feed ingredients, feeds and faecal waste, crude protein (CP = 6.25 × *N*) was determined by the Kjeldahl method (ISO 5983-1979), and moisture (ISO 6496-1983) and ash (ISO 5984-1978) determined gravimetrically after drying for 4 h at 105 °C and after combustion for 16 h at 450 °C, respectively. Lipid levels in feed ingredients were determined by the Soxhlet method using petroleum benzene extraction (AOCS Ba-38). Lipid levels in diets were determined by the Bligh and Dyer extraction method (Bligh & Dyer 1959), followed by determination of the oxidation state of the oil by the analysis of peroxide number (AOCS

**Table 3** Weaning diets' total amino acid composition (g $kg^{-1}$ diet dry matter)

| Feed processing | AG | CPW | AG | AG | AG | AG |
|---|---|---|---|---|---|---|
| Feed name | FM | CMM + SM | CMM + SM | FM + SM | FM + KH | CMM + KH |
| Diet number | D1 | D2 | D3 | D4 | D5 | D6 |
| Asparaginic acid + Asparagine | 59 | 65 | 60 | 49 | 52 | 56 |
| Glutaminic acid + Glutamine | 88 | 95 | 89 | 74 | 79 | 84 |
| Hydroxyproline | 6 | 2 | 2 | 5 | 6 | 3 |
| Serine | 29 | 31 | 29 | 27 | 27 | 28 |
| Glycine | 44 | 44 | 41 | 47 | 45 | 39 |
| Histidine | 14 | 16 | 16 | 14 | 14 | 15 |
| Arginine | 41 | 46 | 43 | 47 | 48 | 43 |
| Threonine | 27 | 30 | 28 | 26 | 27 | 28 |
| Alanine | 42 | 43 | 39 | 39 | 42 | 39 |
| Proline | 29 | 27 | 27 | 30 | 29 | 25 |
| Tyrosine | 26 | 26 | 24 | 24 | 26 | 26 |
| Valine | 32 | 34 | 33 | 31 | 32 | 33 |
| Methionine | 20 | 22 | 21 | 19 | 19 | 21 |
| Isoleucine | 28 | 32 | 30 | 27 | 27 | 29 |
| Leucine | 49 | 53 | 50 | 46 | 47 | 50 |
| Phenylalanine | 25 | 28 | 27 | 24 | 23 | 26 |
| Lysine | 52 | 62 | 56 | 46 | 49 | 55 |
| Total amino acids | 612 | 656 | 614 | 576 | 593 | 602 |

Cd 8b-90) and anisidine number (AOCS cd 18-90). The diets were also analysed for their content in the chemical antioxidant ethoxyquin (AOAC 963.07) and crude energy (ISO 9831). For total amino acid profile determination, samples were hydrolysed in 6 M HCl for 22 h at 110 °C and analysed by HPLC using a fluorescence detection technique (Cohen & Michaud 1993). The total levels of tryptophan and cysteine in the diets were not determined. Free amino acids, taurine and anserine were analysed as described in Bidlingmeyer *et al.* (1987). The water-soluble fraction of the marine protein meals and 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. Total phosphorous (P) was determined by a spectrophotometric method (ISO 6491-1998). All analyses were performed in duplicate. If differences between parallels exceeded standardized values, new duplicate analyses were carried out according to accredited procedures.

### *Statistics*

One-way ANOVA and correlation statistical analyses were performed using Microsoft Excel and IBM SPSS statistics 21 (IBM Corp., Armonk, NY, USA). Data were checked for homogeneity of variances by the Levene test and, where necessary, transformed *via* arcsine function. When significant differences between groups were identified, multiple comparisons among means were made using the Tukey test. Differences were considered significant at the level of $P < 0.05$.

