Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Anna Sigrid Norberg Aase

Nutritional condition of ballan wrasse (*Labrus bergylta*) larvae reared on different live feed diets – an investigation of gut and liver histology

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Supervisor: Elin Kjørsvik
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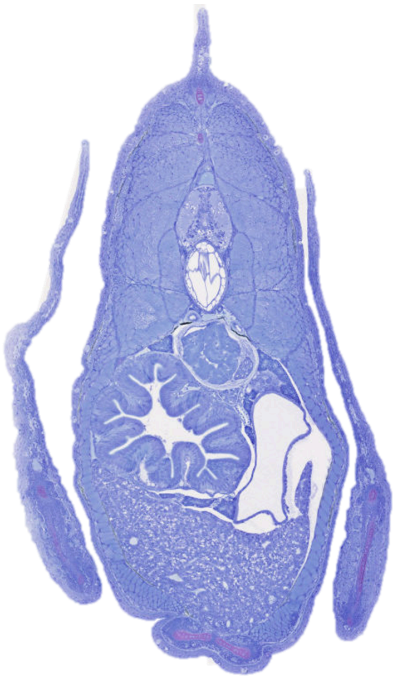

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Abstract

The threat of salmon lice remains the largest obstacle for further growth of the Norwegian salmon aquaculture industry, where large amounts of delousing operations are performed every year to keep the lice at bay. The use of ballan wrasse as a biological delousing agent has been increasing in popularity over the last years in the salmon aquaculture industry due to a high delousing efficiency, no resistance development and smaller effects on salmon welfare compared to chemical and mechanical delousing. The demand has mainly been met by fisheries, however, there is a large interest in successful aquaculture of the species to secure year-round availability of high quality fish. The rearing of ballan wrasse has proven difficult, with bottlenecks occurring in the larval stages. As for many other marine fish, ballan wrasse larvae are small and little developed upon hatching and do not accept inert feed. Thus, live feed must be supplied in adequate quantities and qualities to secure growth and survival. The traditional feeding regime of rotifers followed by *Artemia* before weaning onto formulated feed seems to be suboptimal for the ballan wrasse larvae, where large mortalities, low growth and stress tolerance and deformities are prevalent in cultivated larvae. Improved growth and development has been observed when the fish are fed on copepods during the larval stages compared to rotifers and *Artemia*, likely due to the nutritional composition which is more adapted to the requirements of marine fish larvae. Copepods have recently become commercially available, in addition to a new live feed organism, namely cryopreserved cirriped nauplii. These nauplii have been suggested to have a similar nutritional composition as copepod, and thus may be able to replace rotifers and *Artemia* as live prey in the rearing of ballan wrasse. Thus, the present study was conducted to evaluate the effect of feeding ballan wrasse larvae with copepods and/or cirripeds compared to the traditional diet of rotifers followed by *Artemia.* A start-feeding experiment was performed, where ballan wrasse larvae were fed either copepods, experimental small cirripeds or rotifers from 4-18 dph and either *Artemia* or large cirripeds from 18-32 dph, followed by weaning onto the same formulated feed from 32-48 dph. As the gut and liver in developing fish larvae respond sensitively to dietary changes, histological examination of biomarkers of nutritional condition in the gut and liver tissue was performed. In addition, growth and survival were assessed. The different live feed organisms varied greatly in their quality as live feed for ballan wrasse larvae, where feeding with copepods as the first live feed gave significantly better survival than larvae fed

either rotifers or experimental small cirripeds as their first diet regardless of the second live feed offered. Growth was similar between larvae fed copepods and rotifers, but larvae receiving experimental small cirripeds were significantly smaller than all other treatments throughout the experimental period. The nutritional condition of the larvae was reflected in the gut and liver histology, where feeding with copepods followed by *Artemia* resulted in a rapid development of both gut and liver tissue during the live feed period, but weaning to formulated feed induced some degeneration of the gut tissue in the form of shortened microvilli. Copepods followed by large cirripeds resulted in similar liver histology but a slightly slower development of gut tissue during the live feed period compared to the other copepod group, but weaning onto formulated feed showed pronounced effects in the form of large hepatocytes and amount of glycogen vacuoles in the liver, and taller villi, epithelial height and microvillus height than the other treatments. Although larvae fed experimental cirripeds displayed signs of starvation after the first live feed period in the form of a reduced hepatonuclear size in the liver as well as shortened gut epithelial height and microvilli, signs of starvation were reduced after the second live feed period. Larvae fed rotifers followed by *Artemia* displayed histopathological alterations such as accumulation of lipid droplets in the liver tissue and enterocytes as well as small and indistinct hepatocyte cells with shrunken nuclei after weaning to formulated feed. Most measured parameters of the gut and liver could be related to standard length with some variation between treatments. The relationship between gut and liver histology and growth rate was less clear.

It is concluded that feeding with copepods resulted in the best growth, survival and nutritional condition of ballan wrasse larvae, and thus may be a viable option to replace rotifers in aquaculture of the species. The best results were obtained by feeding copepods followed by cirripeds, where a large surface area of the gut as well as high amount of energy reserves in the liver indicated a high metabolic capability and resistance to starvation, as well as a good utilization of the formulated feed.

Sammendrag

Lakselusproblematikk fortsetter å være den største hindringen for fortsatt vekst i den norske laksenæringen, hvor mange avlusningsoperasjoner blir gjennomført hvert år for å holde lusetallene nede. Bruk av berggylt som en biologisk avlusningsmetode har økt i popularitet de siste årene i lakseoppdrettsnæringen, grunnet en høy avlusningseffektivitet, ingen resistensutvikling og mindre effekter på laksevelferd sammenlignet med kjemisk og mekanisk avlusning. Behovet for berggylt har stort sett blitt møtt av fiskeri, men det er stor interesse for å oppdrette arten ettersom dette vil sikre en mer stabil tilgang året rundt i tillegg til en jevnere kvalitet på fisken. Oppdrett av berggylt har vært vanskelig, der flaskehalser finner sted i larvestadiene. Liksom mange andre marine fiskearter er berggyltlarver små og lite utviklet når de klekker fra eggene, og de klarer ikke å spise formulert fôr. Dermed må passende mengder levendefôr med god nok kvalitet gis for å sikre vekst og overlevelse. Tradisjonelle startfôringsregimer med bruk av rotatorier etterfulgt av *Artemia* før tilvenning til formulert fôr virker å være suboptimale for berggyltlarver, der høy dødelighet, lav vekst og stresstoleranse i tillegg til deformiteter ofte er tilfellet. Bedre vekst og utvikling har derimot blitt sett i berggyltlarver som har blitt fôret på copepoder under larvestadiene, sannsynligvis fordi copepoder er et naturlig fôr for marine fiskelarver og godt tilpasset larvenes ernæringsbehov. Copepoder har nylig blitt tilgjengelig på det kommersielle markedet, i tillegg til en ny levendefôrorganisme, nemlig kryopreserverte rurnauplier. Disse naupliene sies å ha lignende ernæringsprofil som copepoder, og kan dermed muligens være en kandidat for å erstatte rotatorier og *Artemia* som levendefôr for berggylt. Dermed ble denne studien gjennomført med det formål å evaluere effektene av å fôre berggyltlarver med copepoder og/eller rurnauplier sammenlignet med den tradisjonelle dietten som består av rotatorier og *Artemia*. Et startfôringsforsøk ble gjennomført, der berggyltlarver ble fôret enten copepoder, eksperimentelle små rurnauplier eller rotatorier fra 4-18 dager etter klekking (dph), etterfulgt av enten *Artemia* eller store rurnauplier fra 18-32 dph, før alle larver ble tilvent det samme formulerte fôret fra 32-48 dph. Ettersom tarmen og leveren in fiskelarver under utvikling responderer sensitivt til endringer i diett ble histologiske undersøkelser av biomarkører for næringsstatus i lever og tarm utført. Vekst og overlevelse ble også vurdert. De ulike levendefôrorganismene varierte stort i kvalitet som fôr til berggyltlarver, der fôring med copepoder som det første levendefôret gav en signifikant bedre overlevelse sammenlignet

