Research and development to optimise hatchery production of ballan wrasse (*Labrus bergylta*): bacterial control and nutritional aspects

ANTONIOS EMM. CHALARIS

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University of Stirling, Scotland

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Declaration

This thesis has been composed in its entirely by the candidate. Except where specifically acknowledged the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of the candidate: ______________________________

(Antonios Chalaris)

Signature of the supervisor: ______________________________

(Prof. Hervé Migaud)

Date: ______________________________

Abstract

Farmed ballan wrasse (*Labrus bergylta*) is recognised an efficient biological control of sea lice (*Lepeophtheirus salmonis*) infections in farmed Atlantic salmon (*Salmo salar*). Wild caught harvesting is putting increased pressure on wild stocks to meet growing demand, therefore, the production of disease free, farmed populations is a priority. Two main bottlenecks to be addressed are low larvae survival rates and slow juvenile growth during the hatchery phase. A first study compared egg disinfection protocols using bronopol as an alternative to the commercial use of formalin. Bronopol concentration of 100ppm applied for 240min on the first day of egg incubation followed by daily treatment with 25ppm for 30min is recommended for disinfection to control bacterial growth without affecting egg survival and hatching. A follow up study investigated the need for bacterial control at early larvae stages. Results showed positive effects of synergetic use of probiotics and bronopol on survival rate and microbial analyses identified opportunistic bacteria in the rearing environment and fish. The work then compared commercially available diets developed for weaning and on growing of other marine fish species. Benchmarking experiments confirmed the importance of developing ballan wrasse tailored diets based on specific raw materials, and provided a stepping stone to rationalise growth potential, feed intake, digestive and liver function, against which to compare in future studies. Clear differences in survival and growth were observed during weaning with improved survival in larvae weaned onto a diet including shrimp and cod fillet meal. During on-growing, results indicated improved FCR and growth compared to previous studies, potentially reducing time required to reach deployment size from 20–24 month to 16–19 month period. Overall, this study provides new knowledge that can be directly applied to help tackle major bottlenecks in commercial exploitation of farmed ballan wrasse.

Keywords: Cleaner fish, egg disinfection, larviculture, diets, *Bacillus*, bronopol, digestive functions.

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List of abbreviations

Below is the list of the most commonly used abbreviations in the text. Other abbreviated terms are explained in the text.

AA	Amino Acid(s)
ADC	Apparent Digestibility Coefficient
ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
AP	Alkaline phosphatase
ARA	Arachidonic acid (20:4n-6)
AST	Alanine transaminase
BBM	Brush border membrane
BW	Body (wet) weight
CFU	Colony forming units
DD	Degree days
DHA	Docosahexaenoic acid (22:6n-3)
DM	Dry matter
DPH	Day post hatch
DW	Dry weight
EAA	Essential Amino Acid(s)
ED	Eye diameter
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid(s)
FAME	fatty acid methyl esters

FCR	Feed Conversion Ratio
GSI	Gonadosomatic index
HSI	Hepatosomatic Index
HUFA	Highly Unsaturated Fatty Acid(s)
K	Foulton’s Condition Factor
LAP	Leucine aminopeptidase
Leu-ala	Leucine–alanine peptidase
LOQ	Limit of quantification
^{o}C	Degrees centigrade
PUFA	Poly-Unsaturated Fatty Acid(s)
SD	Standard deviation of the mean
SGR	Specific Growth Rate
SL	Standard length
SL	Standard length
TGC	Thermal Growth Coefficient
TL	Total length
VSI	Viscerosomatic Index

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Chapter 1: General introduction

1.1 Biology and ecology of ballan wrasse

Ballan wrasse belongs to the Actinopterygii class, Perciformes order and Labridae family (Luna, 2014). It is the largest north European Labridae species and it can reach up to 60cm long, a weight of 4 kg and 29 year old (Dipper et al., 1977), although lengths of 30-50 cm are more common (Sayer & Treasurer, 1996). This predominately demersal species inhabits the stony and rocky shores along the Eastern Atlantic from Morocco to Norway (Quignard & Pras, 1986) (Fig. 1.1).

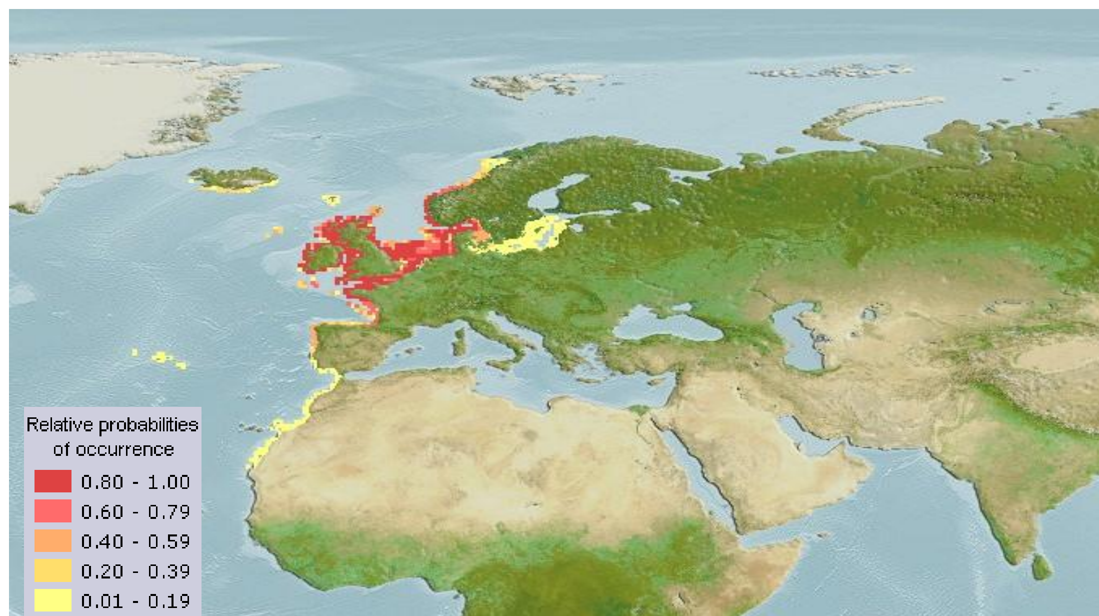

Figure 1.1: Natural range of ballan wrasse (source: www.aquamaps.org).

Ballan wrasse growth and feeding intensity has been clearly associated with prevailing seasonal temperature (Darwall et al., 1992). Additionally, ballan wrasse feeding activity shows a seasonal variation with a peak in feed intensity around August, which coincides with raised water temperatures, in excess of 14 ºC and follows the end of the spawning season (Dipper et al., 1977). The same authors reported, based on an extensive sampling of wild fish, that ballan wrasse is a stomachless, slow growing species with 0.62 mean intestine length to fish length ratio (Fig. 1.2), classifying them as omnivorous, towards

carnivorous, species, with nutrient absorption relying mainly on the intestine and in alkaline conditions (Kjørsvik et al., 2014). In general, stomachless fish species digestion of proteins, peptides, carbohydrates and lipids occur through digestive enzymes excreted from pancreas and intestinal mucosa in an alkaline pH (Yonge & Russell, 1972). Herbivorous species are reported to have intestine lengths longer than their body length in contrary with the carnivores with intestine lengths shorter than their body length. The only exception to this pattern is species belong in the order of Beloniformes, where despite their short intestine and stomachless tracts, appear to cover a very wide dietary spectrum (Day et al., 2011). Analysing stomachs contents, ballan wrasse feeding habits vary and "tend towards the carnivorous side of an omnivorous diet" with a clear preference to molluscs (mainly), decapods and isopods (Dipper et al., 1977). Dipper et al. (1977) also highlights the fact that feeding habits changes according to food availability and migration patterns. In particular, young fish (0 – 10 cm) naturally mainly feed on isopods and amphipods (found on the intertidal pools) and older fish expand the dietary variety due to their improved digestive abilities. A study by Figueiredo et al. (2005) showed that ballan wrasse natural diet includes primarily decapods, echinoderms and gastropods. In all cases, the natural feed selection could be summarised as being relatively low in lipids and easily digestible (Hamre et al., 2013a).

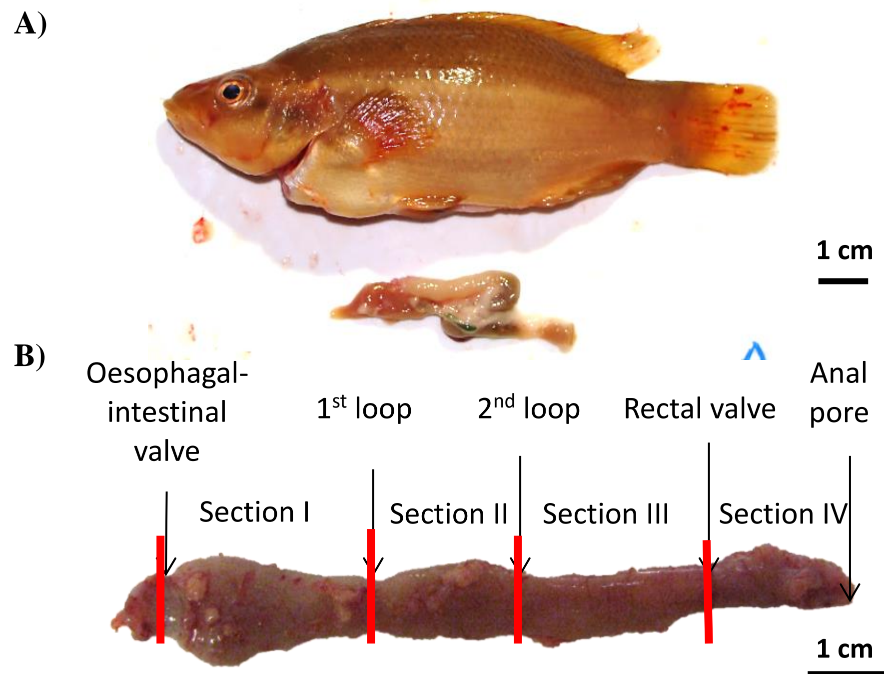

Figure 1.2: (A) Image showing "Z" shaped farmed ballan wrasse juvenile intestine structure; B) Ballan wrasse stretched intestine divided in to four sections: proximal (I), middle (II), distal (III) and rectal (IV). Scale bar = 1cm.

Reproduction in ballan wrasse is seasonal (Elofsson et al., 1999, Müncaster et al., 2008; Grant et al., 2016a), which is a common strategy among temperate fish. Seasonality in temperate fish plays a key role for the success of reproduction and seasonal cues regulate most physiological events, such as synchronisation of spawning (Migaud et al., 2010). It should be also taken into consideration, that synchronisation can be affected significantly by a range of environmental (light, temperature, salinity), nutritional (feed quality, quantity) and social (sex ratio, size structure and dominance/hierarchy) factors (Migaud et al., 2013) but as yet, the significance of such parameters on the spawning of ballan wrasse

remains unknown. In general, captive ballan wrasse broodstock appear to reliably produce naturally fertilised eggs in captivity without showing any notable reproductive malfunctions (Grant et al., 2016a). It naturally attains sexual maturation somewhere between three to nine years of age depending on location within the natural range (Dipper & Pullin, 1979; Villegas – Ríos et al., 2013b), releasing the eggs on gravel or rocky substrate (Darwall et al., 1992).

Ballan wrasse is a protogynous (Elofsson et al., 1999; Müncaster et al., 2008) monandric (lacking primary males), hermaphrodite (Dipper & Pullin, 1979; Elofsson et al., 1999). While showing a great diversity in external colour and pattern phenotypes they exhibit no distinct sexual dimorphism or dichromatism (Dipper & Pullin, 1979, Villegas-Ríos et al., 2013b) and as such it has been suggested that the expressed colour variance could be more clearly associated with the individuals chosen habitat background colour (Wheeler, 1969). As such, there remains limited validated methods to reliably identify the genders. To date, very little is known about protogynous sex change in temperate and high latitude species. It remains unclear which parameters triggers the sex change in ballan wrasse, but is believed to rely on social interactions, in particular when the male dies the dominant female substitutes the male (Sjölander et al., 1972; Dipper et al., 1977; Dipper & Pullin 1979; Hilldén 1984). The natural sex ratio has been reported to be 0.08 males to females in the wild (Dipper et al., 1977). Being protogynous hermaphrodites in the wild males tend to be bigger than the females (Dipper et al., 1977; Müncaster et al., 2013; Villegas-Ríos et al., 2013b; Leclercq et al., 2014a). Leclercq et al. (2014a) states that morphometric assessment is the most effective method of sexing ballan wrasse with 91% accuracy. In particular, female ballan wrasse appear to be smaller (body weight, length, height and condition factor) than the males, showing fish over 38 cm long and 850 g in weight is most likely to

be male. Thus, discriminant morphometric assessment can act as a useful tool for sex determination of ballan wrasse in the farm environment.

The females are described as being group-synchronous multiple batch spawners, based on both histological evidence (Müncaster et al., 2010; Villegas-Rios et al., 2013b) as well as direct observation of spawning productivity over a spawning season and subsequent assignment of parentage using microsatellite markers (Grant et al., 2016a). Spawning generally occurs between April and June, when both day light and temperature are increasing, and peak in May (Müncaster et al., 2008, 2010). During this season, the sex steroid levels are elevated in mature fish (Müncaster et al., 2008) and the gonadosomatic index (I_G) increases significantly from 0.86 ± 0.05 to 11.66 ± 1.23 in mature females (Müncaster et al., 2010). During the spawning season itself Grant et al. (2016a) presented evidence of a clear spawning rhythm with 3-5 spawning periods inclusive of spawning windows lasting 1-9 days followed by interspawning intervals of 8-12 days. Villegas-Rios et al. (201) suggests that, batch fecundity of ballan wrasse could range between 11-135,000 eggs per female (not corrected for female size) which is comparable to the estimated egg number recorded per spawning event (25-74,000 eggs) reported by Grant et al. (2016a). However, accurate egg counting remains a challenge due to current egg collection methods which is discussed further below.

1.2 Use of cleaner fish in the salmon industry

The current worldwide production of farmed Atlantic salmon (*Salmo salar*) exceeds 2,000,000 tonnes annually (FAO 2014). The ectoparasitic copepod sea lice (*Lepeophtheirus salmonis*) continue to be a major health and economic threat for the farmed Atlantic salmon industry (Treasurer 1996; Costello, 2009; Aaen et al., 2015). The

average cost in affected countries is estimated to be at least 6 % of production value and in Scotland, it was suggested to be in the region of 7-10% (Costello, 2009). Various medicinal, physical and biological methods have been used over the years. Additional methods involving farm management areas synchronised with fallowing and treatments as well as strategic control measures at key time points have also been applied (The Scottish Government, 2017, http://www.gov.scot/Resource/0039/00399444.pdf). Medicinal treatments including the use of azamethiphos, dichlorvos and hydrogen peroxide baths as well as in-feed medicines such as emamectin benzoate (SLICE®; MSD Animal Health, UK), diflubenzuron or teflubenzuron (Rae, 2002), have been reported to have a negative environmental impact, and furthermore resistance in sea lice to these treatments can be developed which ultimately impacts on the public perceptions of salmon farming and the wider sector of aquaculture. Therefore, the focus is moving towards the validation of novel non medicinal treatments methods which includes submerged laser technology which detects and removes sea lice from salmon skin (developed by Stingray, Norway; en.stingray.no); raised water temperature baths (30 – 34 °C), (Thermolicer, developed by Steinsvik, Borway; www.steinsvik.no) and stock behavioural manipulations achieved by altering lighting and feeding systems in the salmon cages (Frenzl et al., 2014). Amongst all of the methods being developed, the production and application of cleaner fish is the closest to the market place and providing a natural biological control to the problem of sea lice.

The first observation of delousing activity from wrasse has been reported by Åsmund Bjordal (1988) and the use of wrasse as cleaner fish on salmon farms in Norway and Scotland has proven to be an effective sea lice treatment since the early 90’s (Costello, 1996). Five wrasse species are common in British water (Bron & Treasurer, 1992; Treasurer, 1994), but ballan wrasse (*Labrus bergylta*) is of particular interest to the salmon

industry as cleaner fish, due to its significant delousing efficiency (Sayer & Treasurer, 1996). Ballan wrasse, although not new in terms of being a biological control species in salmonid farming (Bjordal, 1988), is a relatively new species in the temperate aquaculture sector. Commercial interest has grown rapidly over recent years to reduce the fishing pressure on wild wrasse stocks (Skiftesvik et al., 2013; Leclercq et al., 2014b). In Norway only, the total number of wild and farmed cleaner fish used escalated from almost 2,5 M (valued for 9 M NOK) in 1998 up to 26,5 M in 2015 (valued for 370 M NOK) (Norwegian Directorate of Fisheries statistics, 2016). However, out of the 26.5 M used in 2015 only 15 M were farmed. The latest annual production figure of farmed cleaner fish in Norway showed 1.5 M ballan wrasse and 14 M lumpfish (*Cyclopterus lumpus*), an alternative cleaner fish species (Norwegian Directorate of Fisheries statistics, 2016). The farming of cleanerfish is commonly believed to be the preferred future supply route as part of a sustainable sea lice management plan, with the salmon industry targeting the exclusive use of farmed cleaner fish by 2020.

Imsland et al. (2014a) reported clear signs of grazing activity on salmon lice from lumpfish, providing a suitable cold-water cleanerfish alternative. With small size lumpfish (50g) showing higher delousing activity than larger ones (>350g) (Imsland et al., 2014b) and with way higher growth rates (6 months to deployment size of 20g) compared to ballan wrasse (20-24 months to deployment size of 40g), lumpfish farming provided a faster supply of cleanerfish. It has been shown that use of lumpfish can be effective, but has also been shown that are numerous disease challenges which are continually being discovered that need to be faced, such as *Tenacibaculum maritimum,* a worldwide pathogen (Småge et al., 2016). More recent anecdotal evidences from the industry in Norway and UK also show that lumpfish are very susceptible for bacterial diseases such as Atypical *Aeromonas*

salmonicida, *Vibrio* sp. and *Pasteurella* sp., resulting to high mortalities and also potentially acting as vector of disease for farmed salmon.

The effective farming of ballan wrasse has become a high priority to reduce the pressure on wild stocks and provide a reliable supply of disease free cleaner fish to the salmon industry. In addition, cases of pathogen transfer from wild caught wrasse to salmon have been reported, including potential emerge of new disease. This has been demonstrated by an outbreak of VHS (viral haemorrhagic septicaemia) in wrasse being introduced to salmon farms in Shetland (Murray, 2014). Farmed ballan wrasse have shown similar efficacy to wild counterparts in sea cages (Skiftesvik et al., 2013) and their high delousing activity was confirmed and quantified in tanks (Leclercq et al., 2014b), offering a more environmentally friendly, effective and potentially cost effective alternative method to the use of chemotherapeutants (Costello, 1993). The proposed current best practice would suggest that the salmon industry requires > 45g ballan wrasse (Fig. 1.1) to be effective for sea lice control while avoiding wrasse escapees. However, current hatchery experience would suggest that the time required to produce ballan wrasse from fertilised egg to this size can be as much as 20-24 months (David Patterson, Otter Ferry Seafish, pers. comm.) which needs to be reduced to make the industry viable. As farming of ballan wrasse is relatively new, there are clear gaps in knowledge in many aspects of the species biology and production science. The propagation and rearing of ballan wrasse has progressed in recent years, but as will be outlined below, there is limited theoretical and practical knowledge of hatchery performance. There are numerous perceived production bottlenecks that will require further investigation to develop adequate species specific protocols to make a step change in hatchery performance. Only once these are addressed will a viable farming industry be able to fulfil salmon industry requirements.

1.3 Current commercial practices at Otter Ferry Seafish Ltd.

1.3.1 Broodstock / Egg management

Establishing viable breeding populations is key for a successful hatchery production of year-round supply of high quality eggs. To achieve this, many actions need to be taken, starting with the better understanding of stock's reproductive physiology and spawning performance in captivity, so hatchery techniques can be adjusted to fish breeding strategies. At present, very little is known about ballan wrasse reproductive physiology and spawning activity in both natural and controlled hatchery environment.

Currently, ballan wrasse hatcheries are reliant on wild captured broodstock (Grant et al., 2016a). A number of hatcheries both in the UK and in Norway report holding captive reared fish as potential F1 broodstock but given the slow growth rate of the species no sexually mature farmed broodstock have been reported yet.

Ballan wrasse egg diameter is 950 ± 4 μm and is surrounded by a sticky gelatinous layer 120 ± 2 μm thick with a gap at the micropyle and lack of oil droplet (Grant et al., 2016a,b). In the wild, females will spawn the eggs onto a substrate e.g. exposed rock surface where they are fertilised and then guarded by the male (Sjölander et al., 1972). In a production context a removable artificial spawning substrate must be provided on to which the eggs are released (Grant et al., 2016a) (Fig. 1.3, 1.4). This egg collection method clearly differs from most other marine finfish hatcheries where either the freely spawned pelagic eggs are collected from surface overflows (e.g. Atlantic cod, *Gadus morhua*, Penney et al., 2006; Gilthead seabream *Sparus aurata*, Meseguer et al., 2008), or unfertilised gametes are manually stripped and fertilised artificially for species which do not spawn spontaneously in captivity (e.g. Atlantic halibut, *Hippoglossus hippoglossus*, Brown et al, 2006; seabass, *Dicentrarchus labrax,* Siddique et al., 2017). The spawning substrate preferred is short pile

carpet mats which are placed on the bottom of the broodstock tanks and following successful spawning the mats are removed and incubated in well aerated incubation tanks until eggs are ready to hatch at *circa* 72 degree days (dd) (Ottesen et al., 2012). In an attempt to allow ballan wrasse hatcheries to return to pelagic egg incubation, Grant et al., (2016b) explored the application of enzyme alcalase® as an effective egg degumming treatment. While the authors reported a treatment that did not compromise hatch rates in a small scale research context they failed to validate an effective methodology at a commercial scale and as such all hatcheries continue to incubate eggs on the spawning mats. The use of collection mats, in conjunction with the use of wild caught broodstock and high egg stocking density in the incubation systems require the validation of egg disinfection and overall management methods. The external surface of fish eggs can easily be colonized by bacteria, meaning that there can be simple transference from the broodstock to the incubated eggs and on to the newly hatched larvae (Overton et al., 2010). High egg stocking densities and cross contamination between egg batches can also contribute to the bacterial development (Overton et al., 2010) and therefore, validation of egg disinfection methods is required. At the beginning of this study, the commercial practice in ballan wrasse eggs at Otter Ferry Seafish consisted in treating them with formalin (150 ppm for 60 min) at day 0, followed by a daily static bath of bronopol (25 ppm for 30 min) until 72 degree days when hatching was physically induced by “scrapping” them off the egg collection carpets.

Figure 1.3: Typical set up of a ballan wrasse broodstock tank at Otter Ferry Seafish. About half of tank's total bottom (5.72 m^2) is cover with short pile mats (right side of the figure), where the females lay their eggs and fertilisation takes place. Artificial sea weed are placed on the other held of the tank (left side of the figure) providing a simulation of the natural habitat.

Figure 1.4: Ballan wrasse eggs released on egg collection mats and before their placement in the egg incubator until hatching. Each mat is covering 0.30 m^2 of broodstock tank bottom.

Development of reliable gamete quality determinants remains a problem in aquaculture, especially amongst new species. Different factors can have significant effect on gamete quality including broodstock nutrition, environment, stress and genetics. Therefore, there is little agreement regarding reliable quantitative methods for gamete quality assessment (Migaud et al., 2013). Ballan wrasse, being a new aquaculture species, is no exception to this. In most marine species, the two principal characteristics that are measured after egg collection are total egg volume (also separation of fertilised and unfertilised eggs) and egg diameter (Thorsen et al., 2003). However, in ballan wrasse, due to the current egg collection methods (natural spawning of demersal sticky eggs on egg collection mats, as previously described); egg quantification cannot be assessed volumetrically. Therefore, current commercial protocols at Otter Ferry Seafish in order to assess egg quality are based on egg fertilisation, survival (until hatching) and hatching rates. Classification of egg density on the collection mats could help egg quantification, but accuracy of the method due to its reliance on subjective assessment remains a commercial concern (Grant et al., 2016a). Lack of reliable egg quantification method in ballan wrasse hatcheries results into poor egg and hatched larvae survival estimations, which also affect the later larval rearing stages. While no accurate egg quantification method exists, it is clear that significant losses during incubation appear as the incubation system is completely novel. Therefore, validation of adequate egg disinfection protocols is considered as a priority.

1.3.2 Larval rearing

Limited information is currently available regarding the early development of ballan wrasse. D'Arcy et al. (2012) and Dunaevskaya (2010) presented the embryonic development and also the organ and tissues development respectively. The egg hatching time (50% of total number of fertilised eggs) was reported to be about 62 dd, with time to hatch being inversely related to ambient temperature (D'Arcy et al., 2012). The newly

hatched larvae are on average 3.64 ± 0.05 mm standard length (Dunaevskaya et al., 2010; D'Arcy *et al*., 2012). Dunaevskaya (2010) divided larval development into four stages according to the external morphological characteristics and source of food, at 12 ºC: (1) Yolk sac larva (0-9 dph), (2) Preflexion larva, (10-25 dph); (3) Flexion larva (26-33 dph); (4) Postflexion larva (34-49 dph).

Commercial hatchery production of marine juveniles still relies to a great extent on the supply of costly live prey, such as rotifers, copepods and *Artemia*. The transitional stage of changing from live to dry feed, known as weaning, is a crucial stage in the culture of most marine species. Ballan wrasse larval stages appear to be the most challenging period of hatchery production where the main bottlenecks appear to be associated with bacterial control and weaning performance. In the early years of production Gagnat (2012) reported up to 95 % mortalities early weaning. There tends to be a reliance on already available hatchery protocols and diets, however, it has become apparent that they have to be tailored to ballan wrasse specific needs (Kousoulaki et al., 2014a,b; Bogevik et al., 2015). Until it is shown otherwise, ballan wrasse are currently produced following a routine intensive culture method (Fig. 1.5), which is comparable to larval rearing protocols from other cold water marine species, such as Atlantic cod culture (Hamre et al. 2006). The larvae are offered rotifers (*Brachionus* sp.) (3 – 35 dph) then move onto brine shrimp (*Artemia* sp.) (23 – 58 dph), before the co-feeding started (40 dph) (Kousoulaki et al., 2014a). More recent studies, suggest that use of cultivated copepod (*Acartia tonsa*) nauplii have positive long term effects on growth and viability of ballan wrasse larvae (Øie et al., 2015). However, a major bottleneck in marine fish farming is during the transition from endogenous to exogenous feeding, with low survival rates linked to inadequate weaning diets and one of the main objectives in larval nutrition is the formulation of a compound diet that can substitute as early as possible the use of live preys in hatcheries (Watanabe &

Kiron, 1994). The development of species-specific weaning diets that satisfy physical, digestive and nutritional requirements of marine fish larvae is vital to improve larvae survival and general juvenile robustness in the early stages of production (Cahu & Infante, 2001).

The ontogeny of the digestive tract of marine fish larvae has been well documented in several species (see review by Zambonino Infante et al., 2008). Person-Le Ruyet (1989) described the major changes in the digestive tract before the on-set of exogenous feeding in marine fish larvae. At hatching, it is in a very premature stage (a straight tube) connecting the closed mouth with the anus. No morphological differences take place until the full yolk sac absorption when the intestine differentiates to buccopharynx, foregut, midgut and hindgut. Ballan wrasse shows similar basic mechanisms of development to other teleosts, with appearance of mucous cells in oesophagus, intestine folds and intestinal brush border development after exogenous feeding initiation (Dunaevskaya, 2010). However, differences such as lack of stomach and pyloric caeca, together with some interspecific variability in the timing at which different ontogenic events becames apparent in ballan wrasse. Therefore, there is a need for further investigation on larvae readiness for dry feed initiation and determination of the optimum weaning window.

This co-feeding period has been significantly reduced due to the larviculture advantages obtained the last years, with attempts of early weaning giving variable results. Fletcher et al. (2007) examined *Artemia* replacement with two micro-diets in Atlantic cod, testing 50 % and 100 % substitution between 25 dph (end of rotifer supply) and 70 dph (all larvae were weaned). Despite that the best growth and survival were obtained in treatments containing *Artemia*, co-feeding with micro-diets can generate comparable growth and survival rates, showing the potential of reducing the live feed demands in Atlantic cod culture. On the contrary, Cahu et al. (2003) achieved the first successful total live prey

replacement by a compound diet in sea bass larvae, where feeding larvae directly on an experimental dry diet sustained good growth, survival and skeletal development. Recent studies in white sea bream (*Diplodus sargus*) suggested initiation of weaning diets as early as 20 days post hatch (360 dd) (Guerreiro et al., 2010). In ballan wrasse there is a current lack of a reliable targeted larval rearing protocol and determination of the optimum weaning window. In recent studies the ballan wrasse weaning window is between 40 and 70 dph (Kousoulaki et al., 2014a) or 55 – 70 dph (Bogevik et al., 2015), both conducted at 16.5 ^{o}C. Various parameters can affect the determination of weaning window, but given the capacities of each hatchery (i.e. water temperature), refining of content and feed palatability for the need of each species can make major impacts on larvae survival, growth and robustness.

The larval rearing protocol of Otter Ferry Seafish as described in figure 1.5. In specific, larval rearing is carried out in continuous light photoperiod in dark green 3 or 5m^3 flat bottom tanks using filtered water through 10μm absolute filtration and UV applied post filtration at a dose of 200 MJ/cm^2. Newly hatched larvae are introduced in larval rearing tanks without any aeration until live feed initiation on 6 dph with rotifers. Live feed addition starts with rotifers (*Brachionus plicatilis*) on dph 6 and until 25 dph, followed by brine shrimp (*Artemia salina*) until 80 dph. Weaning starts at 45 dph with co-feeding with *Artemia* for approximately 30 days. This is an unusually long weaning window compared to other marine species such as haddock (*Melanogrammus aeglefinus) (*7 days; Hamlin & Kling, 2001) or cod (10 days; Puvanendran et al., 2006). However, this long transition period seems to be essential for larvae survival at Otter Ferry Seafish.

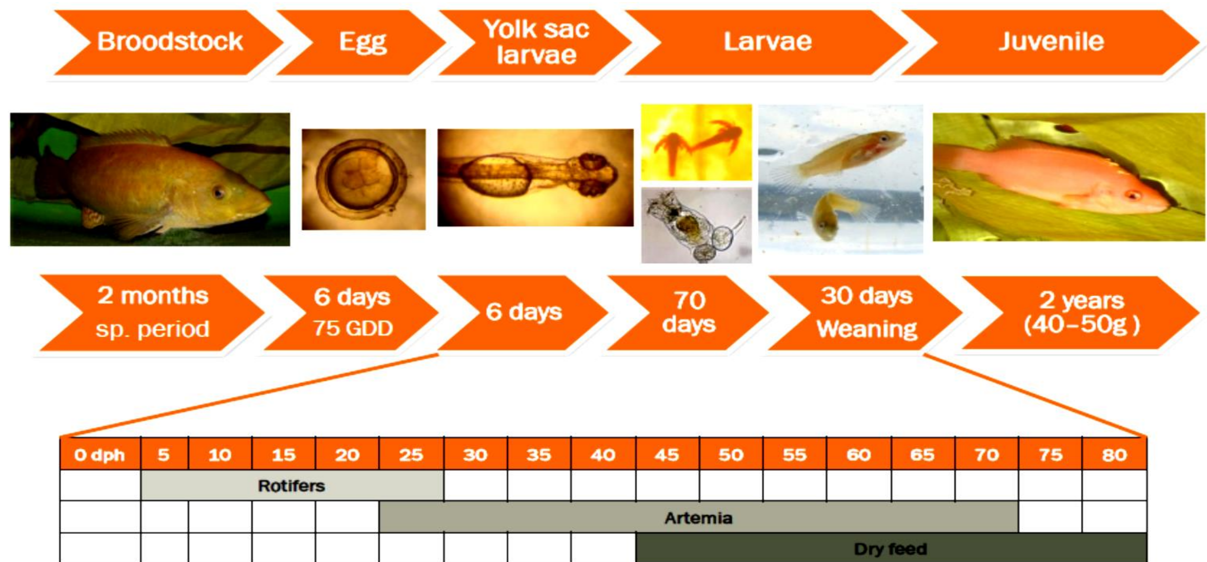

Figure 1.5: Schematic representation of feeding regime at the early stages and overall juvenile production in ballan wrasse farming at Otter Ferry Seafish.

1.3.3 Juvenile production

Slow growth is a key aspect in ballan wrasse commercial exploitation as a cleaner fish species. The time required to reach deployment size (> 45 g) for transfer to sea cages size is 20 – 24 months which is considered too long and costly (David Patterson, Otter Ferry Seafish, pers. comm.). The feeding of ballan wrasse is currently based on diets formulated for other marine temperate aquaculture species such as Atlantic cod due to limited knowledge of ballan wrasse nutritional requirements. However, ballan wrasse natural prey selection indicates the possible requirement for a different diet formulation higher in shrimp meal to other commercial marine carnivorous fish species (Hamre et al., 2013a). The three main drivers to juvenile fish performance are genetics, the environment (e.g. water temperature, light) and nutrition (Valente et al., 2013). It is widely recognised that refinements of nutritional content and feed palatability can make major impacts on growth and robustness of farmed fish and therefore it was a priority of this study.

The hatchery cycle of ballan wrasse at Otter Ferry Seafish, at the beginning of this study, was between 20 and 24 months from fertilised egg to deployment size juvenile (45g, Figure 1.6) using commercially available diets for other marine species. Overall, low survival at the early larval stages and long on-growing period are the apparent main bottlenecks for scaling up the hatchery production of ballan wrasse at Otter Ferry Seafish. In order to achieve this overarching aim, specific areas of interest were identified as priorities for this study.

Figure 1.6: Deployment size (45 g) ballan wrasse juvenile farmed at Otter Ferry Seafish (Scotland, UK).

1.4 Bacterial control in marine hatcheries

In a commercial context losses in production associated with bacterial disease is a common and significant problem (Austin 2006).The majority of bacterial pathogens in marine fish species are aerobic, Gram-negative rods belonging to the Enterobacteriaceceae (e.g.

Aeromonas sp.), Pseudomonadaceae (e.g. *Pseudomonas sp.*) and Vibrionaceae (e.g. *Vibrio sp.*) families (Petty & Francis-Floyd, 2015). There are numerous physical factors that can be associated with disease outbreak these can include (but are not limited to); poor water quality, organic loading of the environment, handling and transport of fish, considerable temperature changes, hypoxia or excessive stress (Petty & Francis-Floyd, 2015). Most aquatic bacteria associated with disease in fish are naturally found in the marine environment as saprophytes which utilise organic and mineral matter. In general, aquatic microorganisms populate a continuous medium with easy access to host surfaces (Olafsen 2001). In nature, fish eggs are exposed to relatively dense bacterial colonization and subsequent growth, but this growth gets amplified in eggs incubated under the intense rearing conditions that the commercial aquaculture requires (Hansen & Olafsen, 1999). Microbial communities in the natural aquatic environment respond quickly to changes in their surrounding environment and therefore a continuous microbial monitoring and adaptation of the microbial control methods is required. Different bacteria survival strategies have been developed through the years to allow adaptation to the changing environmental conditions. Hansen & Olafsen (1999) reviews some examples of marine bacterial species that could not be detected in conventional culture media, as being in a state of dormancy, including *Vibrio cholera*, *Vibrio vulnificus*, other *Vibrio* sp. and *Aeromonas salmonicida* and adaptation of direct viable count methods (using fluorescence microscopy or flow cytometry) is required to quantify them. More recently developed molecular techniques, such as DNA sequencing technologies and bioinformatic analysis, have been developed and give an insight into the complex microbial communities associated with larval rearing water and intestine in response to a variety of factors affecting the host (Ghanbari et al., 2015). Recognition of new uncultured bacterial groups

have been associated with some fish diseases including rainbow trout summer enteritic syndrome for example (Austin, 2011).

The general requirements of marine larvae and the effects of bacterial environment are not fully understood. Low survival rates are observed, especially at the sensitive early larval stages, often without the detection of a known pathogenic organism. In cold-water marine species, such as Atlantic cod, *Vibriosis* has been the most important bacterial disease in juvenile and larval stages, with *Listonella* (*Vibrio*) *anguillarum* dominant amongst pathogenic isolates (Samuelsen et al., 2006). However, *Vibrio spp*. are not frequent on Atlantic cod egg surfaces, but are dominated by members of *Cytophaga* /*Flavobacterium* / *Flexibacter* group (Hansen & Olafsen, 1999). In Atlantic halibut, another cold-water marine species, *Virbio anguillarum*, *Flexibacter ovolyticus* and atypical *Aeromonas salmonicida* have been identified as major bacterial pathogens in larval and juvenile commercial production (Bergh et al., 2001).

Bacteria ingestion can start from as early as the yolk sac stage resulting in establishment of a primary intestinal microflora which persists beyond first feeding. In order to osmoregulate, fish larvae start “drinking”, thus, primed with antigens before the yolk sac is consumed and active feeding commences (Hansen & Olafsen, 1999). Therefore, obtaining microbial control of the larval rearing environment is a key parameter in the success for the commercial exploitation of fish species.

The need for production scale up has led to adoption of intensive rearing, which provides more control over the rearing environment. The required addition of food in intensive culture conditions provides a suitable medium for growth of heterotrophic or opportunistic bacteria. Feed composition affects larval intestinal microflora which may result in growth reduction (Olafsen, 2001). Therefore, it has been reported that treating live prey (*Artemia*

sp.) with authochthonous bacteria before offering it Atlantic halibut larvae can improve survival and growth (Bjornsdottir et al., 2010).

The high host density in commercial aquaculture conditions gives opportunistic organisms a competitive ecological advantage compared to the natural environment. In intensive culture methods, eggs are incubated in incubators with a microflora that differs from that in the sea, and can become heavily colonised within hours post fertilisation (Olafsen, 2001). In particular, fish larvae reared in tanks, with hatching eggs and debris can result in a 1000-fold increase in bacterial load from ambient water through hatching (Olafsen, 2001). Two widely adopted elements to achieve microbial control at the early larval stages of fish are selective and non-selective reduction of bacteria, through addition of probiotics and water filtration / disinfection respectively (Vadstein et al, 1993; Skjermo & Vadstein, 1999).

Disinfection of either water or eggs in aquaculture provides an additional mode of protection. Various forms of water and/or egg disinfection are common practice amongst commercial marine hatcheries targeting bacterial control through minimisation of the bacterial load (Olafsen, 2001). However, total elimination of the bacterial load might disturb the balance of microbial communities and potentially favour rise of opportunistic bacteria (Olafsen, 2001). Various chemicals have been successfully applied as disinfectants in aquaculture, with different pathogen targets and modes of action (biocidal, bactericidal, virucidal and/or fungicidal) including hydrogen peroxide, glytaraldehyde, ozone, iodophors, formaldehyde, paracetic acid, tannic acid, sodium chloride, copper sulphate and bronopol (De Swaef et al., 2015) (Table 1.1). However, most of the previously mentioned chemicals can be toxic with negative effects to eggs and/or hatchery staff and careful control is required while less toxic agents would be desirable (Salvesen et al., 1997; De Swaef et al., 2015).

Table 1.1: Summary of chemical treatments and their effects on marine finfish eggs.

Species	Treatment	Concentration (ppm)	Duration	Application	Treatment replication	Best results obtained / recommended treatment	Reference
Atlantic cod	Bronopol (Pyceze), kickstart(ctrl) sea water (ctrl)	50, 500, 1000 1000 (ctrl)	45sec	Immersion	On stocking	50-500ppm bronopol, 83-89% egg survival as opposed to 48% to control.	Treasurer et al., 2005
Atlantic cod	Bronopol, sea water(ctrl)	36.7	30min	Continuous addition	Once daily for 8 days, for days 0 and 8, none(ctrl)	Daily disinfection, 66% egg survival	Treasurer et al., 2005
Haddock	Bronopol, kickstart(ctrl) sea water (ctrl)	50, 500, 1000 1000 (ctrl)	60sec	Immersion	On stocking	500ppm bronopol, 73.6% egg survival	Treasurer et al., 2005
Haddock	Bronopol, Sea water (ctrl)	50	30min	Static Immersion	Daily	Daily disinfection, 93% egg survival	Treasurer et al., 2005
Haddock	Bronopol, Sea water (ctrl)	50 250	60sec 120sec	Immersion	On stocking	250ppm for 120sec, 93% survival	Treasurer et al., 2005
Atlantic cod	Glutaraldehyde, Iodophor (Actomar K30), Process water (ctrl), Clean sea water (ctrl)	100, 200, 400, 600, 800 10, 50, 100, 150	10min	Immersion	On stocking	100ppm iodophor (84 % survival and hatch) or 400ppm glutaraldehyde (76% egg survival and hatch)	Overton et al., 2010

California yellowtail, white seabass and California halibut	Formalin, Povidone-iodine, Sea water (ctrl)	100 1000 50 100 -	60min 15min 15min 10min 60min	Immersion	On stocking	100ppm for 60min formalin, 77% egg hatching	Stuart et al., 2010
Atlantic cod, Haddock	Hydrogen peroxide, Polyvinylpyrrolidone iodine, Sodium hypochlorite, Antibiotic solution (peniculin/streptomycin, 3:2) Autoclaved seawater (ctrl)	3% 1% 0,1% 0.005% -	5min 24h	Immersion	On stocking	PVP iodine or antibiotic solution, 74% egg hatch for cod and 35% for haddock	Peck et al., 2004
Atlantic halibut	Glytaraldehyde, Seawater (ctrl)	400, 800, 1200	2.5, 5, 10min	Immersion	On stocking	400-800ppm for 5-10min. No differences in survival or hatching	Salvesen et al., 1997
Turbot	Glytaraldehyde, Seawater (ctrl)	400, 800, 1200	2.5, 5, 10min	Immersion	On stocking	400-800ppm for 2.5min. No differences in survival or hatching	Salvesen et al., 1997

Probiotic addition, as a selective enhancement of bacteria, have various applications in hatcheries including the modulation of interactions with the tank environment, improvement of larval immune system, improvement of water quality and stimulation of the larval digestive system (Lauzon et al., 2014). Inclusion of probiotics may confer benefits to the host by providing both nutritional benefit and protection against pathogens (Gatesoupe, 1991, 1994, 2002; Sveindottir et al., 2009; Mandiki et al., 2011; Hauville et al., 2015). An additional element to the bacterial control of larval rearing tanks is the use of clay. Various larviculture protocols recommend the use of turbid conditions to improve larval performance. Recent studies have shown that addition of ceramic clay can have beneficial effects on larval performance which can be attributed to the increase of turbidity and reduction of microbial load by binding to organic matter and bacterial cells (Stuart et al., 2015; Attramadal et al., 2012a; Bjornsdottir et al., 2011; Clayton et al., 2010).

The importance of microbial control in ballan wrasse larval rearing has been recently highlighted by Attramadal et al. (2016), showing that the selective pressure in the system can reduce the opportunities of opportunistic bacteria proliferation and coming also in line with the microbial control techniques in other marine larvae (Skjermo & Vadstein, 1999). Chemical treatments (bronopol and formalin) seem to have a positive impact in first feeding tank hygiene and possibly survival (David Patterson, Otter Ferry Seafish, pers. comm.) with on-site commercial trials showing a significant reduction of bacteria in the larval rearing water 2 hours after low concentration bronopol addition (25 ppm). So far, the only information available regarding ballan wrasse microflora has been published by Birkbeck and Treasurer (2014) reporting Vibrio species (*Vibrio splendidus, Vibrio ichthyoenteri* and *Vibrio pacinii*) isolated from the digestive tract microflora in 150 dph larvae. However, there is a significant lack of information regarding the interaction between marine bacteria and various biota involved during larval rearing, especially in

"new" aquaculture species, such as ballan wrasse. Therefore, determination of bacteria load during the early larvae stages and methods to control them should be a priority aiming maximisation of larvae survival and growth.

1.5 Nutritional requirements

1.5.1 Nutritional requirements in finfish farming

Nutrition plays an important role in the aquaculture industry by influencing fish growth, health, flesh quality, and waste generation. Feed costs often represent 50 % of variable costs of an aquaculture business, affecting significantly the economic returns (NRC, 2011). Development of nutritious, efficiently delivered and cost-effective diets relies on understanding each species nutritional requirements.

1.5.1.1 Proteins

Proteins and amino acids are critical molecules because of the role they play in the structure and metabolism of all living organisms. Proteins are organic material consisting major part of fish tissue (65-75 % of the total on a dry-weight basis). Proteins are digested and release free amino acids to the intestinal tract and distributed by the blood to the organs and tissues, targeting either building of new proteins (for activities such as growth and reproduction) or to maintain existing proteins. Adequate amounts of protein are required to ensure maintenance of body functions and maximise growth. However, higher than required doses of protein will not be utilised to make new proteins and will be converted to energy (Wilson, 2002). Protein digestion appears to be the main determinant of live weight (biomass) gain in fish (Dumas et al., 2007). This close association is related to close association of water with protein, where in lipid deposition a large proportion of triglycerides is stored in tissues by replacing for water and therefore lipid does not always seem to contribute significantly to live weight gain (Dumas et al., 2007). Protein is an

essential but one of the most expensive components in fish feeds and determination of the optimum levels and sources is critical for the formulation of cost-effective feeds. The recommended dietary protein levels (%) for fish species weighing less than 20g vary between 40 % (Nile tilapia, *Oreochromis niloticus*) and 55 % (European sea bass). However, these the requirements are reduced in bigger size fish (600 – 1,500 g) ranging between 28 % (Nile tilapia) and 45 % (European sea bass) (NRC, 2011).

Hydrolysed proteins are generally used to increase bioavailability of protein and lipid to fish larvae and the protein becomes more water-soluble and beneficial for larvae growth and survival (Hamre, 2006). Also, supplementation of micro-diets with moderate levels of protein hydrolysates facilitates the intestinal maturational process, improves survival and increases larval development (Zambonino-Infante et al., 2008). Nevertheless, excessive amount of hydrolysed protein can cause difficulties in digestion (Kvåle et al., 2009). However, this depends on species and on their feeding habits. In Atlantic cod, replacement of the dietary protein with hydrolysed (0 – 400 g kg^{-1}) increased significantly larvae survival, when the same levels of substitution had the opposite results in Atlantic halibut survival (Kvåle et al., 2009). Moderate levels (19 and/or 38%) of hydrolysed proteins promote the digestive development, reduced the malformations and increase survival (only with 19% replacement) in sea bass larvae, when higher incorporation of it (58 %) reduced growth and survival, due to perturbation in digestion (Cahu et al., 1999).

Amino acids can be linked in varying sequences to form a vast variety of proteins with amino acids sequence being the primary structure of protein. The amino acids that an organism can synthesise on its own are denoted as "essential amino acids" (EAAs). Deletion of an EAA could affect significantly growth, while a "nonessential amino acid" (NEAA) would not, advocating that EAA could not be synthesised by the animal and their dietary supplementation is essential (NRC, 2011).