## Results

Ballan wrasse larvae weaned on the experimental moist diet, and all dry agglomerated diets in the present study performed well, except from the fish fed D1 (FM). Most of the fish in these tanks (D1) did not appear to feed and died, whereas the surviving fish had similar growth rate compared with the fish fed D2. By the end of the 2nd month of the weaning trial, fish fed D2 had similar body weight but lower survival rates, although not significantly, compared with the fish fed D3. This may have been an effect of the lack of dietary phospholipids in D2 and the comparatively higher levels of free fatty acids in D2 compared with D3, which are shown to have detrimental effect in larvae performance (Sale *et al.* 2013). At the end of the first trial month, fish fed diets 3–6 appeared larger, although not statistically significantly, compared with those fed the diets D1 and D2. The fish increased their body weight 4–5 times during the last month of the feeding trial (Table 6). At the end of the trial, fish fed the moist- and dry-agglomerated diets D2 and D3, respectively (CMM + SM), were significantly smaller compared with the fish fed the FM + SM and FM + KH diets D4 and D5 and smaller but not significantly compared to those fed D6

**Table 4** Weaning diets' free amino acid composition (g $kg^{-1}$ diet dry matter)

| Feed processing | AG | CPW | AG | AG | AG | AG |
|---|---|---|---|---|---|---|
| Feed name | FM | CMM + SM | CMM + SM | FM + SM | FM + KH | CMM + KH |
| Diet number | D1 | D2 | D3 | D4 | D5 | D6 |
| Asparaginic acid | 0.17 | 0.76 | 0.39 | 0.30 | 0.36 | 0.78 |
| Glutaminic acid | 1.39 | 5.71 | 2.11 | 1.57 | 1.91 | 2.85 |
| Hydroxyproline | 0.01 | 0.43 | 0.10 | 0.01 | 0.01 | 0.05 |
| Serine | 0.22 | 3.53 | 0.71 | 0.85 | 0.81 | 0.81 |
| Asparagine | 0.03 | 0.00 | nd | 0.21 | 0.59 | 0.15 |
| Glycine | 1.11 | 14.66 | 7.68 | 8.19 | 4.64 | 3.91 |
| Glutamine | 0.16 | 6.14 | 2.72 | 2.62 | 0.50 | 3.79 |
| 3-amino-propionic acid | 0.39 | 0.80 | 1.50 | 0.10 | 1.98 | 0.47 |
| Taurine | 5.44 | 8.03 | 7.04 | 7.52 | 9.20 | 7.77 |
| Histidine | 0.11 | 0.51 | 0.29 | 0.13 | nd | nd |
| 4-amino-butanic acid | 0.00 | 0.00 | nd | 0.09 | 0.03 | 6.10 |
| Citrulline | 0.26 | 0.97 | 0.11 | 0.11 | 0.09 | 0.08 |
| Threonine | 0.23 | 3.07 | 0.54 | 0.51 | 1.11 | 1.18 |
| Alanine | 2.89 | 21.30 | 4.88 | 4.73 | 5.53 | 5.53 |
| Carnosine | 0.04 | 1.38 | 0.27 | 0.41 | 0.62 | 0.35 |
| Arginine | 0.38 | 5.89 | 3.17 | 4.11 | 4.60 | 4.21 |
| Proline | 0.53 | 6.93 | 3.99 | 3.78 | 1.27 | 1.47 |
| Anserine | 2.06 | 3.33 | 4.09 | 0.40 | 0.14 | 4.60 |
| Creatinine | 3.47 | 0.16 | 0.64 | 3.52 | 4.01 | 1.46 |
| Tyrosine | 0.22 | 1.13 | 0.42 | 0.47 | 1.26 | 1.14 |
| Valine | 0.66 | 3.79 | 0.67 | 0.81 | 1.50 | 1.31 |
| Methionine | 0.03 | 2.20 | 0.38 | 0.29 | 1.10 | 1.07 |
| Isoleucine | 0.36 | 3.07 | 0.56 | 0.68 | 1.51 | 1.42 |
| Leucine | 0.79 | 4.32 | 0.93 | 1.06 | 3.31 | 3.09 |
| Phenylalanine | 0.34 | 2.07 | 0.44 | 0.49 | 1.60 | 1.45 |
| Tryptophane | 0.07 | 0.43 | 0.12 | 0.11 | 0.16 | 0.18 |
| Ornithine | 0.14 | 1.58 | 0.27 | 0.35 | 0.23 | 0.18 |
| Lysine | 0.73 | 6.95 | 2.31 | 2.02 | 3.11 | 3.23 |
| Total free amino acids | 22.21 | 109.15 | 46.32 | 45.43 | 51.20 | 58.62 |