med rotatorier og rurnauplier, uavhengig av hvilken fôrorganisme som ble gitt etterpå. Veksten var lignende mellom larver gitt copepoder og rotatorier, men larver som fikk eksperimentelle små rur var mindre enn larver gitt andre fôr gjennom hele den eksperimentelle perioden. Næringsstatus i lever og tarm ble reflektert i histologiske funn, der fôring med copepoder etterfulgt av *Artemia* resulterte i en rask utvikling av både lever- og tarmvev mens larvene fikk levendefôr, mens tilvenning til formulert fôr induserte noe degenerasjon av tarmvevet i form av kortere microvilli. Copepoder etterfulgt av store rurnauplier resulterte i en lignende leverhistologi, men litt saktere utvikling av tarmvev gjennom levendefôrperioden sammenlignet med den andre copepodegruppen. Tilvenning til formulert fôr gav derimot uttalte effekter i denne gruppen, der store hepatocytter og store mengder av glykogenvakuoler ble sett i leveren, samtidig som høyere tarmtotter, epitelhøyde og microvilli ble observert i tarmen. Larver gitt eksperimentelle små rurnauplier viste noen tegn til sult etter den første levendefôrperioden, i form av redusert leverkjernestørrelse og kortere tarmepitel og microvilli. Disse tegnene var ikke sett etter den andre levendefôrperioden. I larver fôret på rotatorier etterfulgt av *Artemia* ble det observert histopatologiske endringer som akkumulering av lipiddråper i levervevet og enterocyttene i tillegg til små og utydelige hepatocyttceller med krympede kjerner etter tilvenning til formulert fôr. De fleste målte parametre i lever og tarm kunne relateres til standard lengde, med noe variasjon mellom fôringsgruppene. Forholdet mellom vekstrate og lever- og tarmparametre var derimot noe utydelig. Det konkluderes med at å fôre copepoder gav den beste veksten, overlevelsen og næringsstatusen i berggyltlarver, og kan dermed være et alternativ til å erstatte rotatorier i oppdrettet av arten. De beste resultatene ble sett når fôring med copepoder ble etterfulgt av å gi store rurnauplier, der et stort overflateareal i tarmen i tillegg til en stor mengde overskuddsenergi i leveren indikerte en høy metabolsk aktivitet og motstandsdyktighet mot sult, så vel som en god utnyttelse av det formulerte fôret.

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Abbreviations

AA	Amino acid – organic compound containing both amino and carboxylic acid functional groups. May exist in free form or combine to form proteins
ARA	Arachidonic acid (20:4n6) – a polyunsaturated omega-6 fatty acid essential for marine fish larvae
DHA	Docosahexaenoic acid (22:6n3) – a polyunsaturated omega-3 fatty acid essential for marine fish larvae
Dph	Days post hatching – number of days since a fish larva hatched from its egg
DW	Weight of larvae after being dried at 60 C for 48 hours, here given in µg
DWI	Daily weight increase (%) – percentage of mean daily increase in dry weight over a given time interval. Derived from the SGR
EFA	Essential fatty acid – fatty acid that cannot be synthesized and must be supplied by diet
EPA	Eicosapentaenoic acid (20:5n3) – a polyunsaturated omega-3 fatty acid essential for marine fish larvae
FA	Fatty acid – carboxylic acid with a long aliphatic chain, which may be saturated (no double bonds) or unsaturated (one or more double bonds). Major component in lipids
FAA	Free amino acid – amino acid not bound by peptide bonds
HUFA	Highly unsaturated fatty acid – fatty acid containing three or more double bonds in the molecule. Classified as omega-3 or omega-6 depending on the position of the first double bond
LC-PUFA	Long chain polyunsaturated fatty acid – polyunsaturated fatty acid with 20 or more carbon atoms and 3 or more double bonds
NL	Neutral lipid – simple lipids formed by dehydration of one of more fatty acids by alcohol. Often storage lipids, such as triglycerides

PAS	Periodic acid Schiff – a staining method used to detect polysaccharides and mucosubstances in tissues
PBS	Phosphate buffered saline – a water based salt solution commonly used in biological research
PFA	Paraformaldehyde – a fixative used to preserve biological specimens and tissue
PL	Phospholipid – a class of lipid. Consists of a phosphate group, a glycerol molecule and two fatty acids. Key components of all cell membranes
SGR	Specific growth rate – rate of mean daily increase in dry weight over a given time interval
SL	Standard length – length of fish measured from snout to caudal peduncle (or tip of notochord in the case of pre-flexion larvae)
TAG	Triacylglycerol – a neutral lipid derived from glycerol and three fatty acids.
TB	Toluidine blue – a basic dye with a high affinity for acidic tissue components

1. Introduction

1.1 Norwegian salmon production and the use of cleaner fish as a biological delousing agent

In a world where the ever growing human population is putting pressure on global food production, the demand for seafood is increasing. The role of aquaculture has long been considered crucial in the overall increased growth in seafood production and it has been the most rapidly growing food production industry for several decades (Asche et al., 2013; FAO, 2020; Kumar & Engle, 2016). Within the aquaculture sector, the production of salmonid fishes has been among the most successful (Asche et al., 2013). Cultivated species include, but are not limited to, Atlantic salmon (*Salmo salar*), Arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorchynchus mykiss*), where Atlantic salmon is by far the species with the highest production volume (Asche et al., 2013). Norway has been the lead producing country in the salmon industry since its inception, and is responsible for approximately half of all salmonid production worldwide (Asche et al., 2013). In 2020, Norwegian salmon farms sold approximately 1,38 million tonnes of Atlantic salmon where over 95% was exported for a value of 70 billion NOK (FAO, 2020; Norwegian Directorate of Fisheries, 2022a). The salmon industry has however for several decades been challenged by the infestation of salmonids by salmon lice (*Lepeophtheirus salmonis* Krøyer, 1838), caligid copepod ectoparasites feeding off the skin, mucus and blood of salmonids (Iversen et al., 2015; Torrissen et al., 2013). These parasites may cause a range of negative consequences for the fish, including stress; anemia; osmotic imbalance; behavioral changes; secondary infections and death (Costello, 2006; Tully & Nolan, 2002; Wagner & McKinley, 2004). In addition, it has been suggested that sea lice may act as potential reservoirs and vectors for fish pathogens (Gonçalves et al., 2020). The parasitic infestation by salmon lice may also cause adverse ecological effects, in the case of migrating natural populations of salmon and trout smolts being infected when swimming close to open net pens in the sea (Costello, 2009; Dempster et al., 2021; Torrissen et al., 2013). Although a naturally occurring parasite to salmonids, the high density of cultivated salmon in open net pens offers opportunities for high proliferation rates of the parasites as there is a large amount of hosts concentrated over a small area (Torrissen et al., 2013). By effects on mortality rates, growth and feed conversion rates on the salmon as well as strict

governmental requirements on allowable amounts of lice within farms with following high amounts of delousing operations (Larsen & Vormedal, 2021; Norwegian Ministry of Trade, Industry and Fisheries, 2015; Overton et al., 2019), treatment and prevention of salmon lice is directly responsible for large economic costs and losses in the salmon industry (Costello, 2009; Iversen et al., 2020). Thus, there is a need for effective delousing methods to prevent both economical losses as well as fish welfare problems and ecological issues.