1.5.1.2 Lipids

Lipids are, along the proteins, the major organic materials of fish tissue playing an important role as sources of metabolic energy for growth (Tocher, 2003). Lipids are the favourite source of metabolic energy in fish, particularly in the case of marine carnivorous fish where carbohydrates play a lesser important role as energy source (Watanabe, 1998). In terms of energy, lipids are the most energy rich of all classes of nutrients with gross energy value of 9.5 kcal / g, compared with protein (5.6 kcal / g) and carbohydrates (4.1 kcal / g).

Fatty acids (FA) are major components of most lipids and have functional roles as a source of metabolic energy (as in TAG), structural components (as in membrane PL) and as precursors of bioactive molecules (Sargent et al., 2002; Tocher, 2003). The fatty acids of fish species are rich in ω3 long chain, highly unsaturated fatty acids (n-3 HUFA) which have critical roles in animal nutrition and physiological processes (Tocher, 2003). The importance of providing the right blend of sufficient amounts of fatty acids for successful production of marine finfish is well appreciated (Tocher, 2003, Sargent et al., 2002). All fatty acids can act as energy sources, but some specific LC-PUFA also have a number of essential roles in fish metabolism (NRC, 2011).

Numerous studies have proven that marine fish requirements in essential fatty acids (EFAs) varies with dietary lipid levels and dietary DHA/EPA ratio (Tocher, 2010). The importance of n-3 highly unsaturated fatty acids (n-3 HUFA, especially DHA and EPA) has been well reported in marine finfish with effects on growth and other important developmental and physiological functions (Tocher, 2003). Since marine fish cannot synthesize LC-PUFAs from their 18-carbon precursor fatty acids such as linolenic acid (18:3n-3) and linoleic acid (18:2n-6), dietary addition of these fatty acids is essential for fish development (Tocher, 2003). Additionally, moderate dietary DHA/EPA can enhance

fish growth performances (Xu et al., 2016). Many studies have shown that the fatty acids composition of marine fish tissues, to a huge extent, reflects that of the diet (Jobling et al., 2008; Olsen et al., 2015). Beneficial dietary DHA/EPA ratios can vary between species. For instance, DHA/EPA ratio of 1.53 – 2.08 have been shown beneficial for growth of the Japanese seabass (*Lateolabrax japonicus*) juveniles (Xu et al., 2016), while ratio of 0.5 appeared to be sufficient to enhance growth in gilthead seabream juveniles (Ibeas et al., 1997).

Lipids can be classified into lipid classes having various roles and functions. The lipid classes dominating fish tissues and feeds are triacyglycerols (TAG), phosphoglycerides or phospholipids (PL), sphingolipids, sterols and wax esters (Sargent et al., 2002).

Sterols, tetracyclic hydrocarbon alcohol compounds are the most important simple lipids with cholesterol being the most commonly found sterol in fish (NRC, 2011). The most important neutral lipid is triacylglycerol (TAG), which primarily serves as energy storage. Dietary inclusion of marine TAG can increase growth in marine species, such as sea bass (Cahu et al., 2003). Excess dietary fatty acids are exported from the liver in the form of lipoproteins and accumulated and stored in the form of TAG in specific lipid storage sites (Tocher, 2003). TAG is the main form in which lipids are stored in fish tissues, stored in most species in mesenteric adipose tissue fundamentally as a source of long-term energy (Sargent et al., 2002). However, in gadoids, such as Atlantic cod, large amounts of lipid are stored in their livers (Jobling et al., 2008).

Phospholipids (PL) are polar lipids, and besides serving as a source of energy, they are important structural and functional components of cell membranes and brain and eye tissue (Sargent et al., 1993 ; Watanabe & Kiron, 1994 ; Furuita et al., 1998 ; Sargent et al., 2002). Diets containing high and moderate levels of DHA and EPA in the phospholipids fraction

can enhance growth and intestinal maturation in Atlantic cod (World et al., 2007). Similarly sea bass can utilise more efficiently dietary phospholipids than neutral lipid fractions resulting to enhanced survival, growth and fish quality (Cahu et al., 2003). Compared to TAG the PLs are more easily digested, and their presence may enhance digestion of other lipids in addition to being a key component of the lipoproteins transporting nutrients after uptake by the enterocytes (Coutteau et al., 1997; Tocher et al., 2008).

1.5.1.3 Carbohydrates

The main importance of carbohydrates in fish feeds relies to their attractive physical characteristics and supply of energy at low cost. Carbohydrates in fish feed range from easily digestible mono-, di- and oligosaccharides to insoluble and indigestible hemicelluloses and cellulose, with sources from seaweed, algae and plankton until refined grain and soybean products. The commercial need for cheaper diets have initiated the use of cheaper feed ingredients with higher carbohydrates contents. The primary role of carbohydrates in fish diets is energy sources and binding sources (starches, pectins and hemicelluloses) (Krogdahl et al., 2005). Digestion is the main limiting factor of efficient utilisation of starch for fish growth. Improvement in carbohydrates digestion efficiency can be achieved by providing it in an available form in the diet. Starch gelatinisation by either heat treatment of the specific feed ingredient or extrusion of whole feed has been demonstrated to improve digestion efficiency. The highest recommended levels of digestible starch inclusion in feed varies between 15-25 % in salmonids and marine species and up to 50 % in omnivorous species (NRC, 2011).

1.5.1.4 Minerals

Dietary inclusion of minerals in fish is limited compared to most other nutrient groups. The metabolism of minerals by fish can be influenced not only by the dietary intake but also by

the concentrations of dissolved ions in the water (Moyle & Cech, 2000). Supply of adequate dietary mineral composition at a relatively high concentrations is required to achieve formation of skeletal structures and other hard tissues, electron transfer, regulation of acid : base equilibrium, the production of membrane potentials and osmoregulation (NRC, 2011). The most commonly found macrominerals in fish are calcium, chlorine, magnesium, phosphorus, potassium and sodium. Microminerals are required in then diet in much smaller quantities than macrominerals and act as important elements in hormones and enzymes. The most commonly found microminerals are chromium, copper, iodine, iron, manganese, selenium and zinc (NRC, 2011).

1.5.1.5 Vitamins

Vitamins are organic compounds distinct from all other nutritional groups that are essential in trace amounts to be included in diets for normal fish growth, reproduction and health (NRC, 2011). Vitamins are divided in to groups: (1) water soluble (vitamins C and B-vitamins) and (2) fat soluble (vitamins A, D, E and K). Water soluble vitamins have unique coenzyme functions in cellular metabolism being involved in numerous biochemical reactions (NRC, 2011). As an example, the di-phosphatase form of thiamin, thiamin pyrophosphate (TPP) is an essential cofactor for number essential enzymatic steps in energy production. Thiamin deficiency can cause convulsion, reduction of appetite, dark pigmentation and mortalities in rainbow trout (Morito et al., 1986). Fat soluble vitamins are absorbed in the intestine together with the dietary lipids. Animals usually store fat-soluble vitamins in specific cell comportments. However, when dietary supply is in excess, storage of fat-soluble vitamins is taking place as simple accumulation in lipid compartment causing toxic conditions (hypervitaminosis). The body level of fat soluble vitamins often chances in line the increasing supplementation to far above the fish requirement and lower

concentrations of these vitamins in farmed than in wild fish does not show certainly deficiency (Hamre et al., 1997).

1.5.1.6 Additives

Various factors can affect feed palatability and appetency in fish species, including appetite stimulators and attractants (Kasumyan & Doving, 2003). Feedstuffs of marine origin are noted for their high palatability as ingredients to fish feeds. Giullaume & Metailler (2001) have classified fish attractants in to three main groups consisting of: (a) L-amino acids, (b) betaine or other molecules with pentavalent nitrogen atom, and (c) nucleosides and nucleotides, with nucleotides being more effective. Marine origin attractants suit these categories due to fish solubles are rich in nucleotides, fish protein hydrolysates contain free amino acids, and invertebrate meals have in excess nitrogenous bases (NRC, 2011). Krill and shrimp meal are widely used as appetite stimulators for cold water species such as rainbow trout (*Oncorhynchus mykiss*) (Oikawa & March, 1997) and Atlantic cod (Tibbetts et al., 2011). These attractants also highly depend on certain free amino acids (e.g. alanine, glutamate, proline and serine) which can act as appetite stimulators (Li et al., 2009).

In the recent years, studies have shown the potential value of probiotics inclusion in fish feeds to enhance growth, immunity, and resistance to diseases (NRC, 2011). Introduction of probiotic bacteria, such as *Bacillus sp*., causes competition for nutrients, produces growth inhibitors or quench cell-to-cell communication that allows for settling within biofilms (Schreier et al., 2010). Additionally, probiotics can be a source of nutrients and enzymatic contribution to digestion, direct uptake of dissolved organic material mediated by the bacteria, enhance the immune response against pathogenic organisms and provide antiviral effects (Ringø et al., 2010). Inclusion of probiotics may confer benefits to the host by providing both nutritional benefits and protection against pathogens as suggested in

many other species including turbot (*Scophthalmus maximus*) (Gatesoupe 1991; Gatesoupe 1994), pollack (*Pollachius pollachius*) (Gatesoupe 2002), Atlantic cod (Sveinsdóttir et al., 2009) and Eurasian perch (*Perca fluviatilis*) (Mandiki et al., 2011). Potential probiotics tested in cold water fish species include Gram-positive (e.g. lactic acid bacteria, *Brochothrix thermosphacta*, *Arthrobacter*, *Bacillus* and *Kocuria spp.*) and Gram-negative (e.g. *Aeromonas*, *Glyconobacter*, *Paracoccus*, *Phaeobacter*, Phenylobacterium, Photobacterium, Pseudomonas, Pseudoalteromonas, Psychrobacter, *Shewanella* and *Vibrio spp*.) bacteria, but also yeasts such as *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Rhodotorula glutinis* (Lauzon et al., 2014).

Supplementation of non-nutritional additives to improve fish health and immunity has become an effective practice to reduce dependence of chemotherapeutics to treat diseases in aquatic animals (NRC, 2011). Compounds with anti-inflammatory properties can be obtained from other living organisms or artificially manufactured as synthetic chemicals. A common anti-inflammatory additive in fish feeds is the inclusion of algae. *Schizochytrium limacinum* previously shown to have positive growth effects in rainbow trout (Song et al., 2007) which may be mediated by its immunomodulatory properties. Spirulina has been found to improve overall intestine health and growth in common carp (*Cyprinus carpio*) (Nandeesha et al., 1998). Another substance that its inclusion in fish feeds has been proven beneficial is brewer's yeast, a single cell bacterial protein source. It has been shown in carp (*Cyprinus carpio*) (Marković et al., 2012) and Atlantic salmon (Urán et al., 2008; Romarheim et al., 2011) that inclusion of a single cell bacterial protein source appears to offer protection from soy bean meal enteritis while also promoting increase in absorptive surface areas.

1.5.2 Nutritional requirements in ballan wrasse

A key driver for fish growth is the dietary nutrient availability (Valente et al., 2013). At the beginning of this study, there were no indicators available of nutrient absorption efficacy and metabolism in relation to diet formulation to improve growth and intestinal health of farmed ballan wrasse stocks. Therefore, assessment of the effects on survival, growth and intestinal health of pre-existing diets for other cold water marine species was a priority. Ballan wrasse natural diet can be summarised as being relatively low in lipids and easily digestible (Hamre et al., 2013a) and a clear preference to molluscs, decapods and isopods (see section 1.2) (Dipper et al., 1977). Its natural prey selection indicates the possible requirement for a different diet formulation to other commercial marine fish species, rich in krill and shrimp meal and easily digestible (Hamre et al., 2013a). Hamre et al. (2013a) reported some of the nutritional requirements of ballan wrasse analysing the nutrient composition in whole body farmed juveniles and the female gonads from farmed and wild broodstock fish. It was found that the optimum macronutrient composition in diets for broodstock and juvenile ballan wrasse is 65% dietary protein, 12% lipid and 16% carbohydrate. It should be also taken into consideration that this study was based on maximum lengthwise growth only, without assessing other parameters (e.g. intestine health, deformities, etc.). However, extrapolation of this information for the development of larval stage diets can be risky and will not have necessarily the same results as in bigger fish, due to possible formulated feeds leakage and different nutritional requirements for different developmental stages (Hamre, 2006). After identifying the natural feed selection of a species, the next step is to understand the nutrient utilisation mechanisms (digestion, absorption and assimilation) and screening the nutrient requirements to facilitate its domestication. However, the choice of nutrient sources varies during fish lifecycle, feed strategies, geographical location and environment. To date, limited information is available

regarding ballan wrasse nutritional requirements, diet formulation and hatchery performance with the existing commercial marine species diets providing inadequate results to ballan wrasse hatchery performance. This highlights the need to develop ballan wrasse-specific diets, alternative to fish meal raw materials (i.e. krill and shrimp meal) and new formulations should be tested to improve feed palatability, digestibility and growth through nutritionally and economically balanced diets.

Various factors can affect feed palatability and appetency in fish species, including appetite stimulators and attractants (Kasumyan & Doving, 2003). Krill and shrimp meal are widely used as appetite stimulators for cold water species such as rainbow trout (*Oncorhynchus mykiss*) (Oikawa & March, 1997) and Atlantic cod (Tibbetts et al., 2011). These attractants also highly depend on certain free amino acids (e.g. alanine, glutamate, proline and serine) which can act as appetite stimulators (Li et al., 2009). It has been hypothesised that other currently available marine diets might be inadequate for ballan wrasse due to different nutritional requirements and lack of attractants, which results in reduced ingestion and growth.

Cahu & Zambonino-Infante (2001) reviewed that digestive functions are efficient before the onset of exogenous feeding, with newly hatched larvae undergoing a series of morphological and cellular transformations before being ready for this. Several parameters (water temperature, food availability and composition) can delay or accelerate these processes. For instance, most cold water species seem to follow similar developmental patterns, but natural environment temperature of each species can affect them significantly (Zambonino-Infante & Cahu, 2001). Also, Yúfera & Darias (2007) summarise that contribution of exogenous enzymes from live prey is very controversial and highly depended on the individual species.

The development of species-specific weaning diet is an essential larval nutrition objective, which can be achieved by producing diets attractive enough for the larvae and fulfilling the physical, digestive and nutritional feed requirements of the larvae (Cahu & Zambonino-Infante, 2001). A wider understanding is also required of ontogenetic development, activation of the digestive enzymes and diets digestibility to provide the adequate diets (Kolkovski, 2001). Adaptation of feeding diets and protocols to the fish larvae digestive and assimilation capacities is key for a successful weaning process in marine species. Weaning diets acceptance can be affected by various non-nutritional parameters, including environmental (temperature, water quality and tank design) and diet's visual detection (Person-le-Ruyet, 1989). Adaptation of the rearing conditions is required to meet the larval requirements for optimum detection of live feed and weaning diets (Hamre et al., 2013b).

Kolkovski (2001) reported that the poor performance of weaning diets (referring to poor larval survival and growth) in various carnivorous fish larvae is due to inadequate incorporation of nutrients from the larvae. This is mainly due to not fully developed enzymatic activities and, in specific, the absence of pepsin-like enzyme activity. Therefore, several weaning diets were built on the supply of exogenous enzymes. However, Cahu & Zambonino-Infante (2001) reviewed that digestive functions are efficient before the onset of exogenous feeding, with newly hatched larvae undergoing a series of morphological and cellular transformations before being ready for this. Diet composition can indeed enhance, stop or delay the maturational process of digestive enzymes (Zambonino-Infante & Cahu, 2001). Andrade et al. (2012) tried different weaning regimes in red porgy, *Pagrus pagrus*, including direct weaning. This strategy failed due to nutritional restrictions and delay of the maturation of digestive system, concluding that for developing weaning diets for red porgy it should be taken into consideration: particle size, water stability, nutritionally well-balanced ingredients and level of digestibility.

Partial replacement of fish meal by krill meal has proven to be beneficial in other cold water species. Feeding activity and consequently growth performance were improved significantly after partly replacing fish meal by freeze dried krill meal (*Euphausia superba*) in Atlantic cod and Atlantic halibut (Tibbetts et al., 2011). However, high levels of fish meal replacement (over 25 – 50%) may not result in significant growth differences and consequently can be a non-cost-effective solution (Tibbetts et al., 2011). Ballan wrasse weaning success highly depends on raw material quality (Kousoulaki et al., 2014a) and especially fish meal quality (Bogevik et al., 2015). It has been reported by Kousoulaki et al. (2014) that ballan wrasse larvae fed weaning diets containing krill hydrolysate resulted in highest body weight larvae compared to larvae fed fish, cod muscle and shrimp meal. Given also that ballan wrasse prefer easily digestible feed sources (Hamre et al., 2013a; Dipper et al., 1977), dietary inclusion of hydrolysed proteins can be considered as promising element in ballan wrasse diet development.

The availability of diets that meet fish larval nutritional requirements has been a major constraint to the expansion of many marine fish species production with subsequent poor survival at the early life stages (Tilseth, 1990). The development of species-specific weaning diets that satisfy physical, digestive and nutritional requirements of marine fish larvae is vital to improve larvae survival and general juvenile robustness in the early stages of production (Cahu & Infante, 2001). Major losses during weaning in ballan wrasse require further investigation on a suitable weaning diet that provide adequate larvae survival and growth. Following the larvae stage, and when the larvae complete their metamorphosis to fully active feeders with completed scales and working fins they can be called juveniles.

Recent studies on intestine health of ballan wrasse using diets with different moisture levels reported increased inflammation scores across the intestine (primarily in the most

distal end) regardless of the diets used (Krogdahl et al., 2014). A nutritionally imbalanced diet or raw material source and type may cause intestine inflammation and therefore impair immunity and functionality of the intestinal mucosa (Merrifield et al., 2011). Intestine inflammation has a high metabolic cost, through repair and maintenance of the enterocytes, resulting in possible decreased in weight gain, feed intake and growth efficiency (Rasković et al., 2011). Given the importance of intestine health, various diagnostic methods have been developed adopting semi-quantitative scoring systems, histochemical and morphometric assessments (Rasković et al., 2011). To monitor the dietary effects on intestine health and connect with fish growth, a number of enteritis scoring systems have been adopted (Urán et al., 2008; Martinez-Rubio et al., 2013). Morphometric analyses of the intestinal tract such as thickness of the mucosa, submucosa and enterocyte thickness have been used as indicators of nutrient absorption efficacy and metabolism in relation to diet formulation to improve growth and fish heath in captive stocks (Buddington et al., 1977). In general, very limited information is currently available regarding the ballan wrasse intestine health and how different diets can affect it, making it a significant element to be considered in diet development.

1.6 Research aims

The overall aim of this thesis was to undertake a body of research targeted scaling up and further developing the hatchery technology of ballan wrasse in Otter Ferry Seafish. The research encompassed two main aspects: management of microbial communities to improve survival in the egg/larval production followed by the testing and optimisation of feeds to improve juvenile growth performance.

The specific aims of this study were:

i) To develop a commercial disinfection protocol that provides eggs with the highest level of disinfection whilst maximising survival and hatching rates;

ii) To assess the effect of different bacterial control strategies on survival, growth and bacterial load in early larval stages;

iii) To benchmark the impact of co-feeding with four different micro-diets (three commercially available and one experimental) on larvae survival and growth performance;

iv) To benchmark the impact of four commercial feed formulations on juvenile growth performance and investigate their subsequent impacts on liver fatty acid composition, intestinal health (intestine histomorphology and inflammation scoring) and digestive functions (digestive enzymes activity and nutrients digestibility);

v) To assess the impact of four diets with increasing moisture levels including a diet containing agar on juvenile growth performance and investigate their subsequent impacts on digestive enzymes, intestine and liver health.

Chapter 2: Surface disinfection of ballan wrasse (*Labrus bergylta*) eggs with bronopol: evaluation of concentration, contact time and commercial application.

2.1 Introduction

Egg disinfection is an important and common practice among marine hatcheries, used as a disease management tool to ultimately help reduce mortalities and thus improve the hatchery productivity (De Swaef et al., 2015). The external surface of fish eggs can easily be colonized by bacteria, meaning that there can be transmission from the broodstock to the incubated eggs and on to the newly hatched larvae (Overton et al., 2010). High egg stocking densities and cross contamination between egg batches can also contribute to the bacterial development (Overton et al., 2010). A variety of chemicals are available for egg disinfection in aquaculture species with different pathogen targets and modes of action (biocidal, bactericidal, virucidal and/or fungicidal) including hydrogen peroxide, glytaraldehyde, ozone, iodophors, formaldehyde, paracetic acid, tannic acid, sodium chloride, copper sulphate and bronopol (De Swaef et al., 2015). The efficiency of various chemicals relies on their non-selective reduction of bacteria in the egg incubation and later larvae rearing systems (Vadstein et al., 1993). For instance, glutaraldehyde disinfection of Atlantic halibut and turbot eggs can have a positive effect on hatching and singificnat improvement on viability of yolk-sac larvae. Application of 400-800 ppm glutaraldehyde for 5-10 minutes was considered optimal for halibut, when lower contact time (2.5 minutes) was required to achieve adequate effects (Salvesen et al., 1997). Additionally, application of 100 ppm iodophor or 400 ppm glutaraldehyde for 10 minutes to cod eggs resulted antibacterial and antiviral effects, but without having any effects on egg survival. However, glutaraldehyde treatments improved the later survival of yolk sac larvae (Overton et al., 2010). Formalin and povidone-iodine have been tested as egg disinfection chemicals on California yellow-tail (*Seriola lalandi*), white seabass (*Atractoscion nobilis*) and California halibut (*Paralichthys californicus*), with the best the best balance between bacterial control and egg viability in all three species obtained by applying formalin 100

ppm for 60 minutes (Stuart et al., 2010). Can et al. (2010) suggested that glutaraldehyde offers optimum egg antibacterial and hatching rate balance in in gilthead sea bream, red porgy (*Pagrus pagrus*) and common dentex when applied in 200 ppm between 4 and 8 minutes. In the same study, hydroxide peroxide and iodine treatments of 300 ppm for 10-15 minutes and 300 ppm for 15-20 minutes respectively balanced reduction of bacterial load and hatching rates.

Most of the above chemical treatments can be toxic with negative effects to eggs and careful control is required while less toxic agents would be desirable (Salvesen et al., 1997; De Swaef et al., 2015). Ballan wrasse are benthic spawners, releasing eggs surrounded by a gelatinous adhesive layer (D'Arcy et al., 2012; Grant et al., 2016a,b). Commercial ballan wrasse hatcheries generally place short pile mats on the bottom of the broodstock tanks that are used as spawning substrates (Grant et al., 2016a). Following successful spawning, the mats are removed and incubated in well aerated incubation tank until the eggs are ready to hatch at *circa* 72 degree days (dd) (Ottesen et al., 2012). This is a novel collection method in comparison to most marine finfish hatcheries where either the freely spawned pelagic eggs are collected from surface overflows (e.g. Atlantic cod, Penney et al., 2006; Gilthead seabream, Meseguer et al., 2008), or unfertilised gametes are manually stripped and fertilised artificially for species which do not spawn spontaneously in captivity (e.g. Atlantic halibut, Brown et al, 2006; seabass, *Dicentrarchus labrax,* Siddique et al., 2017). Therefore, due to a combination of factors including the nature of the ballan wrasse egg collection method, the wild origin of the commercial broodstock (Leclercq et al., 2014a) together with the adhesive gum layer (Grant et al., 2016a,b), new egg disinfection protocols must be validated.

The commercial practice in ballan wrasse eggs at Otter Ferry Seafish prior to this study consisted in treating with formalin (150 ppm for 60 min) at day 0, followed by a daily

static bath of bronopol (25 ppm for 30 min) until 72 degree days when hatching is physically induced by "scraping". Formalin can have antibacterial effects in balance without compromising egg survival or hatching rates in marine species (Stuart et al., 2010). However, preliminary experiments (later preliminary experiment 1) suggested that formalin treatment at concentrations between 200 and 1,000 ppm for 30 minutes can have negative effect on hatching rate in ballan wrasse eggs. In addition, formalin has been classified as carcinogen 1B and mutagen 2 (EC 1272/2008), and therefore alternative treatments should be developed. Dahle et al. (2014) reported the effects of Pyceze and glutaraldehyde in ballan wrasse eggs, suggesting that use of glutaraldehyde provides a good bactericidal effect without adverse effects on egg hatchability. Nevertheless, similar to formalin use, glutaraldehyde should be carefully used due to potential health risk to users.

Bronopol (2-bromo-2-nitro-1,3 propanediol) licensed in the UK under the brand name Pyceze^{TM} (50 % W/V bronopol, Novartis Animal Health UK) can be used against fungal infections especially saprolegnia in Atlantic salmon and rainbow trout farming (Oono & Hatai, 2007). It has also been shown to provide a broad spectrum antibacterial treatment, effective for egg disinfection in cold water marine species like Atlantic cod (Treasurer et al., 2005; Overton et al., 2010) and Atlantic halibut (Birkbeck et al., 2006); as well as being an effective anti-parasitic compound (Shinn et al., 2012).

The aim of this study was to develop a commercial disinfection protocol that provides ballan wrasse egg with the highest level of disinfection whilst maximising survival and hatching rates. To do so, three trials have been performed to 1) determine the bactericidal bronopol concentration and treatment time *in vitro*, 2) determine and validate the bactericidal bronopol concentration and treatment time in a semi-commercial setting and 3) compare the efficacy of the disinfection protocol against the current commercial practice.

2.2 Materials and methods

2.2.1 Broodstock and egg collection

This study was performed during the ambient (May – July) spawning seasons of 2013 (experiments 1 and 2) and 2014 (experiment 3) at Otter Ferry Seafish, Argyll, United Kingdom. Eggs were collected from natural spawning ballan wrasse broodstock (wild caught animals held captive for over 3 years) housed in 3.25 m^3 tanks. Broodstock tanks were on a flow-through system supplied with ambient seawater drawn from 20 m depth of Loch Fyne and filtered through sand-filters.

Water temperature in broodstock tanks during the spawning season fluctuated from 10 to 13 ºC. The tanks were equipped with artificial seaweeds made from green plastic sheets, to simulate species natural habitat. Fish were fed to satiation daily (approximately 3 % of fish biomass) with mussels, prawns and a semi-moist sausage diet (broodstock base mix supplied by BioMar UK). Short pile mats were provided as a spawning substrate for females to lay adhesive eggs on and covering 2.86 m^2, 50 % of tank's bottom area (5.72 m^2). Egg collection mats were removed daily in the morning, quality of the eggs was assessed (fertilisation rate and developmental stage) and incubated in a 1 m^3 tank, with gentle aeration and water with similar level of filtration as broodstock tanks, prior to the start of the experiments. The egg holding system was in constant illumination and in flow-through water system, 15 µm sand filtered, 10 µm absolute filtered and UV applied post filtration at a dose of 200 mJ / cm^2. Temperature and dissolved oxygen of water were measured daily. Temperature was maintained at 11 ± 1 ºC (experiments 1 and 2) and 13 ± 1 ºC (experiment 3). Oxygen levels of the water in the commercial setting experiments (experiments 2 and 3) fluctuated between 85 – 100 % saturation. All collected eggs were at 16 to 128 cells developmental stage at the initiation of the experiments having over 85 %

fertilisation rate and all sampling procedures were conducted in temperature-controlled conditions.

2.2.2 Preliminary work

Prior to the experiments, preliminary work was conducted to get an initial understanding about the impact of bronopol and formalin in hatching rate of ballan wrasse eggs. In the preliminary experiment 1, eggs were gently removed from five different locations of the egg collection mat into petri dishes (30 – 40 eggs / Petri dish), to test in triplicate application of: 25, 50 and 100 ppm bronopol (used as Pyceze^{TM}, bronopol 50 % (W / V); Novartis Animal Health UK) for 30 minutes; 200, 400 and 1000 ppm formalin for 30 minutes; and control (no treatment). Each Petri dish was filled with 20 ml mechanically filtered through a filter unit at 0.2 µm (Ministart, Sartorius stedim, Germany) hatchery sea water following 15 µm sand filtration, 10 µm absolute filtration and UV applied post filtration at a dose of 200 mJ / cm^2. Water in Petri dishes was replaced with the bronopol or formalin concentration or sterile seawater (for the control) during the treatment time (30 minutes) by removing carefully most of the water with a pipette. Eggs were rinsed with sterile sea water (3 x 10 sec) at the end of each treatment and sterile seawater was filled in each Petri dish. The disinfection routine was applied only the day of eggs collection. Control groups were treated with sterile seawater to simulate similar physical handling as treated groups. Petri dishes for were held at temperature controlled conditions at 12 ± 1 ºC.

It became apparent that formalin, even at the lowest tested concentration (200 ppm), could have a significantly negative impact on eggs hatching rate. Therefore, bronopol was chosen to be an adequate candidate for further experimentation.

In the preliminary experiment 2, eggs were gently removed from five different locations of the egg collection mat into three 500 ml glass beakers. From each beaker eggs were taken

to generate twelve petri dishes (30 – 40 eggs / Petri dish), from which nine were used to test bronopol concentration within three different times (after 15, 30 and 60 minutes) and three untreated petri dishes used as controls. Fertilisation rate was calculated individually for each Petri dish. Each concentration of bronopol was prepared in 1 l beakers at three concentrations, 12.5, 25 and 50 ppm. These were compared with the control (no treatment) with three (biological) replicates per treatment. Each solution was tested in triplicate and compared against a triplicate set of control Petri dishes containing 20 ml mechanically filtered through a filter unit at 0.2 μm hatchery sea water. Water in Petri dishes was replaced with the bronopol concentration or sterile seawater (for the control) during the treatment time (15, 30 or 60 minutes) by removing carefully most of the water with a pipette. Eggs were rinsed with sterile sea water (3 x 10 sec) at the end of each treatment and sterile seawater was filled in each Petri dish. The disinfection routine was applied once daily until hatching. Control groups were treated with sterile seawater to simulate similar physical handling as treated groups. Six eggs were randomly sampled in triplicate using a sterile set of tweezers after each treatment at day 0 and 5 for bacterial plating. Petri dishes for both egg development and bacterial plating were held at temperature controlled conditions at 11 ± 1 ^{o}C.

2.2.3 Experiment 1: *In vitro* concentration and contact time determination

Eggs were gently removed from five different locations of the egg collection mat into three 500 ml glass beakers. From each beaker eggs were taken to generate nine petri dishes (30 – 40 eggs / Petri dish), from which six were used to test bronopol concentration within two different times (after 30 and 60 minutes) and three untreated petri dishes used as controls (Fig. 2.1). Fertilisation rate was calculated individually for each Petri dish. Each concentration of bronopol was prepared in 1 l beakers at three concentrations, 50, 250 and 500 ppm. These were compared with the control (no treatment) with three (biological)

replicates per treatment. Each solution was tested in triplicate and compared against a triplicate set of control Petri dishes containing 20 ml mechanically filtered through a filter unit at 0.2 µm hatchery sea water Water in Petri dishes was replaced with the bronopol concentration or sterile seawater (for the control) during the treatment time (30 or 60 minutes) by removing carefully most of the water with a pipette. Eggs were rinsed with sterile sea water (3 x 10 sec) at the end of each treatment and sterile seawater was filled in each Petri dish. The disinfection routine was applied once daily until hatching. Control groups were treated with sterile seawater to simulate similar physical handling as treated groups. Six eggs were randomly sampled in triplicate using a sterile set of tweezers after each treatment at day 0 for bacterial plating. Petri dishes for both egg development and bacterial plating were held at temperature controlled conditions at 11 ± 1 ºC. Bacteria colonies were counted 60 h post plating.

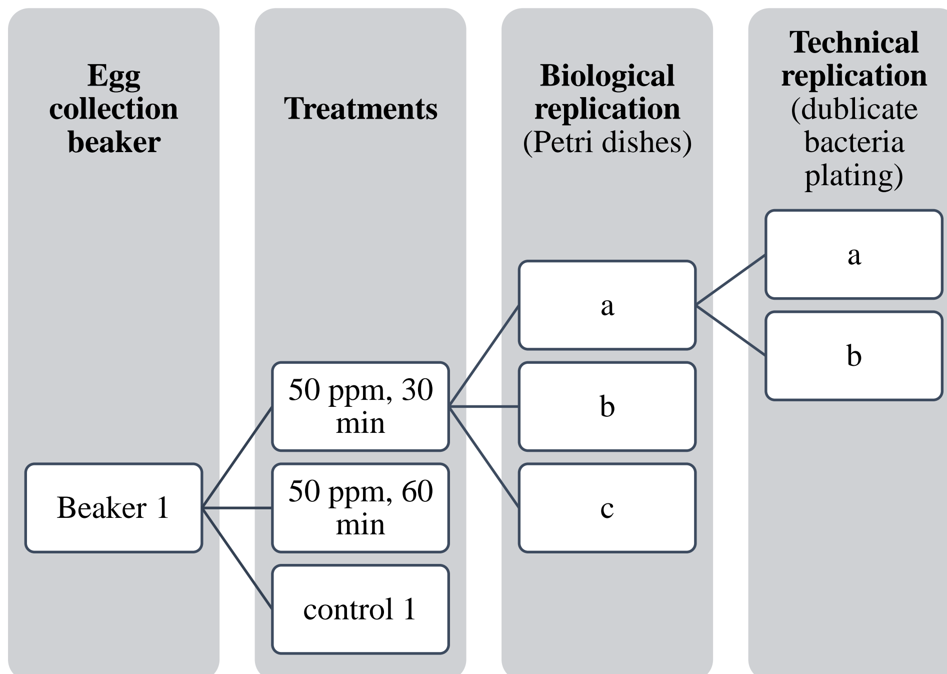

Figure 2.1: Example of experiment 1 sampling structure.

2.2.4 Experiment 2: Concentration and contact time determination in semi-commercial set up

This study was conducted in a semi-commercial system of 4 semi-conical 80 l tanks. The inlet hatchery water of the system was flow-through, running at a mean temperature of 11 ± 1 °C. One egg collection mat was removed from the broodstock tank and four pieces were cut off, with visually comparable egg density on them, of a size approximating 15 cm^2 each. A mat piece was submerged in each experimental tank. A small quantity of water was initially taken from the experimental tanks and well mixed with the total required amount of bronopol, targeting concentrations of 0, 50, 100 and 250 ppm within the incubation tank. During the treatments no water exchange was allowed, providing static conditions. At the end of the all exposure periods (i.e. 0, 30, 60, 120 or 240 min) tested, flow was turned back on allowing a full water exchange in 30 minutes, following producers' recommendations. Application of 25 ppm for 30 minutes was followed daily until hatching. Six eggs were randomly sampled in triplicate using a sterile set of tweezers after each treatment at day 0 for bacterial plating. Petri dishes for both egg development and bacterial plating were held at temperature controlled conditions at 11 ± 1 °C. Bacteria colonies were counted 60 h post plating.

2.2.5 Experiment 3: *In vivo* commercial application

Following the results of experiment 2, treatments with 100 and 250 ppm bronopol for 240 minutes resulted to significant reduction of the bacterial load whilst showing increased hatching and final yield results with respect to the untreated eggs. Treatment with 50 ppm, despite that also resulted reduction in the bacterial load, had similar final yield to the control. Therefore, in experiment 3 treatment with 100 ppm bronopol was considered the best candidate to be tested as an alternative to the current commercial practice, but also the

need for daily disinfection after day 0. This experiment was conducted in the same semi-commercial system as described above, comparing a control group (current hatchery protocol, day 0: formalin (formaldehyde solution 37 % w/w) 150 ppm for 60 min and daily bronopol 25 ppm for 30 min) and two experimental groups (100 ppm bronopol (day 0) for 240 min followed by either daily 25 ppm or every second day 50 ppm bronopol treatment for 30 min). Six eggs were randomly sampled in triplicate using a sterile set of tweezers after each treatment at days 0, 2 and 4 for bacterial plating. Oxygen levels was checked daily. Water samples (100 µl) were taken from each conical in triplicate on days 0, 2 and 4 prior to treatments to assess water bacterial load, where 100 µl tank water were mixed with 900 µl sterile seawater and 20 µl were plated on marine agar. Water temperature of the system was held at 13 ± 1 ºC. Bacteria colonies were counted 48 h post plating.

2.2.6 Bacterial plating

In each experiment, six eggs were sampled in triplicate before and after each treatment and washed gently twice for 10 sec with sterile seawater to remove any organic residuals. Eggs were then homogenised using a mechanised mortar and pestle, with added 400 or 800 µl sterile sea water. A total of 20 µl of the resulting homogenate was inoculated onto three replicate marine agar (Trypticase Soy Agar (TSA) + 2 % NaCl, Oxoid, UK) plates per treatment. Samples were incubated at 11 ± 1 ºC (experiments 1 and 2), 13 ± 1 ºC (experiment 3) and bacteria colonies were counted 48 h (experiment 3) and 60 h (experiments 1 and 2) post plating. Colony forming units (CFU) per ml were calculated as follows:

CFU ml^{-1} = (number of CFU in 20 µl drop / 20) x total homogenate volume

2.2.7 Hatching and survival assessment

The determination of hatching rate in the tanks in experiments 2 and 3 was conducted by adopting the commercial practice of mechanically stimulating (“scraping”) egg hatching at 75 degree days. After scraping eggs off the spawning mat, they were placed in a Petri dish and hatching rate was determined 10 minutes later. In experiments 2 and 3, all eggs were removed from the mats to determine survival and hatching rate. Hence these factors could not be tested statistically as only a total final count of eggs and larvae was available.

The survival, hatching and final yield percentages were calculated as follows:

Survival = ((swimming larvae + unhatched larvae) x 100) / (live larvae + unhatched larvae + dead eggs)

Hatching = (swimming larvae x 100) / (swimming larvae + unhatched eggs)

Final yield = (hatching / 100) x survival

2.2.8 Statistics

Prior to analysis, normality and homogeneity of variance were assessed and transformation was applied when required. Parameters assessed as a percentage were first subjected to arcsine transformation. Egg survival, hatching and number of bacterial colonies were compared using one or two way analysis of variance (ANOVA) and followed by a Dunnett’s *post hoc* test and compared with the control groups with 95% confidence, by using MINITAB® Release 17 (Minitab Ltd., UK). All data are expressed as mean ± standard deviation (SD). The level of significance was set at $P < 0.05$.

2.3 Results

2.3.1 Preliminary work

In the first preliminary experiment, static exposure to a dose of 25 ppm for 30 minutes resulted in a comparable survival and final yield with the control untreated group (Table 2.1). The rest of the treatments resulted to a decrease of the survival and final yield. No effects appeared in the hatching rate of the alive eggs between treated and untreated groups.

In the second preliminary experiment, no differences were found in the survival, hatching or final yield between the treatend and untreated eggs.

Application of both 12,5 and 50 ppm bronopol for 15, 30 and 60 minutes resulted in a statistically reduction of the bacterial load between treated and untreated eggs. Suprisingly, application of 25 ppm for 30 minutes resulted to an increase of the bacterial load, when the rest of the application times (15 and 60 minutes) show no difference (Fig. 2.2).

The preliminary work conducted showed high variation between bronopol treated eggs and it was considered more as natural variation, rather than treatment effect. Increased formalin treatments affected severely eggs survival and final yield, making bronopol the main candidate for the following trials.

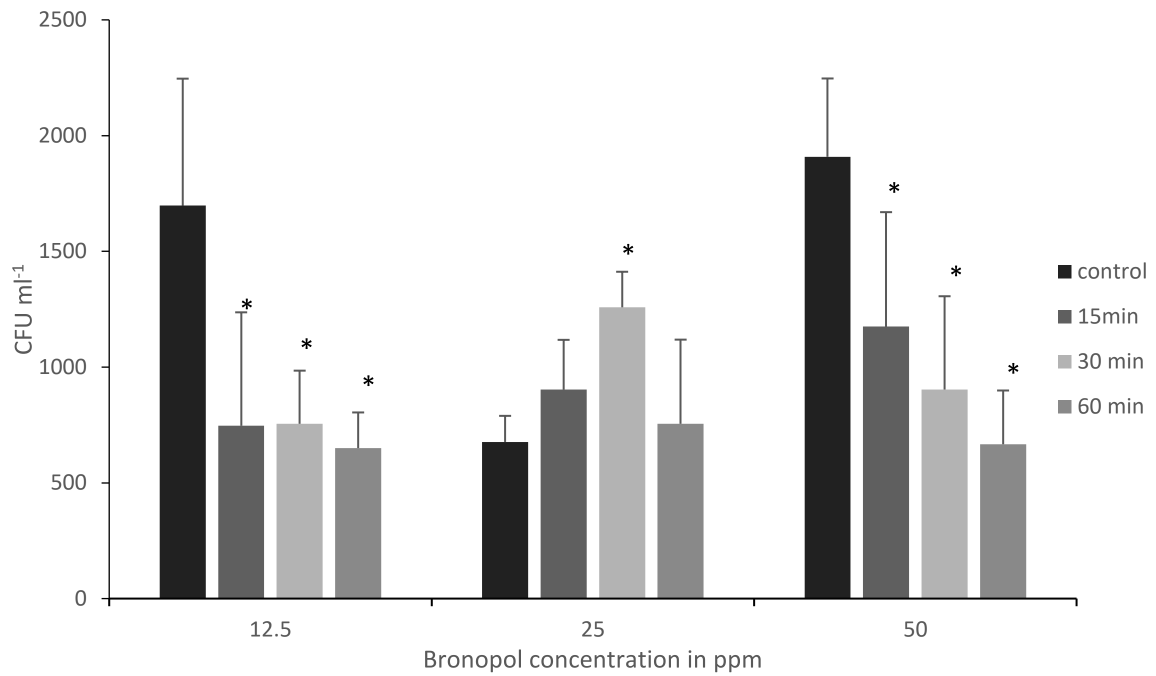

Figure 2.2: Bacteria counts of ballan wrasse eggs after exposed *in vitro* at thee concentration levels of bronopol (12.5, 25 and 50 ppm) and for three durations (15, 30 and 60 min) on day 0 post fertilisation. Data are presented as mean ± SD (n = 3) and asterisks denote significant differences between concentrations in comparison with the individual controls ($P < 0.05$).

Table 2.1: Survival and hatching rates of ballan wrasse eggs following preliminary disinfection treatments. Data are presented as mean ± SD (n = 3) and asteriscs denote significant differences between treatments and the control (no treatment) ($P < 0.05$).

	Treatment	Survival (%)	Hatching (%)	Final yield (%)
Preliminary experiment 1	No treatment	88.6 ± 12.7	93.9 ± 5.4	83.5 ± 15.7
	Bronopol 25 ppm for 30 min	88.1 ± 10.9	97.2 ± 4.8	85.7 ± 12.4
	Bronopol 50 ppm for 30 min	49.3 ± 9.3*	100 ± 0	49.3 ± 9.3*
	Bronopol 100 ppm for 30 min	47.7 ± 5.3*	100 ± 0	47.7 ± 5.3*
	Formalin 200 ppm for 30 min	50.4 ± 10.6*	95.8 ± 7.2	48.3 ± 11.3*
	Formalin 400 ppm for 30 min	8.2 ± 9.8*	33.3 ± 57.7	6.3 ± 11.0*
	Formalin 1000 ppm for 30 min	7.3 ± 3.9*	100 ± 0	7.3 ± 3.9*
Preliminary experiment 2	No treatment	71.7 ± 12.2	52.6 ± 22.4	38.0 ± 19.1
	Bronopol 12.5 ppm for 15 min	74.2 ± 11.6	33.5 ± 6.5	24.8 ± 6.3
	Bronopol 12.5 ppm for 30 min	80.8 ± 8.0	39.4 ± 32.4	30.4 ± 25.1
	Bronopol 12.5 ppm for 60 min	76.7 ± 8.0	22.5 ± 13.9	16.7 ± 8.8
	Bronopol 25 ppm for 15 min	71.4 ± 8.9	41.1 ± 10.4	29.9 ± 11.1
	Bronopol 25 ppm for 30 min	82.1 ± 9.9	39.4 ± 12.9	31.9 ± 9.7
	Bronopol 25 ppm for 60 min	62.0 ± 3.0	52.4 ± 19.9	32.1 ± 11.2
	Bronopol 50 ppm for 15 min	76.0 ± 7.9	42.6 ± 9.9	32.3 ± 7.4
	Bronopol 50 ppm for 30 min	56.2 ± 17.0	47.9 ± 37.0	22.8 ± 11.1
	Bronopol 50 ppm for 30 min	72.9 ± 2.6	70.8 ± 9.0	58.4 ± 10.0

2.3.2 Experiment 1: *In vitro* concentration and contact time determination

Static exposure to a dose of 250 ppm and 500 ppm bronopol for 60 minutes resulted in a statistically significant reduction in egg bacterial loading of 85 % and 75 % with respect to the control respectively (Fig. 2.3). Applications of 50 ppm bronopol for 30 and 60 minutes appeared to reduce the bacterial load, but not significantly (Fig. 2.3). Survival was lower in the 50 ppm for 60 minutes application compared to the control with no significant differences were observed between treatments and the control group in hatching rate and final yield. (Table 2.2). However, treatments with 250 ppm (for both 30 and 60 min) appeared to result in the highest final yield, although not significantly. Plate dublication of the same treatment (technical replicates) showed no differences amongst them, indicating that single plating is adequate for the following experiments.

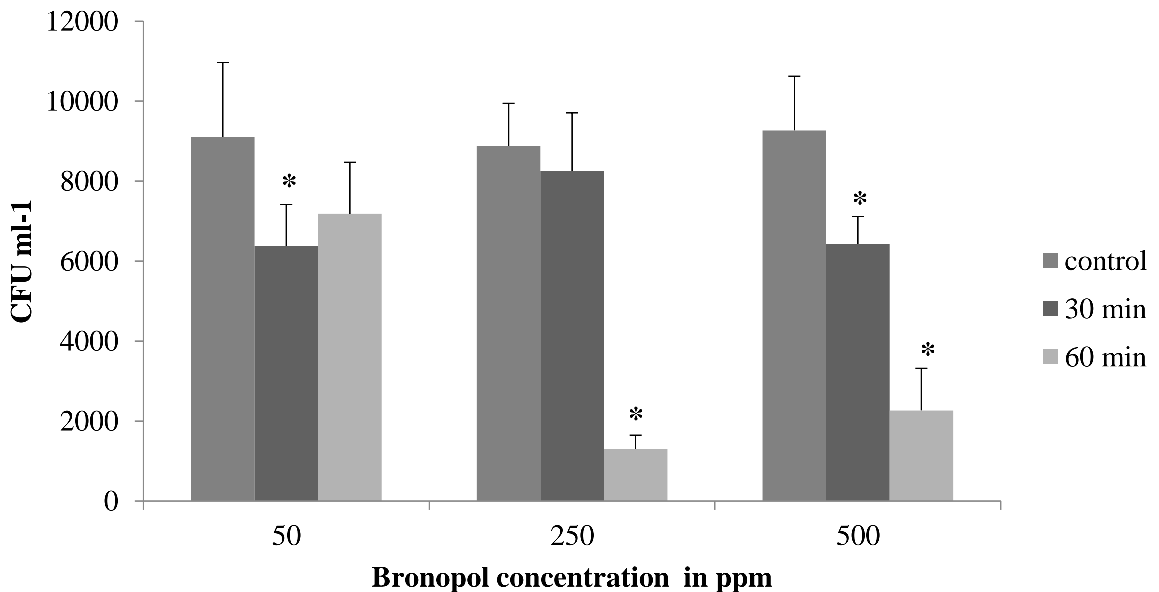

Figure 2.3. Bacteria counts of ballan wrasse eggs after exposed *in vitro* at thee concentration levels of bronopol (50, 250 and 500 ppm) and for two durations (30 and 60 min) on day 0 post fertilisation. Data are presented as mean ± SD (n = 3). Asterisks denote significant differences between concentrations in comparison with the individual controls ($P < 0.05$).