nd = not detected.

(CMM + KH). However, the mortality data after 1 and 2 months had the opposite trend to the growth performance data, with a higher mortality in fish fed the FM diets either added SM or KH compared with the CMM diets. The difference was statistically significant only between fish fed D5 (FM + KH) with the highest mortality and those fed D3 (CMM + SM) with the lowest. Larvae mortality during the first weaning month (M1) accounted for about two-thirds of total weaning mortality (M2).

During the first month of weaning, none of the analysed dietary parameters correlated significantly with fish growth performance. Nevertheless, fish mortality correlated positively with dietary oil oxidation and negatively with total dietary soluble protein and free amino acids (Table 7). During the second month, the mortality rates were lower and similar among the treatments. Final fish body weight showed significantly positive correlation with dietary DHA/EPA ratio, total P and cholesterol, and negative correlation with total dietary fatty acids.

## Discussion

In a preliminary experiment, we observed a significant effect of protein quality in Ballan wrasse larvae weaning performance as well as indications that dry agglomerated feeds perform better than otherwise processed feeds (extruded, moist) (Kousoulaki *et al.* 2011). In the present trial, the moist diet D2 performed equally well compared with the dry agglomerated diet D3 in terms of growth but had higher mortality rates, although not statistically significant. The present trial confirmed the industrial experience, showing that most Ballan wrasse larvae do not accept fish meal-based diets, when no shrimp or krill is present. Even when krill or shrimp was present (D4 and D5), fish given diets containing fish meal had higher mortality rates compared with those without. This effect is intriguing and may be attributed to potential lack of specific appetite-stimulating compounds for Ballan wrasse or the presence of toxic or repellent substances in the used fish meals.