Initially, the use of chemotherapeutants as a delousing agent proved effective, leading to a high chemical usage for salmon lice treatments in the 1990's and 2000's (Denholm et al., 2002; Igboeli et al., 2014; Aaen et al., 2015). However, the use of these chemotherapeutants lead to resistance, resulting in new lice generations chemical treatments were largely ineffective (Igboeli et al., 2014; Aaen et al., 2015). Sublethal and lethal effects of delousing species were also seen on non-target species such as crustaceans (Urbina et al., 2019). Mechanical delousing operations involve crowding and pumping of the fish through fresh or warm water with high pressure nozzles or brushes to flush and spray off the lice (Nilsson et al., 2023; Overton et al., 2019). Although these methods are considered to be highly effective, they are detrimental to fish health and welfare, by both increasing levels of stress hormones, causing mechanical damage in the form of scale loss and ulcerations, and elevating mortality rates (Føre et al., 2018; Moltumyr et al., 2022; Nilsson et al., 2023; Overton et al., 2019). Preventative strategies for maintaining low lice levels, such as snorkel pens, lice skirts and submerged pens are under testing and development (Barrett et al., 2020), but regular delousing still remains necessary to keep the government-mandated limits to lice levels.

Biological delousing is done by use of so-called "cleaner-fish", species employing specialist feeding strategies where they pick lice directly off other fish's skin to eat. These fish are deployed directly in the net pen together with the salmon, and no pumping or crowding of salmon is needed. The use of cleaner-fish does not seem to increase stress levels in the salmon or otherwise impact the salmon health and welfare significantly, with the exception of some antagonism in the case of large wrasse being deployed in the net pen with salmon (Cerbule & Godfroid, 2020; Leclercq et al., 2014). Therefore, the use of cleaner-fish has increased in popularity and there is a high demand from the salmon industry to both capture and cultivate these fishes. Several species of cleaner fish are in use in the Norwegian salmon

industry, where the most popular are lumpfish (*Cyclopterus lumpus*) followed by different species within the *Labridae* or wrasse family. These species include ballan wrasse (*Labrus bergylta*), corkwing wrasse (*Symphodus melops*), goldsinny wrasse (*Ctenolabrus rupestris*) and rock cook (*Centrolabrus exoletus*) (Norwegian Directorate of Fisheries, 2022c). Out of the different wrasses used for biological delousing of salmon, the ballan wrasse is the largest, is an efficient louse-eater and has proven to be the most promising species for aquaculture (Leclercq et al., 2014; Skiftesvik et al., 2013). In addition, several benefits may be gained from combining the use of lumpfish and ballan wrasse as delousing agents in the salmon pens, as the species occupy different niches and habitats in the pen as well as being active at different temperatures (Brooker et al., 2018; Powell et al., 2018).

1.2 Ballan wrasse – biology, ecology and aquaculture

The ballan wrasse is a protogynous hermaphrodite that is distributed along the North Atlantic coastline from Morocco to Norway (Quignard & Pras, 1986). Its main habitats are found in inshore waters amongst rock and algal cover (Darwall et al., 1992), where it lives in colonies with one, dominant male keeping a harem of females (Darwall et al., 1992; Sjölander et al., 1972). When the male dies, a large female will undergo sex change and take place as the new harem leader (Muncaster et al., 2013). The adult fishes show a high variation in color, but generally either have a marbled brownish, greenish or reddish color, or dark orange/reddish with white spots (Villegas-Ríos et al., 2013). The ballan wrasse is considered an important species in its environment with complex life history traits. The fish is able to live to old ages, and the male-female ratio is highly skewed with only about 10% of the populations being males(Dipper et al., 1977; Leclercq et al., 2014; Quignard & Pras, 1986). This ratio is also determined by the size and age of the fishes, with larger, older individuals having a higher probability of being males(Dipper et al., 1977; Leclercq et al., 2014; Muncaster et al., 2013). In addition, ballan wrasse may control populations of other species by feeding on e.g. echinoderms, thus helping to maintain the balance in local ecosystems (Figueiredo et al., 2005). The species has also shown to have a high home-range fidelity (Morel et al., 2013; Villegas-Ríos et al., 2013). Ballan wrasse fisheries may therefore cause several disturbances in local biological communities, leading to a strict regulation of the fisheries for use of the species in salmon aquaculture by Norwegian governmental policies. This, coupled with the

efficacy the fish has shown to remove salmon lice, and the need for a stable year-round production of good quality fish, has led to a high interest in successful aquaculture production of the species.

Although lumpfish is still the highest produced and deployed cleaner-fish in Norway (Norwegian Directorate of Fisheries, 2022a), the amount of cultivated ballan wrasse for use in Norwegian salmon aquaculture has shown an increasing trend over the past years, where in 2020, the percentage of deployed ballan wrasse from aquaculture surpassed that of wild-caught fish for the first time (Norwegian Directorate of Fisheries, 2022a). The sale of both cultivated and wild-caught ballan wrasse in Norway overall is also increasing, simultaneously as the amount of wild caught wrasse and sale of lumpfish is decreasing (Norwegian Directorate of Fisheries, 2022a, 2022b). In line with increased production, there is a rising focus on how to optimize rearing techniques and overcome the bottlenecks associated with production of ballan wrasse. As for several other marine fish species, these bottlenecks are strongly associated with the larval stage, and include high mortalities, slow growth, non-acceptance of inert diets in the early larval stages, a long live feed period and poor quality and viability of produced larvae (Kousoulaki et al., 2015; Lekva & Grøtan, 2018). As an example, cultivated ballan wrasse show a high degree of skeletal deformities and poor stress tolerance (Fjelldal et al., 2021; Kousoulaki et al., 2021; Lekva & Grøtan, 2018). Much can be linked to suboptimal nutrition and a lack of knowledge on nutritional requirements (Kjørsvik et al., 2011; Kousoulaki et al., 2015; Kousoulaki et al., 2021; Lekva & Grøtan, 2018; Øie et al., 2015).