Table 2.2: Survival and hatching rates of ballan wrasse eggs following disinfection treatments. In experiment 1, data are presented as mean ± SD (n = 3) and asteriscs denote significant differences between treatments and the control (no treatment) ($P < 0.05$). In experiments 2 and 3, the total number of eggs was counted (experiment 2: 160 – 190 eggs per treatment; experiment 3: 180 – 220 eggs per treatment), hence survival and hatching rate could not be tested statistically.

	Treatment (bronopol)	Survival (%)	Hatching (%)	Final yield (%)
Experiment 1	No treatment	67.1 ± 12.4	89 ± 12.4	59.0 ± 11.9
	50 ppm for 30 min	60.6 ± 8.4	85.5 ± 6.0	51.8 ± 8.2
	50 ppm for 60 min	36.0 ± 8.7*	63.7 ± 31.7	23.3 ± 14.1
	250 ppm for 30 min	78.2 ± 9.0	91.0 ± 7.9	70.7 ± 2.9
	250 ppm for 60 min	79.0 ± 9.6	93.4 ± 6.2	73.4 ± 4.6
	500 ppm for 30 min	59.6 ± 2.4	100.0 ± 0.0	59.6 ± 2.4
	500 ppm for 60 min	65.6 ± 2.5	100.0 ± 00	65.6 ± 2.5
Experiment 2	No treatment	90.8	79.8	72.4
	50 ppm for 240 min	85.5	81.6	69.7
	100 ppm for 240 min	83.3	97.5	81.3
	250 ppm for 240 min	81.3	98.5	80.0
Experiment 3	No treatment	82.3	88.4	72.7
	Commercial practice (day 0: formalin 150 ppm : 60 min, followed by daily bronopol 25 ppm : 30 min)	79.1	95.6	75.6
	Bronopol daily application (day 0: 100 ppm : 240 min followed by daily 25 ppm : 30 min)	87.0	98.4	85.6
	Bronopol daily application (day 0: 100 ppm : 240 min followed by every 2nd day 50 ppm : 30 min)	80.4	95.8	77.1

2.3.3 Experiment 2: Concentration and contact time determination in semi-commercial set up

With the exception of the control treatment, there was a significant reduction in bacterial loading on the eggs at the end of the study (240 min) with the 100 and 250 ppm treatment resulting in > 90 % reduction in bacterial load compared to control while 50 ppm resulted in 80 % reduction compared to the control (Fig. 2.4). Additionally, significant bacterial load reduction appeared in eggs exposed to: [50 ppm : 120 and 240 min] ; [250 ppm : 30 and 120 min]. Treating eggs with 100 ppm for 60 or 120 minutes and with 250 ppm for 60 minutes elicited a considerable reduction of the bacterial load but not significant ($P = 0.06$) (Fig. 2.4). Doses and durations of bronopol exposure did not affect egg survival in treated eggs, ranging between 81.3 and 85.5 % compared to untreated group (90.8 %) (Table 2.1). Hatching rate suggested a positive relation to the bronopol concentration with hatching of the untreated eggs at 79.8 %, when the treated eggs had 81.6, 97.5 and 98.5 % hatching rate (50, 100 and 250 ppm bronopol treatment respectively) (Table 2.1).

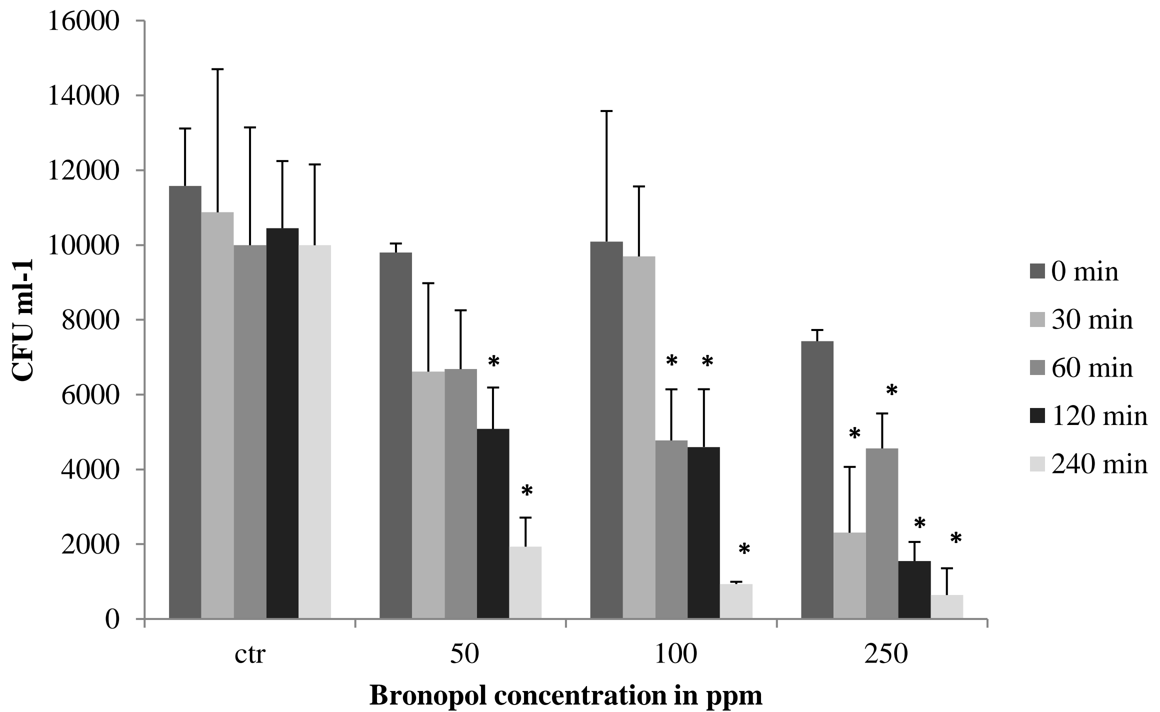

Figure 2.4. Bacteria counts of ballan wrasse eggs treated with three different concentrations of bronopol (50, 100 and 250 ppm) for four different durations (30, 60, 120 and 240 min) at day 0 post fertilisation. Data are presented as mean ± SD (n =3). Asteriscs denote significant differences between bronopol concentrations for a given exposure time and the individual untreated group ($P < 0.05$).

2.3.4 Experiment 3: *In vivo* commercial application

In this experiment, the absolute CFU levels were not comparable between the individual controls of each treatment; therefore data was normalised to the individual controls to allow a meaningful comparison of the treatment effect. Total reduction of bacterial load was achieved after all treatments by day 4, including the control group. Following the commercial protocol, no significant reduction in bacterial level was observed on day 0, however by day 2 (prior to any further treatment) the bacterial load was significantly lower compared to day 0 and reached almost 100 % reduction on day 2 after the bronopol treatment (Fig. 2.5). Both treatments using exclusively bronopol, showed an effective

bactericidal effect (> 90 % reduction in bacterial loading) of the initial 100 ppm after 240 minutes on day 0 and reduced the bacterial load after the first treatment on day 0 (Fig. 2.5). The daily application of 25 ppm bronopol maintained the bacterial reduction on day 2 in contrast to 50 ppm. Similarly, on day 4, the 25 ppm treatment appeared to elicit a higher bacterial reduction compared to the 50 ppm treatment, although not significant. Eggs in the control tank showed no reduction of the bacterial load on day 2, but an 89 % reduction occurred on day 4. No significant bacterial growth (< 200 CFU / ml) was detected in any of the water samples collected from the tanks (Data not shown).

Survival to hatch for the disinfected eggs was 79.1, 87.0 and 80.4 % for the commercial protocol, treatment 1 and 2, respectively) compared with 82.3 % for the untreated eggs (Table 2.1). Hatching was similar for the treated groups (96.6 %), slightly reduced (not significantly) for the untreated group (88.4 %) (Table 2.1).

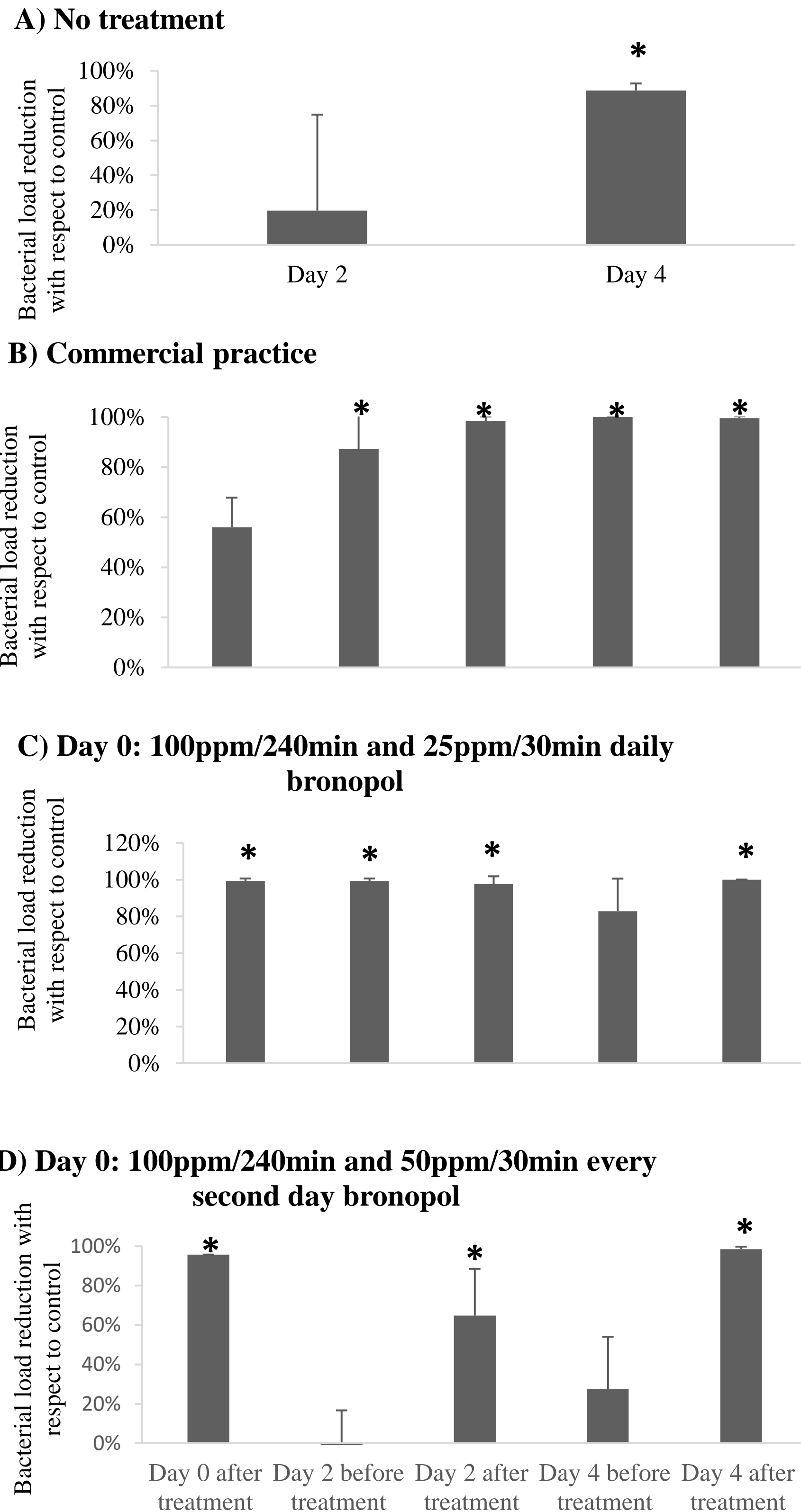

Figure 2.5. Bacterial load reduction over the incubation period when eggs exposed to different bacterial control strategies ((A) no treatment; (B) commercial practice (day 0: formalin 150 ppm for 60 minutes and daily bronopol 25 ppm for 30 min); (C) day 0 : 100

ppm bronopol for 240 minutes followed daily 25 ppm for 30 min; (D) day 0 : 100 ppm bronopol for 240 min followed every second day 50 ppm bronopol treatment for 30 minutes. Samples obtained before and after treatment on days 0; 2 and 4 with respect to the untreated eggs of the each group at day 0. Data are presented as mean ± SD (n = 3). Asterisks denote significant bacterial load reduction in respect to prior-treatment egg bacterial load of the same group ($P < 0.05$).

2.4 Discussion

Egg chemical disinfection is considered an important biosecurity practice in marine finfish hatcheries, targeting control of bacterial infections to ultimately increase final yield of eggs. Results from the present study clearly confirmed the bactericide action of bronopol when used to disinfect ballan wrasse eggs. Interestingly, not only higher doses (up to 500 ppm) and increased exposure time (up to 240 mins) did reduce bacterial load to minimal levels but it also appeared to not negatively impact on survival, hatching success or final yield and even showed improvements in some cases. It should be acknowledged, that given the limited egg availability, experiments 2 and 3 were each performed in four experimental tanks allowing generation of triplicate samples from each tank. This replication can be considered more as "technical" rather than "biological" and results should be extrapolated with caution with further investigation required to verify these conclusions.

Most of the chemical treatments currently used commercially to prevent and control bacteria proliferation in aquaculture (i.e. formalin, glytaraldehyde, iodine, ozone, paracetic acid and copper sulphate) have proven toxicity and can and impact egg survival (Salvesen et al., 1997; De Swaef et al., 2015). The use of such treatment should therefore be optimised and new treatments with reduced potential toxicity should be deployed when

possible. Bronopol is being widely used as a broad-spectrum anti-bacterial and anti-fungal chemical in food production and water disinfection. It has also proven efficacy in freshwater and cold water finfish species, including Nile tilapia (*Oreochromis niloticus*), Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Jantrakajorn & Wongtavatchai 2015; Treasurer et al., 2005; Birkbeck et al., 2006). In addition, bronopol is very unlikely to bioaccumulate, due to its short-lived environmental persistence (De Swaef et al., 2015). Therefore, bronopol was considered as the main candidate in the present study for egg disinfection in ballan wrasse hatcheries.

No bactericidal effect was detected in low concentration treatments of 50 or 100 ppm with less than 120 minutes application, but 250 ppm caused significant reductions in bacterial loads after 30 minutes. Following the initial treatment on day 0, it became apparent (experiment 3) that a low dose daily treatment (25 ppm for 30 min) is also required to suppress bacterial growth until hatching. Nevertheless, the use of long term and low dose bronopol exposure strategy might have the potential to select for resistant strains (Shinn et al., 2012). The results of this study are in agreement with the findings of Birkbeck et al. (2006) who determined the minimum inhibitory concentration (MIC) of bronopol, by using 13 bacteria isolates from halibut and cod eggs, at 64 ppm MIC (for all tested bacteria). In addition the same study showed treatment with 200 ppm bronopol had significant bactericidal effect (killing > 90% of bacteria) when used for 30 min and > 99.99 % of bacteria within 2 hours, when exposure of lower concentrations (100 ppm) showed no bactericidal effect within 1 hour. Importantly, Birkbeck et al. (2006) study was performed at lower water temperature than the present study (10 ^{o}C vs. 11 – 13 ^{o}C), which could explain differences found on bronopol efficacy which is positively related to temperature (Shepherd et al., 1988).

Surprisingly, the bacterial levels in experiment 3 were found significantly reduced (89 %) in the untreated eggs after four days of incubation. At the same time, all treated eggs had a total elimination of the bacterial load. One explanation could be that the use of filtered water, high water exchange and aeration during the incubation period, have been potentially maintaining low bacterial load on the untreated eggs. Similar fluctuations of bacterial load during egg incubation has been recorded by Komar et al. (2004) in bullseye puffer (*Sphoeroides annulatus*) eggs incubated in different levels of treated water (filtration and ultraviolet (UV) sterilisation) with no bacterial load differences occurring between treatments, but bacterial levels exhibiting high fluctuations during the incubation (in a similar pattern for all treatments). The reduction of bacterial load in control untreated groups in conjunction with the lack of significant effect of disinfection on final yield could advocate that additional egg disinfection could be unnecessary. Importantly, ballan wrasse eggs are surrounded by an adhesive gum layer (D'Arcy et al., 2012; Grant et al., 2016a,b) which may have antibacterial properties explaining also the reduced bacterial load in untreated eggs. This would agree with the hypothesis that the cherry salmon (*Oncorhynchus masou*) egg outermost fertilisation layer envelope may have the ability to protect the egg against bacteria, fungi and noxious materials (Kudo & Yazawa 1997). If this was proven in ballan wrasse, the egg degumming method proposed by Grant et al. (2016b) applied in ballan wrasse eggs could potentially be detrimental by increasing their exposure to bacteria. The antibacterial properties of the gum layer could also vary depending on bacterial strains present in the water. Moreover, the guarding behaviour of fringed darter (*Etheostoma crosspterum*) male can inhibit microbial colonisation of eggs by coming in contact or close proximity contributing antimicrobial properties of body mucus, as an effective component of parental care (Knouft et al., 2003). Despite that currently no report states the level of parental care in ballan wrasse, the strong territorial

behaviour observed in males (Sjölander et al., 1972) might imply such a natural prevention of microbial colonization in eggs. The present study assessed the overall bacterial load and bacteria strains identification was not within its scope. Despite the significant reduction of bacterial numbers on egg surface, survival rate of treated eggs was generally similar to untreated eggs, implying that the presence of bacteria on incubating egg surfaces is not detrimental to survival. This contrasts with previous studies which reported significant increase of cod egg survival (83 – 89 %) treated with a single dose of bronopol (immersion at 50 ppm to 500 ppm for 45 s) compared to the untreated groups survival (48 %) (Treasurer et al., 2005). However, the same study did not show a higher survival in haddock (*Melanogrammus aeglefinus*) eggs treated with bronopol (71.6 % and 62.4 % survival in treated and untreated eggs, respectively).

The current study showed that bronopol treatment at a dose > 100 ppm appeared to have a positive effect on egg hatching rate and final yield. In experiment 1, no significant differences were observed between treatments, although eggs treated with the highest dose (500 ppm) showed 100 % hatching rate in comparison compared to 89 % in the untreated eggs. In experiments 2 and 3, the total number of eggs was counted, hence survival could not be tested statistically. Overall, hatching rate was increased by 9 to 21 % in treated eggs compared to the untreated eggs. No effects on hatching time was observed at the highest doses of bronopol (500 ppm) suggesting bronopol did not impact on embryonic development. Similarly, in crayfish (*Pacifastacus leniusculus)*, bronopol was shown to not have any effects on hatching rates even at doses up to 5,000 ppm (González et al., 2013). Similar results were reported in rainbow trout (*Oncorhynchus mykiss*) egg disinfected with oxolinic acid with a clear reduction of egg surface bacteria, but no effects on hatching (Barker et al., 1990). Survival and hatching rates of the eggs treated with 50 ppm bronopol for 60 min (experiment 1) were unexpectedly low (36 ± 8.7 % and 63.7 ± 31.7 %,

respectively). Many other factors could affect egg survival and hatching, such as flow rate, dissolved oxygen, pH or temperature (Barker et al., 1989).

Daily application of bronopol offered a 16% higher final yield compared to the untreated group (experiment 3). This percentage increase can have a considerable impact on the production of a new aquaculture species, such as ballan wrasse. Assuming that an egg incubation system for ballan wrasse eggs consists of two 3.25 m^3 tanks with egg disinfection taking place the first day of collection (100 ppm), followed by daily application until hatch (25 ppm in daily basis) and the hatchery produces eggs every day during three photoperiod regimes (2 months each), this will required daily use of 325 ml (100 ppm) and 81 ml (25 ppm) of bronopol or 650 ml and 162 ml of Pyceze (the commercially available of bronopol). This will result in requirement of 146 l Pyceze (180 days x 812 ml) per spawning season, with a commercial value of approximately £4,800 (£33 / l of Pyceze).

Taken together, the present study developed and validated a commercial egg disinfection protocol for ballan wrasse hatcheries using bronopol, which offers an alternative to the commercial practice of formalin use. Concentration of 100 ppm for 240 min on day 0 of egg incubation followed by daily treatment with 25 ppm for 30 min is recommended to disinfect eggs of farmed ballan wrasse to supress bacterial growth without affecting egg survival and egg hatching rate. Potential antimicrobial property of the egg gum layer is suggested which warrants further investigations. A way that these properties could be investigated is by following the degumming procedure proposed by Grant et al. (2016b) and compare the bacterial load of a ballan wrasse egg before and after the degumming procedure, but also follow up the effects on the survival and development of newly hatched larvae. Also identifying the bacteria strains present that could have not been possibly cultured using the methods of this study, by using molecular techniques such as 16s rDNA

sequencing identification. This will allow to determine where loses appear at the next life stages could be related to egg incubation. In addition, given the variability of the bacterial loads between the control groups and lack of biological replicates in parts of this study, future studies should validate egg disinfection experiments by assessing protocols in different egg batches and increasing the robustness of the results by utilising as many biological replicates as possible.

Chapter 3: Effects of a commercial *Bacillus* sp. probiotic mix and bronopol on survival, growth and bacterial load in ballan wrasse (*Labrus bergylta*) larvae.

3.1 Introduction

Bronopol action in ballan wrasse eggs has been proved in chapter 2, offering significant antibacterial effects on egg surface. The need for daily application became apparent to maintain reduced bacterial levels, although no evidences show the necessity of it to improve or even maintain egg survival or hatching. Nonetheless, given the high losses at the later early larval stages, microbial control needs to be further investigated, as being a potential cause of the increased larval mortalities in ballan wrasse larviculture.

Bacterial control at the early fish larval stages is a key parameter for finfish hatcheries successful production. Following the egg disinfection study in ballan wrasse in chapter 2, the next hatchery stage is the understanding and control of bacteria at the early larvae stages. Bacteria populations play in important role both in egg and larval stages in finfish culture (Hansen & Olafsen, 1999). Larval rearing protocols for commercial marine fish larvae have been improved over the last decades through better understanding of the species-specific nutritional and husbandry requirements (Vadstein & Skjemo, 1993; Hansen & Olafsen, 1999; Kolkovski, 2001; Hamre et al., 2013b). While wild caught ballan wrasse has been used since the 80's as a cleaner fish to control sea lice in the Atlantic salmon cages, it can be considered as a new species in aquaculture. High survival fluctuations with often very low values are commonly observed during the early life stages in cold water marine species, such as Atlantic cod (5.2 – 42 % between 0 – 27 dph; Lauzon et al., 2010), Atlantic halibut (0 – 75 % between 0 – 51dph; Bjornsdottir et al., 2011) and turbot (77.8 – 91.1 % between 0 – 80dph; Dagá et al., 2013). Ballan wrasse production also shows high survival variation amongst batches during the early stages, but still being an aquaculture species in its infancy the survival does not often exceed the 10 % (David Patterson, Otter Ferry Seafish, pers. comm.). These losses have been attributed to bacterial infections due to the lack of microbial control in the first feeding tanks.

Live feed is in general a major source of bacteria introduction to the larvae rearing tanks (Vadstein & Skjemo, 1993; Blancheton et al., 2013). Following the commercial hatchery protocol at Otter Ferry Seafish, intensive ballan wrasse larvae conditions requires live feeds until approximately 90 days post hatch (dph) and includes addition of rotifers (*Brachionus plicatilis*) (until 25 dph) and brine shrimp (*Artemia salina*) (until 90 dph) (David Patterson, Otter Ferry Seafish). Microbial control of the eggs, larvae and their rearing environment is therefore a priority for the success for the commercial exploitation of ballan wrasse.

Bacteria ingestion can start from as early as the yolk sac stage resulting in the establishment of a primary intestinal microflora which persists beyond first feeding. In order to osmoregulate, fish larvae start “drinking” before the yolk sac is consumed and bacteria thus enter the digestive tract before active feeding commences (Hansen & Olafsen, 1999). So far, very little on ballan wrasse microflora has been published with *Vibrio* species (*Vibrio splendidus, Vibrio ichthyoenteri and Vibrio pacinii*) isolated from the digestive tract microflora of 150 dph larvae (Birkbeck & Treasurer, 2014). Two commonly used strategies to achieve microbial control at the early larval stages in fish are selective through the addition of probiotics and non-selective through the reduction of bacteria and water disinfection (Vadstein & Skjemo, 1993; Skjermo & Vadstein, 1999).

Probiotic addition as a selective enhancement of bacteria has various applications in hatcheries including the modulation of interactions with the tank environment, improvement of larval immune system, improvement of water quality and stimulation of larval digestive system (Lauzon et al., 2014). Inclusion of probiotics may confer benefits to the host by providing both nutritional benefits and protection against pathogens as suggested in many other species including turbot (*Scophthalmus maximus*) (Gatesoupe, 1991; Gatesoupe, 1994), pollack (*Pollachius pollachius*) (Gatesoupe, 2002), Atlantic cod

(Sveinsdóttir et al., 2009) and Eurasian perch (*Perca fluviatilis*) (Mandiki et al., 2011). *Bacillus* sp. is a spore-forming bacteria, which enables longer term survival in condition that could otherwise be detrimental to the vegetative cell (Nicholson et al., 2000). Nevertheless, *bacillus spp.* can germinate under favourable conditions with appropriate nutrients and water (Moir, 2006). The efficiency of *Bacillus* sp. has been tested, amongst *o*thers, in rainbow trout (*Oncorhynchus mykiss*) to improve protection against multiple pathogens including *Aeromonas* (Newaj-Fyzul et al., 2007) and in European sea bass against *Vibrio* (Touraki et al., 2012).

Bronopol (2-bromo-2-nitro-1,3 propanediol) can be used as Pyceze™ (Novartis Animal Health UK), a commercial product licensed in the UK containing 50 % W/V bronopol initially used against fungal infections in Atlantic salmon and rainbow trout. Bronopol offers a proven efficiency against fungi (Pottinger & Day, 1999; Oono & Hatai, 2007) but also parasites (Picón-Camacho et al., 2012; Shinn et al., 2012) and bacteria (Treasurer et al., 2005; Birkbeck, 2006). Antibacterial action of bronopol is believed to act through catalytic oxidation of accessible thiols and creation of free radicals causing cell death (Shepherd et al., 1988). Another agent used to control microbial load in marine fish hatchery tanks is clay. Recent studies have shown that addition of ceramic clay can have beneficial effects on larval performance which can be attributed to the increase of turbidity and reduction of microbial load by binding to organic matter and bacterial cells (Clayton et al., 2010; Bjornsdottir et al., 2011; Attramadal et al., 2012a; Stuart et al., 2016). Various larviculture protocols recommend the use of turbid conditions to improve larval performance. To the author's knowledge, the administration strategy and actions of probiotics and bronopol have not yet been reported in ballan wrasse.

The aim of this study was to assess the effect of different bacterial management strategies on survival, growth and bacterial load in early larval stages of ballan wrasse. Three

different treatments were tested using either: (1) a probiotic commercial product or (2) bronopol or (3) mixture of both, in addition to clay.

3.2 Materials and Methods

3.2.1 Experimental fish and system

This 25-day study was performed at Otter Ferry Seafish, United Kingdom, between the 3rd and 26th of August 2015. Eggs were collected from natural spawning of captive ballan wrasse broodstocks kept captive for over 5 years. Broodstocks were housed in 6 m^3 tanks equipped with recirculation filtration systems at constant temperature of 12 ± 0.8 ºC. Artificial seaweeds made from green plastic sheets were introduced to the tanks to simulate species natural habitat. Fish were fed daily to satiation (approximately 3% of fish biomass) with mussels (*Mytilus edulis*), langoustine (*Nephrops norvegicus*) tails and an extruded commercial pellet (Symbio Wrasse Diet, 6.5 mm diameter; BioMar^Ltd, Grangemouth, Scotland, UK).

Short pile mats were provided as a spawning substrate for females to lay adhesive eggs on and covering approximately 50 % of tank's bottom area. Egg collection mats were removed daily in the morning and held in a 1 m^3 tank with gentle aeration until the start of the experiment. The eggs used for the experiment had a fertilisation rate was 93 ± 2 % and hatching rate estimated at 90 ± 8 %, as routinely done in the commercial hatchery (as described in chapter 2). The egg holding system was under constant illumination with flow-through water, 15 µm sand filtered, 10 µm absolute filtered and UV 200 mJ / cm^3 water supply. Eggs were disinfected according to the commercial protocol of Otter Ferry Seafish, with 150 ppm formalin for 60 minuts at egg collection day, followed by daily application of 25 ppm bronopol for 30 minutes. The eggs were mechanically induced to

hatch at 80 degree days (dd) and the newly hatched larvae were allocated to a commercial 3 m^3 larval rearing tank before their transfer to the experimental system.

The experimental system consisted of 12 x 40 L black flat-bottom tanks and newly hatched larvae (0 dph) were stocked at a volumetrically estimated initial density of 186 larvae/L. The tanks had a flow though water supply with similar level of filtration as described above. Water was introduced at the top of the tank at the rate of 18 % water exchange (0.12 L/min) from 0 dph to 23 dph, increased to 24 % (0.16 l min^{-1}), according to the commercial protocol at Otter Ferry Seafish. Temperature was recorded daily and averaged 13.1 ± 0.7 ^oC throughout the experiment.

The feeding regime was adopted from the commercial ballan wrasse protocol at Otter Ferry Seafish (Fig. 3.1). The larvae were fed with rotifers *Brachionus plicatilis* four times a day (at 9:00 am, 12:00 am, 04:00 pm and 10:00 pm) from 6 to 25 dph at initial daily density of 4 rotifer/ml gradually increased to 12 rotifer/ml previously enriched with ORI-GO (Skretting, France). From 22 dph onwards, *Artemia franciscana* nauplii was co-fed to the tanks three times a day (at 9:00 am, 04:00 pm and 10:00 pm) at a daily density of 2 nauplii / ml previously enriched with LARVIVA Multigain (BioMar, Denmark). The experiment was terminated at the beginning of *Artemia*-feeding period.

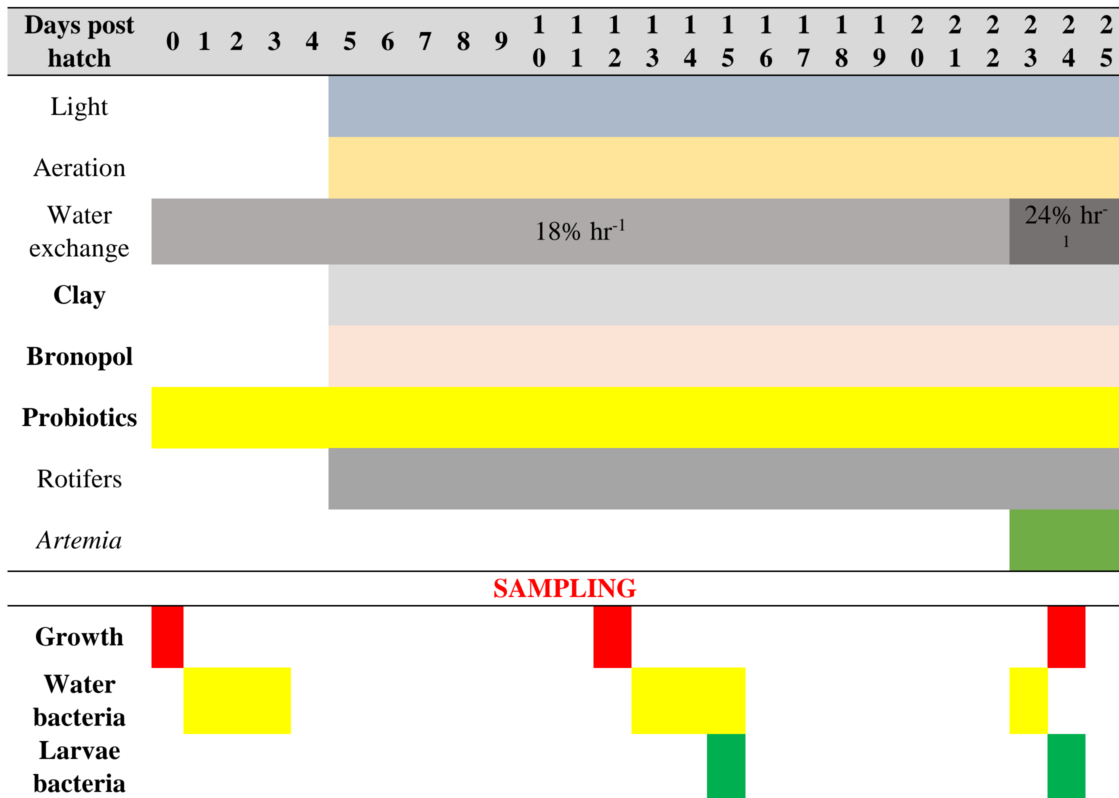

Figure 3.1. Schematic representation of the rearing conditions and sampling times during the 25-day experiment.

3.2.2 Experimental treatments and sampling

Four treatments were tested in triplicate: (1) a commercial *Bacillus* sp. mix (PR) (Sanolife MIC-F, INVE Technologies, Belgium) with 7.5 g m^3 added to the larval rearing tank (according to manufacturer's recommendations) at 8 am, (2) bronopol 25 ppm (BR) (used as Pyceze^{TM}, bronopol 50 % (W / V), Novartis Animal Health UK) added at 9 pm, (3) a combination of (1) and (2) (PR+BR) added at 8 am and 9 pm, respectively, and (4) control group (CTR) with no additional treatments. Ceramic clay was also added in all tanks across treatments at a density of 1.6 g/tank at 9 pm, to mimic hatchery protocol at Otter Ferry Seafish.

3.2.3 Growth and survival assessment

Larval growth was measured at 0, 12 and 24 dph on subsamples of 10 larvae per tank (30/treatment). Larvae were euthanized with MS-222 overdose (400 ppm; Pharmaq^{Ltd}, Hampshire, UK) and photos were taken using a computer controlled digital microscope camera (GXCam3, GT Vision, Suffolk, UK) fitted onto a stereo microscope (GX Stereo microscope, XTL3T, GT Vision, Suffolk, UK). Total length (TL, from the tip of the jaw to end of the tail), standard length (SL, from the tip of jaw to the end of spinal cord), myotome height (MH, from the posterior to the anus) and eye diameter (ED) were determined according to Fletcher *et al*. (2007) to the nearest 0.1 mm. Initial sampling was from a pool of larvae (5 samples of 10-20 larvae per sample), and terminal sampling was with larvae pooled in groups of 10 (3 pools per tank; 9 per treatment) and stored at -20 ^{o}C until freeze drying them to determine the dry weight (DW) to the nearest 0.001 g. At the end of the experiment (24 dph) all larvae were manually counted to determine survival in each tank.

3.2.4 Bacterial analyses

Samples for bacteriological analyses of the water and the larvae were taken randomly in triplicate from one tank of each treatment (the same in every time point). Water samples were taken at 2, 4, 13, 14, 15, 16 and 23 dph before the morning and night live feeds and water treatments, at 7:30 am and 8:30 pm. Samples were platted on Thiosulfate-citrate-bile salts-sucrose (TCBS) (Oxoid, UK) for enumeration of presumptive *Vibrio sp.* and marine agar (Tryptone Soy Agar + 2 % NaCl) for enumeration of all marine heterotrophs. The TCBS petri dishes were incubated at 35 ^{o}C and the marine agar at 12 ^{o}C to ensure growth of psychrophilic bacteria. The colony forming units (CFUs) were counted after 24 h (TCBS) and 72 h (marine agar) after-plating.

Water bacterial levels were analysed by mixing 950 µl of autoclaved sea water with 50 µl of water from each tank. Samples of 20 µl from each tank were plated onto the agar types mentioned above. Larvae (7 larvae per sample; 21 larvae per treatment) were transferred to a solution of 0.1 % benzalkonium chloride for 30 sec, rinsed in autoclaved water to remove the surface bacteria (Munro et al., 1994) and homogenate using a mechanised mortar and pestle in 1 ml of autoclaved seawater. Subsamples of 20 µl from the resulted homogenate and serial dilution were plated in the agar types mentioned above.

3.2.5 Bacterial strains identification

Colonies from the bacteria cultures showing distinct morphologies were sampled and stored in 95 % ethanol for later 16S rDNA sequencing identification. Samples were transferred in PCR tubes with 20 µl of ddH_2O, placed in a thermal cycler (Biometra Gradient, Germany) programmed for 10 min at 95 ^oC and then centrifuged for 1 minute at 21,000 rpm. The supernatant (boil prep.) was used for the PCR. A commercially prepared master mix, MyTaq^{TM} HS Mix (Bioline Reagents$^{Ltd.}$), containing $MgCl_2$, PCR buffer and specific *Taq* enzyme, was used to reduce non-specific amplifications. Each reaction was consisting of 0.3 µl boil prep., 3.75 µl MyTaq^{TM} HS mix, 0.3 µl of each forward and reverse primer, Bac16s_341F (CCTACGGGNGGCWGCAG) and Bac16s_805R (GACTACHVGGGTATCTAATCC), and 2.85 µl ddH_2O. The total reaction volume of 7.5 µl was placed in a thermal cycler (Biometra Gradient, Germany) programmed for 1 min at 95 ^oC, followed by 35 or 40 cycles of 15 sec at 95 ^oC (denaturation), 15 sec at 62 ^oC (annealing), 20 sec at 72 ^oC (extension) and terminated with 2 min at 72 ^oC (final extension). Samples were then held at 20 ^oC. The size of the PCR products was verified using 1µl of the reaction volume on a agarose gel (1% agarose gel in 1 x TAE (Trisma Base-Acetic Acid-EDTA)) buffer were run at 60 volts for 5 minutes followed by 90 Volts for 30 minutes to visualize yield for size and purity. To purify DNA, prior to sequencing, a

rapid PCR clean-up enzyme set (New England BioLabs; E2622) product was used by adding 1 μl of each enzyme (Exonuclease 1 and Rasp) to the 5 μl of DNA product. The total volume of 7 μl was placed in a thermal cycling conditions programmed for 5 minutes at 37 °C (incubation time) and 10 minutes at 80 °C (heat activation). The amplified DNA fragments of each strain were sequenced by GATC Biotech (Germany) using both the forward and reverse primers. The sequencing results were used for homology searches by standard Nucleotide BLAST (Basic Local Alignment Search Tool), https://blast.ncbi.nlm.nih.gov, focusing on database for 16S ribosomal RNA sequences.

3.2.6 Statistical analyses

Prior to analysis, normality and homogeneity of variance were assessed and datasets were transformed when required (square-root, logarithmic or power transformation). Parameters assessed as portion of percentages were first subjected to arcsine transformation. Statistics in water and larvae bacterial samples could not be performed due to single biological replicates. Morphometrics, dry weights and survival were compared using one way ANOVA. All ANOVA analyses followed by a –Dunnet's *post hoc* test and compared with the control untreated groups with 95 % confidence, by using MINITAB® Release 17 (Minitab Ltd., UK). All data are presented as mean ± standard deviation (SD).

3.3 Results

3.3.1 Survival

The survival was significantly higher only for the bronopol and probiotics treated group (4.4 ± 1.6 %) compared to the control group (0.7 ± 0.1 %) (Fig. 3.2).

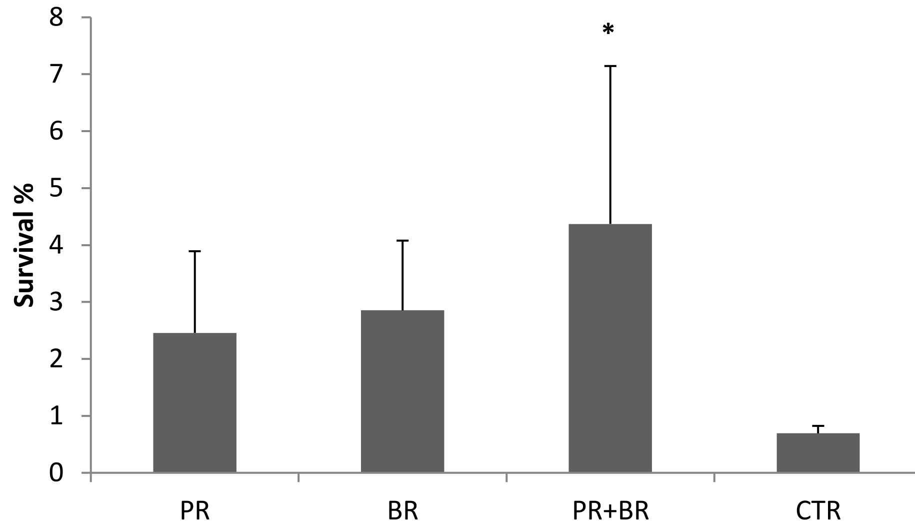

Figure 3.2. Effect of probiotic mix (PR), bronopol (BR), probiotic mix together with bronopol (PR+BR) and no supplementation (CTR) on the survival of ballan wrasse larvae from hatching to the end of the 25-day experiment. Data are presented as mean ± SD (n = 3). Asterisk indicates significant differences in comparison with the control group.

3.3.2 Growth

3.3.3 Dry weight and Morphometric analyses

At the end of the experiment, no significant differences in larvae dry weight were observed between treatments with 262 ± 144 µg, 471 ± 698 µg, 403 ± 489 µg and 450 ± 517 µg for the PR, BR, PR+BR and CTR treatments, respectively (Data not shown). CTR larvae had significantly greater standard length (5.38 ± 0.76 mm) and condition index (8.51 ± 1.67) than all other treatments and myotome height (0.47 ± 0.15 mm) comparable to the BR larvae (0.43 ± 0.12 mm), but higher to PR + BR and PR (0.40 ± 0.10 mm and 0.42 ± 0.11 mm respectively) (Fig. 3.3.). No differences were found in larvae eye diameter amongst treatments (0.43 ± 0.01 mm).

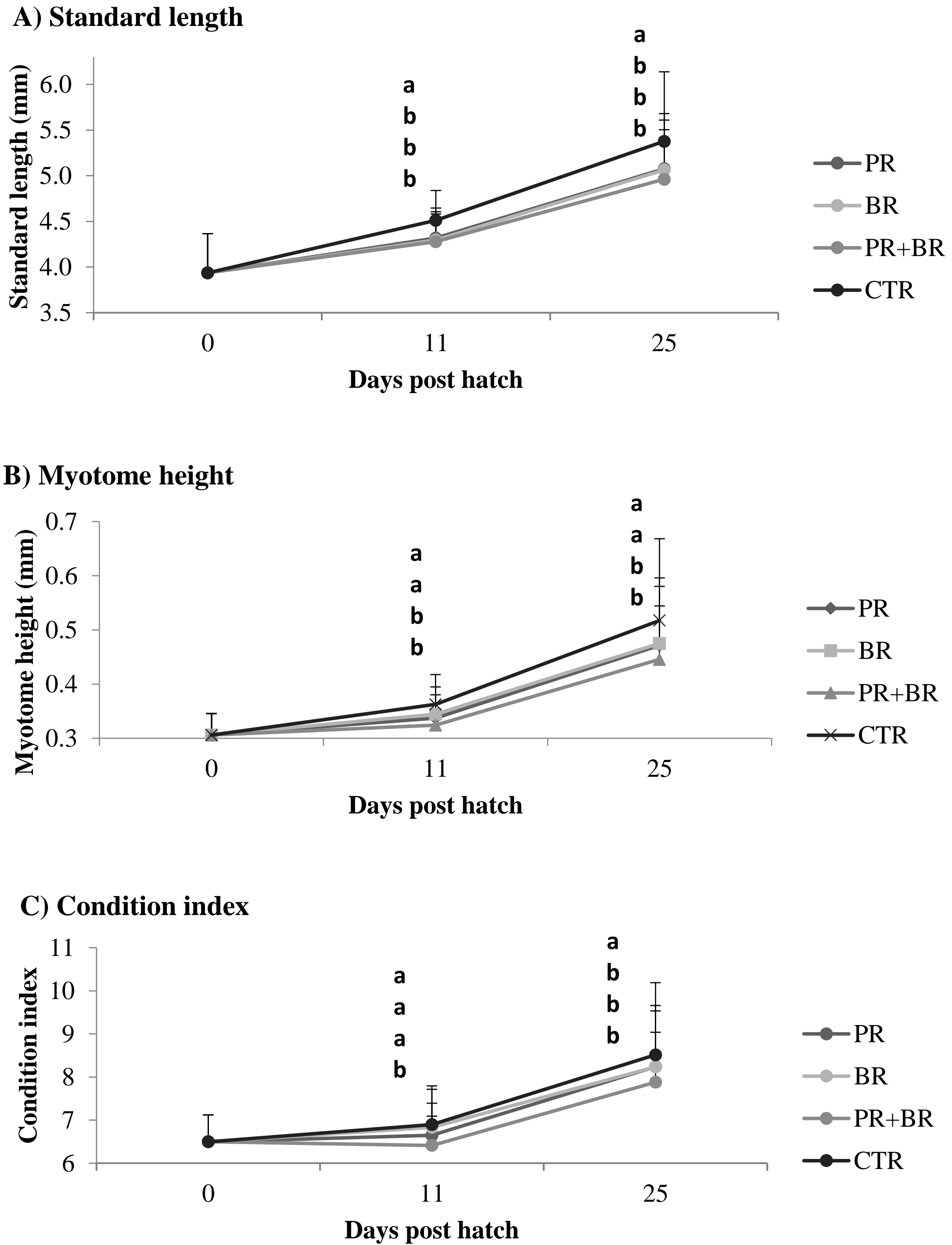

Figure 3.3. Standard length (A), myotome height (B) and condition index (C) of larvae (n = 30 larvae per tank and time point) exposed to different bacterial management strategies during the experiment. Data are presented as mean ± SD (n = 3). Letters indicate significant differences between treatments and the control (CTR) at any given time.

3.3.4 Bacterial analyses

The bacterial counts of water samples showed a general trend of suppressed CFU values for all treatments after 15 dph and 14 dph for marine and TCBS agar, respectively, but with no obvious differences amongst treatments became apparent (Fig. 3.4). Similarly, in the bacterial counts of the larvae homogenates, no obvious differences became apparent between treatments or time points (Fig. 3.5).