**Table 5** Weaning diets' lipid class and fatty acid composition (g $kg^{-1}$ extracted fat)

| Feed processing | AG | CPW | AG | AG | AG | AG |
|---|---|---|---|---|---|---|
| Feed name | FM | CMM + SM | CMM + SM | FM + SM | FM + KH | CMM + KH |
| Diet number | D1 | D2 | D3 | D4 | D5 | D6 |
| Total saturated fatty acids | 163 | 139 | 171 | 165 | 169 | 179 |
| Total monosaturated fatty acids | 268 | 390 | 445 | 393 | 395 | 457 |
| Total PUFA (n-6) fatty acids | 106 | 27 | 40 | 25 | 26 | 40 |
| Total PUFA (n-3) fatty acids | 124 | 270 | 137 | 154 | 150 | 135 |
| Total PUFA fatty acids | 232 | 302 | 180 | 182 | 179 | 178 |
| Sum fatty acids | 663 | 831 | 796 | 740 | 743 | 814 |
| 14:0 | 26 | 27 | 51 | 46 | 49 | 57 |
| 16:0 | 116 | 91 | 104 | 105 | 108 | 108 |
| 18:0 | 19 | 21 | 14 | 12 | 11 | 13 |
| 20:0 | 1 | nd | 1 | 1 | 1 | 1 |
| 22:0 | 1 | nd | 1 | 1 | nd | nd |
| 16:1 n-7 | 24 | 55 | 31 | 30 | 28 | 30 |
| 18:1 (n-9) + (n-7) + (n-5) | 116 | 172 | 122 | 118 | 115 | 121 |
| 20:1 (n-9) + (n-7) | 53 | 97 | 110 | 94 | 96 | 114 |
| 22:1 (n-11) + (n-9) + (n-7) | 70 | 62 | 175 | 144 | 149 | 185 |
| 24:1 n-9 | 5 | 4 | 7 | 7 | 7 | 7 |
| 16:2 n-4 | 1 | 3 | 2 | 2 | 2 | 2 |
| 16:3 n-4 | 1 | 2 | 1 | 1 | 1 | 1 |
| 18:2 n-6 | 102 | 16 | 32 | 18 | 22 | 34 |
| 18:3 n-6 | 1 | 1 | 1 | 1 | nd | 1 |
| 20:2 n-6 | 1 | 2 | 2 | 1 | 1 | 1 |
| 20:3 n-6 | nd | 1 | 1 | 1 | 1 | 1 |
| 20:4 n-6 (ARA) | 2 | 7 | 4 | 4 | 2 | 3 |
| 18:3 n-3 | 16 | 6 | 8 | 6 | 7 | 8 |
| 18:4 n-3 | 8 | 17 | 10 | 9 | 9 | 11 |
| 20:3 n-3 | 1 | 1 | nd | 1 | nd | nd |
| 20:4 n-3 | 2 | 5 | 3 | 3 | 3 | 3 |
| 20:5 n-3 (EPA) | 34 | 92 | 45 | 48 | 44 | 41 |
| 21:5 n-3 | 1 | 3 | 1 | 1 | 1 | 1 |
| 22:5 n-3 | 4 | 11 | 6 | 5 | 5 | 6 |
| 22:6 n-3 (DHA) | 58 | 135 | 64 | 81 | 81 | 65 |
| Triglycerides | 78 | 310 | 380 | 46 | 530 | 650 |
| Diglycerides | 40 | 20 | 8 | 28 | nd | 19 |
| Monoglycerides | 23 | nd | nd | 25 | nd | nd |
| Free fatty acids | 380 | 450 | 36 | 390 | 13 | 100 |
| Cholesterol | 33 | 25 | 16 | 46 | 39 | 25 |
| Cholesterol esters | nd | nd | nd | 6 | nd | nd |
| Phosphatidylethanolamine | 35 | nd | 19 | 22 | 26 | 29 |
| Phosphatidilcholine | 80 | nd | 300 | 110 | 130 | nd |
| Lyso-phosphatidylcholine | nd | nd | 28 | 18 | 10 | 14 |
| Total polar lipids | 123 | 7 | 345 | 146 | 163 | 72 |
| Total neutral lipids | 559 | 805 | 443 | 538 | 588 | 798 |
| Total sum lipids | 681 | 812 | 788 | 684 | 751 | 870 |
| DHA/EPA | 1.7 | 1.5 | 1.42 | 1.69 | 1.84 | 1.59 |

nd = not detected.

In the first-feeding larval stages of fish, fish protein hydrolysates and other marine water-soluble compounds are commonly used as feeding attractants (Berge & Storebakken 1996; Yilmaz 2005). In the present study, the presence of soluble protein and thus free amino acids of particularly crustacean origin appears crucial in terms of feed intake and survival in first feeding Ballan wrasse larvae with formulated diets. Certain free amino acids are known to act as attractants stimulating fish appetite and facilitating detection of pray (Hara 1982; Caprio 1984). The appetite-stimulating free amino acids are not the same for all fish species (Li *et al.* 2009), and they are not yet identified in Ballan wrasse. Free amino acids and metabolites found at high levels in krill hydrolysate such as taurine, creatinine, glycine, glutaminic acid, lysine, arginine, leucine, alanine and proline are also abundant in fish meal water solubles.