In the wild, marine fish larvae are highly sensitive to both starvation and predation (Balon, 1986; Hunter, 1981). Therefore, the organs that are most necessary for growth and survival, namely those organs involved in capture, ingestion and digestion of food as well as swimming musculature and skeletal structures for both hunting prey and evasion of predators, are prioritized first in larval growth - a process called allometric growth (Osse & Van den Boogaart, 2004). This development is, along with other parameters, dependent on nutrition (Gagnat et al., 2016). Therefore, the study of these organs and how they are developing may shed light on how the nutritional requirements of the larvae are being met.

1.3 The fish larval digestive system – function and development

1.3.1 The digestive system of teleost fish

The digestive system of teleost fish consists of the gastrointestinal tract, or alimentary canal, divided into mouth, pharynx, oesophagus, stomach, intestine and rectum, as well as accessory digestive glands and organs – namely the liver, pancreas and gallbladder (Bone & Moore, 2008; Kryvi & Poppe, 2016; Wilson & Castro, 2010). Pyloric caeca are also often present in species with a functional stomach, but are always absent in agastric fish (Bone & Moore, 2008). Although the main functions of the digestive system are acquisition, breakdown, assimilation, uptake and transport of nutrients (Bakke et al., 2010; Bone & Moore, 2008), the system also aids in immune and barrier function, osmoregulation, metabolism and hormone secretion (Bakke et al., 2010; Jutfelt, 2011; Wallace et al., 2005). In general, the alimentary canal consists of four concentric layers: the innermost layer, called the mucosa, includes epithelial cells facing the digestive tract lumen apically, and a surrounding layer of lamina propria, which consists of vascularized loose connective tissue. Surrounding the mucosa is the submucosa, which contains blood vessels and lymph, nerves and connective tissue. The next layer is the muscularis, which includes striated and smooth muscle innervated with enteric neurons which regulate intestinal motility. Finally, the outermost layer is the serosa, which is composed of simple flat epithelium (Lazo et al., 2011; Wallace et al., 2005). The structure of the four cell layers may vary along the alimentary canal, and not all layers are present at all regions of the tract (Lazo et al., 2011). For example, the epithelial cells of the mucosa may be differentiated into secretory cells or absorptive cells, among other cell types, according to the functionality of the region (Lazo et al., 2011). The different regions of the alimentary canal perform different functions, where the mouth and pharynx are involved in capture and mechanical processing of food, oesophagus, stomach and intestine in chemical digestion, and rectum in elimination of wastes (Wilson & Castro, 2010). The accessory digestive organs, i.e. the liver, pancreas and gallbladder, aid the gastrointestinal tract in numerous ways in digestion, and also have important functions in maintaining homeostasis. The liver is a central digestive organ which is not only involved in nutrient metabolism, conversion, mobilization, storage and transport, but also in detoxification and production of bile, which aids in emulsification of lipids in the intestine

(Akiyoshi & Inoue, 2004; Bakke et al., 2010; Hoehne-Reitan & Kjørsvik, 2004; Lazo et al., 2011; Zambonino-Infante et al., 2008). It is a glandular organ that is mainly structured into parenchymal hepatocyte cells that are anastomosed and arranged around a central vein; sinusoids, which are capillary networks transporting nutrients and oxygen; and bile canaliculi as well as bile ducts for transportation of bile (Akiyoshi & Inoue, 2004; Hoehne-Reitan & Kjørsvik, 2004; Lazo et al., 2011). Bile is produced in the hepatocytes, is transported to the gall bladder via the bile canaliculi and stored there until it is secreted into the intestinal lumen as a response to food present in the gut by way of the bile duct (Hoehne-Reitan & Kjorsvik, 2004). Assembled nutrients, on the other hand, are stored in the liver hepatocytes as either glycogen or lipids (Lazo et al., 2011). The amount of hepatic nutrient storage is interspecific, and may differ over the course of development (Hoehne-Reitan & Kjørsvik, 2004). The pancreas can be divided into exocrine and endocrine pancreas, where the exocrine part is mainly responsible for the production and secretion of digestive enzymes, and the endocrine part, called islets of Langerhans, secrete hormones (Lazo et al., 2011).

1.3.2 Development of the digestive system in ballan wrasse

The development of organs and tissues associated with the digestive system in fish follows the same general pattern, but timing and function may vary between species (Lazo et al., 2011). The level of development of the digestive tract at the onset of exogenous feeding is dependent on the amount of endogenous nutrients up until that point, and can be separated into three modes: precocial, altricial and agastric (Kjørsvik et al., 2004; Rønnestad et al., 2013). Precocial fish, e.g. wolffish (*Anarhichas lupus*) and Atlantic salmon (*Salmo salar),* hatch from large eggs and have a fully differentiated digestive system with a functional stomach at the time of first feeding (Falk-Petersen & Hansen, 2001; Gorodilov, 1996) Altricial fish, including cod and halibut as well as many other marine species, have an undifferentiated alimentary canal with no functional stomach at hatching, as it appears during metamorphosis (Gomes et al., 2014; Kamisaka & Rønnestad, 2011). Agastric fish remain stomachless throughout their life span (Kjørsvik et al., 2004; Rønnestad et al., 2013). The ballan wrasse is agastric, however its alimentary canal closely resembles that of altricial fish larvae during early development (Dunaevskaya et al., 2012; Norland et al., 2022), whereupon at hatching, the alimentary canal is a straight tube, dorsally attached to the yolk sac closed in both ends

with undifferentiated epithelium along its length (Dunaevskaya et al., 2012; Govoni et al., 1986; Lazo et al., 2011), which differentiates into buccopharynx, foregut, midgut and hindgut. Two valves separate the tract between oesophagus and foregut as well as between midgut and hindgut, and mouth and anus opens right before the onset of first feeding (Dunaevskaya et al., 2012). Over the course of the larval period, the alimentary canal elongates and increases in surface area by folding of the mucosa into villi and development of a brush border apically toward the gastrointestinal lumen, and one intestinal loop is formed (Dunaevskaya et al., 2012; Norland et al., 2022). The mucosa of the alimentary canal differentiates into cuboid epithelium scattered with goblet cells in the oesophagus, and enterocytes, goblet cells, enteroendocrine cells and rodlet cells in the intestine (Dunaevskaya et al., 2012; Gagnat et al., 2016; Lazo et al., 2011; Norland et al., 2022). The liver is not present at hatching, but by onset of exogenous feeding primordial liver tissue is apparent as a cluster of rounded cells close to the yolk sac (Dunaevskaya et al., 2012; Gagnat et al., 2016), and it quickly develops into a cohesive organ which increases in size and both elongates ventrally toward the hindgut and surrounds the midgut dorsally (Gagnat et al., 2016; Norland et al., 2022). Glycogen storage in the hepatocytes can be seen after first feeding (Dunaevskaya et al., 2012; Gagnat et al., 2016). The gall bladder is apparent before first feeding, and increases in size during development (Dunaevskaya et al., 2012). An incipient pancreas can be seen after hatching, with acinar cells, zymogen granules and a primary islet of Langerhans appearing right before onset of exogenous feeding. The pancreas also increases in size during development, and spreads into the liver tissue forming a diffuse hepatopancreas during metamorphosis (Dunaevskaya et al., 2012; Gagnat et al., 2016).