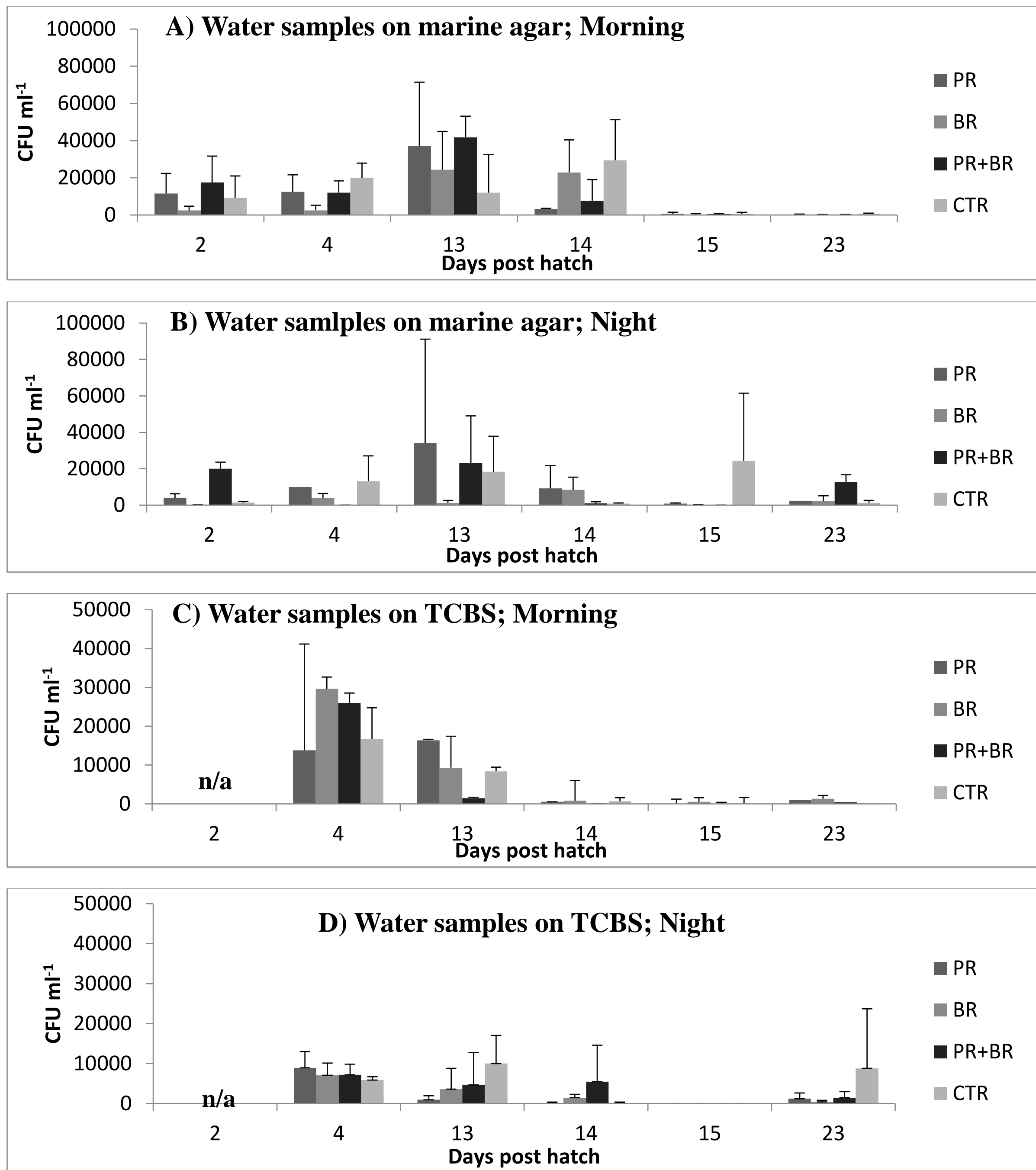

Figure 3.4. Number of CFUs per ml of tank water from larvae treated with probiotics mix (PR), bronopol (BR), probiotics and bronopol (PR+BR) and without any supplementation (CTR). Water samples collected in morning (A) (C) and night (B) (D) times were plated on marine agar (A) (B) and TCBS (C) (D) substrates (n = 1, n = 3 in technical replication).

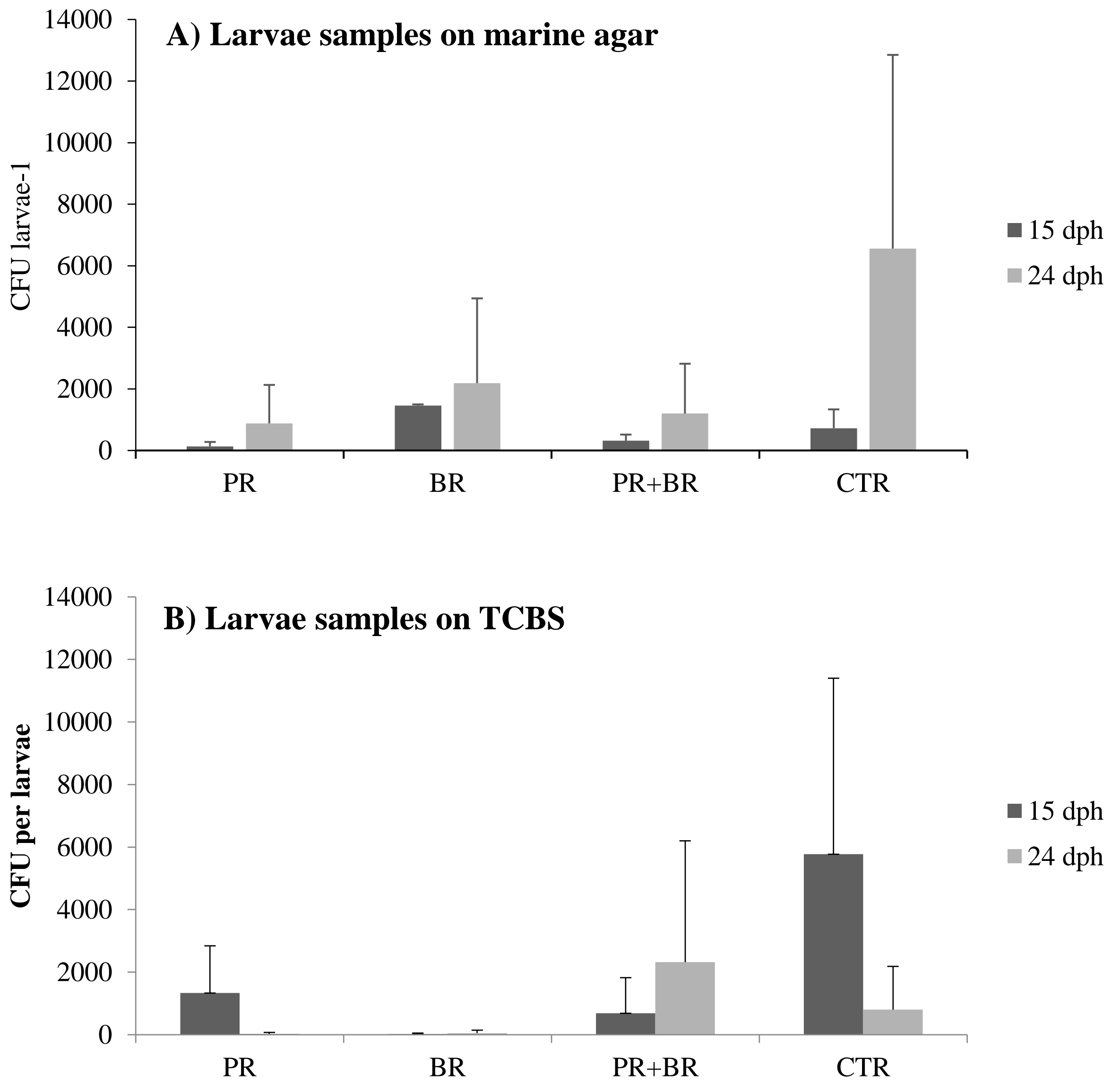

Figure 3.5. Effect of the administration of probiotic mix (PR), bronopol (BR), probiotic mix together with bronopol (PR+BR) and without any supplementation (CTR) to number of CFUs per sampled larvae at 15 dph and 24 dph plated on marine agar (A) and TCBS (B) substrates (n = 1, n = 3 technical replication).

3.3.5 Strains identification

The total 54 BLAST searches of the 16S rRNA gene sequences were taken from cultured bacteria colonies showing distinct morphologies on the marine and TCBS agar plates. Among these, 37 strains obtained from the water samples and 17 from surface disinfected

larvae. The overall dominating genera were *Vibrio* (41 strains of which 32 belong to *Vibrio* harveyi species group), *Pseudoalteromonas* (8 strains) and *Shewanella* (5 strains). The remaining 9 searches did not give certain species identification.

Generally, not a clear pattern became apparent across treatments. The common marine species *Vibrio* sp., *V. anguillarum* was only been clearly identified in one larvae sample in the PR+BR group. From the water samples, 37 bacteria strains were found, including 33 which belong to *Vibrio* (29 of them to *Vibrio* harveyi species group) and 4 to *Pseudoalteromonas* genera. From the surface disinfected larvae sampled at 15 and 24 dph, 17 strains were obtained, including 8 *Vibrio* (8 strains, in which 3 from the *Vibrio* harveyi group), 5 *Shewanella* and 4 *Pseudoalteromonas*. Bacterial belonging to genus *Vibrio* were detected at all water samples and larvae, apart from the BRON treatment larvae were three out of four isolates belong to *Shewanella* and one to *Pseudoalteromonas* genera. Additionally, all isolates from the PR+BR treatment exclusively belonged to *Vibrio* genus.

3.4 Discussion

This study clearly suggested a beneficial effect of the bronopol treatment with the addition of probiotics on survival of early larvae stages ballan wrasse. Larvae survival was higher in groups treated with probiotics and bronopol (PR+BR) with respect to the control and comparable for the groups treated with either bronopol (BR) or probiotics (PR). This significant difference in survival between the CTR and PR+BR treatment clearly highlights the need for applying bacterial control methods to the early life stages of ballan wrasse larviculture. This need for bacterial control at the early larvae stages has been shown in cold water marine fish larvae such as Atlantic cod (Attramadal et al., 2012b; Lauzon et al., 2014), Atlantic halibut (Skjermo et al., 1997; Bjornsdottir et al., 2010) and turbot (Munro

et al., 1994; Skjermo et al., 1997). Tilseth (1990) stated that mass mortality of cold-water marine fish larvae is associated with bacterial infections during first feeding. This is due to the extended incubation and yolk-sac periods in cold water species and negative interactions between larvae and bacteria (Tilseth, 1990). Various fish pathogenic bacteria have developed mechanisms to allow them adapting to fluctuations in nutrient availability (Hansen 1999). Proliferation of opportunistic fish pathogenic bacteria is common in sea water and can also cause increased larvae mortalities, by taking advantage of the ecological changes introduced when the water is used in aquaculture (Skjermo et al., 1999). Commercial aquaculture systems tent to increase carrying capacity of the system, often allowing proliferation of opportunistic bacteria (Vadstein et al., 1993). This is caused mainly due to large removal of bacteria of the incoming water, without controlled recolonisation of the microbial balance. As Vadstein et al. (1993) describes, r-selected species dominate in pioneer, more unstable and low biological control communities, where K-selected species dominate in mature, more stable systems and with high biological control communities. However, while a bacterium might be pathogenic to one species, it can be beneficial to another; making selective bacterial control playing an important role to the development of species-specific probiotics in aquaculture (Hai, 2015). Some bacteria species generate a wide range of antagonistic and antibiotic compounds that can be valuable as probiotics and be used as an alternative solution to antibiotics prophylaxis and chemicals (Hai, 2015). Together with the selective (e.g. probiotics) or non-selective (e.g. chemical disinfectants) proposed by Vadstein et al. (1993), microbial matured water can also provide a solution for microbial control with creation of a high diversity bacterial flora environment dominated by non-opportunistic bacteria (Sjermo et al., 1999). This exposure of larvae to a fairly diverse bacterial flora can increase survival and growth (Skjermo et al., 1999) and is also commonly found amongst marine hatcheries. *Shewanella*, *Vibrio* and

Pseudoalteromonas were the main bacteria genera identified in this study. The literature suggested various roles and uses depending on the species within these groups. All of them are commonly found Gram-negative bacteria and occasionally also used as probiotics in aquaculture (Hai, 2015). In the present study only some of the culturable bacteria were identified which could be assigned predominantly to genus. It should also be taken into consideration that many other factors may affect the population of endogenous microbiota, including genetic, nutritional and environmental factors (Ringø et al., 2010). Very limited information is available on ballan wrasse bacterial flora to date as for most other marine fish species. Birkbeck & Treasurer (2014) reported isolation of three *Vibrio* sp. (*V. splendidus*, *V. ichtyoenteri* and *V. pacinni*) from the digestive tract of ballan wrasse larvae reared in commercial conditions. *Vibrio* have also been reported in corkwing wrasse, *Symphodus melops,* and goldsinny wrasse, *Ctenolabrus rupestis,* (Gravningen et al., 1996; Bergh & Samuelsen, 2007). *Vibrio* and *Pseudoalteromonas* genera are commonly isolated from the intestinal flora of cold water marine species, such as Atlantic cod, Atlantic halibut and turbot (*Scophthalmus maximus*) (Hansen & Olafsen, 1999). *Vibrio* sp. are the most common and potentially pathogenic bacteria in marine aquaculture (Chatterjee & Haldar, 2012), commonly found in Atlantic cod (Reid et al., 2009), Senegalese sole *Solea senegalensis* (Chabrillón et al., 2005) and salmonids (Zhang & Austin, 2000), but also in marine invertebrates (Austin & Zhang, 2006). *Vibrio* sp. have also been used as probiotics in aquaculture. For instance, *V. alginolyticus* for Atlantic salmon (Austin et al., 1995), *V. proteolyticus* for turbot (Deschrijver and Ollever 2000), *V. fluvialis* for *Penaeus japonicus* (El-Sersy et al., 2006) and *Penaeus monodon* (Alavandi et al., 2004). The bacteria the most prevalent in this study was *V. Harveyi*, which is a major causal agent of luminous vibriosis in a wide range of marine organisms (Zhang & Austin, 2000), especially in shrimp *Litopanaeus vannamei* and *Penaeus monodon*. *V.Harveyi* can also cause vibriosis during

the early larval stages of fish and shellfish (Thompson et al., 2004), which might explain the low survival rates of this study. *V. Harveyi* has been detected in all water samples and larvae apart from the larvae treated exclusively with bronopol (BR). The bacterial strain *B. subtilis*, which is one of the main components of the probiotic mix used in this study, has antimicrobial activity against *Vibrio* sp., including *V. parahaemolyticus* and *V. harveyi* (Balcázar & Rojas-Luna, 2007). Surprisingly, the results of this study also identified *V. parahaemolyticus* and *V. harveyi* in the probiotic treated tanks (PR and PR+BR). The second most prevalent bacteria genus in this study was *Pseudoalteromonas*, which is also commonly dominating the intestine of cold water marine species intestine (Korsnes et al., 2006). The interest of genus *Pseudoalteromonas* is due to a variety of biological functions, including antibacterial, antiviral and probiotic (Pujalte et al., 2007). Use of *Pseudoalteromonas* sp. D41 and *P. gallaeciensis* as probiotics can provide protection against challenge by *V. caralliilyticus* in pacific oyster (*Crassostrea gigas*) and improve survival by 50% and 40% respectively (Kesarcodi-Watson et al., 2012). Bacteria belonging to *Shewanella* genus were detected in larvae of all treatments excluding PR+BR. *Shewanella* is a major spoilage organism commonly found in marine situations which can metabolise a number of free amino acids and sugars, producing odorous compounds, such as hydrogen sulphide (H_2S) (Kaiser et al., 2010). *Shewanella* has been previously found in fish internal organs, but their action remains unclear (Austin, 2006). However, the probiotic action of *Shewanella* has been seen in *Shewanella putrefaciens* were used as probiotic in Senegalese sole (De La Banda et al., 2012; Tapia-Paniagua et al., 2012) and gilthead sea bream (Tapia-Paniagua et al., 2012).

The beneficial use of probiotics in marine fish larvae has been proved in various studies, offering modulation of interactions with the tank environment, improvement of larval immune system, improvement of water quality and stimulation of larval digestive system

(Lauzon et al., 2014). Probiotics can provide nutritional benefits to the host and protection against pathogens (Gatesoupe, 1991; Gatesoupe, 1994; Gatesoupe, 2002; Sveinsdóttir et al. 2009; Mandiki et al., 2011; Hauville et al., 2015). Introduction of probiotic bacteria, including *Bacillus sp*., causes competition for nutrients, produces growth inhibitors or quench cell-to-cell communication that allows for settling within biofilms (Schreier et al., 2010). Additionally, probiotics can be a source of nutrients and enzymatic contribution to digestion, direct uptake of dissolved organic material mediated by the bacteria, enhance the immune response against pathogenic organisms and provide antiviral effects (Ringø et al., 2010). The fact that no *Bacillus* sp. were detected neither in the water or larvae samples could be due to probiotic application method (i.e. dose, timing, etc.), or bacteria selection and isolation method. Compatibility of the probiotic mix and isolation of various *Bacillus* sp. used with the same marine agar has been previously proved positive by Hauville et al. (2015). Various factors, such as probiotic selection, quantity, frequency and method applied should be further tested before any conclusions can be drawn. Under the current experimental conditions, the exclusive use of probiotic (PR) or bronopol (BR) had no impact on larvae survival or growth with respect to the control group. However, the synergetic use of probiotic and bronopol (PR+BR) had a significant positive effect on larvae survival relative to the control. The high survival did not imply higher growth in PR+BR treatment. On the contrary, the control group displayed the highest standard length and condition index compared to the rest of the treatments. These larvae were most of the total very low survivors in the CTR group and possibly the most robust ones. However, dry weights were comparable between all treatments and not showing a clear pattern of higher growing larvae in the control group. Size-dependent mortalities and natural variations in the size in marine larvae is being commonly observed (Garrido et al., 2015), and it can support the hypothesis of having only the most robust larvae surviving in the

CTR group. High variability between replicates values in dry weights might be due to the number of larvae used (3 pool samples of 10 larvae per treatment and per sampling point), suggesting higher number of samples should be used in future studies. Growth differences observed between treatments in this study is more likely to be related to natural fluctuations rather than environmental or bacterial load effects. Therefore, the fact the CTR larvae showed some morphometric differences to the rest of the treatments should be further investigated before any conclusion can be drawn for the treatments effect on larvae growth. Many factors contribute to larvae growth and many rearing aspects remain to be resolved in ballan wrasse, being a new species in aquaculture. The use of *Bacillus spp.* (in the form of commercial product as used in this study) has been previously reported to have a positive impact on growth metabolism and stress response of sea bream larvae administrated by bioencapsulation in live prey together with direct addition to the larvae rearing water or exclusively live prey administration between 3 and 73 dph (Avella et al., 2010). Supplementation of *Bacillus* coagulants improved also growth and immune response of white shrimp (*Penaeus vannamei*) when administered directly into the rearing tank (Wang & Gu, 2010). In contrast, administration of *B. licheniformis*, *B. subtilis* and *B. pumilus* directly to the water had no effects on Eurasian perch (*Perca fluviatilis*) growth and survival (Mandiki et al., 2011). In a recent study by Ramos et al. (2016), a different commercial probiotic mix (PAS-TR®, IMEVE S.A.) based on *B. subtilis* was tested which did not improve growth and immune response in rainbow trout (*Oncorhynchys mykiss*) and brown trout (*Salmo trutta*), and resulted in deleterious effects on intestinal morphology. Contrasting results and arguments have been reported when testing probiotic mix in marine fish larvae, probably due to species-specific requirements and application methods. The use of host-specific strains has been reported as an essential step when evaluating potential probiotic strains (Hansen & Olafsen, 1999). No such information is currently available on

ballan wrasse and further studies are needed towards understanding the intestinal colonisation potential of different probiotic strains. The bacteria genera identified in this study (*Shewanella*, *Vibrio* and *Pseudoalteromonas)* could provide a stepping-stone to develop indigenous probiotics for ballan wrasse. Non-selective reduction of bacteria by disinfection is a key element in bacterial control for the larvae rearing environment in marine aquaculture (Vadstein & Skjemo, 1993; Skjermo & Vadstein, 1999). Bronopol is a broad spectrum antibacterial (Shepherd et al., 1988) with MIC (minimum inhibitory concertation) to *Vibrio,* according to Pyceze manufacturer, being 10 ppm. Birkbeck et al. (2006) reported bronopol MIC against *Vibrio* and *Pseudoalteromonas* isolated from Atlantic halibut and cod eggs to be 1 – 4 and 8 – 32 ppm, respectively. The bronopol concentration used in this study was 25 ppm with no *Pseudoalteromonas* detected in the PR+BR treatment and only one out of twelve isolates from the BR treatment belonged to this genus. Bronopol treated larvae (BR) were the only ones with no signs of *Vibrio* isolates, confirming bronopol action against *Vibrio*. On the contrary, three out of four isolates belonged to *Shewanella* and one to *Pseudoalteromonas*. Bronopol MIC to *Bacillus* sp., according to Pyceze manufacturer, varies between 6.25 to 12.5 ppm. The risk of bronopol inhibitory action towards the probiotic mix used in PR+BR treatment is unlikely since their application gap in the tank of 12 hours allowed a water exchange from 200 up to 290 % day^{-1} (at the end of the trial).

A recent additional component in fish larval rearing bacterial control is the use of clay. Clay has been used over the past years as an alternative strategy to the green water technique in marine fish larval culture, showing significant reduction of the bacterial load in larvae rearing tanks (Clayton et al., 2010; Bjornsdottir et al., 2011; Attramadal et al., 2012a; Stuart et al., 2016). However, despite the reduction of opportunistic bacteria, clay does not always result in enhanced larvae survival. Bjornsdottir et al. (2011) tested the

effects of environmental shading of clay and algae in halibut larvae and despite the significant reduction of bacterial load in the water column and larvae gastroinstestinal tract, survival was not impacted. However, Stuart et al. (2016) demonstrated a reduction of *Vibrio* sp. 14 dph followed by an increase in survival of California yellowtail (*Seriola lalandi*) larvae. In the present study, clay has been added at night to all treatments to adopt the hatchery practice at Otter Ferry Seafish. The BLAST results did not show any consistent differences between bacteria genera across treatments, indicating that under the current experimental set up the use of clay was not significantly affected by probiotic or bronopol addition. Following the once in a day clay addition in all treatments, reduction of the water column bacteria load has been noticed in the morning counts both at the marine agar and TCBS after 15 and 14 dph respectively. Bacterial samples obtained from one tank per treatment as technical replicates. Therefore, no statistical analysis could be performed. This sampling protocol can be masking potential effects and therefore, higher number of biological replicates from all treated tanks and sampling points is recommended for future studies.

Taken together, the results of the present study highlight the importance of microbial environment at the early life stages of ballan wrasse culture. Repeated bronopol treatment with probiotic addition from the yolk-sac stage resulted in an increase of larvae survival, but not growth. Under the current experimental conditions, the exclusive use of probiotic or bronopol had no positive impact in larvae survival or growth. The main bacteria genera isolated from ballan wrasse larvae and tank water were *Vibrio, Pseudomonas* and *Shewanella.* The present study provides the basis for further studies to monitor and control microbial environment, but also to define how bacteria may cause detrimental damage to larvae resulting in high mortalities. The use of probiotics and bronopol as described in this study have been adopted by Otter Ferry Seafish and integrated in the commercial protocol

since the beginning of season in 2016. Following this, a significant increase in larvae survival during the first feeding phase became apparent. Required daily use of 7.5 g / m^3 of MIC-F and 50 ppm of Pyceze (25 ppm of bronopol) in a system using a total of 15 first feeding tanks (average 4 m^3 each) for 75 days (yolksac and first feeding period) in each tank results to a 33.75 kg of MIC-F and 225 L of Pyceze for the entire season resulting in production of 130,000 juvenile ballan wrasse. Considering the cost of MIC-F (£188 / kg) and the Pyceze (£33 / L), a cost of £13,770 will be added to the production cost, adding £0.10 to each deployment size fish. Considering the above cost estimations, bacterial management in ballan wrasse early larval stages needs to be further investigated with development and evaluation of host-specific probiotics during the early ontogenesis of ballan wrasse to provide a cost efficient production of cleanerfish.

Chapter 4: Evaluation of micro-diets for weaning of ballan wrasse (*Labrus bergylta*): Implications on larval survival and growth.

4.1 Introduction

After the yolk sac and bacterial control at the early life stages, ballan wrasse hatchery production main challenge is to develop adequate weaning protocols meeting the dietary requirements passing from live to dry compound diet. As food is the main source of energy and nutrients for fish larvae, optimisation of larval rearing protocols to meet larvae digestive and assimilation capacities is key for the upscaling of commercial production of any new or emerging aquaculture species. Ballan wrasse, although not new in terms of being a biological control species in salmonid farming (Bjordal, 1988), is a relatively new species in the temperate aquaculture sector.

The availability of diets that meet fish larval nutritional requirements has been a major constraint to the expansion of many marine fish species production with subsequent poor survival at the early life stages (Tilseth, 1990). Until recently, ballan wrasse larvae were fed commercially available diets developed for other temperate species, which require transition from live to dry feed, such as Atlantic cod, Atlantic halibut, European sea bass and gilthead sea bream. The development of species-specific weaning diets that satisfy physical, digestive and nutritional requirements of marine fish larvae is vital to improve larvae survival and general juvenile robustness in the early stages of production (Cahu & Infante, 2001). Nutrition can affect mechanisms involved in the development of fish larvae, but availability of feeds, composition, genetic and environmental factors can also play an important role to this (Zambonino Infante & Cahu, 2007). Early weaning in white seabream does not affect intestinal maturation and has a positive impact on the development of brush border indicating the ability of this species to digest food at the early life stages (20 dph) (Guerreiro et al., 2010). On the contrary, previous experiences had showed marine larvae not having a fully functional digestive system the first 2 or 3 weeks of their life (Person-Le Ruyet, 1989). Towards stimulating intestinal maturation and

enhancement of weaning diets utilisation, protein hydrolysate has been included with varying results. While addition of hydrolysed protein increased survival in Atlantic cod, the same doses had the opposite effect in Atlantic halibut by reducing larvae survival (Kvåle et al., 2009). These results could possibly be related to different feeding practice adopted by these two species, with halibut being a slower feeder than cod and therefore allowing a higher leaching in the water-soluble proteins.

Andrade et al. (2012) reported that diet regime can impact red Porgy (*Pagrus pagrus*) larvae performance and physiological status by regulating the enzymatic activity of larvae digestive system and fatty acids composition. As a result, adequate particle size, water stability, nutritionally balanced raw materials and high digestibility were recommended as key factors for weaning diet development. The effects of specific raw ingredients, such as krill phospholipids can influence larvae digestive enzymes activity and development. Suboptimal levels of krill phospholipids can be detrimental for sea bream larvae growth performance, intestinal development, dietary lipid utilisation, stress resistance (Saleh et al., 2012). Additional elements, such as inclusion of probiotics can also be beneficial in weaning diets. For instance, inclusion of bioencapsulated probiotics resulted in higher survival and growth in turbot larvae (Dagá et al., 2013).

Live prey contrasts in many aspects with artificial diets including attractiveness, texture, digestibility and nutritional value (Hamre et al., 2013b). It is believed that studies using dose-response designs on larvae nutritional requirements are lacking and commercially available diets have deficiencies in some nutritional factors (such as amino acids profile similar to larval body, level and source of phospholipids, fatty acid composition, lipid classes and protein) and/or characteristics which are vital for larvae digestion and nutrient availability (Hamre et al., 2013b). In addition, microparticulate diets uptake by larvae can be affected by environmental conditions (temperature, water quality, light and tank design)

(Person Le Ruyet 1989). Ballan wrasse natural diet is primarily non-piscivorous with a clear preference for molluscs, decapods and isopods (Dipper et al., 1977) which are relatively low in lipids and easily digestible (Hamre et al., 2013a). At the time that this study was conducted no information was available on ballan wrasse larvae nutrition and weaning diet formulation. Anecdotal evidence indicated a weaning diet produced by Nofima as being suitable for ballan wrasse weaning. However, this was a custom made and based on expensive raw materials. Therefore, the need for assessing the performance of other well-established weaning diets formulations for marine fish became a commercial priority. Nevertheless, apart from the optimum composition of the weaning diet, other parameters play also a key role in larvae survival, growth and digestive capacity, such as the weaning time frame (Fletcher et al., 2007; Guerreiro et al., 2010). Such data of weaning protocol in ballan wrasse larviculture are currently lacking. Current commercial practice at Otter Ferry Seafish relies on a weaning period of 30 days, which appears to be beneficial to achieve gradual transition to dry feed with the lowest possible larvae losses. However, this is an unusually long co-feeding period compared with other cold water marine species such as haddock (*Melanogrammus aeglefinus*) (7 days; Hamlin & Kling, 2001) or cod (10 days; Puvanendran et al., 2006).

Hamre et al. (2013a) has proposed that juvenile ballan wrasse diets should contain 65 % protein, 12 % lipid and 16 % carbohydrate to meet macronutrient needs and promote ballan wrasse growth. However, this result has been based on juvenile ballan wrasse, which does not guarantee suitability for larvae. Other studies have shown that larvae weaning success can be impacted by the raw materials used (Kousoulaki et al., 2014a) and the fish meal quality (Bogevik et al., 2015). It was suggested that the increased inclusion of ethoxyquin in the diet, a widely used antioxidant in fish feeds, can have negative effects on weaning performance of ballan wrasse larvae fed fish meal based diets. This can be related to

increased ethoxyquin sensitivity at the early life stages of ballan wrasse compared to later juvenile stages (Bogevik et al., 2015). The aim of this study was to benchmark the impact of co-feeding with four different micro-diets (three commercially available and one experimental) on ballan wrasse larvae survival and growth performance. This study provided a stepping-stone to Otter Ferry Seafish identifying a diet that provides the best results using the current commercial production weaning protocol.

4.2 Materials and Methods

4.2.1 Egg collection and larvae rearing

This study was conducted between the 17th of October 2013 and the 4th of December 2013. Eggs were obtained from natural spawning of captive wild caught ballan wrasse broodstock stocked in 3.25 m^3 tanks at Otter Ferry Seafish under a delayed photoperiod regime (July until September as opposed to ambient spawning April to June). Broodstock tanks were equipped with artificial seaweed made from green plastic sheets, to simulate species natural habitat. Temperature fluctuated between 8 – 13 ºC during the year and fish were fed daily to satiation with mussels (*Mytilus edulis*), langoustines (*Nephrops norvegicus*) and a semi moist commercial broodstock mix (BioMar, Grangemouth, UK) in a sausage form (6 cm length; 3 cm wide) produced on-site containing 46 % moisture, 12 % oil, 26 % protein, and 5% ash and targeting DHA/EPA and EPA/ARA ratios of 1 and 1.5 respectively.

Eggs were collected on spawning substrates (short pile dark green mats of 0.30 m^2 each) and covering 50 % of the total 5.72m^2 bottom area of the broodstock tank. Before moving the spawning mats to the incubation unit, they were disinfected with 150 ppm formalin for 60 min, followed by 100 ppm Pyceze daily for 30 min during the incubation period (6 – 7 days at 12 ºC). Both egg incubation and larval rearing systems were flow through with

pumped seawater sand filtered (10 µm) and UV treated (200 mJ / cm^2). At about 75 degree days (dd, 6 days post collection at 12 °C), hatching of eggs was mechanically induced by “scrapping” them off the carpet in to a separate hatching tank (0.8 m^3), as routinely done in the commercial hatchery, resulting in an estimated hatching rate of 90 ± 8 %. All hatched larvae (n = 30,000 – 75,000 larvae per batch) were transferred to a commercial 2 m^3 circular tank. Due to limited number of larvae per spawning days, three different batches (i.e. consecutive spawning days with maximum four days difference between the first and last batch) were pooled in the same commercial tank (n = 150,000 larvae in total). Larvae were kept in the commercial tank following the hatchery larval rearing protocol. In particular, the rotifers used prior to the experiment were produced in a batch culture system (72 hour turnover batches), fed a commercial production microalgae concentrate *Nannochloropsis* (Nanno 3600, Reed Mariculture Inc, CA, USA) which was diluted and introduced to the cultures 3 times a day (2.5 ml / M rotifers) and enriched with RotiGrow *plus* (Reed Mariculture Inc., CA, USA) (0.25 g / M rotifers) 2 days prior to their introduction to the first feeding tank. Rotifers were initially introduced to the first feeding tank 4 times a day from 3 dph through 26 dph beginning at 20 M / day / tank targeting rotifer density of 4 rotifer / ml. Number of offered rotifer gradually increased to 12 rotifers / ml by 19 dph and co-feeding with *Artemi*a started at 23 dph.

4.2.2 Experimental system and set up

At the start of the trial larvae at a median age of 60 days post hatch (dph) (*n.b.* total range was 58 – 62 dph, due to the use of three egg batches) larvae were stocked at a density of 8 larvae / L (based on volumetric estimation) in 12 x 80 L black semi-conical experimental tanks (water volume was set to 63 L). Weaning diet treatments were randomly assigned to each tank such that four micro-diets (Table 4.1) were tested in triplicate (BioMar Prowean 300, 0.15 – 0.4 mm, BioMar, Grangemouth, UK; Nofima 0.3 – 0.6, Nofima, Norway;

Otohime B1, 0.25 – 36 mm, Marubeni Nisshin Feed Co, Japan; Skretting Labrus 0, crumble, Skretting, France) (Table 4.1). Proximate analysis of the diets was performed in duplicate at the Institute of Aquaculture, University of Stirling and gross energy content was calculated based on energy contents of carbohydrates (4.1 kcal / g), fats (9.3 kcal / g) and proteins (5.4 kcal / g) (Table 4.1). The flow rate was individually controlled for each tank with 0.4 L / min initially (36 % water exchange / hour) gradually increased to 0.5 L / min (48 % exchange / hour) between 91 and 95 dph. Water supply was filtered as described above. Temperature (11.4 ± 0.6 ºC) and oxygen (85.0 ± 5.0 % saturation) were recorded daily.

Artemia were enriched with a commercial enrichment product (BioMar Multigain, BioMar, Grangemouth, UK) at 0.2 g / L dose three a day (9:00 am, 4:00 pm and 10:00 pm) and fed to the larvae at 9.5 nauplii.ml^{-1} three times a day (at 9:00 am, 4:00 pm and 10:00 pm) and gradually reduced to 1.5 nauplii.ml^{-1} from 93 dph. Co-feeding started two days after larvae were placed in the experimental system (62 dph) and it lasted until 93 dph when *Artemia* supply stopped (Fig. 4.1).

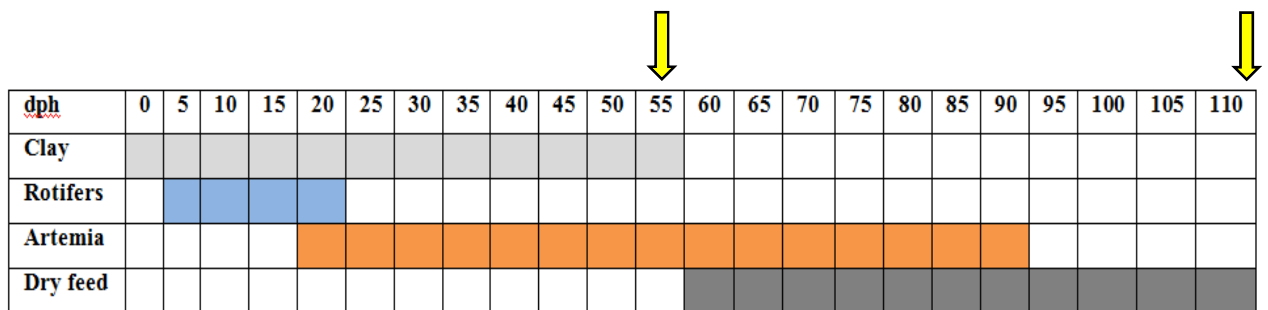

Figure 4.1. Commercial larval rearing protocol for ballan wrasse larvae at Otter Ferry Seafish. Arrows indicate the beginning and end of this study and also the larvae sampling points.

Dry feed was supplied with 24 hours automatic feeders and hand feeding (5 times / day) to ensure feeding to satiation. Tanks were siphoned and mortalities were removed on a daily basis to maintain tank hygiene and assess survival rates. The experiment was terminated at 108 dph when larvae were fully weaned for 15 days (Fig. 4.1).

To limit the number of larvae sampled, growth assessment was only performed at the start (60 dph, 90 larvae from the commercial tank) and end (108 dph, 30 larvae / experimental tank) of the experiment. Larvae were randomly selected from each tank by siphoning and euthanised using an overdose (400 ppm) of tricaine methanesulfonate (MS-222). Individual larvae wet (individually only at the end of the trial) and dry weights (in pools of 10 at the beginning and end of the trial, n = 10 per tank, 30 per treatment) were assessed to the nearest 0.01 g and 0.001 g respectively. In addition, photos of the larvae were taken using a computer controlled digital microscope camera (GXCam3, GT Vision, Suffolk, UK)) fitted onto a stereo microscope (GX Stereo microscope, XTL3T, GT Vision, Suffolk, UK). Total length (from the tip of the jaw to end of the tail), standard length (from the tip of jaw to the end of spinal cord), myotome height (from the posterior to the anus) and eye diameter were determined according to Fletcher *et al*. (2007) to the nearest 0.1 mm (Fig. 4.2). Condition index of larvae was calculated using, condition index = (myotome height (mm) / standard length (mm)) * 100.

At the experiment end, the total number of remaining larvae were counted in each tank to estimate survival rates.

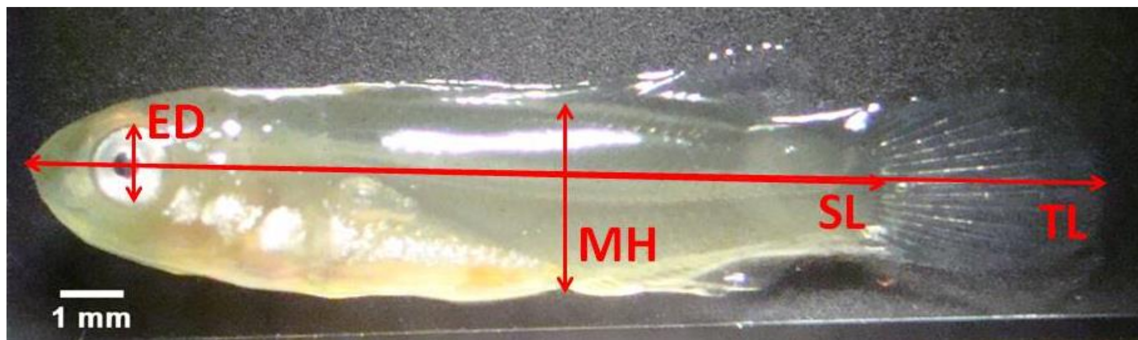

Figure 4.2. Ballan wrasse morphometric measurements through image analysis for total length (TL), standard length (SL), myotome height (MH) and eye diameter (ED).

Table 4.1. Formulation and proximate composition of the four experimental micro-diets.

	BioMar	**Nofima**	**Otohime**	**Skretting**
Size (mm)[1]	0.15 – 0.4	0.3 – 0.6	0.25 – 0.36	0 crumble
Protein (%)[2]	55.81	54.20	58.77	52.48
Lipids (%)[2]	11.96	11.56	14.42	14.76
Moisture (%)[2]	3.89	8.02	6.21	8.53
Ash (%)[2]	10.77	13.83	13.73	12.31
Gross Energy (MJ/kg)[3]	20.28	18.87	20.07	19.65
Ingredients[1]	Fish meal, krill meal, hydrolysed fish protein, wheat gluten, wheat, algae, lecithin, monocalcium phosphate, betaine, DL-methionine, yeast extract, Vitamins mix	Shrimp meal, fish meal (cod meal + herring stick water), krill hydrolysate, squid meal, krill oil, algae, yeast extract, soy lecithin, wheat, , vitamin and mineral mix	Fish meal, krill meal, squid meal, vitamin and mineral mix, potato starch, plant gum, wheat flour, inorganic calcium phosphate, guar gum, betaine	Fish meal, shrimp meal, wheat, wheat gluten, micro components, yeast glucans Macroguard, vitamin and mineral mix, pigments

[1] As communicated by the manufacturer

[2] As determined by direct measurement of feed samples by Nutritional Analytical Services at the University of Stirling, Institute of Aquaculture, Stirling, Scotland

[3] Calculated.

4.2.3 Statistical analysis

Prior to analysis, normality and homogeneity of variance were assessed and datasets were transformed when required (square-root, logarithmic or power transformation). Parameters assessed as percentages were first subjected to arcsine transformation. Data were compared by one way analysis of variance followed by a Tukey *post hoc* test with 95 % confidence, using MINITAB® Release 17 (Minitab Ltd., UK). Data are expressed as means ± standard deviation (SD). The level of significance was set at $P < 0.05$. Survival curves were estimated using survival analysis by Kaplan-Meier method followed by curve comparison by Wilcoxon long-rank test.

4.3 Results

4.3.1 Survival

There was no apparent notable mortality records across all treatments from day 60 to 97 (1 – 2 larvae / day / tank), when *Artemia* was supplied (Fig. 4.3 & 4.4). Thereafter a rapid increase in observed mortality was apparent in all treatments. In the 15 days in which dry feed only was offered > 70 % mortality was recorded in all treatments. At the study's end the highest survival was observed for larvae fed Nofima diet (22.3 ± 2.1 %), but comparable results were obtained using Otohime diet (14.6 ± 1.9 %) (Fig. 4.4). Significantly lower survivals were observed for Skretting (11.5 ± 3.1 %) and BioMar diets (5.6 ± 0.9 %) compared to Nofima diet (Fig. 4.4).

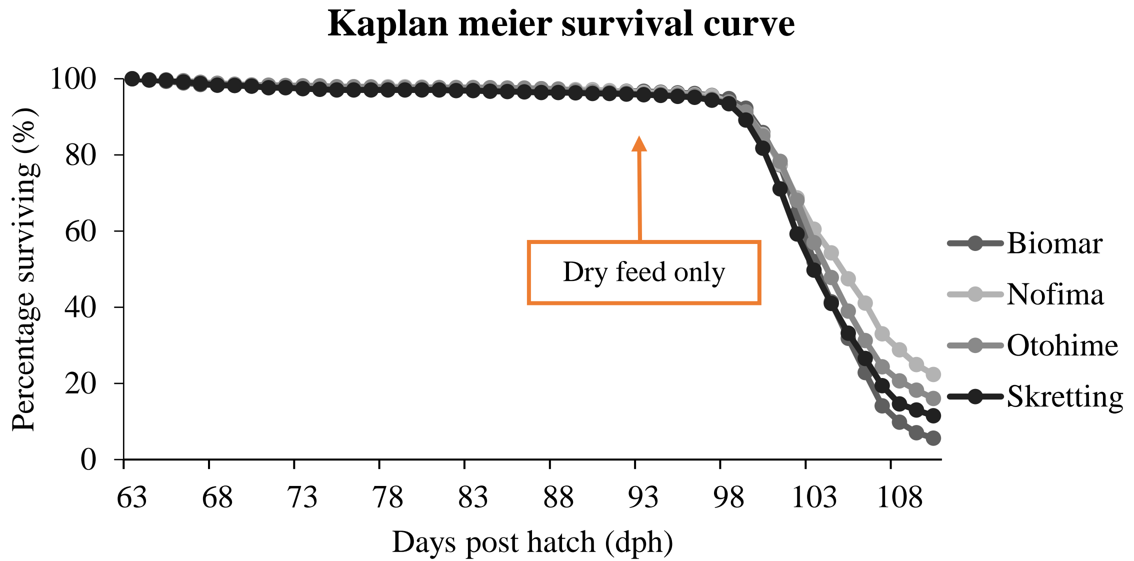

Figure 4.3. Daily estimated, as on mortalities collected, Kaplan meier survival curve (%) based on daily mortalities collected of ballan wrasse larvae from 60 until 108 dph fed four different micro-diets from 93 dph onwards.

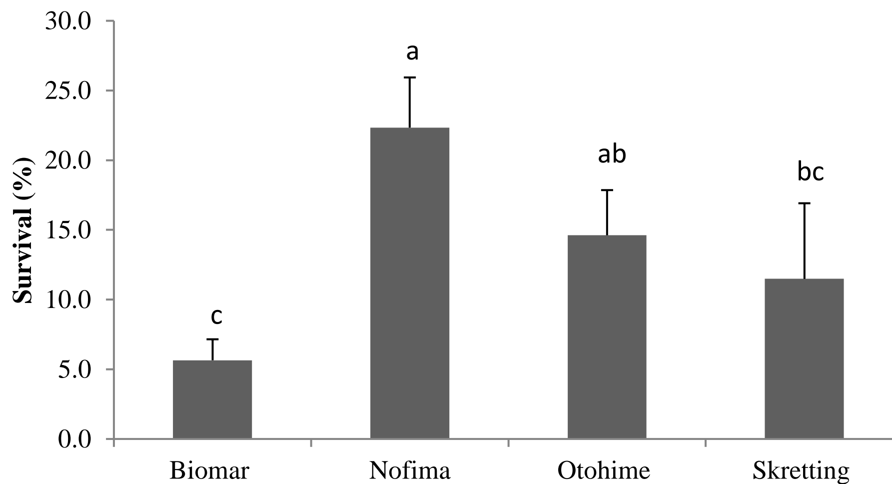

Figure 4.4. Survival rates (%) of ballan wrasse larvae from the start (60 dph) until the end of the study (108 dph) fed four different micro-diets (BioMar, Nofima, Otohime and Skretting) from 93 dph onwards. Data are presented as mean ± SD (n = 3). Letters indicate significant differences between diets ($P < 0.05$).

4.3.2 Larval growth

Larvae size between the different treatments at the end of the experiment showed the following pattern: Nofima > Otohime = Skretting > BioMar fish. This pattern became apparent in TL, MH and ED with larger TL recorded for Nofima diet (22.19 ± 3.13 mm) and lower for BioMar diet (19.01 ± 1.27 mm). MH was increased in larvae fed the Nofima and BioMar diets (4.03 ± 0.77 mm and 3.05 ± 0.30 mm, respectively) and ED was larger in larvae fed Nofima diet (1.70 ± 0.22 mm) and lower in BioMar (1.58 ± 0.10 mm). Weight of Nofima fish were significantly higher (wet weight: 0.17 ± 0.08 g; dry weight: 0.03 ± 0.015 g) than fish fed the other micro-diets and weight of BioMar fish was the smallest (wet weight: 0.07 ± 0.03 g; dry weight: 0.012 ± 0.005 g) (Fig. 4.5). However, condition index in fish fed the Nofima diet was significantly higher (22.59 ± 1.55) compared to the other treatments (<21) (Fig. 4.5). No significant differences were found in larvae standard length (16.3 ± 2.1 mm) between diets.