**Table 6** Ballan wrasse weaning performance (final body weight) fed weaning diets of different protein quality (raw material source) after 1 month (1) and 2 months (2) feeding (values are mean ± SD, $n = 3$)

| Feed processing | AG | CPW | AG | AG | AG | AG | |
|---|---|---|---|---|---|---|---|
| Feed name | FM | CMM + SM | CMM + SM | FM + SM | FM + KH | CMM + KH | |
| Diet number | D1 | D2 | D3 | D4 | D5 | D6 | One-way ANOVA (P) |
| BW1[1] (g) | 0.11 ± 0.02 | 0.11 ± 0.02 | 0.14 ± 0.03 | 0.15 ± 0.02 | 0.16 ± 0.04 | 0.17 ± 0.03 | ≤0.1 |
| BW2[2] (g) | | 0.59 ± 0.02$^{a}$ | 0.57 ± 0.06$^{a}$ | 0.70 ± 0.04$^{bc}$ | 0.74 ± 0.03$^{c}$ | 0.62 ± 0.03$^{ab}$ | ≤0.01 |
| M1[3] (%) | 51 ± 6$^{c}$ | 24 ± 8$^{ab}$ | 17 ± 12$^{a}$ | 39 ± 7$^{bc}$ | 39 ± 1$^{bc}$ | 22 ± 1$^{ab}$ | ≤0.001 |
| M2[4] (%) | | 35 ± 9$^{ab}$ | 23 ± 15$^{a}$ | 47 ± 8$^{ab}$ | 53 ± 0$^{b}$ | 33 ± 12$^{ab}$ | ≤0.05 |

Values with different superscript letter are significantly different by Tukey test ($P \leq 0.05$).
[1] Fish body weight after 1 month.
[2] Fish body weight after 2 months.
[3] Total mortality after 1 month.
[4] Total mortality after 2 months.

However, there are some free amino acids, such as 3-amino-propionic acid, proline, tyrosine, isoleucine, glycine, carnosine, serine, asparagine, valine, leucine, phenylalanine, 4-amino-butanic acid, arginine, ornithin, alanine, tryptophane and aspartic acid, found at much higher relative amounts in krill hydrolysate compared with fish meal (Kousoulaki *et al.* 2013). Some salmonid fish species possess specific amino acid receptors for some of those and can thus detect them initiating thereby appetite and feeding (Hara & Marui 1984; Yamashita *et al.* 2006). The lower mortality rate in Ballan wrasse larvae fed diets with crustaceans could thus be related to the presence of these free amino acids, which may have stimulated increased feed consumption.

Potential reduced feed intake and thus higher mortality rates of the fish fed the fish meal-based diets could also be related to the chemical content of antioxidants, and a somewhat higher lipid oxidation level in the present fish meal ingredient compared with the processed ingredients from cod, shrimp and krill (Laohabanjong *et al.* 2009), although it did not seem to affect Ballan wrasse larvae body growth rates during weaning. Several studies report negative effects of dietary oxidized lipids on fish performance (Tocher *et al.* 2003; Zhong *et al.* 2007; Gao *et al.* 2012a,b). Especially during early juvenile stages, it is considered important to avoid lipid oxidation, which may cause disease and subsequent mortalities of marine fish (Tocher *et al.* 2002).

**Table 7** Significant correlations between Ballan wrasse weaning performance and specific dietary chemical characteristics

| | Dietary component | Linear corellation | $r^2$ | $n$ | $P$ |
|---|---|---|---|---|---|
| 1 month | Totox[1] + EQ[2] versus mortality | + | 0.5 | 18 | <0.05 |
| 1 month | Free amino acids versus mortality | − | 0.41 | 18 | <0.05 |
| 1 month | Soluble protein versus mortality | − | 0.34 | 18 | <0.05 |
| 2 months | DHA/EPA versus growth | + | 0.79 | 15 | <0.01 |
| 2 months | P versus growth | + | 0.69 | 15 | <0.01 |
| 2 months | Cholesterol versus growth | + | 0.66 | 15 | <0.01 |
| 2 months | Fatty acids versus growth | − | 0.60 | 15 | <0.05 |

[1] Total lipid oxidation = anisidine + peroxide number.
[2] Ethoxyquine.