1.4 Fish larval nutrition and diets used in ballan wrasse larval cultivation

The development, growth and survival of fish larvae is highly dependent on nutrition, in addition to environmental factors (Gisbert et al., 2008; Kjørsvik et al., 2011). A larval diet containing all essential nutrients promote proper growth and development in fish larvae, while unbalanced or sub-optimal diets may lead to poor growth, lowered survival rates, skeletal deformities, and, in the case of flatfish, affect eye-migration as well as pigmentation (Busch et al., 2010; Cahu et al., 2003; Hamre & Harboe, 2008; Hamre et al., 2002; Jafari et al., 2021; Karlsen et al., 2015; Kjørsvik et al., 2009; Koedijk et al., 2010; Næss et al., 1995; Payne

& Rippingale, 2000; Shields et al., 1999; Øie et al., 2015). This is due to the rapid and energy-demanding growth and differentiation processes the fish larvae undergo from hatching until metamorphosis, where they are highly sensitive to nutritional stress (Gisbert et al., 2008). The diet also needs to be suited to the developmental stage of the digestive system in the larvae, as this will vary over the course of ontogeny (Rønnestad et al., 2013; Zambonino-Infante et al., 2008). Thus, when cultivating fish larvae, knowledge of the nutritional requirements together with the developmental stage of the digestive and metabolic systems in the specific species is of a high importance when selecting larval diets and determining feeding regimes (Gisbert et al., 2008; Kjørsvik et al., 2011).

Much the same as for adults and juveniles, fish larvae have a high quantitative demand for fatty acids (FA). Marine fish larvae require in particular three highly unsaturated fatty acids (HUFAs): docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), which are essential for the larvae and must be supplied by the diet (Sargent et al., 1999). DHA, EPA and ARA are necessary for the normal growth and development of marine fish larvae, being important structural components in cellular membranes and precursors for bioactive molecules (Sargent et al., 1999). In addition to the essential fatty acids (EFAs) needing to be supplied in sufficient quantities, the ratio of the fatty acids in relation to one another as well as the lipid class they are incorporated into are also of high importance (Izquierdo et al., 2000; Sargent et al., 1999). Although the optimal ratio of DHA:EPA:ARA is species-specific, it is approximated to be around 2:1 for DHA:EPA and 4:1 for EPA:ARA for most marine species (Izquierdo & Koven, 2011). Furthermore, the EFAs seem to be easier for the larvae to digest and utilize for growth and development when incorporated into polar lipids, such as phospholipids (PLs), compared to neutral lipids (NLs), such as triacylglycerols (TAGs) (Izquierdo et al., 2000; Sargent et al., 1999), where several studies have linked an increased amount of FAs in the PL fraction compared to the NL fraction to better growth, development, nutritional condition and survival in marine fish larvae (Cahu et al., 2003; Fontagne et al., 1998; Gisbert et al., 2005; Jafari et al., 2021; Kjørsvik et al., 2009; Morais et al., 2007; Olsen et al., 2014; Wold et al., 2009). This seems to be due to fish larvae having a limited ability to synthesize PLs de novo, and thus needing to have them supplied from the diet to be able to grow and develop properly. (Coutteau et al., 1997; Sargent et al.,

1999; Tocher et al., 2008)

Fish larvae also have a high dietary demand for amino acids, as they are both a major energy source and fundamental for growth (Conceição et al., 1997; Fyhn, 1989; Rønnestad et al., 1999). Although marine fish eggs contains a large pool of amino acids (AAs), these are depleted during yolk resorption and an exogenous supply is necessary at first feeding (Rønnestad et al., 1999). In the early larval stages, free amino acids (FAAs) seem to be absorbed and assimilated at a higher rate in the larval gut than peptides or protein bound amino acids, likely due to the low proteolytic capacity of fish larvae at first feeding (Rønnestad et al., 2000; Rønnestad et al., 1999; Rønnestad et al., 2003). In addition, the solubility of proteins affects the larval uptake as water-soluble protein seems to be better digested in the larval gut than insoluble proteins (Carvalho et al., 2003).

Currently, the most common start feeding diet in the production of ballan wrasse larvae is rotifers followed by *Artemia* before weaning onto formulated feed (Kousoulaki et al., 2018; Lekva & Grøtan, 2018). This diet however seems to be suboptimal for the sensitive larvae when compared to offering a first feeding diet of natural zooplankton (Øie et al., 2015). Although often preferred over natural zooplankton due to their ease of cultivation, standardized production protocols and cost-effectiveness (Bengtson et al., 1991; Lubzens & Zmora, 2003; Sorgeloos, 1980), both rotifers and *Artemia* are naturally poor in HUFAs, and need to be enriched in order to deliver appropriate amounts of EFAs necessary for larval growth and development (Conceição et al., 2010). In addition, although rotifers and Artemia have a high amount of crude protein out of which approximately 50% is water-soluble (Carvalho et al., 2003; Conceição et al., 2010; Srivastava et al., 2006), the amount of FAAs and amino acid profiles may not be suited to several fish larval species (Conceição et al., 2010).

Natural zooplankton, in particular copepods, as a live prey diet for fish larvae has long been established as a “gold standard” in larval rearing, as this is the natural diet that most fish larvae are adapted to (Hunter, 1981), and copepods are high in all the essential nutrients needed for larval growth and development (van der Meeren et al., 2008). In particular, the amount of EFAs in the PL fraction is beneficial, as well as the the amount of FAAs, which are all much higher than in rotifers and *Artemia* (van der Meeren et al., 2008). Indeed, supplying

copepods as the first feed has previously resulted in both higher growth rates, survival, improved stress tolerance and both digestive system as well as skeletal development compared to feeding rotifers in ballan wrasse (Gagnat et al., 2016; Romundstad, 2015; Øie et al., 2015). Previous concerns regarding the use of copepods have centered around variable availability, cost and the risk of introducing pathogens into the larval rearing facility when using extensively cultivated or harvested copepods (Olsen, 2004). In the recent years, however, intensively farmed copepods have become commercially available, where the closed facility, disinfection protocols and establishment of in-house breeding animals able to produce dormant eggs year-round is said to eliminate most of the aforementioned problems (CFEED AS, 2022; Gagnat & Giebichenstein, 2021). In addition, a completely new live prey solution involving the use of cryopreserved cirripede, i.e. barnacle, nauplii has now also been introduced on the aquaculture market. These species are claimed to be high in EFAs and a more natural prey to marine fish larvae, similarly to copepods (Planktonic AS, 2022; Tokle, 2021). For these new live feeds to be accepted and utilized commercially in rearing of ballan wrasse larvae, however, further testing of their effects on growth, survival and viability of the larvae needs to be performed.