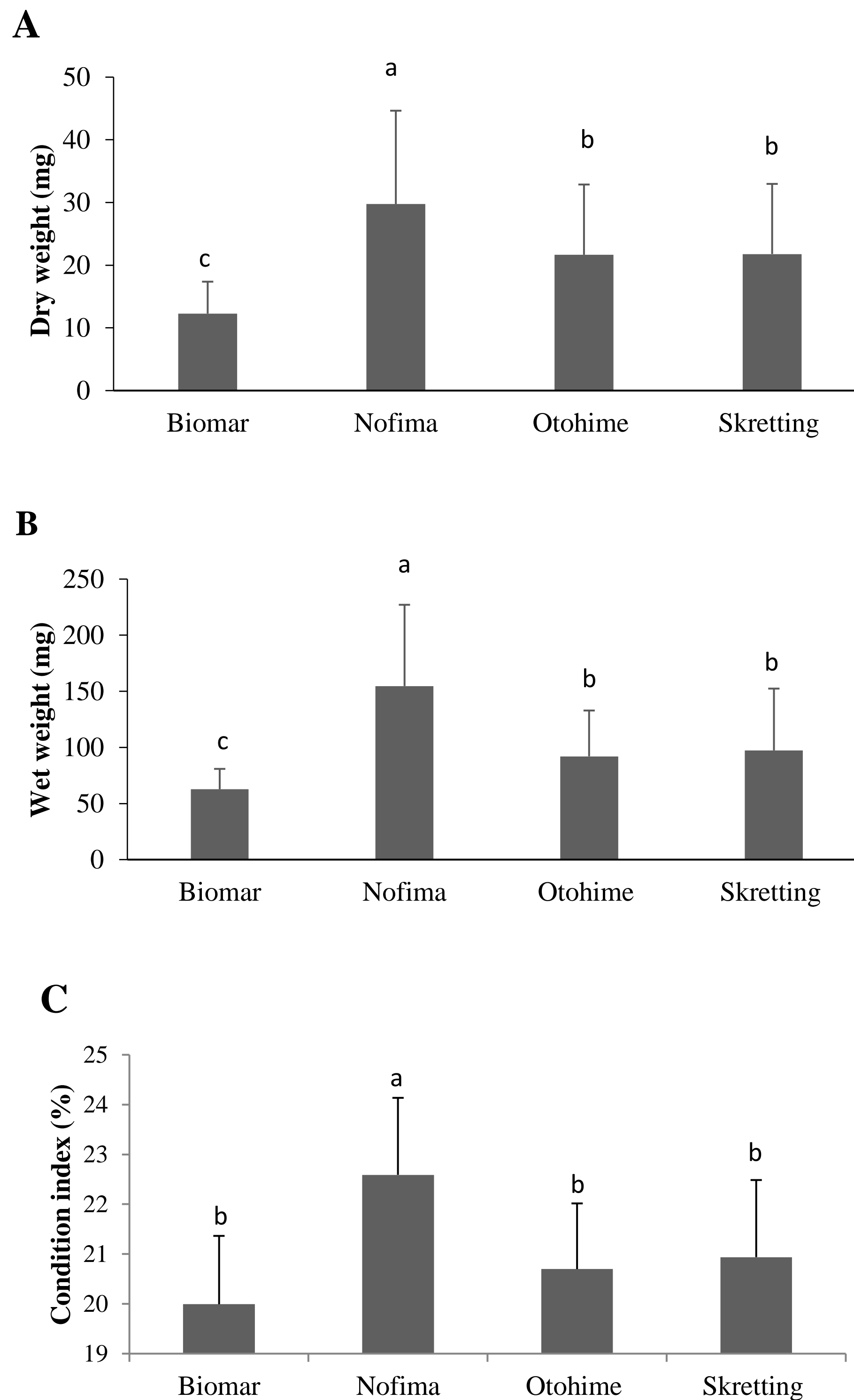

Figure 4.5. Effects of four micro-diets on (A) dry weight, (B) wet weight and (C) condition index of ballan wrasse larvae at the end of the 48-day study. Data are presented as mean ± SD (n = 3, 30 larvae per replicate). Letters indicate significant differences between diets (P < 0.05).

4.3.3 Cost-benefit analysis

BioMar, Otohime and Skretting are within the same price band (£15, 12.5 and 15.3 per kilo). On the contrary, Nofima as being an experimental diet, costs almost six times higher (£81.6 per kilo) than the other commercially available diets used. Considering that the amount of feed offered is similar between diets, the feed cost of achieving 1% survival for each diet has been estimated (Table 4.2). The cost per survival unit increases significantly making Nofima being one of the least cost-effective diet with Otohime being at the other end of the spectrum.

Table 4.2: Cost-benefit analysis of the feeds used.

Diet	Survival (%)	Cost (£ per kilo)	Cost / survival ratio
BioMar	5.6	15	2.67
Nofima	22.3	81.6	3.65
Otohime	14.6	12.5	0.85
Skretting	11.5	15.3	1.33

4.4 Discussion

One of the main restrictions for upscaling commercial production of temperate marine fish larvae is the low survival during first feeding and weaning due to suboptimal environmental and nutritional conditions (Tilseth, 1990). Ballan wrasse farming is very much in its infancy and very little is known on the species nutritional requirements and overall hatchery performance. This study assessed the effects of four micro-diets on survival and growth during weaning of ballan wrasse larvae, demonstrating the need for developing a species-specific diet. Larvae fed the commercially available weaning diets

(produced by BioMar, Otohime and Skretting) showed a trend for reduced performances (survival and growth) than larvae fed the experimental diet (produced by Nofima). Indeed, the best survival was achieved with Nofima diet (22.3 ± 2.1 %), an experimental diet developed for ballan wrasse weaning, although survival in larvae fed the Otohime diet was not significantly different (14.6 ± 1.9 %).

Survival rates (ranging from 5 to 22 %) obtained in the present study can be considered as low compared to other recently published weaning studies (Kousoulaki et al., 2014a; Bogevik et al., 2015). A possible explanation for the reduced survival rates found in the present study could be the timing of weaning from live feed to artificial micro-diets, but also water temperature which have not yet been standardised in ballan wrasse. The highest survival achieved in the study conducted by Kousoulaki et al. (2014a) was 77 % (from 40 through 100 dph) when weaning was performed between 40 – 70 dph and final larvae BW was 0.57 g. The study by Bogevik et al. (2015) showed 50 % survival (from 40 through 70 dph) when weaning between 55 – 70 dph and final larvae BW was 0.11 – 0.14 g. Both studies were conducted at 16 ± 0.5 ºC, in contrast to the present study which was performed at 11.4 ± 0.6 ºC and with a weaning window of between 62 and 93 dph. This might show that the weaning period applied at Otter Ferry Seafish and in this study could have been too long and with negative effects on weaning success. Acceptance of dry feed in marine larvae can be affected by different parameters, such as environmental conditions (temperature, water quality and tank design), diet nutritional balance and visual detection (Person Le Ruyet, 1989). Newly hatched larvae undergo a series of morphological transformation before the onset of exogenous feeding (Zambonino-Infante & Cahu, 2001), therefore parameters such as digestive capacity should be investigated extensively for the initiation of dry feed (weaning window). In some cases, early weaning had positive effects on survival and/or growth as shown in Red Porgy (*Pagrus pagrus*) (Andrade et al., 2012)

or sea bass *(Dicentrarchus labrax)* where early weaning (before 20 dph) resulted in comparable survival to the live feed treatment, but reduced growth (Cahu & Infante, 1994). White sea bream (*Diplodus sargus*) larvae have the ability to digest food at early life stages with early weaning having a positive role on intestinal maturation, but reducing digestive capacity (Guerreiro et al., 2010). However, it was also shown that sea bass larvae are able to modulate their digestive enzyme activity in response to a change in diet even when weaned early, but with the weaning diet delaying some age dependent digestive processes, as would malnutrition (Cahu & Infante, 1994). Higher water temperatures increase larvae metabolism and activity level and potentially also weaning success by improving readiness of accepting a dry diet (Appelbaum, 1989), reducing the weaning time required. Studies have also suggested that premature weaning can have negative effects on larvae performance due to their immature digestive system with reduced enzymatic activity and even absence of pepsin-like enzyme activity, resulting in low assimilation of nutrients (Kolkovski, 2001). Contrasting results and arguments have been reported when testing different weaning strategies in marine fish larvae, probably due to species-specific requirements. In general, for the feasibility of fish larviculture, it is important to start weaning to dry feed as early as possible, taking into consideration that production of live feed is a major expense (Person Le Ruyet, 1993). Currently, no information is available about the optimum weaning window or water temperature for ballan wrasse. Both need to be research priorities for the optimisation of larval hatchery production.

A nutritionally suitable weaning feed is key to successful larviculture (Watanabe & Kiron, 1994) with diet composition being able to enhance, suppress or delay the development of the digestive system and activity of larvae digestive enzymes (Cahu & Zambonino-Infante, 2001). The gross macro-nutrient composition of the experimental diets of this study did not show any consistent differences amongst them that may explain their

performance. This said, the main difference between Nofima and the other diets is the source of fish meal being from cod meal and herring stick water in addition to krill hydrolysate, squid meal and krill oil. This is in agreement with a recent study from Kousoulaki et al. (2014a), using a comparable composition diet produced by Nofima, which reported that the weaning success of ballan wrasse larvae depends on specific raw materials, such as shrimp or krill. The same study suggested that the combination of fish meal or cod muscle meal with shrimp and krill hydrolysate can result in the best weaning performance of ballan wrasse, possibly due to the presence of specific appetite-stimulating compounds. Krill hydrolysate contains higher levels of certain free amino acids (taurine, creatinine, glycine, glutaminic acid, lysine, arginine, leucine, alanine and proline) compared with fish meal. Four common types of attractants, which can boost feeding behaviour in marine fish, are low-molecular-weight metabolites including free amino acids, quaternary ammonium compounds, nucleotides or nucleosides and organic acids (Carr et al., 1996). However, no data is available on the content of the diets tested in the present study in these attractant molecules and therefore no assumptions can be made without further studies. There is evidence that inclusion of water soluble fraction of marine protein sources (stickwater) in fish feeds can improve fish feed intake and growth (Kousoulaki et al., 2009; Kousoulaki et al., 2012). However, the reasons behind the effects of stickwater in fish feeds have not been properly addressed yet. In this study, stickwater was included only in the Nofima diet. It has also been reported that ethoxyquin, commonly used as an antioxidant in commercial feed production, might have a negative effect on ballan wrasse weaning (Bogevik et al., 2015). Nevertheless, inclusion of high quality ingredients together with either reduced ethoxyquin levels or alternative antioxidants in the feed can provide positive results. In particular, survival in fish fed fish meal based diets containing ethoxyquin was less than 30 % compared to 50 % in fish fed the ethoxyquin-

free diet (Bogevik et al., 2015). Ethoxyquin levels in the diets used in this study were not measured, so potential association with larvae survival should be further explored. In agreement with this, the highest survival achieved in this study was obtained using diet based on non-commercial and high quality fish meal.

The source of raw materials and lipid source in each feed has a significant impact in larval development. The effect of lipid classes, such as the ratio between neutral lipids (NL) and phospholipids (PL) is more important than to the total dietary lipid content (Cahu, 2003). The importance of dietary PL levels in larvae is higher than in juveniles, given that are extremely sensitive to PL deficiency (Coutteau et al., 1997). A high source of phospholipids is the krill oil, which is typically composed of at least 20% EPA and DHA and 40% PL (Massrieh, 2008). Krill oil is also rich in astxanthin, which provides a natural protection against oxidation. It has been previously reported by Kousoulaki et al. (2014b) that ballan wrasse larvae weaned with a diet free of fish meal or whole fish meal hydrolysate (such as the Nofima diet used in this study) might lead to severe head deformities, recommending a diet alteration, as soon as weaning to dry diet is terminated, towards fishmeal or stickwater enhanced diet together with krill or shrimp meal. Despite deformities assessment was not within the scope of the current study, no noticeable head or body deformities were observed. Inclusion of soy lecithin in Nofima diet might have helped towards the apparent lack of deformities, given that is a rich source of phosphatidylinositol which can reduce deformities in fish larvae (Geurden et al., 1997; Hansen et al., 2011).

Muscle growth at the early larvae stages enact to a great extent growth potentials, viability and quality of the later juvenile stages (Valente et al., 2013). Muscle fibres vary between families and strains, but also are plastic in their growth response to environmental conditions, such as diet, exercise, light and temperature regimes (Johnston, 1999). In this

study, significant growth differences were found between fish DW, MH and ED, following this pattern: Nofima > Otohime = Skretting > BioMar. However, only fish fed the Nofima diet had significantly higher condition index compared to the other treatments and no differences in SL were apparent between treatments. It has been previously reported that diets promoting fast growth rate were related to a higher contribution of hyperplasia in the axial muscle of Atlantic cod (Galloway et al., 1999), describing initial recruitment of the white fibres at the dorsal and ventral apices of the myotome at the onset of exogenous feeding (SL = 4.5 mm). Despite that both hypertrophy and hyperplasia occurred, increased somatic growth was associated with increased white fibre hyperplasia. Nevertheless, Vo et al. (2016) tested the diet effects on Atlantic cod larvae muscle growth, and reported that different red and white muscles growth phases coincided with the different metamorphosis stages. Comparable environmental parameters amongst the treatments of the current study demonstrated the clear diet effects on ballan wrasse larvae non-longitudinal muscle growth (possibly due to fibre hyperplasia). Despite that no information is currently available on ballan wrasse muscle growth, hyperplasia and hypertrophy dynamics can be interesting aspects for future research.

This study showed a trend for reduced survival and growth in larvae fed the commercially available diets developed for other species highlighting the importance to development species-specific diets for ballan wrasse. Larvae weaned on Nofima diet showed better survival and growth performance compared to the other diets tested, although the underlying reasons behind this effect remain unclear. Current results suggested Nofima diet is a suitable micro-diet for ballan wrasse weaning. However, the highest post-weaning survival achieved in this study (22.3 %) was considerably lower compared to other recent studies on ballan wrasse (77 %, Kousoulaki et al., 2014a; 50 %, Bogevik et al., 2015). Future work should focus on investigating the optimum environmental conditions for

ballan wrasse larviculture that will allow a successful and smooth transition from live to dry weaning diet. A starting point would be assessment of the two currently used water temperatures in ballan wrasse hatcheries (11 and 16 ºC) testing three weaning windows (1, 3 and 6 weeks) in a triplicate system (18 tanks in total). Close monitoring of digestive enzymatic activity, intestinal morphology and nutrient assimilation will help understanding the functional maturation of tissues and organs and ultimately understand the underlying mechanisms behind the larval performances. This knowledge will provide a stepping-stone for the further investigation of nutritional requirements. Future studies should not also overlook assessing inclusion levels of expensive raw ingredients, such as krill meal and cod fillet meal, contributing to the development of a cost-effective weaning diet for ballan wrasse.

Chapter 5: Effects of on-growing diets on farmed ballan wrasse (*Labrus bergylta*) feed intake, growth performance, liver fatty acid composition, intestine function and nutrient digestibility.

5.1 Introduction

The next biggest challenge in ballan wrasse farming after survival at the early life stages is reduced growth as juveniles with the time required to reach deployment to sea cages size (e.g. > 45g) being 20 – 24 months from egg to deployment, based on commercial data at Otter Ferry Seafish (David Patterson, Otterferry Seafish, pers. comm.). Shortening the grow-out window is therefore a priority and it is believed this can be achieved through improved nutrition.

Diets providing the correct nutritional requirements are critical to ensure optimal growth and survival of farmed fish (Valente et al., 2013). The feeding of ballan wrasse is currently based on diets formulated for other marine temperate aquaculture species such as Atlantic cod due to limited knowledge of ballan wrasse nutritional requirements. However, ballan wrasse natural prey selection indicates the possible requirement for a different diet formulation to other commercial marine fish species (Hamre et al., 2013a). Ballan wrasse is an stomachless fish with relatively low growth rate and an overall intestine length / body length ratio of 0.62, which reflects the omnivorous diet of the species inclining towards carnivorous with non-piscivorous preferences (Dipper et al., 1977). Studies in farmed ballan wrasse nutrition have suggested that 65 % protein, 12 % lipid and 16 % carbohydrate are optimum for juveniles (1.3 – 13 g, reared at 12 – 16 ºC flow through system) and inclusion of specific raw materials (e.g. shrimp, krill and cod muscle meal) promote growth and survival in larval stages (Hamre et al., 2013b; Kousoulaki et al., 2015).

All vertebrates have an absolute dietary requirement for specific PUFA, with dietary deficiency causing reduced growth and reproduction and eventually death (Das, 2006). These PUFA are termed “essential fatty acids” (EFA), which include members of n-6 and n-3 series (Das, 2006). To determination of optimal dietary amounts of EFA is required for normal growth and development in fish (Tocher, 2010). Prevention of EFA deficiency,

which will cause pathologies, can be characterised as fundamental to determine whether a FA is essential for a given species in absolute and relative terms (Tocher, 2010). For instance, seabream juveniles fed diets containing the same amount of n-3 HUFA but different EPA/DHA ratios exhibit different growth rates (Ibeas et al., 1997). Various factors can affect feed palatability and appetite in fish species, including appetite stimulators and attractants (Kasumyan & Doving, 2003). Krill and shrimp meal are widely used as appetite stimulators for cold water species such as rainbow trout (*Oncorhynchus mykiss*) (Oikawa & March, 1997) and Atlantic cod (Tibbetts et al., 2011). These attractants also highly depend on certain free amino acids (e.g. alanine, glutamate, proline and serine) which can act as appetite stimulators (Li et al., 2009). Given ballan wrasse natural diet (Dipper et al., 1977), it can be hypothesised that other currently available marine diets might be inadequate for ballan wrasse due to different nutritional requirements and lack of attractants.

In general, very limited knowledge is available on the digestive system of stomachless species (Day et al., 2011; Clements et al., 2014) and even less in ballan wrasse. In addition, they are currently very limited indicators available of nutrient absorption efficacy and metabolism in relation to diet formulation to improve growth and intestinal health of farmed ballan wrasse stocks. Krogdahl et al. (2014) described ballan wrasse intestine as short and simple which lies in the abdomen in one loop surrounded by the other internal organs with similar general organisation similar to Atlantic salmon. It has also being noted that the ability of ballan wrasse juveniles to handle diets with different composition and moisture, due to their flexible intestine (Helland et al., 2014). Nevertheless, as farming of ballan wrasse is relatively new, there are key gaps in knowledge in many aspects of the species biology. A better understanding of nutrient utilisation (digestion, absorption and assimilation) and requirements is a priority for the scaling up of wrasse production.

A nutritionally imbalanced diet or inclusion of anti-nutritional factors and/or toxins, have been reported as factors promoting intestinal inflammation in fish (Merrifield et al., 2011). Intestinal inflammation has a high metabolic cost, through repair and maintenance of enterocytes, with consequent negative effects on feed intake and growth. Due to the importance of intestine health, various histological methods have been developed including semi-quantitative scoring systems, histochemical and morphometric assessments in order to identify and score intestinal inflammation (Urán, 2008; Raskovic et al., 2011). Kjørsvik et al. (2014) reported a quantitative description of ballan wrasse digestive tract defining it as very long, slender and heavily branched villi in anterior and midgut sections, with villi becoming gradually shorted and less branchy towards the posterior intestine. In general, ballan wrasse intestine has been characterised as highly efficient and flexible being able to handle diets with different composition and under different feeding regimes (Kjørsvik et al. 2014; Helland et al., 2014).

The objectives of this study were to assess the impact of four different commercial feed formulations on juvenile ballan wrasse growth performances and investigate their subsequent impacts on liver fatty acid composition, intestinal health (intestine histomorphology and inflammation scoring) and digestive functions (digestive enzymes activity and nutrients digestibility). Diet A was a diet that was routinely used for the on-growing window at Otter Ferry Seafish, diet B was a tailored recipe to the currently know needs of ballan wrasse, diet C was a standard commercial marine fish formulation and diet D was based on marine polychaetes, as an alternative raw material to fish meal. The aim was to provide baseline data for the development of an on-growing feed that promote growth in ballan wrasse leading to shorter grow out window in the hatchery, but also to understand basic nutrient assimilation features.

5.2 Materials and Methods

5.2.1 Experimental fish and system

The 90-day trial was conducted at Otter Ferry Seafish (Argyll, Scotland, UK) between May until July 2013 using 2012 year class ballan wrasse. Fish were previously reared under continuous artificial light as a commercial standard (24 LL) and fed a standard on-growing diet (Pre-trial; ø 2.0 mm extruded wrasse pellets, BioMar, Grangemouth, UK). The experimental system consisted in 8 tanks (0.9 m radius x 0.4 m depth, 1 m^3) flow-through with temperature kept constant at 11.5 ± 0.7 °C throughout the trial and 24LL. Artificial seaweed was placed in each tank, covering ¼ of the tank surface, to provide cover and simulate the species natural habitat. Seaweed and small-mesh bottom drains were removed daily following uneaten food recovery and before feed initiation for cleaning the tanks. Dissolved oxygen concentration was checked daily and remained > 85 % saturation throughout the study.

5.2.2 Experimental diets and feeding

Fish were randomly stocked in the eight experimental tanks (132 per tank, mean weight of 38.5 ± 7.7 g, initial stocking density of 5 kg / m^3). Four diets (diets A – D) were tested in duplicate with tank designation being selected at random. The formulation of diet A and B was based on the current nutritional requirements of ballan wrasse (Hamre et al., 2013; Kousoulaki et al., 2015) partly replacing fish meal with krill and shrimp meal, diet C was a commonly used marine species diet and diet D was based on marine polychaetes. Diets A, B and D contained yttrium oxide (Y_2O_3) as a digestibility marker for digestibility analyses. All diets also reportedly contained anti-inflammatory and/or probiotic additives (Table 5.1).

Fish were fed 24 h using electric disc feeders (1 % biomass day^{-1}) in addition to hand feeding *ad libitum* twice a day (9:00 am and 4:00 pm), recording the amount of feed offered in each tank (weighted to the nearest 0.01g). Prior to the start of the trial and for each experimental diet, a known number of dry pellets (n = 100 pellets; triplicate count) were weighted in order to calculate the mean-weight of an individual pellet (diet A = 0.0035 ± 0.0001g; diet B =0.0089 ± 0.0002 g; diet C = 0.0087 ± 0.0002 g; diet D = 0.0065± 0.0003 g). Feed waste left at the bottom of each tank was siphoned daily (trying to avoid any faecal material) into a bucket. The excess water of the recovered feed was removed through a fine mesh and then weighted to the nearest 0.01 g. Three sub-samples were separated, weighted and the number of pellets manually counted to calculate feed recovery rate based on the estimated number of pellets distributed and recovered. The protocol was repeated three-times for each experimental diet. Feed recovery rate was high for diet A (98 ± 2%), diet B (99 ± 4%) and diet C (96 ± 5 % but lower for diet D (72 ± 6%). This value was then corrected by pellet number to determine feed intake in all treatments except for diet D due to low stability in water.

5.2.3 Sampling and analyses

Fish were sampled at the start (T0) and 90 days later at the end of the trial (T1) when 30 individual fish per tank were randomly sampled and anaesthetised (40 ppm; MS-222, Pharmaq®) to assess body weight (BW ± 0.01g) and total length (TL ± 0.1 cm). A sub-sample of fish was euthanised at T0 (n = 10 fish from stock) and at T1 (n = 5 / tank, 10 per treatment) by overdose of anaesthetic (400 ppm; MS-222, Pharmaq®) followed by destruction of the brain. Fish were not starved prior to sampling in order to maintain intestinal morphological structure reflecting normal feeding conditions (Baeverfjord & Krogdahl, 2000; Chen et al., 2007). Viscera were excised and weighted (VW; ± 0.01 g). Liver weight (LW; ± 0.01 g), remaining intestinal weight (IW; ± 0.01 g) and intestine

length (IL; ± 0.1 cm) were also recorded. The intestine was dissected from the junction with the bucco-pharingal cavity to the anal opening, sectioned at the 1st and 2nd loop and rectal valve to provide 4 individual sections further cut into 2 sub-samples for analyses (Fig. 5.1). Only sections 1 and 3 were processed. From each section, one sub-section was used to measure pH (MColorpHast, pH 6.5 – 10, Merck®) and digestive enzyme activity (tissue immediately snap frozen in liquid nitrogen) while the second sub-section was rinsed of faecal material in 4 °C saline solution prior to storing in Serra fixative for histological processing.

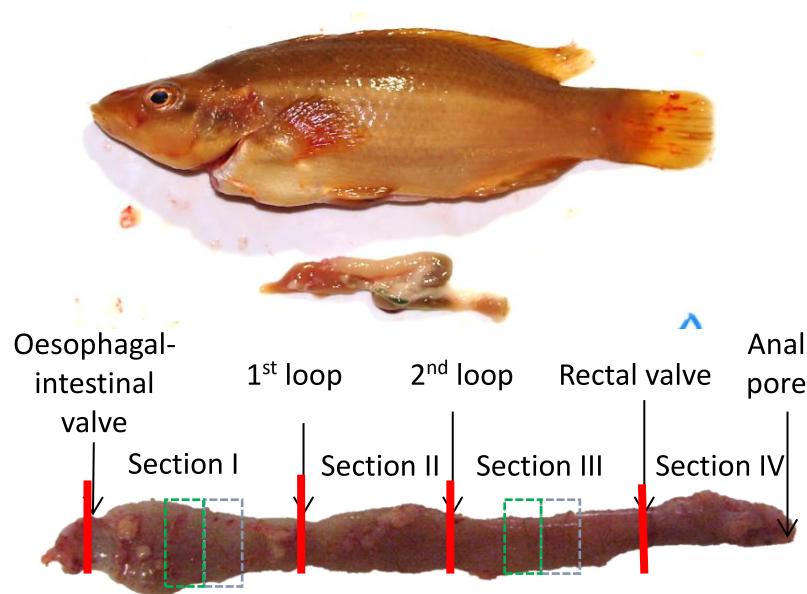

Figure 5.1. Image showing “Z” shaped intestine and sampling method used after extraction of ballan wrasse intestine. Each doted box represents are of tissue removed for histological (in green) and enzymatic analysis (in blue).

5.2.3.1 Growth and morphometric assessment

Fulton’s condition factor (K), Specific growth rate (SGR, % day^{-1}), biological feed conversion ratio (bFCR), thermal growth coefficient (TGC), hepatosomatic index (HSI, %) and viscerosomatic index (VSI, %) were calculated using standard methods, where, BW_0 and BW_1 are the initial and final mean fish weights, FI is the feed intake, FE is the feed

efficiency, WG is the weight gained, MB is the mortalities biomass,dd is the number of degree days and IL/TL is the ratio between intestine and total body length:

Foulton's condition factor (K) = 100 (BW / TL^3)

SGR (% day-1) = [((ln (BW_1) – ln (BW_0)) / total days] x 100

Average metabolic gained BW = ((BW_1/1000 x BW_0/1000)$^{0.5}$)$^{0.8}$

bFCR = daily FI (g) / (WG x No of remaining fish) + MB (g))

FE = BW gain / total dry matter intake

TGC = (($BW_1^{1/3}$ – $BW_0^{1/3}$) x 1000) / dd

HSI (%) = (LW, g / BW) x 100

VSI (%) = (viscera weight, g / BW) x 100

IL/TL = intestine / total body length

5.2.3.2 Proximate, lipid, lipid classes and mineral analyses

Proximate and lipid analyses of the diets and liver samples were performed in duplicate at the Institute of Aquaculture, University of Stirling. Prior to analysis, samples were minced and blended to ensure homogeneity. The analyses of dry matter (110 ºC for 24 h) and ash (600 ºC for 16 h) were performed according to standard laboratory procedures (AOAC 2000). Crude protein was determined by Kjeldahl analysis (Persson, 2008) (nitrogen x 6.25; Kjeltec Autoanalyser, Tecator, Höganäs, Sweden). Crude lipid was determined as described in Karalazos et al. (2007). Lipid extraction was carried out following the protocol outlined by Folch (1957) and fatty acid analysis following the protocol described by Christie (1982) adapted by Tocher & Harvie (1988) by subjecting the lipid fraction to acid-catalysed transesterification resulting in fatty-acid-methyl-esters (FAMEs) which were purified by thin-layer chromatography on silica-coated glass plates using the developing solvent iso-hexane:diethyl ether (90:1 v/v) with 0.01 % BHT (as antioxidant).

Fatty acids composition was determined by gas-liquid chromatography after preparation of FAMEs according to Morrison & Smith (1964).

The FA liver composition was assessed at the beginning (n = 8 fish prior to tank distribution) and at the end of the experiment (n = 5 fish per tank, 10 per treatment). Lipid classes were separated by high-performance thin-layer chromatography on silica gel 60 plates, using single-dimension double-development method as described in Tocher & Harvie (1988). Minerals contents were determined after placing diets in nitric acid (69 %) and then in a microwave (MARSXpress, CEM) for 40 minutes. Following this, digests were transferred into a volumetric flask and made up into x25 dilutions using distilled water. Final samples were analysed by using spectrophotometry as described in Smedley et al. (2016).

The analysed proximate composition of the four experimental diets is shown in table 5.1. The moisture content of diet B was 78.2, 82.2 and 74.4 % higher compared to diets A, C and D, respectively. The protein, lipid, carbohydrates and ash content varied from 51.5 to 58.9, 10.7 to 14.6, 12.3 to 17.1 and 8.6 to 13.5 %, respectively. Mineral levels varied between diets, with major differences occurring in sodium, phosphorus, manganese and iron (Table 5.1.).

Total PUFA was higher for diet A. Diet D exhibited the highest ARA/EPA and DHA/EPA ratio followed by diets C, B and A (Table 5.2). The general trend of lipid classes composition showed (Table 5.3).

Table 5.1. Feed formulation (% of inclusion as communicated by the manufacturer), proximate and mineral compositions (% of wet weight basis) of the experimental diets.

	Diet A	**Diet B**	**Diet C**	**Diet D**
Size (mm)	2	1.5	2.3	2
Feed formulation				
Fish meal (%)	24	0	*	0
Cod muscle meal (%)	0	22	0	0
Krill meal (%)	40	0	*	0
Shrimp meal (%)	0	27	0	0
Polychaetes meal (%)	0	0	0	*
Squid meal (%)	0	0	*	*
Krill hydrolysate (%)	0	4	0	0
Krill oil (%)	0	4	0	0
Analysed composition				
Moisture (%)	4.6	8.2	4.5	4.7
Protein (%)	57.0	56.4	51.5	58.9
Lipid (%)	12.7	12.1	14.6	10.7
Ash (%)	9.2	11.1	13.5	8.6
Carbohydrates (%)	16.5	12.3	15.8	17.1
Minerals				
Sodium (μg/g)	165.7	82.6	94.9	105.7
Magnesium (μg/g)	39.3	36.7	25.4	61.5
Phosphorus (μg/g)	167.5	134.5	197.9	61.5
Potassium (μg/g)	95.5	74.5	109.5	65.8
Vanadium (μg/g)	0.01	0.00	0.02	0.01
Chromium (μg/g)	0.01	0.01	0.00	0.08
Manganese (μg/g)	1.1	0.3	0.4	0.6
Iron (μg/g)	2.8	1.3	8.9	8.5
Cobalt (μg/g)	0.01	0.01	0.01	0.04
Nickel (μg/g)	0.01	0.01	0.01	0.04
Copper (μg/g)	0.3	0.3	0.1	0.9
Zinc (μg/g)	2.6	2.9	2.0	3.4
Additives	Bactocell100® Aquagest®	Stick water SP1 Alltech® Aquatate Alltech®		Brewers yeast Sprirulina

* Represents unknown percentages

Table 5.2. Fatty acid composition (% by weight of total fatty acids) of the experimental diets.

Fatty acid	Diet A	Diet B	Diet C	Diet D
14:0	8.14 ± 0.10^{a}	5.20 ± 0.03^{b}	5.44 ± 0.09^{b}	3.21 ± 0.02^{c}
15:0	0.37 ± 0.01^{c}	0.39 ± 0.01^{c}	0.49 ± 0.00^{a}	0.46 ± 0.00^{b}
16:0	18.94 ± 0.22^{b}	20.44 ± 0.35^{a}	16.98 ± 0.22^{c}	18.18 ± 0.09^{b}
18:0	1.68 ± 0.02^{d}	2.49 ± 0.04^{c}	3.44 ± 0.05^{b}	4.27 ± 0.01^{a}
20:0	0.13 ± 0.01^{ab}	0.06 ± 0.08^{b}	0.20 ± 0.00^{ab}	0.29 ± 0.00^{a}
22:0	<LOQ	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}	0.23 ± 0.00^{a}
24:0	<LOQ	<LOQ	0.12 ± 0.04	0.13 ± 0.03
Total saturated	**29.34 ± 0.34^{a}**	**28.67 ± 0.44^{a}**	**26.77 ± 0.40^{b}**	**26.77 ± 0.11^{b}**
16:1n-9	0.16 ± 0.00^{b}	0.16 ± 0.00^{b}	0.23 ± 0.00^{a}	0.23 ± 0.02^{a}
16:1n-7	6.37 ± 0.03^{a}	5.93 ± 0.00^{b}	6.43 ± 0.02^{a}	3.24 ± 0.04^{c}
18:1n-9	11.81 ± 0.04^{c}	12.95 ± 0.14^{b}	12.03 ± 0.07^{c}	14.42 ± 0.02^{a}
18:1n-7	5.00 ± 0.08^{a}	4.83 ± 0.02^{a}	3.60 ± 0.08^{b}	2.82 ± 0.06^{c}
20:1n-11	<LOQ	0.14 ± 0.21^{c}	3.43 ± 0.00^{a}	1.17 ± 0.03^{b}
20:1n-9	5.13 ± 0.05^{a}	3.99 ± 0.20^{b}	2.30 ± 0.02^{c}	5.57 ± 0.02^{a}
20:1n-7	0.38 ± 0.00^{b}	0.64 ± 0.00^{a}	0.32 ± 0.00^{bc}	0.27 ± 0.03^{c}
22:1n-11	5.34 ± 0.01^{b}	3.40 ± 0.04^{d}	5.14 ± 0.02^{c}	5.78 ± 0.00^{a}
22:1n-9	0.90 ± 0.02^{b}	1.26 ± 0.01^{a}	0.83 ± 0.04^{b}	0.55 ± 0.01^{c}
24:1n-9	0.42 ± 0.02^{c}	0.31 ± 0.02^{d}	0.84 ± 0.01^{a}	0.52 ±0.03^{b}
Total monounsaturated	**35.51 ± 0.02^{a}**	**33.60 ± 0.08^{c}**	**35.16 ± 0.27^{ab}**	**34.57 ± 0.10^{b}**
18:2n-6	6.11 ± 0.04^{c}	8.21 ± 0.08^{b}	3.12 ± 0.01^{d}	18.31 ± 0.02^{a}
18:3n-6	0.17 ± 0.01^{b}	0.09 ± 0.00^{c}	0.18 ± 0.01^{b}	0.28 ± 0.00^{a}
20:2n-6	0.14 ± 0.01^{c}	0.17 ± 0.01^{bc}	0.22 ± 0.01^{b}	1.15 ± 0.02^{a}
20:3n-6	0.12 ± 0.08	0.14 ± 0.06	0.14 ± 0.00	0.12 ± 0.01
20:4n-6 (ARA)	0.40 ± 0.00^{d}	0.58 ± 0.00^{c}	1.05 ± 0.02^{a}	0.95 ± 0.01^{b}
22:4n-6	<LOQ	<LOQ	0.09 ± 0.01^{b}	0.46 ± 0.01^{a}
22:5n-6	<LOQ	0.34 ± 0.02^{a}	0.35 ± 0.01^{a}	0.15 ± 0.0^{b}
Total n-6 PUFA	**6.94 ± 0.13^{c}**	**9.54 ± 0.03^{b}**	**5.15 ± 0.04^{d}**	**21.42 ± 0.03^{a}**
18:3n-3	1.67 ± 0.01^{b}	1.26 ± 0.01^{c}	0.96 ± 0.01^{d}	1.91 ± 0.02^{a}
18:4n-3	3.47 ± 0.02^{a}	2.21 ± 0.05^{b}	2.10 ± 0.04^{b}	0.82 ± 0.01^{c}
20:3n-3	0.16 ± 0.00^{ab}	0.06 ± 0.09^{b}	0.12 ± 0.00^{b}	0.30 ± 0.00^{a}
20:4n-3	0.39 ± 0.00^{b}	0.31 ± 0.00^{c}	0.67 ± 0.01^{a}	0.42 ± 0.01^{b}
20:5n-3 (EPA)	11.82 ± 0.39^{a}	11.77 ± 0.17^{a}	11.67 ± 0.23^{a}	5.22 ± 0.09^{b}
22:5n-3	0.64 ± 0.01^{c}	0.44 ± 0.02^{d}	1.84 ± 0.04^{a}	1.13 ± 0.03^{b}
22:6n-3 (DHA)	8.38 ± 0.04^{c}	11.25 ± 0.20^{b}	13.29 ± 0.29^{a}	6.98 ± 0.10^{d}
Total n-3 PUFA	**26.53 ± 0.22^{b}**	**27.30 ± 0.54^{b}**	**30.66 ± 0.62^{a}**	**16.77 ± 0.22^{c}**
16:2	0.54 ± 0.01^{b}	0.39 ± 0.02^{c}	0.62 ± 0.00^{a}	0.20 ± 0.00^{d}
16:3	0.31 ± 0.00^{b}	0.13 ± 0.01^{c}	0.60 ± 0.00^{a}	0.11 ±0.02^{c}
16:4	0.84 ± 0.02^{b}	0.38 ± 0.02^{c}	1.03 ± 0.01^{a}	0.15 ± 0.01^{d}
Total PUFA	**35.16 ± 0.32^{b}**	**37.73 ± 0.52^{a}**	**38.07 ± 0.67^{a}**	**38.66 ± 0.21^{a}**
ARA/EPA ratio	**0.03 ± 0.00^{d}**	**0.05 ± 0.00^{c}**	**0.09 ± 0.00^{b}**	**0.18 ± 0.00^{a}**
DHA/EPA ratio	**0.71 ± 0.01^{d}**	**0.96 ± 0.00^{c}**	**1.14 ± 0.00^{b}**	**1.34 ± 0.00^{a}**

Limit of quantification (LOQ) for fatty acid analysis is 0.06%

Table 5.3. Lipid classes (% by weight of total fatty acids) of the experimental diets.

Lipid classes	Diet A	Diet B	Diet C	Diet D
LPC	2.0 ± 0.2	2.4 ± 0.2	1.5 ± 0.1	0.4 ± 0.0
UNK 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.1
SM	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	1.4 ± 0.2
PC	10.7 ± 0.8	12.3 ± 0.2	7.9 ± 0.1	5.6 ± 0.0
PI 1	4.8 ± 0.3	2.6 ± 0.4	0.9 ± 0.1	3.6 ± 0.1
PI 2	2.8 ± 0.0	1.6 ± 0.2	3.5 ± 0.7	2.6 ± 0.0
PE 1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 0.1
PE 2	6.8 ± 0.0	4.3 ± 0.7	6.2 ± 0.1	1.2 ± 0.1
DGDG	1.3 ± 0.0	3.5 ± 1.0	0.0 ± 0.0	1.0 ± 0.0
GLY	4.4 ± 0.2	5.1 ± 1.3	1.6 ± 0.3	3.1 ± 0.1
UNK 2	2.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.4
MGDG	6.7 ± 0.2	7.1 ± 0.9	9.9 ± 0.7	11.8 ± 0.6
Total polar	**41.6 ± 0.6**	**38.7 ± 2.3**	**32.4 ± 1.9**	**38.9 ± 0.1**
DAG	3.3 ± 0.1	3.9 ± 0.7	3.8 ± 0.1	3.0 ± 0.0
STER	15.6 ± 0.1	10.9 ± 0.6	12.8 ± 0.5	14.1 ± 0.5
FFA	16.5 ± 0.1	14.4 ± 1.3	11.9 ± 0.8	21.7 ± 0.6
TAG	17.3 ± 0.7	26.3 ± 4.1	32.3 ± 0.7	16.5 ± 0.7
SE	5.8 ± 0.1	5.8 ± 0.9	6.9 ± 1.9	5.8 ± 0.4
Total neutral	**58.4 ± 0.6**	**61.3 ± 2.3**	**67.6 ± 1.9**	**61.1 ± 0.1**

Table 5.4. Amino acids (%) of the experimental diets.

Amino acids	Diet A	Diet B
EAA (%)		
Arginine	3.3	4
Histidine	1.31	1.15
Isoleucine	2.5	2.43
Leucine	4.29	4.13
Lycine	3.92	5.09
Methionine	1.33	1.99
Phenylalanine	2.61	2.24
Threonine	2.3	2.34
Valine	2.76	2.61
NEAA (%)		
Alanine	3	3.26
Aspartic acid	5.21	5.56
Cystein + cystine	0.578	0.528
Glutamic acid	9.47	8.1
Glycine	2.81	3.64
Hydroxyproline	<0.05	<0.05
Ornithine	<0.05	<0.05
Proline	2.75	2.46
Serine	2.5	2.47
Tyrocine	2.03	1.81

5.2.3.3 Histological processing, morphometric analyses and enteritis scoring

Histological processing of the intestine was performed at the Institute of Aquaculture, University of Stirling, Stirling, Scotland (UK). Intestinal samples were flushed with saline prior to their fixation in Serra fixative and were dehydrated according to Bancroft and Stevens (1982) and embedded in paraffin. After hardening the wax, blocks were sectioned in 5 μm sequential transverse sections and placed on glass slides. Two slides were generated from each intestine section (eight slides per fish) in order to stain each section with either haematoxylin-eosin (H and E) or a combination of Periodic Acid Schiff (PAS) and Alcian blue (8GX, pH 2.5) to provide optimal differentiation between acid and neutral mucins respectively from other tissues. Preliminary sequential sectioning showed no significant differences between slides, validating the use of a single slide per stain. Six fish were randomly chosen from each diet (three per replicate tank), out of which the first and third intestine sections were analysed as described below.

Morphometric analyses were performed using a microscope (Olympus BX51, Hamburg, Germany) with an attached micro digital camera (Axiocam MRc5, Carl Zeiss, Cambridge, UK) connected to a PC and an image analysis software (Aperio Image Scope v12.1) according to Lundstedt et al. (2004) (Table 5.5). For intestine sections 1 and 3, the following parameters were measured: a) external diameter (six measurements taken at a perpendicular angle; mm), b) thickness of sub-mucosa layer (six measurements taken at random location per section; mm), c) total thickness of the muscularis layer, d) thickness of mucosa epithelium, e) acid and neutral mucins (total number of cells per mm^2 from six random villous per section) and f) number of mucosal folds counted from the base (Fig. 5.2). Each section was subjectively scored (scale 1 – 5) to quantify severity of potential enteritis according to Knudsen et al. (2008) and Urán (2008) and the overall enteritis score was calculated.

Table 5.5. Histological scoring system of morphological changes used to characterise changes induced by enteritis (adapted from Urán et al., 2008).

Score	Parameter
Lamina propria (LP) of simple folds	
1	Normal size LP
2	Increased size LP
3	Medium size LP
4	Large size LP
5	Largest size LP
Mucus cells (MC)	
1	Scattered MC
2	Increased number and sparsely distributed MC
3	Diffused number widely spread MC
4	Densely grouped MC
5	Highly abundant and tightly-packed MC
Connective tissue (CT)	
1	Very little CT between base of folds and circular muscle
2	Slightly increased amount of CT beneath some of the MF
3	Clear increase of CT below all MF
4	Thick layer of CT beneath high percentage of MF
5	Extremely thick layer of CT beneath some MF
Mucosal folds (MF)	
1	Simple and complex MF appear long and thin
2	Simple MF have medium length, while the complex MF appear thicker
3	Simple MF had short to medium length, while complex MF are stubby
4	Simple MF are thick and short, while thick and stubby complex MF are prevalent
5	Both complex and simple MF appear very short and stubby

Overall Scoring	
1-2	Normal morphology
3	Clear signs of inflammation
4-5	Chronic symptoms of enteritis

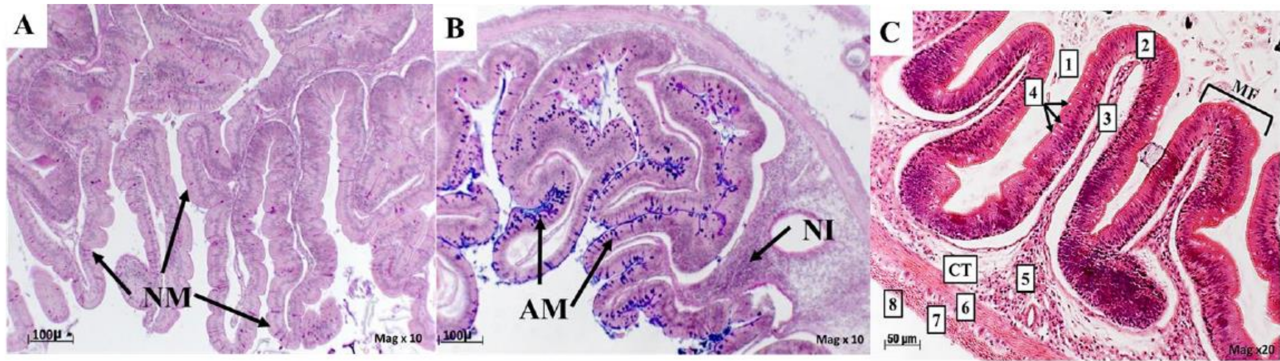

Figure 5.2. Transverse sections of juvenile ballan wrasse intestine. (A) First section of intestine (Alcian blue / PAS, mag x 10) showing a high number of neutral mucins (NM). (B) Third section of intestine (Alcial blue / PAS, mag x 10) showing a high amount of acid mucins (AM) stained deep blue and signs of inflammation through neutrophil infiltration (NI). (C) Second section of intestine (H and E, mag x 20) showing the simple structural organisation; 1, lumen; 2, columnar epithelium; 3, lamina propria; 4, mucous cells; 5, *Stratum granulosum*; 6, Circular muscle; 7, longitudinal muscle; 8, serosa; MF, mucosal fold; CT, connective tissue.

5.2.3.4 Digestive enzyme analyses

The enzyme analyses of the intestine were performed at the Functional Physiology of Marine Organisms Unit at Ifremer, Brest, France. From the four intestinal sections, sections 1 and 3 were used for the digestive enzyme analyses as for previous analyses. After partial thawing and weighing of intestine sections from each replicate tank (n = 2), samples from three fish were pooled to reach the sufficient amount for analysis (i.e. 80 – 100 mg). From the intestinal segment, trypsin activity was measured according to Holm et al. (1988). From the brush border membrane (BBM), alkaline phosphatase (AP), leucine aminopeptidase (LAP) and leucine-alanine peptidase (leu–ala) activities were assayed according to Bessey *et al*. (1946), Maroux *et al*. (1973) and Nicholson & Kim (1975),

respectively. Protein was determined according to the Bradford procedure (Bradford, 1976). Enzyme activity results were expressed as specific activities (U / mg^{-1} protein).

5.2.3.5 Digestibility analyses

Thirty individual fish from tanks fed the diets containing yttrium oxide (diets A, B and D) were anaesthetised at the end of the trial and fecal samples were hand stripped from the distal part of the intestine. However, it became apparent that the collected material (0.08 – 0.56 g dry matter per tank) was insufficient to be analysed (minimum dry matter amount required of 1.7 g.) for most of the diets and no further analysis was performed with these samples. Therefore, the fecal samples were collected manually with a net prior to first daily hand feeding every morning for 3 days. The fecal samples from each treatment were pooled from both replicate tanks to achieve a sufficient amount and kept at – 20 ºC until chemical analysis performed. Fecal material for diet D was still insufficient and only protein and crude fat apparent digestibility coefficients (ADC) were analysed. The tanks were cleaned after sampling at 9:00 am and 4:00 pm. Samples were analysed by Eurofins, Moss, Norway. The nutrients ADC were calculated as follows: ADC (%) = 100 – [100((%/Y_{diet}/%Y_{faeces}) x (%N_{faeces}/%N_{diet}))], where, Y is the Yttrium oxide (Y_2O_3) and N the nutrient.