Nevertheless, Ballan wrasse larvae body growth rates in the present study was equally good or better among the surviving fish fed the diets containing fish meal (D4 and D5) compared with those fed the cod muscle meal-based diets (D2, D3 and D6). The higher growth rates of the fish fed the FM-based diets may be related to the lower biomass density in the respective rearing tanks by the end of the first month of weaning or to potential growth-promoting compounds present in fish meal (Kousoulaki *et al.* 2009) and absent or present at lower amounts in the CMM-based diets. Although the present diets were not balanced for total P as it was considered adequate, a lower level of total and soluble P occurred in the CMM-based diets compared with the respective FM-based diets, which may have been limiting for optimal larvae growth. The dietary P requirement of Ballan wrasse at different life stages is not yet known. Both P and amino acids are crucial micronutrients for the development of organisms and in soluble or free form, respectively, can be more readily available for larval uptake and utilization (Hamre 2006; Hamre *et al.* 2013a,b). Unlike fish muscle meal, whole marine raw materials such as fish meal, krill meal and shrimp meal contain a variety of water-soluble nitrogenous bioactive compounds with feed intake and growth-stimulating properties such as

nucleotides, trimethylamine oxide (TMAO), creatine and organic acids a.o. (Carr *et al*. 1996; Wu & Bechtel 2012). Although all dry diets were added SW and the total dietary soluble protein was formulated to be similar among the experimental treatments and did not correlate with larvae weaning performance, differences in compositions of micronutrients and/or bioactive compounds that were not analysed may have been decisive in creating the observed differences in larvae performance.

As such, the importance of essential highly unsaturated fatty acids in larvae nutrition is widely recognized, particularly, both docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are required for growth, normal larval development, survival and stress resistance (Izquierdo *et al*. 1989a,b; Mourente *et al*. 1993; Rodriguez *et al*. 1993; Rodriguez 1994; Salhi *et al*. 1994; Izquierdo 1996). Optimal DHA and EPA ratio is believed to be critical for larvae performance. In our study, there was a significant linear correlation between final body weight (growth) and the DHA/EPA ratio in the diet. The combined effect of the high amounts of available phosphorous, DHA/EPA ratio, free amino acids and probably also other bioactive compounds present in whole fish raw materials and krill may be the reason for the better Ballan wrasse weaning growth performance in treatments D4 and D5 and the somehow improved performance of the fish in treatment D6 compared with D3.

In the present study, Ballan wrasse larvae growth correlated negatively also to the total amount of fatty acids (as a% of total extracted fat) and positively to the dietary cholesterol levels. Cholesterol is known to be indispensable for good performance of shrimps (Teshima *et al*. 1997; Forster *et al*. 2010). Cholesterol uptake may facilitate the transport and utilization of fatty acids as well as phospholipids may do (Tocher 2003). Lower fatty acid levels indicate higher levels of other fat-soluble compounds that may have various metabolic functions and could have been beneficial for successful Ballan wrasse larvae weaning.

In conclusion, Ballan wrasse larvae weaning success appears to be largely dependent on the presence in the weaning diets of specific raw materials, probably related to crustaceans, such as shrimp or krill. In the absence of such raw materials, larvae apparently feed very little and eventually die. Fish meal or cod muscle meal combined with either shrimp or krill hydrolysate appear to be good raw material combinations for successful Ballan wrasse weaning diets. Nevertheless, higher weaning mortality rates may be expected, mainly during the first month of feeding with formulated diets, when fish meal is present in the diet. Lipid oxidation metabolites and high dietary neutral lipid levels appear to have negative effect, whereas dietary free amino acids, DHA/EPA ratio, phospholipids, phosphorus and cholesterol appear to have positive effect in the weaning success of Ballan wrasse larvae.

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