1.5 Histological analyses of nutritional condition as a tool for assessing larval quality

Evaluating the nutritional condition of fish larvae can give valuable insights into the nutritional requirements of the species under investigation, and how well these are being met, as dietary imbalances or suboptimal feeding conditions may affect the physical and physiological condition of developing fish larvae in a number of ways. Examples of this include abnormal development; degeneration of muscle and tissues of the digestive tract; altered activity levels of digestive enzymes; and depressed growth and survival. (Catalán, 2003; Ferron & Leggett, 1994; Gisbert et al., 2008; Lazo et al., 2011). Several methodologies exist to investigate the nutritional condition of fish larvae, including measurement of RNA/DNA ratio, digestive enzyme activity and gene expression analysis, but the most accurate indices is regarded to be histological assessment of target tissues and cells (Catalán, 2003; Ferron & Leggett, 1994; Lazo et al., 2011). The liver and gut are considered key organs to investigate, as they have been shown to respond rapidly and sensitively to starvation and/or a suboptimal diet (Kjørsvik et al., 2011). For instance, food deprivation may result in depletion of nutrient storage in the

liver hepatocytes with small, indistinct central hepatocyte nuclei as well as a reduced hepatonuclear size, biliary dysfunctions in the form of hepatic cholestasis and hypertrophy of bile canaliculi as well sinusoidal dilation (Gisbert et al., 2008) (Table 1.1.). In contrast, the livers of well fed-larvae have a well-organized tissue with large hepatocytes, where the nuclei are large and distinct, and are peripherally displaced by numerous nutrient-containing vacuoles (Gisbert et al., 2008) (Table 1.1.). In the larval gut, suboptimal diets or starvation may lead to reduced height of the gut epithelium, fewer and shorter muscosal folds, or villi, as well as degeneration of microvilli (Gisbert et al., 2008) (Table 1.1.), while diets containing appropriate amounts of essential nutrients may lead to a deeply convoluted and folded gut mucosa, with a thick intestinal wall and a dense brush border toward the gut lumen (Gisbert et al., 2008) (Table 1.1.). As fish larvae are completely dependent on a well-functioning digestive system for further growth, it is of a high importance to identify any signs of pathological alterations or degenerations in digestive organs caused by dietary imbalances when attempting to find suitable diets. Thus, assessing nutritional condition by use of histological indices in the gut and liver may be useful tools in the establishment of improved start-feeding protocols in larval rearing.

Table 1.1 Histological cellular criteria used for assessment of nutritional condition of gut and liver in fish larvae. Obtained and modified from Gisbert et al. (2008) as well as Di Pane et al (2020) and references therein.

Tissue	Condition		
	Degraded	Average	Healthy
Liver	Nearly all nuclei pycnotic and dark with clumped chromatin; cytoplasm lacks texture; intracellular vacuoles absent; cells small and indistinct, may be disjunctive.	At least 50% of cell nuclei with dark granules and situated medially; nearly 50% of cytoplasm granular; intracellular vacuoles reduced or absent; boundaries of most hepatocytes visible.	Nuclei distinct and often displaced laterally; cytoplasm lightly stained with abundant intracellular vacuoles; boundaries of hepatocytes prominent and liver is well-structured.
Gut	Mucosal cell height reduced by >50% in height; some loss of	Mucosal cells reduced by 25 to 50% in height; some	Mucosa deeply convoluted and mosaic; mucosal cells compact, pronounced in

	striations in bordering microvilli; cells may be disjunctive	loss of striations in bordering microvilli	height, with distinct nuclei; many microvilli

1.6 Aims and hypotheses of the study

The present study was motivated by the intensification of ballan wrasse aquaculture, and the following need for knowledge on the nutritional requirements during the larval stage, as this is a bottleneck for increased production. Histological assessment of gut and liver tissue is considered a highly accurate method to investigate nutritional condition of developing fish larvae. The findings from the present study may help elucidate how different diets affect the gut and liver tissue in developing ballan wrasse larvae, and if this can be related to growth. The study aim was therefore to evaluate the nutritional condition of ballan wrasse larvae fed different live feed organisms by assessing growth and histological biomarkers in the gut and liver.

To meet the aim, a start-feeding experiment was conducted, where four different live feed diets were offered ballan wrasse larvae, followed by weaning all groups onto the same formulated feed. Earlier research on this species have proven that the traditional diet of rotifers and *Artemia* are a suboptimal first feed, while copepods have led to improved growth, survival and development of the liver and gut in the larvae (Gagnat et al., 2016; Romundstad, 2015; Øie et al., 2015). In the aquaculture industry, natural zooplankton as alternatives to rotifers and *Artemia* have only recently become available on the commercial market, and testing of these diets is necessary to evaluate their suitability for use in larval rearing. Thus, in the present study, one control group of ballan wrasse larvae was fed the industry standard diet of rotifers followed by *Artemia,* while the other three groups were fed either copepods or experimental small cirripeds as the first live feed followed by either large cirripeds or *Artemia*.

To evaluate the dietary effects on growth and survival, larval dry weight (DW) and standard length (SL) were measured, and the percentage of daily weight increase (DWI) and mortality

coefficients were calculated. To assess the effect of diet on gut and liver histology, visual examination of morphology of gut and liver tissue in histological sections were performed. In addition, stereological parameters in the gut and liver were chosen for assessment of nutritional condition. These were a) villus height, b) epithelial height and c) microvillus height for the gut, as well as d) hepatocyte cell area size, e) hepatocyte nucleus area size and f) surface area fractions of vacuoles in the liver. The parameters were measured on digitalized transverse sections of the foregut and liver.

The hypotheses were formulated as follows:

H_1 Gut and liver histology reflect nutritional condition in larval ballan wrasse (*L. bergylta*)
H_2 The tested live feeds differ in their quality as feed for larval ballan wrasse and result in differences in nutritional condition
H_3 Gut and liver histology can be related to growth.

2. Materials and methods

2.1 Start feeding experiment

To evaluate and compare effects of different live feed diets on growth, survival and nutritional status of liver and gut in ballan wrasse larvae, a start-feeding experiment was conducted from onset of exogenous feeding until weaning onto formulated diet was completed. The start-feeding experiment was carried out in the CodTech fish larval rearing laboratory at NTNU Centre for Fisheries and Aquaculture (SeaLab) over a time period of 48 days, from late February to early April 2020.

2.1.2 Larval rearing

Newly hatched ballan wrasse larvae were acquired from MOWI Stord and flown to Trondheim via airplane in plastic bags supplied with O_2-supersaturated 34 ppt seawater holding a temperature of 11.9 °C. A total of 10 plastic bags containing live larvae were shipped, where each bag contained approximately 73 600 larvae, leading to a number of 736 000 larvae in

total. Larvae from two successive hatching batches were shipped, where the youngest had hatched the night before (0 days post hatching, dph) and the oldest had hatched two nights before transportation (1 day post hatching, dph), respectively. The supplied larvae came from eggs spawned by wild-caught, photoperiod-manipulated broodstock, and were incubated at 11°C prior to hatching. Of the 10 plastic bags containing approximately equal amounts of larvae, 4 contained the youngest larvae (0 dph) and the remaining 6 contained the oldest (1 dph).