5.2.4 Statistical analysis

Prior to analysis, normality and homogeneity of variance were assessed and datasets with non-normal distribution and/or heterogenous variance were transformed (square-root, logarithmic or power transformation). All results expressed as a percentage were first arcsine transformed. One-way analysis of variance (ANOVA) was used to test for diets effects in all assessed parameters and two-way analysis of variance was used to analyse enzymatic activity and pH data to test for effect of diets and intestinal sections. Data

comparison followed by a Tukey *post hoc* test with 95 % confidence, by using MINITAB® Release 17 (Minitab Ltd., UK). All data are expressed as mean ± standard deviation (SD).

5.3 Results

5.3.1 Growth and feed utilisation

No significant differences in body-size parameters (body weight, total length and condition factor) between tanks were observed at the start of the trial and between replicates at the end of the trial (Table 4). Fish fed diets A and B were significantly larger at the end of the trial (final weight of 53.70 ± 11.27 and 56.38 ± 10.41 g, respectively) than fish fed diets C and D (46.65 ± 10 and 38.6 ± 7.88 g, respectively). Fish fed diet C were also significantly larger than diet D, which the last ones did not grow during the 90 days of the experiment. Fish length was comparable for fish fed diets A, B and C (13.86 ± 0.91, 14.23 ± 0.80 and 13.68 ± 0.92 cm respectively), but fish fed diet D (12.98 ± 0.76 cm) were significantly smaller than for diet B. With respect to Fulton's condition factor, fish on diets A and B had a significantly higher condition compared to those fed diets C &D (1.99 ± 0.16; 1.94 ± 0.14 for diets A and B, compared with 1.80 ± 0.16; 1.74 ± 0.16 for diets C and D). SGR and TGC values were comparable as was their relationship to the treatments where in SGR, for example, growth rate appeared to be the highest in fish fed diet B (0.44 ± 0.18 % day^{-1}) followed by diet A (0.34 ± 0.16 % day^{-1}), C (0.19 ± 0.16 % day^{-1}) and D (0.02 ± 0.16 % day^{-1}). The overall FCR values showed a trend for more efficient conversion ratio in fish fed diet B (2.07 ± 0.93), followed by diet A (3.43 ± 3.22) while diet C was highly variable thus ended with a mean FCR of 15.05 ± 24.30. FCR could not be determined for diet D due to pellet instability. No differences were found in fish IL / TL ratio or VSI amongst diets. However, fish fed diets A, B and C showed higher HSI compared to fish fed

diet D. Mortalities rates were generally low for all treatments, ranging between 0 – 2% (Table 5.5).

The daily feed intake rate was higher in fish fed diet B (0.38 ± 0.04), followed by diet A (0.36 ± 0.02) and C (0.28 ± 0.03) (Fig. 5.3 & Table 5.5), which was reflected on the feeding efficiency (Table 5.5).

Table 5.5. Growth performance, development and mortality of ballan wrasse fed the four experimental diets for 90 days. Values are resented as mean ± SD (n = 2). Superscript letters indicate significant differences between diets ($P < 0.05$).

	Diet A	**Diet B**	**Diet C**	**Diet D**
Initial body weight (g)	38.88 ± 8.28	38.58 ± 7.13	38.71 ± 7.18	38.81 ± 8.30
Initial body length (cm)	12.71 ± 0.84	12.60 ± 0.70	12.61 ± 0.77	12.52 ± 0.89
Initial condition factor	1.90 ± 0.14	1.89 ± 0.17	1.91 ± 0.14	1.89 ± 0.17
Final body weight (g)	53.70 ± 11.27^{a}	56.38 ± 10.41^{a}	46.65 ± 10.01^{b}	38.60 ± 7.88^{c}
Final body length (cm)	13.86 ± 0.91^{ab}	14.23 ± 0.80^{a}	13.68 ± 0.92^{ab}	12.98 ± 0.76^{b}
Final condition factor	1.99 ± 0.16^{a}	1.94 ± 0.14^{a}	1.80 ± 0.16^{b}	1.74 ± 0.16^{b}
SGR (% / day)	0.34 ± 0.16	0.44 ± 0.18	0.19 ± 0.16	0.02 ± 0.16
TGC	0.34 ± 0.15	0.46 ± 0.19	0.19 ± 0.16	0.03 ± 0.15
FCR	3.43 ± 3.22	2.07 ± 0.93	15.05 ± 24.30	n/a*
FI (g/fish/day)	0.36 ± 0.02	0.38 ± 0.04	0.28 ± 0.03	n/a*
FE	0.0228 ± 0.0003	0.0233 ± 0.001	0.0267 ± 0.0020	n/a*
HSI (%)	1.56 ± 0.42^{a}	1.63 ± 0.55^{a}	1.31 ± 0.24^{a}	1.04 ± 0.20^{b}
VSI (%)	6.71 ± 1.15	6.81 ± 1.59	6.50 ± 1.32	5.29 ± 0.91
IL / TL ratio	0.52 ± 0.17	0.55 ± 0.06	0.60 ± 0.05	0.59 ± 0.10
Mortalities (%)	0 ± 0	0.5 ± 0.01	1.00 ± 0.01	2.00 ± 0.01

* Data could not be calculated, due to pellet instability

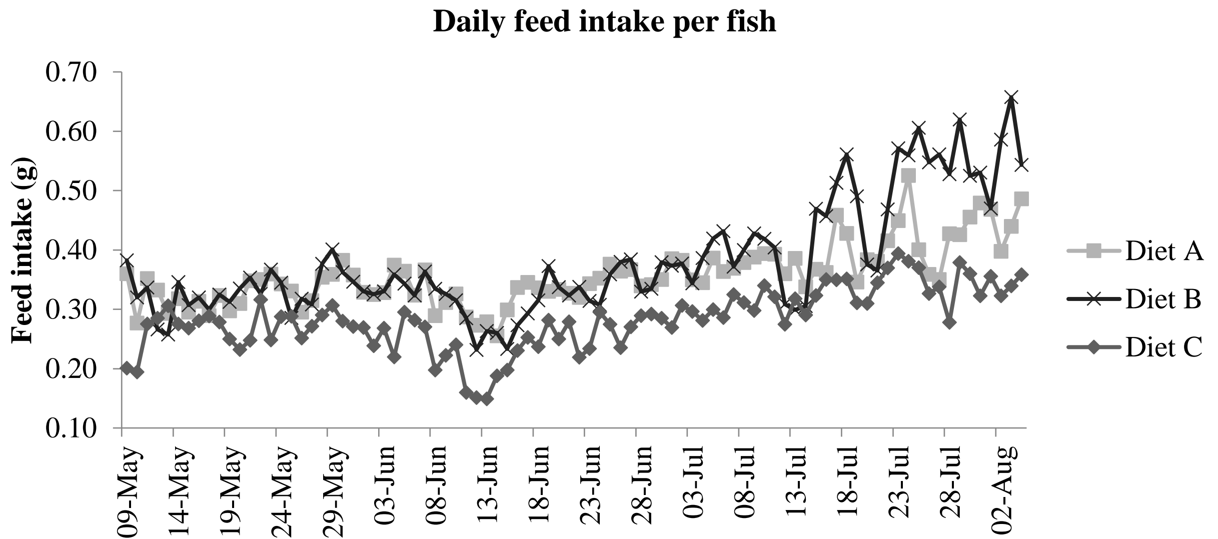

Figure 5.3. Average daily food intake per juvenile ballan wrasse offered three experimental diets during the 3-month experiment. No data available for diet D, due to pellet instability.

5.3.2 Liver fatty acid profile

Palmitic acid (16:0), oleic acid (18:1n-9) and docosahexaenoic acid (DHA) contribute to over 40 % of total liver FA composition, with oleic acid prevailing in all treatments (Table 5.6). No differences were found in liver palmitic acid content between treatments (16.07 ± 1.83 % to 16.48 ± 0.58 % of the total fatty acids). Oleic acid varied from 18.56 ± 2.46 % (diet A) to 26.13 ± 8.09 % (diet D) and DHA from 7.32 ± 1.22 % (diet A) to 12.12 ± 5.01 % (diet C). Typical marine fatty acids, such as EPA, DHA and ARA, clearly reflected the dietary levels of experimental diets. Liver ARA content was almost twice higher in fish fed diets C and D compared to diets A and B. On the contrary, EPA levels were higher for diets A, B and C compared to D, and DHA levels were higher for diet C compared to diet A and comparable for diets B and D.

Table 5.6. Fatty acid composition (% by weight of total fatty acids) of liver from fish fed the four experimental diets. Values are resented as mean ± SD (n = 2). Superscript letters indicate significant differences within the same row ($P < 0.05$).

Fatty acid	Pre-trial diet	Diet A	Diet B	Diet C	Diet D
14:0	5.45 ± 1.24^{ab}	6.68 ± 0.79^{a}	5.01 ± 1.28^{b}	4.87 ± 1.30^{b}	3.00 ± 1.82^{c}
15:0	0.39 ± 0.03	0.39 ± 0.04	0.37 ± 0.05	0.44 ± 0.05	0.41 ± 0.18
16:0	18.73 ± 0.99^{a}	16.44 ± 0.52b	16.48 ± 0.58b	16.08 ± 1.26b	16.07 ± 1.83b
18:0	4.29 ± 0.20^{ab}	3.93 ± 0.45^{b}	4.07 ± 0.29^{b}	4.31 ± 0.40^{ab}	4.94 ± 0.89^{a}
20:0	0.10 ± 0.01	<LOQ	<LOQ	<LOQ	<LOQ
22:0, 24:00	< LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Total saturated	**29.05** ± 0.77^{a}	**27.56 ± 0.93^{ab}**	**26.10 ± 1.22^{bc}**	**25.89 ± 1.01^{bc}**	**24.64 ± 2.82^{c}**
16:1n-9	0.40 ± 0.03^{ab}	0.38 ± 0.03^{b}	0.39 ± 0.06^{ab}	0.42 ± 0.04^{ab}	0.45 ± 0.06^{a}
16:1n-7	5.95 ± 1.41^{a}	7.14 ± 0.45^{a}	6.78 ± 1.41^{a}	6.01 ± 1.35^{a}	3.12 ± 1.77^{b}
18:1n-9	14.04 ± 2.12^{c}	18.56 ± 2.46^{bc}	22.33 ± 3.08^{ab}	20.60 ± 4.84^{abc}	26.13 ± 8.09^{a}
18:1n-7	4.87 ± 0.52^{a}	5.35 ± 0.21^{a}	5.04 ± 0.50^{a}	4.20 ± 0.39^{b}	2.97 ± 0.72^{c}
20:1n-11	< LOQ	0.29 ± 0.04^{b}	0.25 ± 0.09^{b}	1.42 ± 0.51^{a}	0.49 ± 0.48^{b}
20:1n-9	2.74 ± 0.60^{bc}	3.43 ± 0.32^{a}	3.05 ± 0.43^{ab}	2.35 ± 0.50^{bc}	2.55 ± 0.55^{bc}
20:1n-7	0.22 ± 0.03^{bc}	0.28 ± 0.01^{b}	0.39 ± 0.06^{a}	0.24 ± 0.03^{b}	0.18 ± 0.07^{c}
22:1n-11	1.30 ± 0.42	2.14 ± 0.30	1.67 ± 0.27	2.01 ± 0.54	1.80 ± 2.06
22:1n-9	0.37 ± 0.09^{c}	0.50 ± 0.05^{ab}	0.59 ± 0.09^{a}	0.47 ± 0.10^{bc}	0.42 ± 0.09^{bc}
24:1n-9	0.78 ± 0.30^{a}	0.54 ± 0.23^{ab}	0.46 ± 0.14^{b}	0.68 ± 0.12^{ab}	0.73 ± 0.30^{ab}
Total monounsaturated	**30.67** ±4.86^{b}	**38.60 ± 2.55^{a}**	**40.97 ± 1.20^{a}**	**38.39 ± 6.34^{a}**	**38.84 ± 6.43^{a}**
18:2n-6	8.47 ± 0.89^{b}	8.06 ± 0.47^{b}	8.88 ± 0.66^{b}	5.63 ± 1.31^{c}	13.48 ± 2.89^{a}
18:3n-6	0.12 ± 0.03	0.15 ± 0.01	0.10 ± 0.02	0.17 ± 0.07	0.15 ± 0.12
20:2n-6	0.59 ± 0.03^{bc}	0.53 ± 0.05^{bc}	0.61 ± 0.05^{b}	0.51 ± 0.06^{bc}	1.07 ± 0.14^{a}
20:3n-6	0.11 ± 0.01	0.10 ± 0.01	0.16 ± 0.27	0.14 ± 0.02	0.13 ± 0.04
20:4n-6 (ARA)	1.33 ± 0.39^{a}	0.61 ± 0.06^{b}	0.72 ± 0.24^{b}	1.73 ± 0.70^{a}	1.66 ± 0.60^{a}
22:4n-6	< LOQ	< LOQ	<LOQ	<LOQ	<LOQ
22:5n-6	0.16 ± 0.14^{b}	0.09 ± 0.01^{c}	0.24 ± 0.04^{a}	0.25 ± 0.07^{a}	0.16 ± 0.07^{b}
Total n-6 PUFA	**10.79** ± 0.59^{b}	**9.54 ± 0.50^{bc}**	**10.72 ± 0.75^{b}**	**8.46 ± 0.82^{c}**	**16.84 ± 2.73^{a}**
18:3n-3	0.92 ± 0.20^{b}	1.48 ± 0.11^{a}	1.03 ± 0.14^{b}	0.80 ± 0.17^{b}	0.93 ± 0.45^{b}
18:4n-3	0.89 ± 0.24^{c}	2.03 ± 0.18^{a}	1.24 ± 0.23^{b}	1.02 ± 0.22^{bc}	0.40 ± 0.17^{d}
20:3n-3	0.11 ± 0.01^{b}	0.22 ± 0.13a	0.14 ± 0.02ab	0.13 ± 0.03ab	0.15 ± 0.08ab
20:4n-3	0.45 ± 0.07^{ab}	0.52 ± 0.23^{a}	0.44 ± 0.08^{ab}	0.61 ± 0.11^{a}	0.31 ± 0.12^{b}
20:5n-3 (EPA)	12.67 ± 1.44^{a}	10.90 ± 0.75^{b}	9.64 ± 0.53^{b}	10.48 ± 1.83^{b}	6.28 ± 1.39^{c}
22:5n-3	1.09 ± 0.19^{ab}	0.87 ± 0.10^{bc}	0.67 ± 0.11^{c}	1.28 ± 0.30^{a}	1.07 ± 0.39^{ab}
22:6n-3 (DHA)	12.65 ± 4.93^{a}	7.32 ± 1.22^{b}	8.45 ± 1.47^{ab}	12.12 ± 5.01^{ab}	10.04 ± 5.09^{ab}
Total n-3 PUFA	**28.77** ± 5.99^{a}	**23.32 ± 2.10^{abc}**	**21.62 ± 1.54^{bc}**	**26.45 ± 6.70^{ab}**	**19.18 ± 6.22^{bc}**
16:2	0.39 ± 0.12^{ab}	0.48 ± 0.05^{a}	0.33 ± 0.07^{b}	0.41 ± 0.11^{ab}	0.16 ± 0.09^{c}
16:3	0.17 ± 0.07^{ab}	0.19 ± 0.03^{ab}	0.11 ± 0.05^{b}	0.20 ± 0.06^{a}	0.21 ± 0.10^{ab}
16:4	0.16 ± 0.08^{b}	0.30 ± 0.04^{a}	0.15 ± 0.05^{b}	0.20 ± 0.06^{b}	0.13 ± 0.07^{b}
Total PUFA	**40.28** ± 5.29^{a}	**33.84 ± 2.06^{b}**	**32.93 ± 1.40^{b}**	**35.72 ± 6.09^{ab}**	**36.52 ± 5.59^{ab}**
ARA/EPA ratio	**1.08** ± 0.23^{c}	**0.06 ± 0.00^{d}**	**0.08 ± 0.03^{cd}**	**0.16 ± 0.14^{b}**	**0.26 ± 0.05^{a}**
DHA/EPA ratio	**0.98 ±0.26^{b}**	**0.67 ± 0.07^{c}**	**0.88 ± 0.15^{b}**	**1.12 ± 0.26^{ab}**	**1.54 ± 0.55^{a}**

Limit of quantification (LOQ) for fatty acid analysis is 0.06%

5.3.3 Intestine morphology

5.3.3.1 Morphometric analysis

The intestinal morphometric analysis did not reveal consistent differences between fish fed the experimental diets, however, some differences were apparent (Table 5.7). No differences in the number of mucosal folds and sub-mucosa layer were observed in both intestine sections. No differences were also found in the muscularis layer thickness and the number of mucus cells in section 3. Fish fed diet A appeared to have thicker intestine external diameter for both sections 1 and 3 and muscularis layer for section 1 compared to the other diets. The number of mucus cells in section 1 was higher in fish fed diet B compared to the other diets. When comparing experimental fish between start and end of the trial, all of them had thinner intestine external diameter and less number of mucus cells in both intestine sections, but also thinner sub-mucosa layer in intestine section 1.

Table 5.7. Morphometric measurements of intestinal sections 1 and 3 from ballan wrasse fed a pre-trial and four experimental diets. Values are resented as mean ± SD (n = 2, 5 fish per replicate). Superscript letters indicate significant differences within the same row (P < 0.05). No subscript letter indicates lack of significant differences amongst treatments.

	Pre-trial diet	Diet A	Diet B	Diet C	Diet D
External Diameter (mm)					
Section 1	5.21 ± 0.59^{a}	3.68 ± 0.55^{b}	3.01 ± 0.54^{c}	3.07 ± 0.51^{c}	2.90 ± 0.34^{c}
Section 3	3.38 ± 0.64^{a}	3.00 ± 0.46^{b}	2.41 ± 0.44^{c}	2.44 ± 0.53^{c}	2.27 ± 0.44^{c}
Number of muscosal folds					
Section 1	29.33 ± 2.07	30.38 ± 0.04	32.90 ± 4.95	32.50 ± 0.03	30.44 ± 0.05
Section 3	21.83 ± 1.72	25.13 ± 4.76	23.89 ± 3.79	26.00 ± 6.00	25.78 ± 3.73
Sub-Mucosa (mm)					
Section 1	0.23 ± 0.09^{a}	0.08 ± 0.04	0.09 ± 0.04	0.23 ± 0.09	0.08 ± 0.03
Section 3	0.16 ± 0.06	0.08 ± 0.06	0.08 ± 0.04	0.07 ± 0.04	0.07 ± 0.03
Muscularis (mm)					
Section 1	0.09 ± 0.02^{a}	0.08 ± 0.04^{ab}	0.08 ± 0.03^{ab}	0.07 ± 0.03^{b}	0.07 ± 0.03^{b}
Section 3	0.09 ± 0.02^{a}	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.02
Mucus cells (mm^{-1})					
Section 1	19.00 ± 5.97^{a}	12.53 ± 10.48^{b}	16.93 ± 14.24^{a}	10.33 ± 10.32^{b}	11.03 ± 9.63^{b}
Section 3	36.81 ± 11.97^{a}	23.11 ± 16.58^{bc}	26.46 ± 18.16^{b}	19.63 ± 18.63^{d}	16.69 ± 14.78^{cd}

5.3.3.2 Intestine scoring – inflammation

The overall inflammation score was higher at the start of the trial (section 1: 2.63 ± 0.77; section 3: 3.38 ± 0.65) than at the end of the trial (Table 5.8). Fish sampled from all diets showed significantly lower overall inflammation score at the end of the trial, with scoring ranging from 1.43 ± 0.59 (diet C) to 1.68 ± 0.73 (diet B) in intestine section 1 and 1.50 ± 0.51 (diet D) to 1.70 ± 0.61 (diet B) in intestine section 3. Only the number of mucus cells

in section 1 of the intestine for diet B was comparable between start and end of the trial. No significant differences in intestine inflammation scores were found between the experimental diets.

Table 5.8. Semi – quantitative histological assessment for inflammation level of intestinal sections 1 and 3 from ballan wrasse fed a pre-trial and four experimental diets. Values are resented as mean ± SD (n = 2, 5 fish per replicate). Superscript letters indicate significant differences within the same row (P < 0.05). No subscript letter indicates lack of significant differences amongst treatments.

	Pre-trial diet	**Diet A**	**Diet B**	**Diet C**	**Diet D**
Mucus cells (mm^{-1})					
Section 1	2.50 ± 0.55^{a}	2.00 ± 0.82^{ab}	2.00 ± 0.82^{ab}	1.30 ± 0.48^{b}	1.40 ± 0.70^{b}
Section 3	3.17 ± 0.41	1.30 ± 0.48	1.80 ± 0.63	1.90 ± 0.88	1.33 ± 0.50
Connective tissue					
Section 1	3.17 ± 0.75^{a}	1.60 ± 0.70	2.10 ± 0.74	2.10 ± 0.57	2.00 ± 0.47
Section 3	3.83 ± 0.41^{a}	1.80 ± 0.42	2.10 ± 0.32	2.10 ± 0.74	1.78 ± 0.44
Lamina Propria					
Section 1	1.83 ± 0.41^{a}	1.10 ± 0.32	1.10 ± 0.32	1.00 ± 0.00	1.30 ± 0.67^{a}
Section 3	2.67 ± 0.52^{a}	1.30 ± 0.48	1.30 ± 0.67	1.60 ± 0.52	1.00 ± 0.00
Mucosal folds					
Section 1	3.00 ± 0.63^{a}	1.40 ± 0.52	1.50 ± 0.53	1.30 ± 0.48	1.40 ± 0.52
Section 3	3.83 ± 0.41^{a}	1.90 ± 0.32	1.60 ± 0.52	1.40 ± 0.52	1.89 ± 0.33
Overall score					
Section 1	**2.63 ± 0.77^{a}**	**1.53 ± 0.68**	**1.68 ± 0.73**	**1.43 ± 0.59**	**1.53 ± 0.64**
Section 3	**3.38 ± 0.65^{a}**	**1.55 ± 0.50**	**1.70 ± 0.61**	**1.75 ± 0.71**	**1.50 ± 0.51**

5.3.4 Digestive enzymes and pH

Differences in enzymatic activities amongst the two intestine sections of the fish fed the same diet were recorded only in trypsin and leu-ala activity and for diets A and C, with both diets showing significantly higher activity in intestine section 3 compared to section 1 (Fig. 5.4). Fish fed diet D showed a general suppression of enzymatic activity compared to the rest of the diets. No other differences occurred between sections in fish fed the same diet. Trypsin specific activity in section 1 did not differ significantly between diets, but in section 3 diet A had an almost three-fold higher activity compared to diets B and C and seven-fold compared to diet D (Fig. 5.4A). Alkaline phosphatase (AP) specific activity in section 1 was significantly reduced in fish fed diet D compared to all other diets (Fig. 5.3B). However, the same pattern was not followed in section 3, where fish fed diet A had comparable activity to fish fed diet B but higher compared to the rest (Fig. 5.4B). The cytosolic peptidase leu-ala in section 1 was significantly lower in fish fed diet D than all other diets (Fig. 5.4C). Decreased activity in fish fed diet D was also observed in section 3, but not significantly lower compared to diets B or C, only for diet A. The activity of the leucine aminopeptidase (LAP) in the brush border membrane (BBM) did not show any significant differences between diets within the same intestine section or between sections (Fig. 5.4D).

The intestinal pH was higher in section 3 compared to section 1 for diets A (section 1: 8.6 ± 0.26; section 3: 9.1 ± 0.32) and D (section 1: 8.5 ± 0.37; section 3: 9.2 ± 0.24), but similar for diets B (section 1: 8.7 ± 0.24; section 3: 9.1 ± 0.33) and C (section 1: 8.5 ± 0.45; section 3: 8.8 ± 0.19). No significant differences occurred between diets within each intestine section.

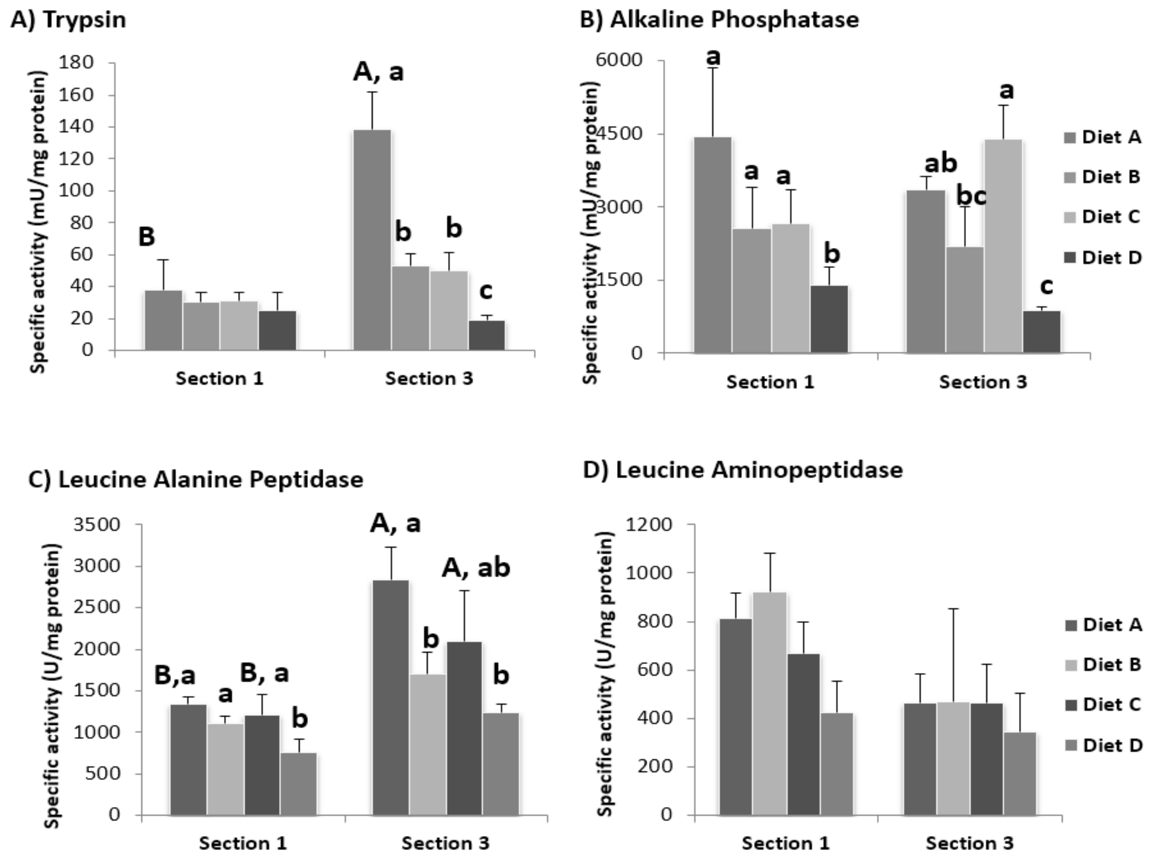

Figure 5.4. Enzyme activities of (A) trypsin, (B) akaline phosphatase (AP), (C) leucine aminopeptidase (LAP) and (D) leucine alanine peptidase (Leu Ala) in intestinal sections 1 and 3 for fish fed the four experimental diets. Upper case letter refers to comparison between intestine sections in the same diet and lower case letters denote significant differences between diets within each intestine section (two-way ANOVA, P < 0.05). Values are resented as mean ± SD (n = 2, 5 fish per replicate).

5.3.5 Digestibility

Apparent digestibility coefficient (ADC) of protein appeared to be similar for diets A, B and C, ranging from 85 to 89 % although no statistical analyses could be done as single values per diet were obtained. ADC of crude fat was similar for diets A and B (85 %), but lower for diet D (73 %). No apparent differences occurred in amino acids ADC between diets A and B (Table 5.9).

Table 5.9. Effect of experimental diets on apparent digestibility coefficient (ADC) (%) for ballan wrasse fed diets A, B or C.

ADC (%)	**Diet A**	**Diet B**	**Diet C**
Protein (%)	89.55	92.77	85.25
Crude fat (%)	85.39	85.59	73.19
EAA (%)			
Arginine	95.07	96.79	
Histidine	91.85	93.32	
Isoleucine	92.95	94.01	
Leucine	94.22	95.28	
Lycine	95.20	96.91	
Methionine	94.20	97.33	
Phenylalanine	93.62	94.21	
Threonine	91.80	93.30	
Valine	92.32	93.64	
NEAA(%)			
Alanine	92.76	94.61	
Aspartic acid	90.81	91.73	
Cystein + cystine	83.16	86.04	
Glutamic acid	95.28	95.80	
Glycine	89.08	92.94	
Hydroxyproline	<LOQ	<LOQ	
Ornithine	<LOQ	<LOQ	
Proline	94.58	94.53	
Serine	91.62	92.35	
Tyrocine	91.99	92.61	

Limit of quantification (LOQ) for amino acids analysis is 0.05%

5.4 Discussion

The aims of this study were to compare growth performances, liver fatty acid composition, intestinal health and digestive function in farmed ballan wrasse fed different experimental and commercial diets. To date, very little information is available on ballan wrasse nutritional requirements, diet formulation and hatchery performances while there is a clear need to develop ballan wrasse-specific diets to promote feed palatability and growth, without compromising intestinal health.

On-farm experiences come in line with previous reports (Dipper et al., 1977) describing ballan wrasse as a slow growing species. Growth performances for other marine species farmed in similar temperatures and size as the experimental fish of this study have been previously reported with Atlantic cod having SGR 1.2 – 1.8 % day $^{-1}$ and Atlantic halibut 0.5 – 1.1 % day^{-1} (Tibbetts et al., 2011). In the present study, SGR values were ranging from 0.02 ± 0.16 (diet D) to 0.44 ± 0.18 % day $^{-1}$ (diet B). SGR value from fish fed diet A (0.34 ± 0.16 % day $^{-1}$) was similar to what has been reported in commercial conditions of Otter Ferry Seafish· (0.35 % day $^{-1}$) (David Patterson, Otter Ferry Seafish, pers. comm.), with similar size fish and using a comparable diet, but also with the results obtained by Helland et al. (2014) using farmed ballan wrasse between 25 – 45 g and getting overall SGR of 0.44 – 0.52 % day^{-1}. However, fish fed diet B had 23 % higher SGR compared to the commercial conditions. It should be acknowledged that water temperature during this study was maintained at 11.5 ± 0.7 °C compared to commercial conditions which fluctuates between 10 and 13 °C. The effects of thermal environment on fish growth have also been previously reported in other cold water marine species (e.g. Atlantic cod, Arnason et al., 2009). The enhanced growth reported in this study can be attributed to feed utilisation and/or lower stocking densities that were used during the experiment (> 5 kg/m^3) compared to commercial conditions (usually > 15 kg/m^3). Reduced stocking density

has been reported to have a positive impact on growth in other cold water marine fish species (e.g. Atlantic cod, Björnsson et al., 2012). Condition factor values for ballan wrasse juveniles have been previously reported to be between 1.52 and 1.85 (Hamre et al., 2013a), which are lower than the best performing fish in this study fish fed diets A and B (1.99 ± 0.16 and 1.94 ± 0.14 respectively), but similar to fish fed diets C and D (1.80 ± 0.16 and 1.74 and 0.16 respectively). Higher condition factor in diets A and B is also in line with the feed intake in these groups. Fulton's condition factor has been used in the literature to describe a two-dimensional relationship between weight and length by translating it into a single statistic which can indicate the energy reserves of a fish (Árnason et al., 2009). Growth in length and weight can indicate accumulation and restoration of energy reserves or synthesis of structural molecules respectively (Counture et al., 1998). FCR measured in the present study can be considered as sub-optimal (minimum of 2.07 ± 0.93 in diet B) when compared to other marine fish species such as Atlantic cod (0.90 ± 0.03, Hixson et al., 2016), but lower compared to the commercial conditions of Otter Ferry Seafish (approximately values between 3 and 4). Increased FCR does not seem to be due to lack of stomach or reduced digestive capabilities since lack of stomach does not seem to affect other stomachless fish digestive capabilities (Day et al., 2011) and ballan wrasse in this study have shown similar digestive capacity to other gastric species. However, ballan wrasse natural diet is based on easily digestible feed sources (Dipper et al., 1977; Hamre et al., 2013a), which might not be totally adopted on the experimental diets. Also, it should be taken into consideration the importance of protein, raw material quality and feeds' physical characteristics which have been previously highlighted by Kousoulaki et al. (2014) that affect significantly growth performance in ballan wrasse juveniles. Indeed, knowledge on ballan wrasse digestive capabilities is in its infancy and further development on species-specific diets is clearly required.

Nutrient availability is one of the main factors that can modulate growth in fish (Valente et al., 2013). Free amino acids present in crustacean raw materials, such as krill meal and shrimp meal (included in diets A and B, and possibly in diet C to an unknown level) can have high attractant properties (Kousoulaki et al., 2015), with potential beneficial effect on feeding, intestinal assimilation and subsequently growth in fish. Indeed, an increased feeding behaviour has been reported in cold water marine species when fed a diet containing high quality squid, krill, fish or crustacean meals as well as hydro-lysates (Tibbetts et al., 2011). Feed intake in ballan wrasse larvae has been also attributed to choose and quality of raw materials. This effect can be related to potential lack of specific attractants for ballan wrasse or the presence of toxic or repellent substances in fish meals (Bogevik et al., 2015; Kousoulaki et al., 2015). In the present study, diets showing best growth performance (diets A and B) contained a high portion of either krill and/or shrimp derivatives as fish meal/oil replacement. Diet B was contained cod fillet fish meal, shrimp meal, krill hydrolysate and krill oil known to act as attractants and feeding stimulants (Tibbetts et al., 2011; Hamre et al., 2013; Helland et al., 2014). Diet C was a fish meal based diet and diet D was based on polychaete worms as a complete fish meal replacement. However, diet D resulted in poorest growth despite comparable intestinal health, fish condition factor and diet proximate composition. This could be attributed to a variety of reasons (i.e. unknown nutritional deficiencies, interactions, etc.), but clear data and intake digestion are lacking to advocate against a polychaete based diet for juvenile ballan wrasse. Diet B had twice higher moisture content compared to the other diets. Increased feed moisture level has also been proven to increase feed intake and growth in various fish species such as common dentex (*Dentex dentex*) (Efthimiou et al., 1994) and Korean rock fish (*Sebastes schlegelii*) (Lee et al., 2000). In a recent study by Helland 2014, it was suggested that higher moisture content may relate to improved feed digestibility and

absorption in ballan wrasse intestine, so subsequently contributing to enhanced growth performance. Given the differences in the experimental feeds in formulation, raw materials and additives, defining species nutritional requirements was not within the scope of this study.

Dietary inclusion of minerals in fish is limited compared to most other nutrient groups. The metabolism of minerals by fish can be influenced not only by the dietary intake but also by the concentrations of dissolved ions in the water (Moyle & Cech, 2000). Supply of adequate dietary mineral composition at a relatively high concentrations is required to achieve formation of skeletal structures and other hard tissues, electron transfer, regulation of acid : base equilibrium, the production of membrane potentials and osmoregulation (NRC, 2011). The most commonly found macrominerals in fish are calcium, chlorine, magnesium, phosphorus, potassium and sodium. Microminerals are required in then diet in much smaller quantities than macrominerals and act as important elements in hormones and enzymes. The most commonly found microminerals are chromium, copper, iodine, iron, manganese, selenium and zinc (NRC, 2011). In this study, the most distinct difference in mineral composition between the diets appeared in sodium, phosphorus, manganese and iron. The highest sodium levels were detected in diet A, followed by diets D, C and B (165.7, 105.7, 94.9 and 82.6 μg/g respectively). Supplementation of sodium chloride is being used as practical supplementation to increase fish growth, especially in freshwater species with the possible explanation of increasing amino acid absorption and reducing osmoregulatory stress (NRC, 2011). European sea bass showed higher growth and feed efficiency in freshwater with addition of 3% of sodium chloride to their diet (Eroldogan et al., 2005). Phosphorus supplementation in fish diets is critical due to its presence in the water and utilisation by fish is limited. Dietary deficiency in phosphorus can impact fish metabolism with negative effects on fish growth, feed conversion and skeletal

malformation related with reduced mineralisation of hard tissues (Sugiura et al., 2004). Dietary levels of phosphorus were similar levels for diets A, B and C (167.5, 134.5 and 197.9 µg/g) but significantly lower in diet D (61.5 µg/g), which might also be related to the reduced growth performance of fish fed diet D. Dietary phosphorus levels in other fish species has been reported to range from 33 to 150 µg/g (NRC, 2011). Dietary deficiencies in manganese can result poor growth, skeletal deformities, cataracts and reduced disease resistance. Given that the manganese content of sea water in very low (0.01 mg/L), dietary supplementation is recommended for fish (NRC, 2011). Unexpectedly, fish in this study which performed the highest growth were fed the diets with the highest and lowest manganese content (diet A, 1.1 µg/g; diet B, 0.3 µg/g respectively). Typically, manganese inclusion in fish diets ranges from 8 to 50 µg/g (Lall, 2002). Iron dietary levels in diets C and D (8.9 and 8.6 µg/g) were 6 and 3 times higher than in diets A and B (2.8 and 1.3 µg/g) respectively. Iron is an fundamental element in the cellular respiratory process through its oxidation-reduction activity and electron transfer with currently known requirements for fish ranging between 30 and 170 mg/g (Lall, 2002). Despite that no obvious abnormalities were observed within this study that can be related to minerals dietary inclusion, anecdotal evidences from the commercial production at Otter Ferry Seafish indicate increasing levels of nephrocalcinosis on kidney of ballan wrasse broodstock and juveniles. In nature compounds differing in electric charge may bind with minerals forming stable compounds that are less soluble in water. However, the acidic condition within fish stomach consents dissociation of the compounds into salts which can easily be absorbed in the intestine (NRC, 2011). Ballan wrasse is a stomachless species with alkaline digestive system, reducing the bioavailability of minerals and also some minerals may interact after being released into the intestine by forming insoluble precipitates. Numerous studies have proven that juvenile marine fish requirements in essential fatty acids (EFAs) varies with dietary

lipid levels and dietary DHA/EPA ratio (Tocher 2010). The importance of n-3 highly unsaturated fatty acids (n-3 HUFA, especially DHA and EPA) has been well reported in marine finfish with effects on growth and other important developmental and physiological functions (Tocher 2003). Since marine fish cannot synthesize LC-PUFAs from their 18-carbon precursor fatty acids such as linolenic acid (18:3n-3) and linoleic acid (18:2n-6), dietary addition of these fatty acids is essential for fish development (Tocher 2003). Also, moderate dietary DHA/EPA can enhance fish growth performances (Xu et al., 2016). However, the optimal dietary lipid and FA content is not known for ballan wrasse. Many studies have shown that the fatty acids composition of marine fish tissues, to a huge extent, reflects that of the diet (Jobling et al., 2008; Olsen et al., 2015). It is also the case in this study with some fatty acids (14:0, 18:1n-7, 20:3n-6, 18:4n-3, ARA and EPA) showing similar liver patterns than dietary inclusion. Some key FA showed liver lipid content modified according to FA dietary content. In particular, increased ARA dietary levels in diets C and D resulted increased fish liver FA levels fed those diets compared to fish fed diets A and B. Similarly, fish fed reduced diet D with reduced dietary EPA showed the lowest EPA levels in their livers compared with the rest of the fish fed the other diets. On the contrary, despite the different DHA dietary levels, no differences were found amongst livers at the end of the study. Beneficial dietary DHA/EPA ratios can vary between species. For instance, DHA/EPA ratio of 1.53 – 2.08 have been shown beneficial for growth of the Japanese seabass (*Lateolabrax japonicus*) juveniles (Xu et al., 2016), while ratio of 0.5 appeared to be sufficient to enhance growth in gilthead seabream juveniles (Ibeas et al., 1997). Fish fed diets A and B resulted in enhanced growth however these diets had lower dietary DHA/EPA levels (0.71 ± 0.01 and 0.96 ± 0.00, respectively) compared to diets C and D (1.14 ± 0.00 and 1.34 ± 0.00 respectively), with liver ratios following similar patterns at the end of the trial.

Diets containing high and moderate levels of DHA and EPA in the phospholipids fraction can enhance growth and intestinal maturation in cod (World et al., 2007). Similarly, sea bass can utilise more efficiently dietary phospholipids than neutral lipid fractions resulting to enhanced survival, growth and fish quality (Cahu et al., 2003). The experimental diets A, B and D of this study had similar total polar lipid levels (41.6 ± 0.6, 38.7 ± 2.3 and 38.9 ± 0.1 % of total lipids) between them, but higher compared to diet C (32.4 ± 1.9). Lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and phatidylethanolamine (PE 2) were from two to five times higher in diets A and B compared to diet D. On the contrary, PE 1 was only detected in diet D. However, no consistent differences among polar levels can clearly justify fish performance. Sterols, tetracyclic hydrocarbon alcohol compounds are the most important simple lipids with cholesterol being the most commonly found sterol in fish (NRC, 2011). The most important neutral lipid is triacylglycerol (TAG), which primarily serves as energy storage. Dietary inclusion of marine TAG can increase growth in marine species, such as sea bass (Cahu et al., 2003). The biggest difference observed between diet A and D which had also most half the amount of TAG compared to diet C (17.3 ± 0.7; 16.5 ± 0.7; 32.3 ± 0.7, respectively). However, the experimental diets in this study had different nutritional composition between each other. Thus, any assumptions about nutritional requirements should be extrapolated with caution. Future studies should assess the effects of comparable composition diets with increasing lipid levels to comprehensively evaluate FA metabolism in ballan wrasse.

The lack of stomach seems to not restrict fish trophic preference, as demonstrated by the wide trophic spectrum of stomachless species displaying a range of diet preferences (Day et al., 2011), confirming ballan wrasse as dynamic and adaptable to different feeding regimes (Helland et al., 2014). Stomachless fish species digestion of proteins, peptides, carbohydrates and lipids occur through digestive enzymes excreted from pancreas and

intestinal mucosa in an alkaline pH (Yonge & Russell, 1972). Stomachless species lack a stomach and therefore pepsin secretion which does not appear to impact on feed digestion in fish, since other proteolytic enzymes secreted by the pancreas can play similar roles in protein digestion (Zambonino-Infante & Cahu 2010). Very little knowledge is available of the digestive physiology of ballan wrasse including changes throughout the life cycle and the impact of environmental factors (e.g. temperature, diets). As mentioned before, intestinal health is broadly suggested to elicit higher growth performance both directly through improved digestive function such as absorption, and indirectly due to metabolic cost of inflammation. In the present study, no consistent differences in intestinal health were observed between fish fed the experimental diets. However, all fish showed reduced inflammation levels at the end of the trial compared to the start reflecting the pre-trial diet. This can possibly be explained by the inclusion of anti-inflammatory additives in all experimental diets, which was the only formulation difference between diet A and the pre-trial diet. Diets A, B and D contained anti-inflammatory additives, such as Bactocell®, brewer's yeast, Spirulina and micro algae (*Schizochytrium limacinum*), which are commonly used to reduce inflammatory response in other fish species (Song et al., 2007). Diet C also contained immuno stimulants although details could not be obtained from the feed producer. It should not be discounted that all diets had different composition between each other and many other factors could have affected this reduction in inflammation levels. Furthermore, the histological method used to score the inflammation levels (Urán et al., 2008) has been originally developed for salmon and was adopted as a guideline for this study.

The intestine length to total body length ratio (IL/TL) of the experimental fish did not vary between diets. The results confirmed an IL/TL ratio similar to wild caught ballan wrasse (0.62 according to Dipper et al., 1977), but also with similar size ballan wrasse (0.37

according to Kjørsvik et al., 2014) which agree with the omnivorous diet of the species. However, ballan wrasse intestine is shorter than in other temperate marine omnivorous species such as Atlantic cod (1.1 ± 0.1 IL/TL, Vargas et al., 2016).

Digestion and absorption of nutrients are highly dependent on the enzymatic activity of the intestine, and especially on the enzymes located in the BBM (Zambonino Infante & Cahu 2001). As it has been previously mentioned, stomachless species lack a stomach and therefore pepsin secretion which does not appear to impact on feed digestion in fish, since other proteolytic enzymes secreted by the pancreas can play similar roles in protein digestion (Zambonino-Infante & Cahu 2010). In this study, trypsin specific activity in the intestine section 3 of fish fed diet D were significantly lower than the other groups. Unfortunately, the precise formulation of raw ingredients in diet D is unknown and therefore this reduced activity, likely related to the disruption of microvilli structure, can not be related to specific ingredients or potential anti-nutritional factors. The higher activity found in diet A suggests a longer "life span" of trypsin with this diet and in consequence a higher peptide and amino acid availability in fish fed this diet. This said, amino acid digestibility did not indicate differences between fish fed diets A and B. Diet B did not contain higher amount of protein compared to the rest of the diets, but a percentage of it was sourced from krill hydrolysate. Inclusion of hydrolysed proteins in fish feeds can boost digestibility of artificial diets and result in enhanced digestive enzyme activity and growth (Cahu et al., 2004; Kotzamanis et al., 2007; Kvåle et al., 2009). The findings of the current study are in agreement with previous work by Krogdahl et al. (2014) which stated that quality of dietary protein in ballan wrasse juveniles can regulate secretion of trypsin. However, the elevated activity pattern at the third section of the intestine compared to the anterior described by Krogdahl et al. (2014) was only observed in fish fed diet A in this study, with the rest of the treatments showing similar activity across the first and third

intestine sections analysed. Intestinal enzymes involved in the final step of digestion are located in the BBM of enterocytes, including AP, γ-glytamyl transpeptidase, N-aminopeptidase and maltase (Cahu & Infante 2001). AP activities in BBM of enterocytes are often used as an indicator of microvilli integrity and general marker of nutrient absorption (Wahnon & Cogan 1992). AP activity in ballan wrasse was much lower compared to other marine species of similar size. In particular, sea bream fed fish meal based diets, showed from 4 times (diet A; intestine section 1) up to 20 times (diet D; intestine section 3) higher AP activity (Silva et al., 2010). Double amount of n-3 PUFA levels in diets A and B, in comparison to diet D, can possibly be reflected to the fish elevated intestinal AP activity of those groups. Cahu et al. (2000) described a similar pattern in sea bass and concluded that AP activity differences were due to alterations in intestinal membrane fluidity reliant mainly on long chain fatty acids. AP activity in carp (*Cyprinus carpio*), another stomachless species with similar intestinal morphology, has been correlated to lipid and protein absorption (Villanueva et al., 1997). Reduced PUFA and increase in monounsaturated fatty acid in BBM, with a simultaneous reduction in BBM enzymatic activity, can reflect inadequate nutrition in mammals and raise concerns for suppressed intestinal functions (Cahu et al., 2000). In the present study, AP activity was comparable in both intestine sections in fish fed the same diet , when in carp most of the AP activity was detected at the anterior part of the intestine (Villanueva et al., 1997). It has been reported that, dietary lipid levels can affect the fatty acid composition of seabass BBM through modifying their fluidity and suppress BBM enzymatic activity (Cahu et al., 2000). In this study, fish fed diet D, which had significantly lower contents of dietary n-3 PUFAs compared to the other diets, showed reduced AP activity in the BBM. Therefore, reduced amounts of n-3 PUFA in ballan wrasse on-growing diets might result in reduced growth performances. It must be acknowledged that fish in the present study were not

starved prior to sampling to maintain the structural integrity of the intestine, which might have affected the amount of secreted enzymes.

Ballan wrasse intestine has been previously reported to be alkaline (Krogdahl et al., 2014; Helland et al., 2014). Similarly, this study showed an alkaline environment in the intestine, with pH higher in section 3 compared to section 1 for diets A and D, but similar for diets B and C. pH values ranged from 8.5 to 8.6 in intestinal section 1 and from 8.8 to 9.2 in section 3. Previous studies by Krogdahl et al. (2014) and Helland et al. (2014) have also showed this elevated pH trend from the first towards the third intestine section of ballan wrasse juveniles, with pH values ranging between 7.6 – 8.5. The values of this study are higher compared to other stomachless species like cunner (*Tautogolabrus Adspersus*) (pH ranging from 7 to 8.5, Chao, 1973). These pH differences indicate a different digestive strategy and potentially intestinal microhabitat preferences to ballan wrasse, which need to be further investigated and understand the digestive processes.

Ballan wrasse digestive system has been previously characterised as highly efficient, given its high intestinal surface area (Kjørsvik et al., 2014). Likewise in this study, digestibility of protein, crude fat and AA were almost all higher than 85 % ADC. Protein and lipid ADCs were similar for diets A and B, but lower in diet D. ADC values are in agreement with several other studies reporting that protein digestibility largely determines protein retention and growth (Valente et al., 2013). Ballan wrasse in this study fed diets A and B appeared to have similar ADCs than other marine species like sharsnout seabream (*Diplodus puntazzo*) (Adamidou et al., 2011) and Atlantic cod (Tibbetts et al., 2006), which suggest similar digestive capacity to other gastric species. Krogdahl et al. (2014) reported that ballan wrasse exhibited higher nutrient digestibility at the posterior (3rd and 4th) (75 – 90 %) than the anterior intestinal sections (1st and 2nd) (55 – 74 %), with highest ADC values for polyunsaturated fatty acids, followed by starch and protein. On the

contrary, monounsaturated and saturated fatty acids showed the lowest digestibility values. In addition, Krogdahl et al. (2014) highlighted underlined improved digestibility in fish fed higher moisture diets compared with dry ones. The results of this study showed high protein digestibility, but space for further improvement in lipid digestibility. .

This benchmark study gave a baseline of a suitable diet for the on-growing phase of hatchery production of ballan wrasse without compromising its intestinal health or digestive functions. The best performing diet of this study can offer a 23 % shorter grow out period compared to the currently commercially used diet and 80 % compared to the fish meal diet tested. Thus, the currently required 20 – 24 month period to reach the deployment threshold size of 45 g can potentially be reduced to 16 – 19 months. The results support that inclusion of specific raw materials can improve significantly growth compared to other marine diets based on fish meal. Diet based on polychaetes elicited reduced growth and suppressed intestinal digestive functions. Further research should focus on obtaining quantitative nutrient requirements, exploring the growth potentials and providing the foundation for the development of cost-effective formulations for ballan wrasse. Improvement of feed utilisation should be an element for research by assessing the whole-body tissue composition after feeding juveniles with different proximate compositions and determine retention of dietary protein, lipid and energy. Protein is a typically preferred form of weight gain rather than lipid deposition in aquatic organisms (NRC, 2011). Given that the requirement for easily digestible feed sources in ballan wrasse, different inclusion levels of hydrolysed protein would be an initial step for further trials. Additionally, the use of expensive raw materials, primarily to increase feed palatability, can potentially be reduced by assessing alternative ways of improving feed ingestion, such as alternative raw ingredients and feed physical characteristics.

Chapter 6: Effect of feed moisture level and agar inclusion on ballan wrasse, *Labrus bergylta*, feed intake, growth performances, digestive enzymes, intestine and liver health.

6.1 Introduction

As farming of ballan wrasse is relatively new, there are clear gaps in knowledge in many aspects of the species biology. One of the main constraints of using farmed wrasse is the time required to reach deployment size (e.g. > 45g), which based on the current commercial hatchery data is 20-24 months from egg to deployment (David Patterson, Otter Ferry Seafish, pers. comm). Shortening the grow-out window is therefore a commercial priority and it is believed this can be achieved through improved nutrition.

A key driver for fish growth is the dietary nutrient availability (Valente et al., 2013). Ballan wrasse are stomachless species and nutrient absorption relies mainly on the intestine (Kjørsvik et al., 2014). While slow growth is a natural characteristic for ballan wrasse (Dipper et al., 1977; Skiftesvik et al., 2013) affecting significantly species commercial exploitation, very little has been published regarding its growth enhancement. Hamre et al. (2013a) suggested that the optimum diet for juvenile growth, (from about 0.5 – 1 g onwards), consists of 65 % protein, 12 % lipids and 16 % carbohydrate. Ballan wrasse natural diet is naturally non-piscivorous, low in lipids derived from easily digestible feed sources (Dipper et al., 1977; Deady & Fives, 1995; Figueiredo et al., 2005; Hamre et al., 2013a). Decapods, bivalves and gastropods are a main part of ballan wrasse natural diet (Deady & Fives, 1995) containing as much as 82 % moisture (Ovissipour et al., 2013). However, current hatchery ballan wrasse diets are considered as "dry" containing low moisture levels (approximately 5 % moisture). The previously conducted benchmark feed trial at Otter Ferry Seafish highlighted the importance of using a ballan wrasse-specific diet enhanced with appetite-stimulants and based on non-piscivorous raw material inclusion (Chapter 5). To ensure the highest growth potential is achieved further nutritional investigations were required.

Water intake in teleost marine fish is vital to avoid dehydration by osmoregulation (Whittamore 2012), which in turn increases the performance of different fish species including Korean rockfish (*Sebastes schegeli*) (Lee et al., 2000), turbot (*Scophthalmus maximus*) (Grove et al., 2001), dentex (*Dentex dentex*) (Chatzifotis et al., 2005) and olive founder (*Paralichthys olivaceus*) (Kim et al., 2011). Fish fed with increased moisture diets would need to take on less water, resulting in a reduction of their swimming rate and an increase in energy allocating for body mass growth (Przybyla et al., 2014). Kristiansen & Cliff Rankin (2001) hypothesized that the dry food of around 10 % moisture needs to be manually moistened to around 75 % for rainbow trout, to match the same moisture levels of wild preys, before being absorbed. In particular, food that is eaten by rainbow trout went from a moisture of 10 to 56 %, before rising even higher to a moisture of 65 % in the digestive tract before the digestion process began (Kristiansen & Cliff Rankin, 2001). A combination of improved growth, greater nutrient retention and a higher digestibility was observed in turbot fed diets with increased moisture contents of between 75 – 82 % in the form of squid pieces (Grove et al., 2001). Lee et al. (2000) also suggested that increased moisture can enhance growth in Korean rock fish (*Sebastes schlegelii*) fed diets of 36 % moisture. Chatzifotis et al. (2005) found that growth was significantly increased in dentex fed 20 % moisture diets for 151 days than fish fed 7 and 40 %. Another dietary moisture study conducted on juvenile dentex, showed that survival, FCR and SGR were all considerably improved in fish fed moistened (50-52 %) pellets compared to dry (8-10 %) pellets (Efthimiou et al., 1994). Increase in diet moisture content can be considered as the least expensive manipulation in fish feeds with potentially great benefits on nutrients uptake and fish growth.

An additional aspect in ballan wrasse successful use is the development of a post-deployment diet in the sea cages to maintain their health and welfare without

compromising delousing activity over the salmon production cycle. A gel form diet containing 60 % moisture has been developed based on manufactured dry-feed component and agar, which is water stable for up to 7 days without significant nutrient leaching and importantly, well accepted by ballan wrasse (Leclercq et al., 2015). However, parameters such as feed intake, growth and health impacts of feeding this agar based diet have not been previously assessed.

The objectives of this study were to assess the impact of four diets with increasing moisture levels including a diet containing agar on juvenile ballan wrasse growth performances and investigate their subsequent impacts on digestive enzymes, intestine and liver health. The starting hypothesis was that increased moisture in the diet would promote growth through enhanced nutrient assimilation. In addition, the diet formulation containing agar used for wrasse deployed in salmon cages was tested to confirm that juvenile wrasse can be conditioned in the hatchery pre-deployment.

6.2 Materials and Methods

6.2.1 Experimental fish and system

The 90-day trial was conducted at Otter Ferry Seafish (Argyll, Scotland, UK) between May and July 2015 using 2014 year class farmed ballan wrasse. Fish were previously reared under continuous artificial light as a commercial standard (24 LL) and fed a standard on-growing diet (Pre-trial; ø 1.5 mm extruded wrasse pellets containing 4.6 % moisture, BioMar, Grangemouth, UK). The experimental system consisted in 8 tanks (0.9 m radius x 0.4 m depth, 1 m^3) flow-through with temperature kept constant at 11.7 ± 0.6 ºC throughout the trial and 24 LL. Artificial sea weed was placed in each tank, covering ¼ of the tank surface, to provide cover and simulate the species natural habitat. Seaweed and small-mesh bottom drains were removed daily following uneaten food recovery and before

feed initiation for brushing the tanks. Dissolved oxygen concentration was checked daily and never dropped below 85% saturation.

6.2.2 Experimental diets and feeding

Fish were randomly stocked in the eight experimental tanks (847 fish per tank, mean weight of 10.2 ± 1.9 g, initial stocking density of 8.7 kg / m^3). Four diets (Diets A – D) were tested in duplicate (triplication was not possible due to limited fish and tank availability): (A) 5 % moisture commercial extruded pellet (comparable composition diet to diet A in chapter 5), (B) 25 % moisture commercial extruded pellet (same as diet A) hand soaked in fresh water (0.32 : 1 water W/ W) for 10 mins twice a day and prior to hand feeding at 08:00 am and 16:30 pm, (C) 16 % moisture content commercial extruded pellet with a proprietary plasticising agent to enhance pellet feed stability at the elevated moisture level **a**nd (D) 60 % moisture agar block feed (agar base). Diet D was produced on-site according to Leclercq et al. (2015) by grinding the commercial pellet (diet A) and binded with 20 g / L agar solution (2 % of the water volume) at 1 / 1.6 (w/v) ratio and offered as blocks on static feeding stations (n = 4 per tank). Each feeding station consisted of a rectangular plastic platform 18 x 13 x 3 cm, offering access from the five sides of agar block. However, it became apparent that the blocks produced with the initial agar inclusion of 2 % were too hard for the fish of this size to feed and ingest it and after day 16 the agar inclusion was reduced to 1 % (day 17 to day 19). It then became apparent that the 1 % inclusion was not water stable therefore agar inclusion was increased to 1.5 % (day 20 onwards). Pellet diets A, B and C were commercial extruded pellets (ø 1.5 mm) derived from the same feedstock base mix to ensure comparable nutritional composition manufactured by BioMar (Grangemouth, UK). All diets also contained a proprietary "health promotion" additives package, the further details of which cannot be released for due to a confidentiality agreement.

Fish were fed 24 h using electric round feeders in addition to hand feeding *ad libitum* twice a day (9:00 am and 4:00 pm) (diets A, B and C) or using static feeding stations (n = 4 per tank) to hold the agar blocks of diet D (continuous feeding). Feed waste left at the bottom of each tank was siphoned daily (weighted to the nearest 0.01 g) to determine feed intake, similar to as described in chapter 5. Prior to the start of the trial and for each experimental pellet diet, a known number of dry pellets (n = 100 pellets; triplicate count) were weighted in order to calculate the mean-weight of an individual pellet (diet A = 0.0028 ± 0.0002g; diet B = 0.0036 ± 0.0001g; diet C = 0.0042 ± 0.0002g). Feed waste left at the bottom of each tank was siphoned daily (trying to avoid any faecal material) into a bucket. The excess water of the recovered feed was removed through a fine mesh and then weighted to the nearest 0.01g. Three sub-samples were separated, weighted and the number of pellets manually counted to calculate feed recovery rate based on the estimated number of pellets distributed and recovered. The protocol was repeated three-times for each experimental diet. This value was then corrected by pellet number to determine feed intake in diets A, B and C. Diet D was offered within four “feeding stations” per tank and each station was replaced with newly prepared blocks every four days. Diet D was prepared every four days, following the established protocol developed by Leclercq et al. (2015). The jelly mix was poured in the feeding stations and left to set at room temperature for minimum of 12 h before deployment in the tanks. Each feeding station was weighted prior to deployment and at subsequent daily intervals they were re-weighed to determine feed intake. In each tank, two of the feeding stations were placed next to the artificial seaweeds and two on the opposite side; all at mid depth.

The analysed proximate composition of the four experimental diets is shown in table 6.1. The moisture content of diets A, B, C and D was 5.1, 25.4, 16.3 and 58.4 %, respectively.

Protein varied from 25.17 to 58.9 % in wet basis and between 60.5 to 66.1 % in dry basis. Lipid content varied from 4.1 to 14.4 % in wet basis and 9.8 to 15.2 % in dry basis. Carbohydrates and gross energy levels were calculated based on the proximate composition and varied from 9.6 to 14.1 % and from 8.9 to 21.1 MJ/kg respectively in wet basis. In dry basis, carbohydrates were between 13.4 and 22.9 %. FA profile in dry basis was the comparable across treatments.

Table 6.1. Proximate (% of wet weight basis) and fatty acid composition (% by weight of total fatty acids) of the experimental diets. Single and double asterisk denote data expression as wet or dry (calculated) weight respectively. Carbohydrates and gross energy were calculated based on the proximate composition. Data are presented as means ± SD.

	Diet A		**Diet B**		**Diet C**		**Diet D**	
Proximate composition	*WW**	*DW***	*WW**	*DW***	*WW**	*DW***	*WW**	*DW***
Moisture (%)	5.13 ± 0.10		25.44 ± 0.15		16.35 ± 0.09		58.42 ± 2.34	
Protein (%)	58.96 ± 0.49	62.15 ± 0.51	49.32 ± 0.32	66.15 ± 0.43	49.38 ± 0.07	59.03 ± 0.08	25.17 ± 0.76	60.53 ± 1.82
Lipid (%)	14.44 ± 0.06	15.22 ± 0.06	7.84 ± 0.62	10.52 ± 0.83	11.87 ± 0.04	14.19 ± 0.05	4.08 ± 0.27	9.81 ± 0.66
Ash (%)	8.72 ± 0.03	9.19 ± 0.04	6.79 ± 0.03	9.11 ± 0.04	8.29 ± 0.04	9.91 ± 0.04	2.77 ± 0.17	6.66 ± 0.41
Carbohydrates (%)	12.75 ± 0.61	13.45 ± 0.53	10.61 ± 0.19	14.23 ± 0.44	14.10 ± 0.05	16.86 ± 0.17	9.56 ± 1.69	22.99 ± 1.56
Gross Energy (MJ/kg)	21.13		16.01		18.20		8.91	
Fatty acids composition								
14:0	8.28	8.73	6.51	8.73	7.49	8.95	3.63	8.73
15:0	0.50	0.53	0.39	0.53	0.49	0.59	0.22	0.53
16:0	21.55	22.72	16.94	22.72	20.42	24.41	9.45	22.72
18:0	1.92	2.02	1.51	2.02	1.93	2.31	0.84	2.02
20:0	0.12	0.13	0.09	0.13	0.13	0.16	0.05	0.13
22:0	0.12	0.13	0.09	0.13	0.12	0.14	0.05	0.13
24:0	0.10	0.11	0.08	0.11	0.10	0.12	0.04	0.11
Total saturated	**32.59**	**34.35**	**25.61**	**34.35**	**30.69**	**36.69**	**14.28**	**34.35**
16:1n-9	0.19	0.20	0.15	0.20	0.28	0.33	0.08	0.20
16:1n-7	5.60	5.90	4.40	5.90	4.99	5.97	2.45	5.90
18:1n-9	11.89	12.53	9.34	12.53	11.72	14.01	5.21	12.53
18:1n-7	5.00	5.27	3.93	5.27	4.57	5.46	2.19	5.27
20:1n-11	0.46	0.48	0.36	0.48	0.53	0.63	0.20	0.48
20:1n-9	2.39	2.52	1.88	2.52	2.75	3.29	1.05	2.52
20:1n-7	0.25	0.26	0.20	0.26	0.23	0.27	0.11	0.26
22:1n-11	3.08	3.25	2.42	3.25	3.78	4.52	1.35	3.25
22:1n-9	0.55	0.58	0.43	0.58	0.49	0.59	0.24	0.58
24:1n-9	0.50	0.53	0.39	0.53	0.47	0.56	0.22	0.53
Total monounsaturated	**29.91**	**31.53**	**23.51**	**31.53**	**29.81**	**35.64**	**13.11**	**31.53**

18:2n-6	8.53	8.99	6.70	8.99	8.54	10.21	3.74	8.99
18:3n-6	0.12	0.13	0.09	0.13	0.14	0.17	0.05	0.13
20:2n-6	0.13	0.14	0.10	0.14	0.15	0.18	0.06	0.14
20:3n-6	0.13	0.14	0.10	0.14	0.15	0.18	0.06	0.14
20:4n-6 (ARA)	0.47	0.50	0.37	0.50	0.48	0.57	0.21	0.50
22:4n-6	0.08	0.08	0.06	0.08	0.07	0.08	0.04	0.08
22:5n-6	0.09	0.09	0.07	0.09	0.11	0.13	0.04	0.09
Total n-6 PUFA	**9.56**	**10.08**	**7.51**	**10.08**	**9.66**	**11.55**	**4.19**	**10.08**
18:3n-3	1.40	1.48	1.10	1.48	1.62	1.94	0.61	1.48
18:4n-3	2.30	2.42	1.81	2.42	2.83	3.38	1.01	2.42
20:3n-3	0.11	0.12	0.09	0.12	0.14	0.17	0.05	0.12
20:4n-3	0.35	0.37	0.28	0.37	0.37	0.44	0.15	0.37
20:5n-3 (EPA)	11.62	12.25	9.13	12.25	11.59	13.86	5.09	12.25
21:5n-3	0.39	0.41	0.31	0.41	0.41	0.49	0.17	0.41
22:5n-3	0.49	0.52	0.39	0.52	0.55	0.66	0.21	0.52
22:6n-3 (DHA)	9.85	10.38	7.74	10.38	10.89	13.02	4.32	10.38
Total n-3 PUFA	**26.51**	**27.94**	**20.83**	**27.94**	**28.40**	**33.95**	**11.62**	**27.94**
16:2	0.59	0.62	0.46	0.62	0.56	0.67	0.26	0.62
16:3	0.18	0.19	0.14	0.19	0.19	0.23	0.08	0.19
16:4	0.66	0.70	0.52	0.70	0.69	0.82	0.29	0.70
Total PUFA	**37.50**	**39.53**	**29.47**	**39.53**	**39.50**	**47.22**	**16.44**	**39.53**
ARA/EPA ratio	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**
DHA/EPA ratio	**0.85**	**0.85**	**0.85**	**0.85**	**0.94**	**0.94**	**0.85**	**0.85**

6.2.3 Settling velocity and nutrient leaching

Using a modified method described in Chen et al. (1999), a 80 cm length and 10 cm diameter tube (to ensure elimination of drag effects from the side-walls) was used to test the settling velocities of the experimental pellet diets (Diets A, B and C; n = 20 pellets $diet^{-1}$). The tube was placed securely in a vertical position and marked every 5 cm along its length. The tube was filled with water from the experimental tanks to ensure salinity and temperature coherence. The pellets were carefully laid to the water surface and the settling velocity (V_{set}) was manually determined as the time required for a pellet to sink between 5 cm below the surface and 10 cm below the tube bottom (to avoid shear effects).

Prior to the main trial, a 4-day nutrient leaching trial was also performed using the agar based diet (diet D) by testing four jelly blocks made containing 1, 1.2, 1.5 and 2 % agar inclusion and within a 100 L seawater tank at 12 ºC with a water exchange equivalent to experimental fish tank conditions. One sample was obtained from each block at 24 h intervals over a 4-day assessment to quantify oil, protein, ash, vitamin C and E. Nutrient loss was expressed as percentage loss in relation to the total starting content of each jelly block. Nutrient leaching was analysed by the Nutritional Analytical Service of the Institute of Aquaculture (Stirling, UK) on duplicated assay.

6.2.4 Sampling and analyses

Fish weight measurements were performed at the beginning of the trial (T0), 45 days later (T1) and the end (T2) (n = 80 / tank / sampling point, 160 / treatment) on randomly selected and anaesthetised 40 ppm; MS-222, Pharmaq®) fish. Fish were individually measured for their body weight (BM; ± 0.01g) and body length (BL; ± 0.1cm). A sub-sample of fish was euthanized immediately prior to allocation to experimental tanks (24

fish from pool) and at T2 (n =12 / tank, 24 per treatment) by overdose of anaesthetic (400 ppm; MS-222, Pharmaq®). The fish were dissected, viscera were excised to measure total viscera (VW; ± 0.01 g) and liver weight (LW; ± 0.01 g). The intestine was dissected from the bucco-pharingal cavity to the anal opening and either immediately snap frozen in liquid nitrogen for later enzymatic analysis (1 fish/tank at T0 and T2, 8/treatment) or fixed in Serra fixative (6:3:1 ratio of ethanol, 10 % formalin and acetic acid respectively)for later histological processing (T0, n = 8 fish; T2, n = 4 / tank, 8 per treatment). The liver was stored in Serra fixative for histological processing.

6.2.5 Growth and morphometric assessment

Fulton's condition factor (K), specific growth rate (SGR, % day^{-1}), biological feed conversion ratio (bFCR), feed efficiency (FE), thermal growth coefficient (TGC), hepatosomatic index (HSI, %),andviscerosomatic index (VSI, %), were calculated using standard methods:

$K = 100 (BW / TL^3)$

SGR (% day^{-1}) = [((ln (BW1) – ln (BW0)) / total days] x 100

bFCR = daily FI (g) / (WG x No of remaining fish) + mortalities biomass, g

FE = BW gain / total dry matter intake

TGC = (BW1$^{1/3}$ – BW0$^{1/3)}$ x (1000 / DD)

HSI (%) = (liver weight, g / BW) x 100

VSI (%) = (viscera weight, g / BW) x 100

where, BW0 and BW2 are the initial and final mean fish weights, FI is the feed intake, WG is the weight gained and DD is the number of degree days.

6.2.6 Proximate, lipid and vitamins analyses

Proximate, vitamins and lipid analyses of the diet samples were performed in duplicate at the Institute of Aquaculture, University of Stirling, Stirling, Scotland (UK). The analyses of dry matter (110 ºC for 24h) and ash (600 ºC for 16 h) were performed according to standard laboratory procedures (AOAC 2000). Crude protein was determined by Kjeldahl analysis (Persson, 2008) (nitrogen x 6.25; Kjeltec Autoanalyser, Tecator, Högänas, Sweden). Crude lipid was determined as described in Karalazos et al. (2007). Lipid extraction was carried out following the protocol outlined by Folch (1957) and fatty acid analysis following the protocol described by Christie (1982) and Tocher & Harvie (1988). Vitamins C and E calculated according to Hofman et al. (Roche Vitamins Ltd.) and McMurrey et al. (1980), respectively.

6.2.7 Histological processing, enteritis scoring and liver adiposity

Histological processing of the intestine and liver were performed at the Institute of Aquaculture, University of Stirling, Stirling, Scotland (UK). The intestine was sectioned at the 1st and 2nd loop and rectal valve to provide 4 individual sections, as described in chapter 5. The 3rd section of intestinal samples fixed in Serra fixative, were dehydrated according to Bancroft & Stevens (1982) and embedded in paraffin. After hardening the wax, blocks were sectioned in one 5 µm transversal section per fish analysed (T0: n = 8; T1: 4 per tank, 8 per treatment) and placed on glass slides. Each section was stained with haematoxylin-eosin (H and E). Each section was subjectively scored (scale 1 – 5) using a microscope (Olympus BX51, Hamburg, Germany) to quantify severity of potential enteritis according to Urán (2008). Each slide was scored by three independent assessors and the overall enteritis score was calculated by averaging the three individual scores. Liver adipose cells scoring was measured using the same microscope as enteritis and

according to Martinez-Rubio et al. (2013) (Table 6.2). Similarly to enteritis scoring, analysis of each slide gave an individual score with each slide being scored triple-blinded and the overall score being calculated by averaging the 3 individual scores.

Table 6.2: Liver histological scoring protocol of morphometric changes to identify adipose cells according to Martinez-Rubio et al. (2013).

Liver adipose scoring protocol	
Score	*Parameter*
0	Formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
1	Formation of vacuoles in the cytoplasm, involving less than 25% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
2	Formation of vacuoles in the cytoplasm, involving less than 50% of the hepatocytes and including less than 50% of the area of the individual hepatocytes
3	Formation of vacuoles in the cytoplasm, involving less than 75% of the hepatocytes and including less than 75% of the area of the individual hepatocytes
4	Formation of vacuoles in the cytoplasm, involving less than 90% of the hepatocytes and including less than 80% of the area of the individual hepatocytes
5	Formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes

6.2.8 Digestive enzymes analyses

The enzyme analyses of the intestine and liver were performed at the Functional Physiology of Marine Organisms Unit at Ifremer, Brest, France. Intestine and liver samples were partly thawed individually and weighted prior to analysis. From the intestinal segment, trypsin, alkaline phosphatase (AP) and leucine-alanine (leu-ala) activity were analysed according to Holm et al. (1988), Bessey et al. (1946) and Nicholson & Kim

(1975) respectively. From the brush border membrane (BBM), alkaline phosphatase (PA) and leucine aminopeptidase (LAP) were performed according to Bessey et al., (1946) and Maroux et al. (1973) respectively. Liver alanine transaminase (ALT) and asparate transaminase (AST) were analysed using spectrophotometry according to Huang et al. (2006). Protein was determined according to the Bradford procedure (Bradford, 1976). Intestine enzyme activity results were expressed as specific activities (U / mg^{-1} protein) and liver enzyme activity as total activities (mU / g liver).

6.2.9 Statistical analysis

Prior to analysis, normality and homogeneity of variance were assessed and transformation was applied when required and datasets with non-normal distribution and/or heterogenous variance were transformed (square-root, logarithmic or power transformation). All results expressed as a percentage (survival, SGR, HIS and VSI) were first arcsine transformed. One-way analysis of variance (ANOVA) was used to test for diets effects in all assessed parameters. Data were compared by one way analysis of variance and followed by a Tukey *post hoc* test with 95% confidence, by using MINITAB® Release 17 (Minitab Ltd., UK). All data are expressed as mean ± standard deviation (SD). The level of significance was set at $P < 0.05$.

6.3 Results

6.3.1 Settling velocity and nutrient leaching

Settling velocity was compared for the pelleted diets only (diets A, B and C) (Fig. 6.1). All diets exhibited significantly different velocities from each other, with diet A having the fastest settling velocity (0.30 ± 0.02 cm sec^{-1}), followed by diet B (0.25 ± 0.02 cm sec^{-1})

and diet C (0.23 ± 0.02 cm sec^{-1}). Results from the 4-day nutrient leaching trial on the agar block (diet D) showed a reduction in protein content of 12.9, 14.9, 7.5 and 7.7 % and oil content of -6, -11.8, 13.5 and 6.1 % in agar blocks containing 1, 1.2, 1.5 and 2 % respectively by day 4 (Fig. 6.2). By day 4, vitamin C was reduced by 50, 35, 30 and 48 % and vitamin E by 47, 41, 18 and 31 % in jelly blocks containing 1, 1.2, 1.5 and 2 % agar respectively. Ash content loss varied between -0.75 and -4.12 %, with no apparent differences between samples.

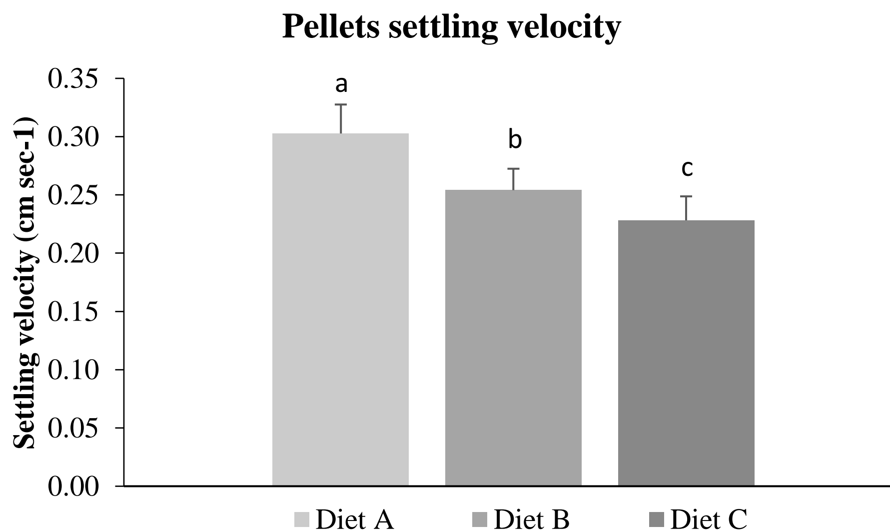

Figure 6.1. Settling velocities of the experimental pellet feeds (diets A, B and C). Values are resented as mean ± SD (n = 20). Superscript letters indicate significant differences between diets ($P < 0.05$).

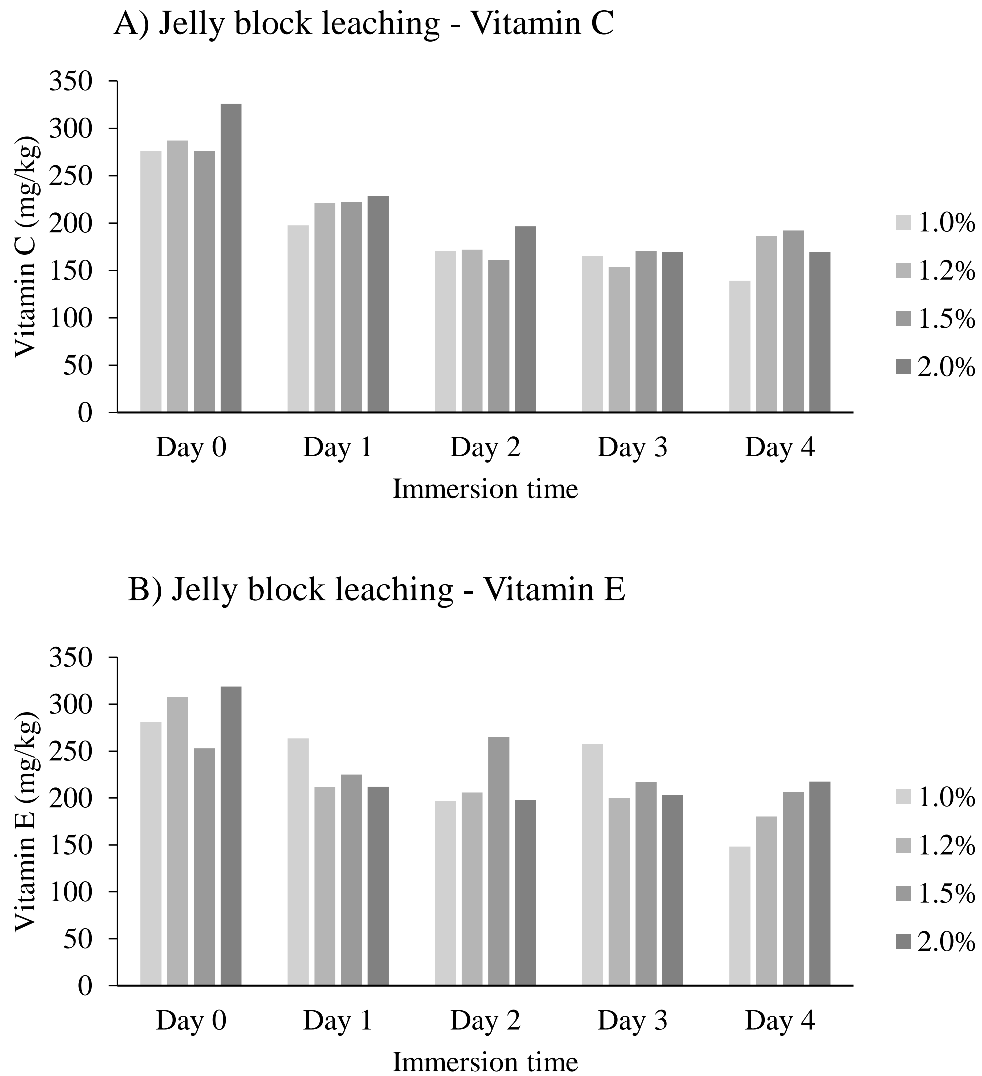

Figure 6.2. Leaching time of vitamins C (A) and E (B) from diet D jelly blocks with different agar inclusion rates (1, 1.2, 1.5 and 2 %) tested during a 4-day immersion trial in hatchery water. Single values did not allow conduction of statistical analysis (n = 1).

6.3.2 Survival, growth and feed utilisation

Fish promptly accepted the experimental pelleted diets A, B and C, resulting in low mortalities of 1.2 ± 0.7, 0.8 ± 0.4 and 1.4 ± 0.5 %, respectively (Table 6.3). However, the

lack of initial acceptance of the jelly block (diet D) resulted in significantly elevated mortality levels of 23 ± 1.8 % in this treatment (Table 6.3). Shifting from 2% to 1.5% agar inclusion rate in the jelly blocks improved feed ingestion, but possibly fish health has been already compromised by the initial lack of ingestion and mortality rates continued to be higher to the rest of the treatment until the end of the trial (Fig. 6.4). Body-size parameters (body weight, total length and condition factor) were comparable at the beginning of the trial and between replicates at the end of the trial. Following the 90-day experiment, no significant differences in final fish weights and lengths were found between diets A, B and C (22.89 – 23.00 g and 10.76 – 10.83 cm), however fish weight and length for diet D was largely reduced (10.20 ± 2.58 g and 8.94 ± 0.57 cm). Condition factor exhibited similar pattern to final animal sizes with no significant differences between diets A – C (1.70 – 1.82) compared to a reduced condition in diet D (1.40 ± 0.16 for diet D). Likewise, diets A, B and C showed higher SGR (0.88 – 0.94) and TGC (0.62 – 0.66) values compared to diet D (0.01 ± 0.04 and 0.01 ± 0.03, respectively). The overall FCR calculated as feed offered was significantly lower in fish fed diet C (1.46 ± 0.07) compared to diet B (1.81 ± 0.06), with diet A being comparable to both of them (1.61 ± 0.10). However, the FCR expressed as dry matter was lower in fish fed diet C (1.22 ± 0.05) compared to diet A (1.52 ± 0.10), but with diet B (1.35 ± 0.05) being comparable to both (Table 6.3). It became apparent that diet D did not maintain fully its stability, therefore FCR could not be determined for this diet. HSI was higher in fish fed diet B compared to diet D, but comparable to diets A and C. No differences were observed in VSI amongst diets.

Table 6.3. Growth performance, development, feed intake and mortality of ballan wrasse fed the four experimental diets for 90 days. Values are presented as mean ± SD (n = 2). Superscript letters indicate significant differences between diets (P < 0.05).

	Diet A – 5% moisture	Diet B – 25% moisture	Diet C – 16% moisture	Diet D – 60% moisture
Initial body weight (g)	10.20 ± 1.79[a]	10.52 ±1.85[a]	9.97 ± 1.87[a]	10.30 ± 2.03[a]
Initial body length (cm)	8.50 ± 0.49[a]	8.58 ± 0.48[a]	8.43 ± 0.49[a]	8.49 ± 0.54[a]
Initial condition factor	1.65 ± 0.12[a]	1.65 ± 0.12[a]	1.65 ± 0.13[a]	1.66 ± 0.13[a]
Final body weight (g)	22.89 ± 3.85[a]	22.90 ± 4.17[a]	23.00 ± 4.40[a]	10.20 ± 2.58[b]
Final body length (cm)	10.76 ± 0.56[a]	10.78 ± 0.61[a]	10.83 ± 0.71[a]	8.94 ± 0.57[b]
Final condition factor	1.82 ± 0.12[a]	1.82 ± 0.20[a]	1.79 ± 0.13[a]	1.40 ± 0.16[b]
Total weight gain (%)	124 ± 0.11	117 ± 0.03	130 ± 0.04	-0.84 ± 0.04
SGR (% / day)	0.91 ± 0.06[a]	0.88 ± 0.02[a]	0.94 ± 0.02[a]	0.01 ± 0.04[b]
TGC	0.64 ± 0.04[a]	0.62 ± 0.02[a]	0.66 ± 0.01[a]	0.01 ± 0.03[b]
bFCR (as offered)	1.61 ± 0.10[ab]	1.81 ± 0.06[a]	1.46 ± 0.07[b]	n/a*
bFCR (dry matter)	1.52 ± 0.10[a]	1.35 ± 0.05[ab]	1.22 ± 0.05[b]	n/a*
FI (g/fish/day; as offered)	0.23 ± 0.06	0.26 ± 0.07	0.22 ± 0.06	n/a*
FI (g/fish/day; dry matter)	0.22 ± 0.05	0.19 ± 0.05	0.18 ± 0.05	n/a*
FE	0.66 ± 0.04	0.74 ± 0.03	0.82 ± 0.04	n/a*
HSI (%)	1.32 ± 0.28[ab]	1.42 ± 0.21[a]	1.21 ± 0.20[ab]	1.14 ± 0.48[b]
VSI (%)	4.57 ± 0.82[a]	4.99 ± 0.49[a]	4.93 ± 0.63[a]	4.40 ± 1.41[a]
Mortalities (%)	1.18 ± 0.67[b]	0.77 ± 0.42[b]	1.42 ± 0.50[b]	23.32 ± 1.75[a]

* FCR for diet D could not be calculated, due to feed instability

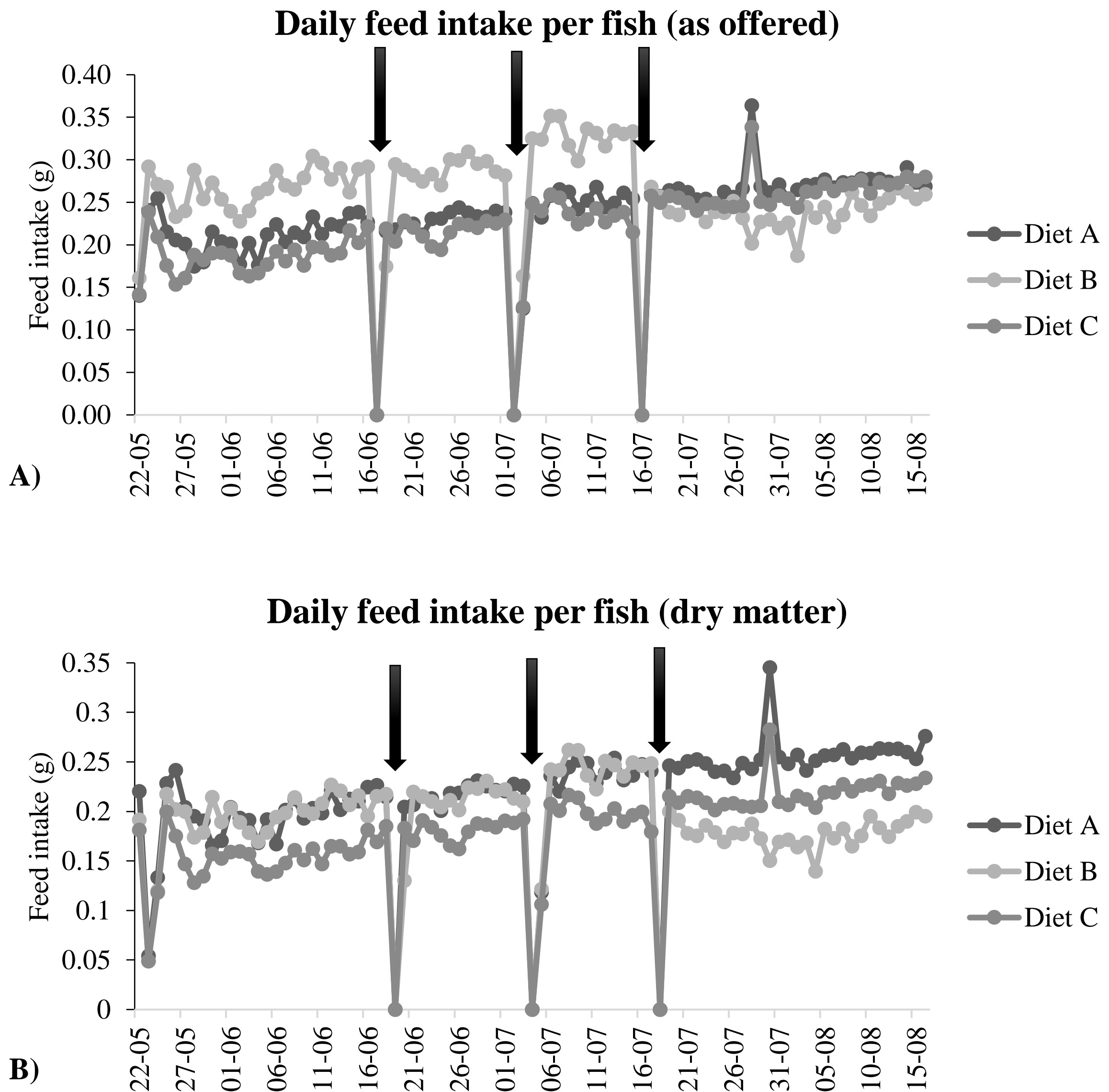

Figure 6.3. Average daily food intake (A) as offered and (B) expressed in dry matter per juvenile ballan wrasse offered three experimental diets during the 3-month experiment. No data were available for diet D, due to pellet instability. Arrows indicate the 24h starvation periods prior to sampling points.

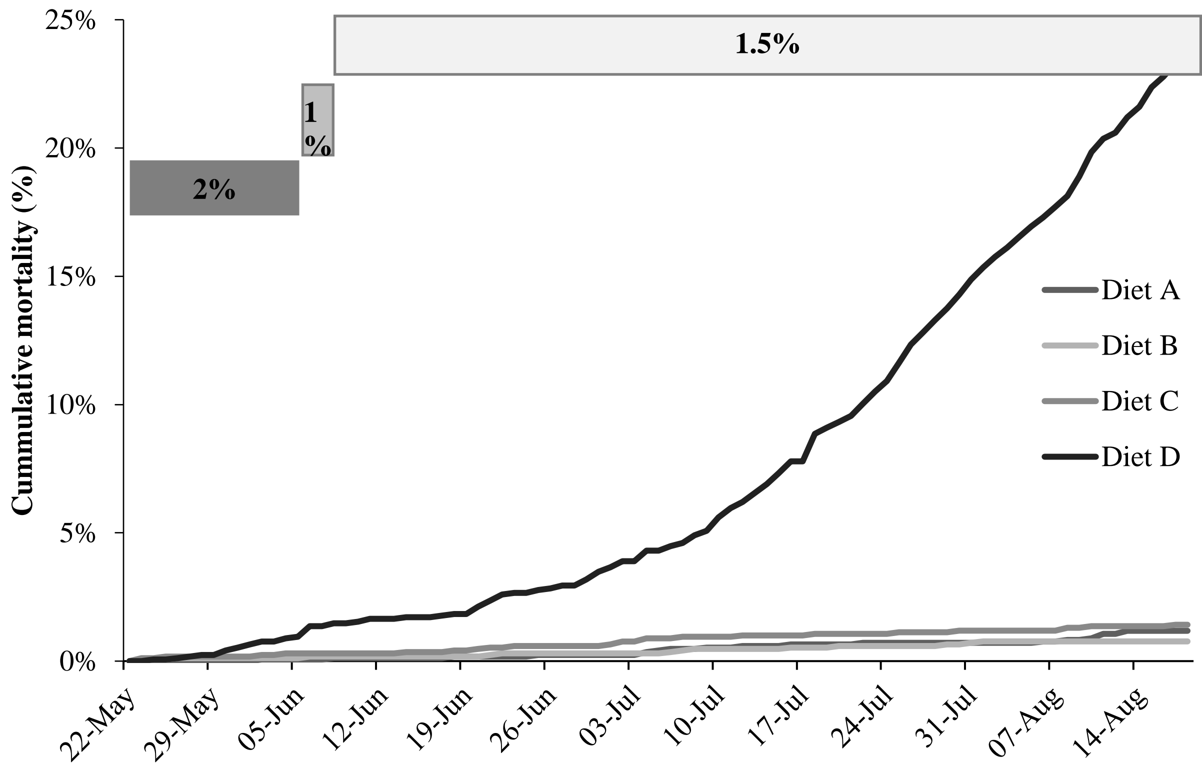

Figure 6.4. Cumulative mortality during the three-month trial. Bars on top of the graph show the three different agar inclusion rates which were tested (1, 1.5 and 2%) in diet D to balance feed ingestion and water stability.

6.3.3 Histological processing, enteritis scoring and liver adipose

Histological processing conducted at T0 and T1 showed no intestine inflammation or differences between treatments. Intestine inflammation score was low at both sampling points. At T0 the overall score from the pool samples was 1.72 ± 0.35 and at T1 comparable for all diets (Diet A: 1.46 ± 0.26; Diet B: 1.36 ± 0.64, Diet C: 1.60 ± 0.38 and Diet D: 1.50 ± 0.29). However, liver adipose score was similar for fish fed diets A, B and C (2.56 ± 0.90, 3.15 ± 1.08 and 2.81 ± 0.87, respectively), but lower for fish fed diet D (0.90 ± 1.13). Given the lack of growth differences amongst the pellet based diets, no further histological processing was performed at T2.

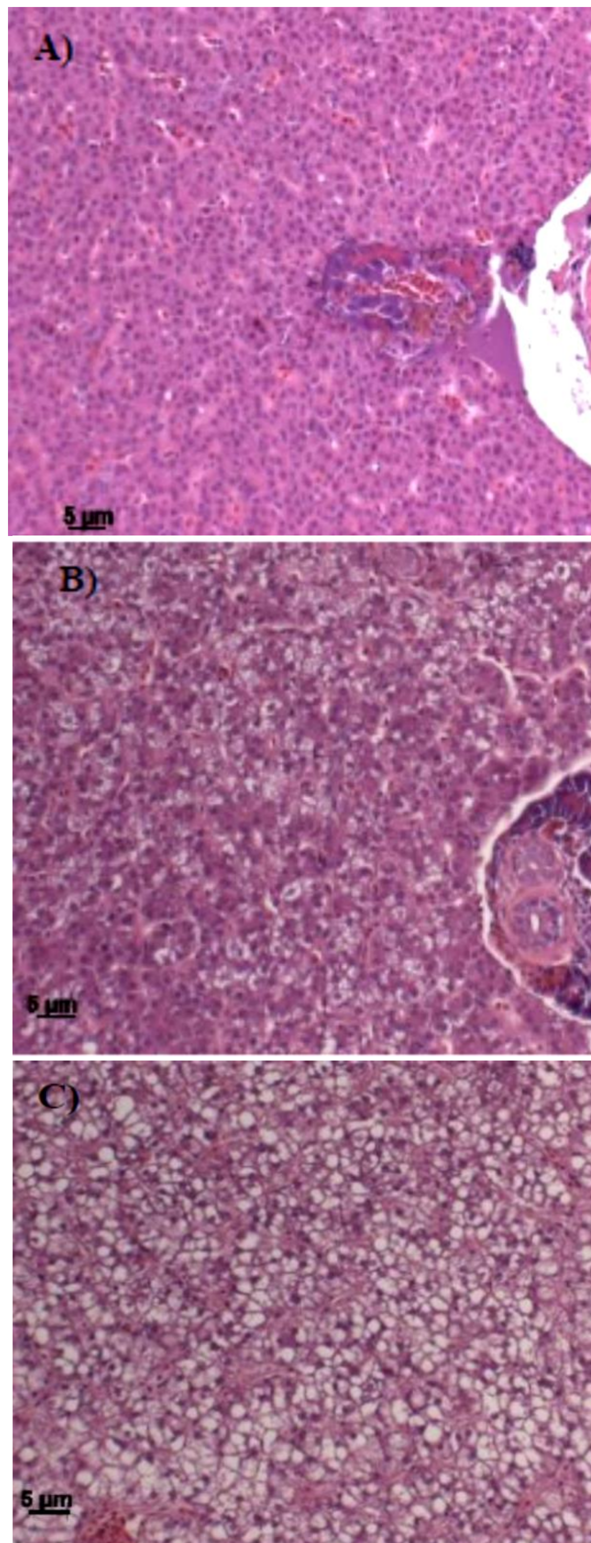

Figure 6.5. Photographic images indicating different scoring of adipose cells found in ballan wrasse liver at the beginning of the trial (T0) adopting the scoring protocol of

Martinez-Rubio et al. (2013). Adipose score of 0 (A), adipose score of 2.83 (B), adipose score of 4.0 (C) (x20). The scale bar corresponds to 5 µm.