Upon arrival to the experimental facility, oxygen concentration, temperature, pH, CO2-concentration and salinity were measured (ProDSS Multiparameter Digital Water Quality Meter, YSI, USA) in the plastic bags before gently transferring the larvae to tanks filled with 12 °C filtered seawater. Dead larvae were allowed to settle to the tank bottom for 20 minutes, before being siphoned out and counted. Survival after transportation were estimated to be 36 and 40% for the 0 and 1 dph old larvae, respectively. Larval density was estimated in the holding tanks by taking out 5 subsamples of 140-160 mL each and counting the number of larvae within each volume. This number was then multiplied by the entire water volume in the tank, giving the total density of larvae. Larvae were then transferred to cylindrical 200L start-feeding tanks (Kunststoff-Spranger GMBH, Germany), equipped with a center outlet pipe fitted with a mesh sieve to prevent escape of larvae and flush out old feed particles, organic matter and debris. An air hose secured to the bottom of the center pipe provided light aeration to ventilate and ensure water circulation in the tank, as well as suspension of live feed particles and larvae. Automatic cleaning devices were used in the tank for consolidation of organic matter, faeces and dead larvae left on the tank bottom and walls which could then easily be removed with a siphon. A schematic drawing of the larval rearing tank is given in Figure 2.1. Seawater used in the experiment had been pumped from 40 meters deep in Trondheimsfjorden, filtered through a sand filter and a 1 µm bag filter for particle removal, continuously degassed to avoid supersaturation of N_2,and heated before entering the larval rearing tanks. LED light tubes (FlexTube SC 4000 K of 75 W, Vanpee AS, Norway) were used to illuminate the tanks. As the density between hatching dates differed, approximately 10200 and 9500 larvae from the hatching batches at 1 and 0 dph, respectively, were added to each larval rearing tank, giving a density of approximately 19700 larvae $tank^{-1}$, or 98.5 larvae L^{-1}.

After transfer to the start-feeding tanks, an acclimation period of 3 days followed. During this period, the experimental facility was kept in complete darkness and tanks containing larvae were not disturbed except to measure water quality parameters and to clean the tanks for debris and organic matter, and removal of dead larvae for counting and survival estimation.

Start feeding commenced at day 4 of the experiment. From this day on, a photoperiod of 24:0 was used, with the light intensity being increased from 100-300 lumen over a period of 10 days and kept constant at 300 lumen throughout the remainder of the experiment. The water temperature was gradually increased from 12 to 16 °C from day 1-23 of the experiment and then kept constant. Likewise, the water exchange rate was increased from 2-8 tank volumes per day over the first 33 days of the experiment then kept constant. To accommodate for increasing sizes of live prey as well as particle debris from formulated diets, sieves with increasing mesh size (64-700 µm) on the center outlet pipe were used.

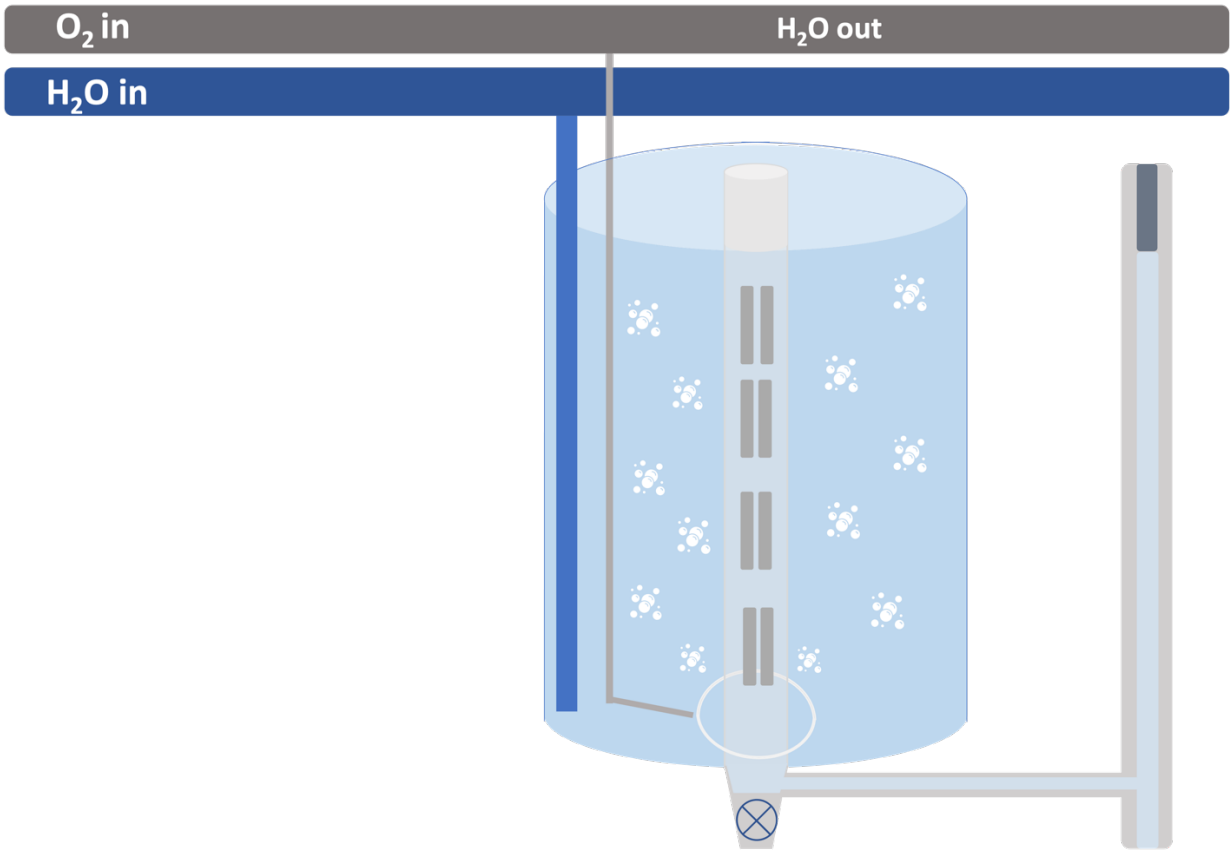
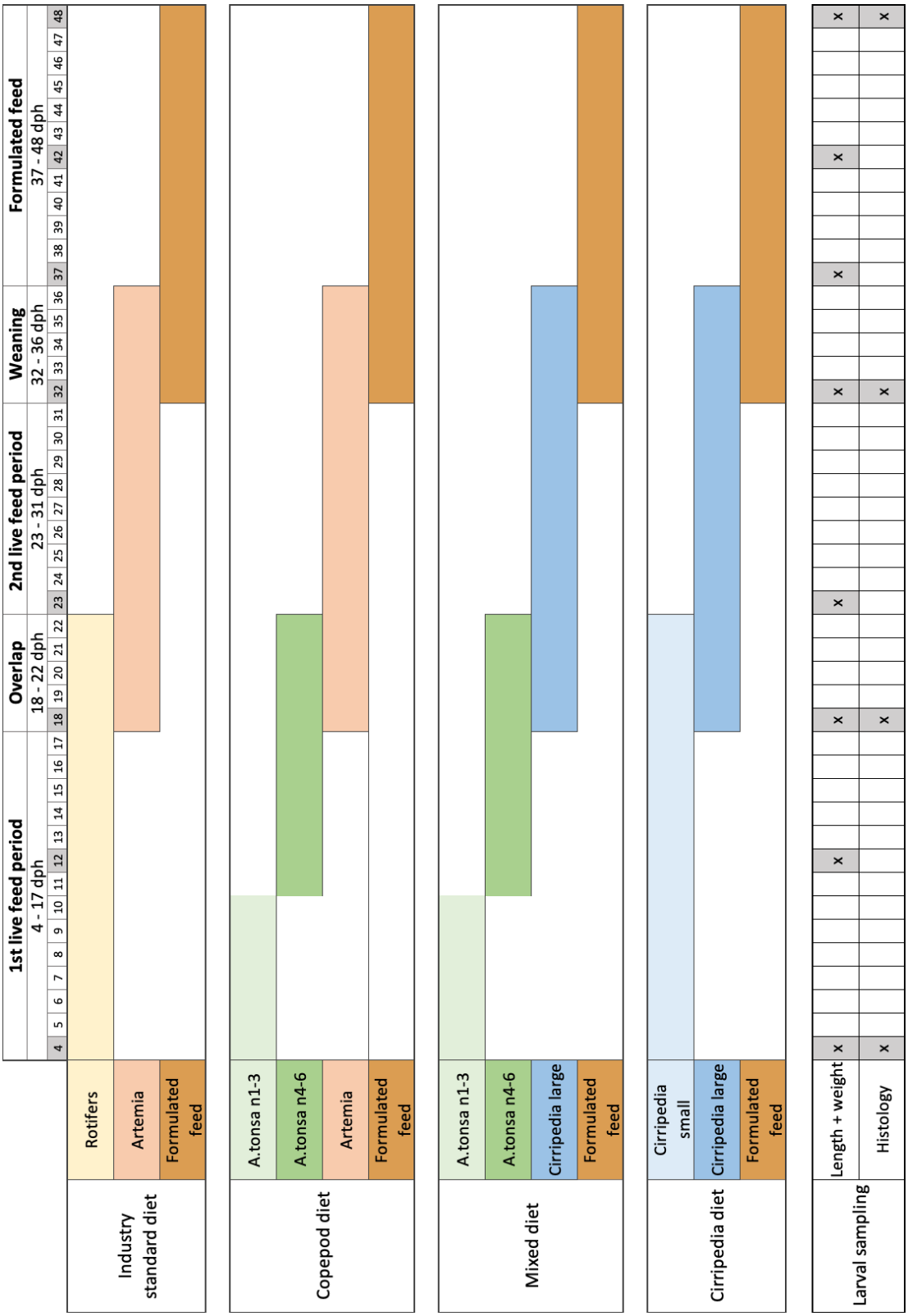