6.3.4 Digestive enzymes

At the end of the 90 days trial, intestine trypsin specific activity was significantly higher for diets A and C compared to diets B and D (Fig. 6.6A). Specific activity of intestinal PAand leu-ala was similar for all treatments. AP specific activities of BBM were 2.9 times higher for diet A compared to diet B, while diets C and D shown comparable activities (Fig. 6.6B). LAP activities were comparable for all treatments (Diet A: 185 ± 109; Diet B: 142 ± 83; Diet C: 145 ± 29 and Diet D: 159 ± 72 mU / mg protein).

Liver transaminase total activities of ALT appeared to increase with moisture content of the diets. ALT total activity was significantly increased in liver from fish fed diet D (0.49 ± 0.13 mU/g liver) compared to diet A (0.24 ± 0.07 mU/g liver) (Fig. 6.6C). However, no significant differences in AST total activity were observed between diets (ranging between 1.3-2.3 mU/g of liver). AST/ALT ratio was significantly lower in fish fed diet D compareto diet B, but not compared to diets A or C (Fig. 6.6D).

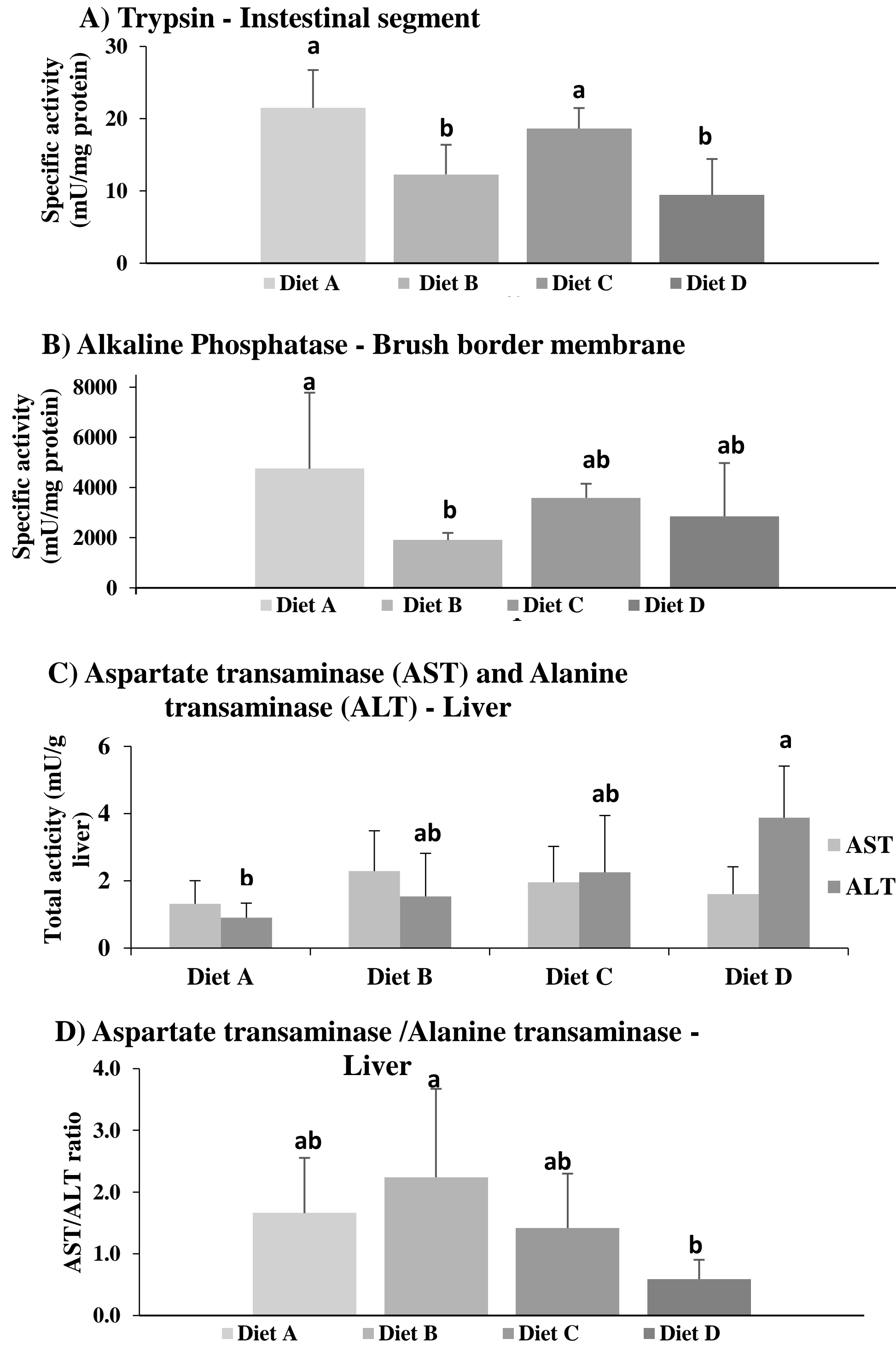

Figure 6.6. Enzyme activities of (A) trypsin in the intestinal segment, (B) alkaline phosphatase (AP) in the brush border membrane (BBM), C) aspartate transaminase (AST) and alanine transaminase (ALT) in the liver and D) AST/ALT ratio for fish fed the four

experimental diets. Superscript letters relate to differences between diets (one-way ANOVA, $P < 0.05$). Values are presented as means ± SD ($n = 2$).

6.4 Discussion

This experiment investigated the impact of four diets with increasing moisture levels including a diet with agar on juvenile ballan wrasse growth performances, digestive enzymes, intestine and liver health. While growth rate of ballan wrasse juveniles did not improve by increasing moisture levels in pelleted diets, a reduction in FCR was found in fish fed diet C containing 16 % moisture compared to diet B hand soaked to 25 % moisture (expressed as offered) and diet A (the current commercial practice) as being the existing commercial practice (expressed as dry matter). The use of diet D exclusively during on-growing in the hatchery significantly hindered growth and resulted in mortalities although fish maintained their weight and no detrimental effects on intestine and liver health or digestive enzymes activity were apparent. Diet D is not considered a commercially relevant hatchery feeding method and so its impacts will be considered separately later.

Ballan wrasse has been characterized to be a slow grown species (Dipper et al., 1977), causing major constrains due to the extended time required to reach deployment size. Commercial evidences at Otter Ferry Seafish suggested that pellet soaking with fresh water increased feed intake and improve growth performances of ballan wrasse juveniles. The previously conducted benchmark study in chapter 5 indicated formulation of diet A as an adequate on-growing commercially available diet for ballan wrasse, giving also the first indications of species growth performances in hatchery environment. No significant growth differences appeared amongst fish fed on pelleted diets A, B and C.

Growth results obtained in this study were the best reported so far with SGR of 0.94 ± 0.02 % / day for diet C. This is a marked improvement, which is about a double fold increase compared to best reported value from the previously conducted benchmark experiment (0.44 ± 0.18 % / day) (Chapter 5). A similar study conducted with bigger size ballan wrasse (i.e. 25 – 46 g) reported SGR of 0.51 and 0.51 in fish fed continuously dry feed and soaked feed respectively (Helland et al., 2014). In addition, TGC values increased by 36 % compared to the previous benchmark study in chapter 5 (current study: 0.66 ± 0.01; benchmark study: 0.46 ± 0.19), with both current and benchmark conducted at the same water temperature (11.6 ± 0.6 ºC). Despite that diet A is currently used as the main on-growing diet at Otter Ferry Seafish, the fastest growing experimental fish fed diet C exhibit approximately 25 % higher SGR compared to similar size (15 – 30 g) commercial fish farmed in similar water temperature at Otter Ferry Seafish (David Patterson, Otter Ferry Seafish, pers. comm.). These differences in growth rates between the two trials could be attributed to various environmental, nutritional and genetic factors. Given that the nutrition was comparable (using diet A as a base diet for all diets), all stocks are originated from the same broodstock population and kept in similar water temperature, it can be assumed that developmental stage might have had a significant effect on fish growth. Fish used for this study had an initial mean body weight of approximately 10 g compared to 38 g for the benchmark trial which may explain differences as larger fish have a smaller relative growth rate (Árnason et al., 2009). In addition, growth rate is highly regulated by the intensity and duration of the mosaic hyperplastic growth, especially at the early life stages, with fast growing fish showing higher hyperplasia compared to slow growing fish (Valente et al., 2013). Therefore, environmental or/and nutritional differences at the early life stages

within the hatchery can potentially imply alterations in growth potentials within each year class.

Increased dietary water content in sea bass might not have had any growth effects, but increased its resistance to *Vibrio anguillarum* infection (Przybyla et al., 2014). *Vibrios* have been reported as pathogens of ballan wrasse, depressing their fitness (Birkbeck & Treasurer, 2014) making underlying health concerns in the previous benchmark to be considered. The best FCR value achieved in this study was with diet C, which was 21 % lower (calculated feed as offered) in fish fed diet C compared to fish fed the standard “dry” diet A. Moreover, FCR achieved with diet C showed a 35 % improved feed utilisation compared with results from the benchmark trial in chapter 5 (2.07 ± 0.93). Despite that increased moisture content has been proved to have positive (Eftimiou et al., 1994) or no effect (Chatzifotis et al., 2005) on marine species FCR, diet C achieved better FCR compared to diet B with higher moisture content, suggesting that also the soaking method might have influenced feed ingestion and potentially palatability. This better feed assimilation of soaked pellets in ballan wrasse has also been reported in ballan wrasse by Krogdahl et al. (2014), where soaked pellets were better absorbed and digested than the dry ones. Feed intake data obtained from the feed as offered confirm that fish fed diet B showed higher feed ingestion compared to diet A and C. However, expressing the feed intake in dry matter basis indicates the lowest feed intake in fish fed diet C signifying higher feeding efficiency in diet C (0.82 ± 0.04) compared to diets A (0.66 ± 0.04) and B (0.74 ± 0.03).

Increasing dietary moisture content has been previously proved beneficial for growth in fish (Efthimiou et al., 1994; Lee, 2000; Grove et al., 2001; Chatzifotis et al., 2005) and FCR (Efthimiou et al., 1994). Turbot has the ability to regulate its intestinal moisture

required to facilitate digestion in dry diets, but potentially also intensifying the metabolic cost (Grove et al., 2001). However, contrasting results have been obtained in turbot (Bromley, 1980) and sea bass (Przybyla et al., 2014) with regards to the effects of dietary moisture levels with, in some cases, no impact on growth or FCR (Bromley, 1980). In the current study, despite a lack of differences in growth between pelleted diets, diet C showed significantly lower FCR 21 % compared to diet B (FCR calculated as feed offered) and to 22 % diet A (FCR calculated as dry feed offered). Diet B contained higher moisture content (25 %) compared to diet C (16 %). However, different strategies were applied to increase the dietary moisture content; diet B was hand soaked with fresh water on-site and diet C moisture level was increased by the feed manufacturer. This moistening method difference might have affected moisture distribution within the pellets and consequently natural characteristics such as settling velocity.

The three pelleted diets exhibited significant differences in velocities with diet A having the fastest settling velocity, followed by diet B and diet C. It became noticeable during this study that ballan wrasse juveniles are mainly pelagic rather than benthic feeders, showing in controlled hatchery environment a clear preference for pellet in the water column rather than the bottom of the tank. Diet C was for longer period of time in the water column and therefore offered better opportunity for fish to ingest it. However, previous studies showed that feed pellet characteristics (size and floatability) did not affect growth or FCR including studies in largemouth bass (*Micropterus salmoides*) (Tidwell et al., 2015). Sinking velocity of diets used in aquaculture can have an impact on the cost of production due to a loss of uneaten food (Piedecausa et al., 2009). Especially in ballan wrasse farming, where diets are based on more expensive raw ingredients than the conventional fish meal ingredients (e.g. krill and shrimp meal), the cost implications can be high. Thus, the

importance of feed settling velocity should be taken into consideration for the development of ballan wrasse on-growing diet.

Fish fed with diet D agar blocks maintained their initial body weight over the 90-day period of this trial, but the initial lack of feed acceptance caused welfare concerns and elevated mortalities. The principle of diet D has been developed by Leclercq et al. (2015) as a supplementary post-deployment in sea cage feed to sea lice to maintain cleaner fish welfare and not as an exclusive hatchery diet. It should also be noticed that this diet has been developed with 52 g fish and not 10 g that were used in this study. However, the reduced growth performance of fish in this study fed diet D raised concerns about agar's leaching blockage, which was potentially reducing feed ingestion or assimilation. The additional 4-day immersion trial in hatchery water, primarily designed to confirm whether agar blocks leaching was leading to reduced ingestion or assimilation, showed no nutrient protein and oil leaching in line with Leclercq et al. (2015). Despite that no leaching occurred in protein, oil or ash content (maximum reduction of 14.9, 13.5 and -0.25 % respectively), water soluble vitamin C shown higher leaching compared to fat soluble vitamin E, which both vitamins play important antioxidant role in fish feeds. Reduced growth performance in fish fed diet D cannot be related explicitly to high moisture content. For its usage as a supplementary feed in sea cages, further research is required to assess feed ingestion and digestibility ensuring maintenance of fish condition.

The condition of the intestine is very important for digestion and absorption of nutrients from the diet passing through the intestine (Kuperman & Kuz'mina, 1994). Based on the semi-quantitative enteritis scoring system developed on the previous benchmark study, the intestine health of all fish in this study appeared to not be severely inflammated. This is likely to be due the addition of a "health promotion" additives package included in all

experimental diets. No further details can be released for due to confidentiality agreement, so no assumptions can be drawn about specific anti-inflammatory additives. Preliminary work on wild ballan wrasse specimen analysed at the Institute of Aquaculture, University of Stirling, Stirling, Scotland (UK) indicated enteritis levels similarly low to this study (1.37 ± 0.18 and 1.6 ± 0.38, respectively). Therefore, it can be suggested the experimental fish had improved healthy and adequate for nutrient absorption intestine.

Regarding liver health and in particular the lipid accumulation, marine teleosts do not seem to have a proliferative response of peroxisomes and artificial diets can possibly lead to liver lipid accumulation. Increased adipose tissue scores indicate liver steatosis, which is described as alteration in the liver due to an excessive intake of dietary lipids and can potentially cause higher mortality rates (Spisni et al., 1998). Currently there is no information available regarding ballan wrasse liver function or health status, with also no clear evidences regarding energy storage organs. This study however, using as guideline a liver adipose tissue scoring system recently developed by Martinez-Rubio et al. (2013) for Atlantic salmon, all ballan wrasse specimen of this study showed adipose scores between 0.90 ± 1.13 (diet D) and 3.15 ± 1.08 (diet B) which are in the mid-range of adipose scoring scale for salmon. A score around 3 – 3.5 in salmon can cause high mortalities and reduced growth in salmon (Roberts, 2012). Based on this assessment the adipose scores for ballan wrasse of this study appear borderline healthy. However, fish with the highest found adipose values appear having normal growth and intestinal functions. Therefore, it can be deemed that the highest adipose scores observed in this study emerge no warning signs of malnutrition or liver damage in ballan wrasse. Fish fed diets A, B and C had adipose scores significantly higher compared to fish fed diet D. Preliminary assessments of adipose scores in wild ballan wrasse showed high variability (0.5 – 2), but a general low adipose score

became apparent; reflecting species whose natural diet is low in lipids, as previously suggested (Dipper et al., 1977; Deady & Fives, 1995; Figueiredo et al., 2005; Hamre et al., 2013a). Lipids are stored in the liver as energy reserves and as such the hepatosomatic index (HSI) is an important indicator of the condition of the fish. In this study, and similarly to the previous benchmark study (chapter 5), slow grown fish had lower HSI. In general, HSI values can vary significantly between marine fish species. For instance, dentex HSI is 1.5 – 3 % (Chatzifotis et al., 2005), while cod is 15 – 20 % (Rosenlund et al., 2004). HSI in fish fed the current experimental diets fluctuated between 1.14 ± 0.48 (diet D) and 1.42 ± 0.21 (diet B), classifying ballan wrasse, based on liver lipid deposition only, as a species with low dietary lipid demands. This study gave a first indication regarding liver condition of farmed ballan wrasse. Further investigations are required to characterise the dietary lipid requirements of the species assessing also whole body lipid stores.

In order to broaden scope of the study, intestinal and liver enzymatic activity was also assessed. Intestinal trypsin specific activity was higher for fish fed diets A and C compared to diets B and D. Higher availability of dietary protein can act as a substrate for trypsin (Giri et al., 2003). Therefore, increased levels of intestinal trypsin could be induced by dietary protein in certain range and consequently improve protein utilisation (Tu et al., 2015). However, dietary protein levels provided in excess can lead to a decrease in intestinal trypsin levels (Debnath et al., 2007; Tu et al., 2015). In this study, high dietary protein levels of fish fed diet A are in line with the high trypsin activity found. However, diet C, while containing the same amount of dietary protein than diet B, showed higher trypsin activity indicating a higher catalytic efficiency (Lemieux & Blier, 2007). AP activities in BBM of enterocytes are often used as an indicator of microvilli integrity and general marker of nutrient absorption (Wahnon et al.,1992). Also in Carp (*Cyprinus*

caprio), as stomachless also fish, AP activity has been hypothesised to be related to protein internalisation or lipid absorption (Villanueva et al., 1997). PA activity was significantly lower in fish fed diet B and appeared lower, but not significantly, in diets C and D compared to diet A. Soaking the pellets with water in diet B may explain the reduction in PA activity, possibly due to lipids (PUFAs) oxidation in the feed affecting the BBM. Comparing the outcomes of this study with a previously conducted benchmark study (chapter 5), similar PA activity were observed in both studies using similar diet formulations (previous benchmark study: 3,519 ± 697 mU / mg protein; current study: 5,559 ± 3,302 mU / mg protein). However, diet B resulted in a 2.9 fold reduction (1,909 ± 284 mU / mg protein). Nevertheless, differences in LAP, which is also located at the BBM, were not evident. Beside the intestinal enzymatic activity analysed, hepatic enzymatic activity was also assessing AST and ALT levels.

The present study is the first to report the enzymatic activity in ballan wrasse liver. ALT and AST are liver enzymes involved in the transfer of amino groups from one specific amino acid to another and are considered as the most important enzymes in amino acids catabolism (Metón et al., 1999). They are good indicators of fish liver function (Ozaki, 1978; Kim & Lee, 2009) and increased levels are usually signs of starvation and malnutrition (Chien et al., 2003). ALT values in the liver showed a positive relation with the moisture content. Elevated ALT levels in fish fed diet D agree with the reduced growth performances under this diet. AST is a less sensitive enzyme to malnutrition and no differences occurred amongst diets. Ballan wrasse liver ALT seem responding clearly to dietary moisture changes, when AST levels remained similar for all treatments. This higher ALT responses to dietary changes compared to AST contradicts to black sea bream response in dietary changes, in which AST seemed to be more active than ALT (Zhang et

al., 2010). However, the positive relation of ALT with the increased of moisture content in this study, might indicate that ballan wrasse were capable of adapting catabolism to dietary moisture content, following a similar response to white sea bream to different protein levels (Sá et al., 2008). The AST/ALT ratio, which is used to assess fish condition, is in line with the growth performances of the fish and were higher for diets A, B and C compared to D.

The present study showed that increased moisture level (16 % moisture) in ballan wrasse diet can enhance FCR up to 22 % and increase feeding efficiency by 19.5% compared to the standard “dry” diet. Nevertheless, the technical implications of a moisture increase should also be considered for a commercial scale feed production, such as physical feed quality and self-life, which were beyond the scope of this study. The potential use of a body weight maintenance agar based diet has been confirmed, but regular body weight monitoring is recommended to ensure adequate welfare in post-deployment to sea cages conditions. This study gives a baseline on liver health and enzymatic activity in ballan wrasse, highlighting the importance of liver functions in diet development. Further research should aim on assessing the impact different hatchery practices on the currently available diets, such as feeding frequency, but also potentially growth potentials from the critically for muscle development larval stages.

Chapter 7: Summary of main experimental findings

Chapter 2: Surface disinfection of ballan wrasse (*Labrus bergylta*) eggs with bronopol: evaluation of concentration, contact time and commercial application.

- Reduction of bacterial load to minimum levels was achieved with high bronopol doses (up to 500 ppm) and extended exposure time (up to 240 mins).
- Best egg disinfection results were obtained when treating eggs with bronopol on day 0 at a concentration of 100 ppm for 240 min followed by daily treatment with 25 ppm for 30 min. Such a disinfection strategy was effective at suppressing bacterial growth without affecting egg survival and egg hatching rate.
- Potential antimicrobial properties of the egg surrounding gum layer was suggested although further studies are required to confirm this hypothesis and identify the nature of such a potential antimicrobial activity.
- Results demonstrated that bronopol treatment could be used for ballan wrasse egg disinfection as an alternative to formalin which is routinely used in commercial hatcheries.

Chapter 3: Effects of a commercial *Bacillus* sp. probiotic mix and bronopol on survival, growth and bacterial load in ballan wrasse (*Labrus bergylta*) larvae.

- Repeated bronopol treatment with probiotic addition from the yolk-sac stage resulted in an increase of larvae survival, but not growth.
- Use of probiotic alone or clay alone (control) had no positive impact on larvae survival however best growth was obtained in the control treatment (clay only).
- Bacteria strains were identified from larvae including *Shewanella*, *Vibrio* and *Pseudoalteromonas* as the main culturable bacteria, however no clear patterns were found amongst disinfection treatments.

- Future studies should develop and evaluate modes of action of host-specific probiotics during the early ontogenesis of ballan wrasse larvae.

Chapter 4: Evaluation of micro-diets for weaning of ballan wrasse (*Labrus bergylta*): Implications on larval survival and growth.

- Larvae weaned on Nofima (0.3 – 0.6 mm, Nofima, Norway) and Otohime diet (B1, 0.25 – 36 mm, Marubeni Nisshin Feed Co, Japan) showed better survival compared to the other diets tested (BioMar Prowean 300, 0.15 – 0.4 mm, BioMar, Grangemouth, UK; Skretting Labrus 0, crumble, Skretting, France). However, the highest growth was observed in fish fed with Nofima diet. Reduced growth and survival in larvae fed the commercially available diets developed for other marine fish species clearly confirms the importance to development species-specific diets based on nutritional requirements and feed preferences.
- Overall, the highest post-weaning survival achieved in this study (up to 22.3 % in larvae fed Nofima diet) was much lower compared to other recent studies on ballan wrasse (up to 77.0 %).
- Future studies should characterise the digestive enzymatic activity, intestine morphology and nutrient assimilation of ballan wrasse in response to different weaning strategies. In addition, longer-term impact of the weaning diets on juvenile growth should be studied.

Chapter 5: Effects of on-growing diets on farmed ballan wrasse (*Labrus bergylta*) feed intake, growth performance, liver fatty acid composition, intestine function and nutrient digestibility.

- Benchmarking of a suitable on-growing diet with inclusion of specific raw materials, which improves by 23 % growth compared to other fish meal based diets and without compromising intestinal health or digestive functions.
- Diet based on polychaetes elicited reduced growth and suppressed intestinal digestive enzymes and FA liver retention, possibly due to presence of anti-nutritional factors.
- When extrapolating the results of the current study, it can be suggested that the best performing diet tested could result in a 23 % shorter grow out period compared to the currently commercially used diet and 80 % compared to the fish meal diet tested. Thus, the currently required 20 – 24 month period to reach deployment size of 45 g could potentially be reduced to 16 – 19 months.
- Future studies should explore factors that increase palatability such as appetite-stimulants naturally occurred in some raw materials and feed physical characteristics.

Chapter 6: Effect of feed moisture level and agar inclusion on ballan wrasse, *Labrus bergylta*, feed intake, growth performances, digestive enzymes, intestine and liver health**.**

- Increased dietary moisture level (16 % moisture) in ballan wrasse decreased FCR by 21 % compared to the standard “dry” diet (5 % moisture).
- Despite an improved FCR, no growth differences became apparent between fish fed pelleted diets containing different moisture levels, indicating better feed assimilation in increased moisture (16 % moisture) level pelleted diet.

- No consistent differences appeared between intestinal enzymes that can be related to moisture levels.
- Description of liver enzymatic activity is given for the first time in ballan wrasse.
- Liver ALT responses showed a positive relation with the moisture content, while AST remained similar for all treatments.
- Liver enzymatic AST/ALT ratio was suppressed in fish fed the agar blocks, while no differences were apparent amongst the other diets.
- Fish condition was maintained using agar blocks although feed intake varied with agar inclusion rate, confirming their potential use as a post-deployment diet.

Chapter 8: General discussion

Commercial interest in farming ballan wrasse has grown rapidly over recent years to reduce the fishing pressure on wild wrasse stocks and due to its proven delousing efficiency on farmed Atlantic salmon (Skiftesvik et al., 2013; Leclercq et al., 2014b), offering a more environmentally friendly, effective and potentially cost effective alternative method to the use of chemotherapeutants (Costello, 1993). The effective farming of ballan wrasse has become a high priority to provide a reliable supply of disease free cleaner fish to the salmon industry.

The overarching aim of this doctorate study was to elucidate and improve farmed ballan wrasse survival, growth and provide reliable hatchery protocols and diet formulations. The work performed was mainly applied with two main research areas: (1) bacterial management at the early life stages (Chapters 2 and 3) and (2) nutritional requirements / diet development at the weaning and on-growing stages of ballan wrasse (Chapters 4, 5 and 6). The context in which this study was conducted was limited to a focus on commercial challenges to be addressed at Otter Ferry Seafish, and by no means covering the range of challenges reported in ballan wrasse farming. It must also be acknowledged that ballan wrasse farming is new, production numbers are very low and therefore, restricted number of sacrificial animals was available for the research project. From the outset of the study, general information on ballan wrasse biology and hatchery performance was very scarce. Therefore, the strategy adopted for most of the experiments in the current thesis was to test existing protocols and products used in other species for egg and larvae disinfection and benchmark diets already commercially available and developed for other marine fish species (e.g. Atlantic cod and Atlantic halibut).

Microbial communities in natural aquatic environment respond quickly to changes in their surrounding environment and therefore a continuous microbial monitoring and adaptation

of the microbial control methods is required in marine fish hatcheries. Different bacteria survival strategies have been developed through evolution to allow adaptation to the changing environmental conditions. Improvement of survival at the early larval stages of ballan wrasse farming was a priority and inadequate bacterial control was believed to contribute significantly to this. It is commonly known that microbial control is a key aspect in aquaculture intensive systems (Olafsen, 2001) and a better understanding of ballan wrasse bacterial load and control was required. The initial investigation of egg disinfection protocols in chapter 2 was based on experiences from cold water marine species (Treasurer et al., 2005; Birkbeck et al., 2006a) and with the aim to use a less harmful chemical to the commercial use of formalin. Experimental results suggested that the surrounding gum layer of the ballan wrasse eggs might already have anti-bacterial properties although this remains speculative at this stage as no characterisation was performed. This needs to be further investigated in line with the potential degumming method using enzyme alcalase® proposed by Grant et al. (2016b). However, in a commercial environment, bacterial control remains a key priority to reduce the risks of transferring non-culturable bacteria with potential long term effects on larvae.

Likewise, results obtained in chapter 3 showed the advantages of bacterial control in larval rearing tanks, by improving significantly survival in comparison to the untreated control groups. Repeated bronopol treatment with chemical probiotic (*Bacillus* sp.) addition from the yolk-sac stage resulted in an increase of larvae survival, but not growth. Lack of bacterial load differences between probiotics and/or bronopol applications might be related to the application methods and possibly to the short timeframe of the experiment. Potential risk of long term resistance to chemical use requires the development of a more sustainable microbial control strategy, such as through the use of probiotics. The interest in the use of

probiotics in aquaculture is somewhat recent and the mechanisms of actions are not fully understood and highly dependent on the probiotic bacterial strains used. Together with the selective (e.g. probiotics) or non-selective (e.g. chemical disinfectants) proposed by Vadstein et al. (1993), microbial matured water can also provide a solution for microbial control with creation of a high diversity bacterial flora environment dominated by non-opportunistic bacteria (Sjermo et al., 1999). This exposure of larvae to a diverse bacterial flora has been used in marine hatcheries and can increase survival and growth (Skjermo et al., 1999). Future studies should develop and evaluate modes of action of host-specific probiotics during the early ontogenesis, but also the use of microbial matured water during the first feeding phase of hatchery production of ballan wrasse.

To achieve biologically and economically efficient larval rearing microbial control protocols, bacteria species identification before and after treatments is required, offering more targeted regimes. Validation of results obtained in the present disinfection and probiotic experiments is required, using higher number of samples and biological replicates (minimum of n = 3, from different egg batches) than in the current study, and assessing different administration methods, such as probiotics addition through live feed. In addition, a better characterisation of the microbial environment as a response to disinfection protocols is required through sequencing and more recently developed molecular tools.

Benchmarking weaning and on-growing diets (Chapters 4 and 5) demonstrated the importance of developing ballan wrasse tailored diets based on specific raw materials (such as krill and shrimp meal) which contain specific appetite-stimulants, as it has also been reported during the timeframe of the present PhD work by Kousoulaki et al. (2014) and Bogevik et al. (2015). Krill hydrolysate contains higher levels of certain free amino acids (taurine, creatinine, glycine, glutaminic acid, lysine, arginine, leucine, alanine and proline)

compared with fish meal. Four common types of attractants, which can boost feeding behaviour in marine fish, are low-molecular-weight metabolites including free amino acids, quaternary ammonium compounds, nucleotides or nucleosides and organic acids (Carr et al., 1996). In addition, krill oil is a high source of phospholipids, which are typically composed of at least 20% EPA and DHA and 40% phospholipids (Massrieh, 2008). The benchmarking of juvenile performance through testing different feed formulations and dietary moisture content (Chapters 5 and 6) represented an important first step to rationalise growth potential, feed intake, digestive function, liver and intestine health and provide data against which to compare future production. However, the inclusion of some of these raw ingredients and superior fish meal quality is costly and might also imply technical difficulties, such as reduced feed shelf-life and lipid oxidation. It must also be acknowledged that diets based on polychaetes elicited reduced growth and suppressed intestinal digestive enzymes, which may suggest the presence of anti-nutritional factors, such as chitin and fluorine (Francis et al., 2001). Further studies should now better characterise the nutritional requirements of ballan wrasse to reach the true growth potential of the species. This should include development of weaning, on-growing and post-deployment diets. Towards this aim, the effects of environmental parameters, such as temperature, also need to be further investigated and optimised. An initial step would be to assess the two currently used water temperatures in ballan wrasse hatcheries (11 and 16 ^{o}C) testing three weaning windows (1, 3 and 6 weeks) in a triplicate system (18 tanks in total). Close monitoring of digestive enzymatic activity, intestinal morphology and nutrient assimilation will help understanding the functional maturation of tissues and organs and ultimately understand the underlying mechanisms behind the larval

performances. This knowledge will provide a stepping-stone for the further investigation of optimum weaning window and nutritional requirements.

A research priority should be the profiling of digestive enzyme activity at the larval stages to determine readiness for weaning diet onset and determination of optimum weaning window. This can be in line with investigating alternative enrichments, but also live feed species, such as cultivated copepods (*Acartia tonsa*) which has been recently proved as a promising live feed candidate for ballan wrasse larviculture (Øie et al., 2015). In addition, the longer term effects of weaning diets should be considered as it is clear that early nutrition can dictate overall fish robustness. To assess, and potentially improve, growth potential, muscle development at the early larvae stages could be further investigated, as it has been previously reported in other farmed fish species (Valente et al., 2013). However, further investigation would be looking at understanding FA metabolism in ballan wrasse and accordingly adjust diets formulation. Furthermore, given that ballan wrasse natural diet is reach in easily digestible sources and the positive results obtained by using cultivated copepods at the early larval stages (Øie et al., 2015), the role phospholipids should be further investigated as potentially key element in fatty acids assimilation. Copepod lipids are characterised by high contents of polar lipids as oppose to rotifers and *Artemia*, which are naturally rich in neutral lipids (Navarro et al., 1999: Dhont et al., 2013). However, the current limited supply of cultivated copepods and the high cost of it require dietary supplementation of phospholipids through alternative ways, such as bioencapsulation with different sources of lecithin (e.g. krill lecithin, soya lecithin). Enrichment strategies using various sources of phospholipids should be tested in the lights of improving larvae fatty acids assimilation, survival and growth. The ultimate goal to achieve a sustainable production of ballan wrasse requires the development of commercially available, cost

effective diets though maximising growth potential while ensuring optimal fish health and welfare and overall robustness post deployment. Considering that current feed cost for ballan wrasse makes up a high production cost, future work on on-growing diets should evaluate the impact of replacing the most expensive raw ingredients (shrimp, krill and cod fillet meal). Experiments can quantify the growth performances and intestinal health in fish fed isonitrogenus and isoenergetic diets with different replacement levels (25, 50, 75 and 100 %) of each ingredient.

The key commercial outcomes of these disinfection and nutritional experiments were significant improvements of ballan wrasse survival and growth performances in hatchery environment. The best egg disinfection protocol based exclusively on bronopol has been successfully adopted commercially at Otter Ferry Seafish from 2015, offering a total replacement of formalin use and enhanced microbial control. The overall survival rates had a significant 12-fold gradual increase between 2012 and 2016, reaching final estimated survival of 3.38 % from fertilised egg to deployment size juvenile (Fig. 8.1, Table 8.1). Substantial improvements were notified at the early stages with first feeding survival rising from 40 % in 2012 to 60 % in 2016 and weaning performance improving from 10 % in 2012 to 50 % after 2014. This PhD study provided a significant contribution to the development of hatchery production in ballan wrasse and an insight of the costs required for the implementation of various tools and dies.

Regarding egg disinfection in chapter 2, daily application of bronopol offered a 16% higher final yield compared to the untreated group. Assuming that an egg incubation system for ballan wrasse eggs consists of two 3.25 m^3 tanks with egg disinfection taking place the first day of collection (100 ppm), followed by daily application until hatch (25 ppm in daily basis) and the hatchery produces eggs every day during three photoperiod

regimes (2 months each), this will required daily use of 325 ml (100 ppm) and 81 ml (25 ppm) of bronopol or 650 ml and 162 ml of Pyceze (the commercially available of bronopol). This will result in requirement of 146 L Pyceze (180 days x 812 ml) per spawning season, with a commercial value of approximately £4,800 (£33 / L Pyceze).

Bacterial control, as described in chapter 3, requires the simultaneous use of probiotics and Pyceze in daily basis to achieve a six-time increase in larvae survival. For instance, daily use of 7.5 g / m^3 of MIC-F and 50 ppm of Pyceze (25 ppm of bronopol) in a system using a total of 15 first feeding tanks (average 4 m^3 each) for 75 days (yolksac and first feeding period) in each tank results to a 33.75 kg of MIC-F and 225 L of Pyceze for the entire season resulting in production of 130,000 juvenile ballan wrasse. Considering the cost of MIC-F (£188 / kg) and the Pyceze (£33 / L), a cost of £13,770 will be added to the production cost, adding £0.10 to each deployment size fish.

The weaning diets tested in chapter 4, BioMar, Otohime and Skretting are within the same price band (£15, 12.5 and 15.3 per kg). On the contrary, Nofima as being an experimental diet, costs almost six times higher (£81.6 per kg) than the other commercially available diets used. Considering that the amount of feed offered is similar between diets, the cost per survival unit increases significantly making Nofima being one of the least cost-effective diet with Otohime being at the other end of the spectrum.

The suggested on-growing diet formulation with specific raw materials (krill and shrimp meal) and increased moisture content (16 %) has been applied at Otter Ferry Seafish and in association with other possible factors (such as water temperature), resulted in a decreased of the hatchery grow out window required to reach deployment size (Fig. 8.2). In particular, a 23 % increase in growth rate has been achieved, which led to a reduction of the hatchery cycle of ballan wrasse from the initial 20 – 24 months in 2011-13 to 16 – 19

month period in 2016. Furthermore, the confirmation of the use of agar based feed blocks as a weight-maintaining post-deployment diet, originally proposed by Leclercq et al. (2015), played a major role to the implementation of new deployment strategy of farmed ballan wrasse in Scotland through hatchery conditioning. The significantly reduced growth performances in fish fed the agar blocks compared to pelleted diets in tanks and suppressed liver enzymatic activities raised concerns and led to further investigations looking at feed digestibility. It was confirmed outside the present PhD work that digestibility of fish fed the agar diet was optimal and therefore concluded that results obtained in the present study were due to fish size (10g) being smaller than deployment size (45g) and the fact that fish were fed exclusively agar diet with no other feed sources as opposed to the cage scenario where ballan wrasse can feed on sea lice as well as many other feed source.

Table 8.1. Ballan wrasse production numbers and survival rates between 2012 and 2016 year class fish presented for each developmental stage.

Year	Values at the end of each stage	Eggs	Larvae		Juveniles		Overall (end of production cycle)
		Fertilised eggs	Yolk sac	First feeding	Weaning	On-growing	
2012	No of fish	2,053,903	657,249	262,900	26,290	5,258	**5,258**
	Survival (%)	80	40	40	10	20	**0.26**
2013	No of fish	2,040,479	734,572	293,829	58,766	11,753	**11,753**
	Survival (%)	90	40	40	20	20	**0.58**
2014	No of fish	2,362,660	956,877	382,751	191,375	38,275	**38,275**
	Survival (%)	90	45	40	50	20	**1.62**
2015	No of fish	4,456,836	1,805,019	902,509	451,255	112,814	**112,814**
	Survival (%)	90	45	50	50	25	2.53
2016	No of fish	4,500,000	2,025,000	1,215,000	607,500	151,875	**151,875**
	Survival (%)	90	50	60	50	25	**3.38**

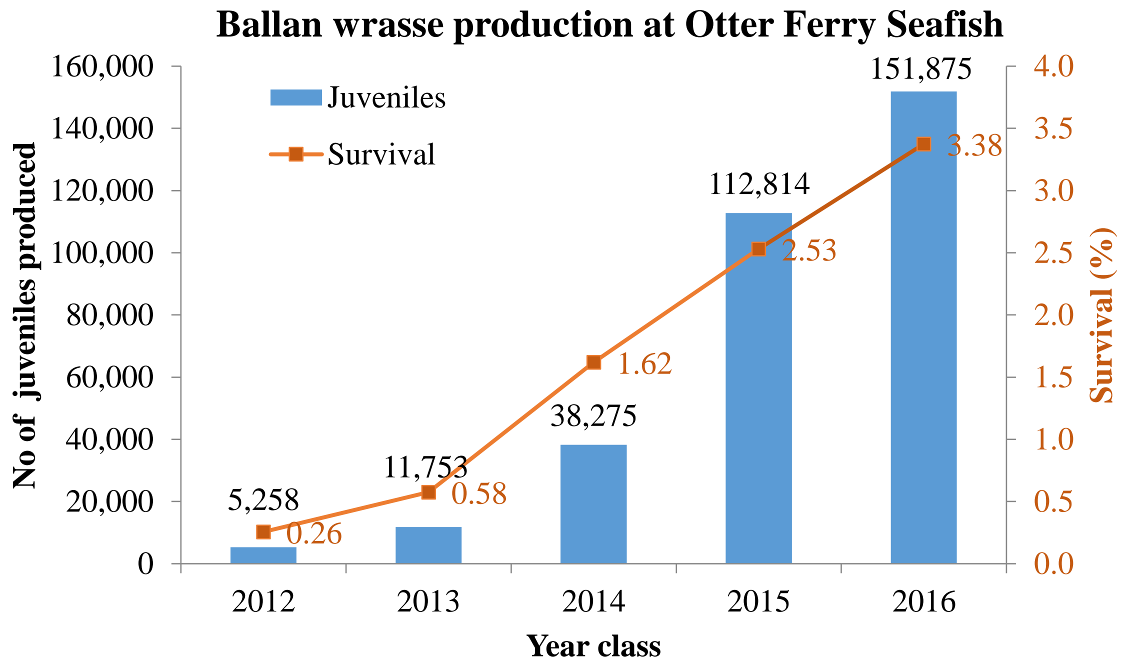

Figure 8.1. Ballan wrasse production and survival rates between 2012 and 2016 year class fish at Otter Ferry Seafish. An overall 12-fold survival increase has been achieved and approximately 300k ballan wrasse juveniles have been produced during the four years of this study.

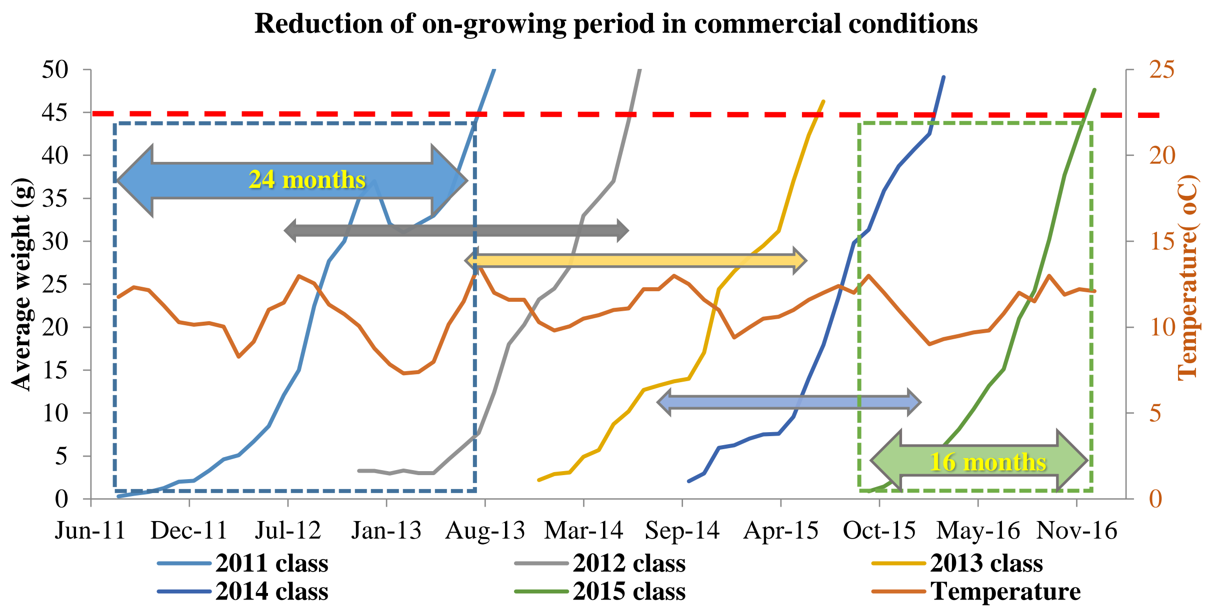

Figure 8.2. Commercial data from Otter Ferry Seafish showing average weights (g) of the leading ballan wrasse juvenile populations between 2011 and 2016 year classes. Dashed horizontal line indicates the minimum deployment size (45 g). The initial 24 month window required to reach the deployment size of 45 g has been shortened by over 30 % to 16 months between 2012 and 2016. Improved diet formulation together with water temperature is considered to be the most important parameters that led to enhanced growth in ballan wrasse farming.

Taken together, the results of this thesis have strong commercial relevance to help towards tackling some of the key bottlenecks limiting expansion of the ballan wrasse farming. These findings highlight the need for a greater understanding of the mechanisms underlying growth and survival performances. What this study clearly demonstrated is the need for adjusting the hatchery protocols and diets to meet the biological needs of ballan wrasse in order to scale up hatchery production of cleaner fish.

Publications

Conference oral presentations

- **Chalaris, A.,** Davie, A. & Migaud, H. 2016. Bacterial management at the early life stages of ballan wrasse (*Labrus bergylta*). “Aquaculture UK 2016”, Aviemore, UK, 25th – 26th May 2016.
- Davie, A., Grant, B., Leclercq E., **Chalaris, A.**, Picci, N., Prat, K. Garcia, M. & Migaud, H. 2014. Broodstock spawning performance and hatchery protocols for ballan wrasse *Labrus bergylta.* International conference “Aquaculture Europe 2014”, San Sebastian, Spain, 14th – 17th October 2014.
- **Chalaris, A.**, Leclercq, E., Zerafa, B., Davie, A. & Migaud, H. 2014. Effects of different weaning and on-growing diets on farmed ballan wrasse *Labrus bergylta* hatchery performance. International conference “Aquaculture Europe 2014”, San Sebastian, Spain, 14th – 17th October 2014.
- Leclercq, E., Grant, B., **Chalaris, A.**, Davie, A. & Migaud, H. 2014. Recent advances in the production and implementation of farmed ballan wrasse (*Labrus bergylta*) in the Scottish salmon industry. International conference “Sea lice 2014”, Portland, Maine, USA, 31st August – 5th September 2014.
- Grant, B., **Chalaris, A.**, Davie, A. & Migaud, H. 2013. Spawning dynamic, egg degumming and disinfection. UK and Norwegian wrasse workshop, Campeltown, Scotland, UK, 3rd October 2013.

Conference poster presentations

- **Chalaris, A.**, Leclercq, E., Zerafa, B., Ryder, D., Zambonino, J., Davie, A. & Migaud, H. 2016. Effects of different on-growing diets, moisture levels and agar inclusion on farmed ballan wrasse (*Labrus bergylta*) hatchery performance. "Aquaculture Europe 2016", Edinburgh, UK, 20th – 23rd September 2016.
- **Chalaris, A**., Leclercq, E., Zerafa, B., Davie, A., Zambonino, J., Kousoulaki, K. & Migaud, H. 2015. Effects of different on-growing diets on farmed ballan wrasse (*Labrus bergylta*) hatchery performance. "Aquaculture Europe 2015", Rotterdam, The Netherlands, 20th – 23rd October 2015.

Commercial magazine articles

- **Chalaris, A.**, 2015. Otter Ferry wrasse project – The next stage. *Fish Farmer* 38 :28 – 32.

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