Figure 2.1: Schematic drawing of larval rearing tank

2.1.2 Feeding regimes

A total of four different experimental diets were compared in the current study. Larvae from each experimental diet were first successively fed two different types of live feed organisms, corresponding to the increasing size of the larva's mouth, before weaning larvae from all diets onto the same formulated feed. This amounted to three different feeding periods, where the larvae were given smaller live prey at the start of the experiment, from 4-18 dph (1st live feed period), larger live prey at the middle of the experiment at 18-32 dph (2nd live feed period), and formulated feed toward the end of the experiment at 32-48 dph (weaning). Before complete transition to another feed type, the new feed was always mixed with the old in an overlap period to allow the larvae to acclimate to the new feed. An overview of the live feeds offered and corresponding time periods is given in table 2.1.

Table 2.1: Overview of start-feeding experimental period in feeding periods, days post hatching and corresponding feeds offered within each dietary group for each feeding period. In addition, sampling dates for growth parameters as well as histology is shown.



The four experimental diets offered were:

1. The Rotifer/*Artemia* diet*,* where rotifers (*Brachionus plicatilis*) were fed during the first live feed period and *Artemia* during the second. Enriched rotifers were fed to the larval tanks treatment in increasing densities of 3-12 individuals ml^{-1} day^{-1} divided into three doses day^{-1}, while enriched *Artemia* nauplii were fed to the larval tanks in densities of 3-9 individuals ml^{-1} day^{-1} divided into three doses day^{-1}.

2. The Copepod/*Artemia* diet, where nauplii (n1-n6) of the calanoid copepod species *Acartia tonsa* were fed to the larvae during the first live feed period and *Artemia* during the second. Copepod nauplii were fed to the larval tanks assigned the Copepod/*Artemia* and Copepod/Cirripedia treatments in densities increasing from 10-20 individuals ml^{-1} day^{-1} divided into three doses day^{-1}, while enriched *Artemia* nauplii were fed to the larval tanks in densities of 3-9 individuals ml^{-1} day^{-1} divided into three doses day^{-1}.

3. The Copepod/Cirriped diet, where where nauplii (n1-n6) of the calanoid copepod species *Acartia tonsa* were fed to the larvae during the first live feed period and cirripeds of the species *Semibalanus balanoides* during the second. Copepod nauplii were fed to the larval tanks assigned the Copepod/*Artemia* and Copepod/Cirripedia treatments in densities increasing from 10-20 individuals ml^{-1} day^{-1} divided into three doses day^{-1}, while large cirripeds were fed in increasing concentrations from 6-12 individuals ml^{-1} day^{1}.

4. The experimental cirriped diet, where experimental small cirripeds (a combination of approximately 50 % *Balanus crenatus* naupliar stages I-II and 50 % *Semibalanus balanoides* naupliar stages I-II) were fed to the larvae during the first live feed period and large cirripeds (*Semibalanus balanoides*, naupliar stages I-II) during the second. Small experimental cirripeds were fed in concentrations of 20 individuals ml^{-1} day^{1} while large cirripeds were fed in increasing concentrations from 6-12 individuals ml^{-1} day^{1}.

For all diets, green water technique involving use of the microalgal species *Rhodomonas baltica* (Cryptophyceae) and Nannochloropsis sp. (Eustigmatophyceae), was applied during the live feed period in order to improve light contrast and visibility of live prey to the fish larvae as well as to improve the microbial conditions in the cultivating tanks. Microalgae was added to the larval tanks in three doses day^{-1} corresponding to the addition of live feed; *R.baltica* was added to the tanks in a concentration of approximately 30 000 cells ml^{-1} day^{-1} (1

mg C L^{-1} day^{-1}), while Nannochloropsis *FrozenPaste* was added to tanks to reach a concentration of approximately 6 mg C L^{-1} day^{-1}.

Toward the end of the second live feed period, at 32 dph, formulated feed (Gemma Micro 150 (Skretting, Norway), Gemma Micro 300 (Skretting, Norway) and AgloNorse Extra 2 (Tromsø Fiskeindustri AS, Norway) was introduced in all tanks. The dry feed was supplied *ad libitum*. From 32 to 37 dph, a mixture of 50% Gemma Micro 150 and 50% AgloNorse Extra 2 was fed to the larvae. From 37 to 42 dph, 25 % Gemma Micro 150, 25 % Gemma Micro 300 and 50 % AgloNorse Extra 2 (Tromsø Fiskeindustri AS, Norway) was administered. From 42 dph to 48 dph, 50% Gemma Micro 300 and 50% AgloNorse Extra 2 was given.

2.1.3 Live feed cultivation

Green water

While *Nannochloropsis* was added to the larval cultivating tanks in the form of commercial frozen paste (Nannochloropsis *FrozenPaste*, BlueBiotech Gmbh, Germany), *R.baltica* was cultivated in-house according to protocols at SINTEF SeaLab (Appendix 3).

Copepods

Calanoid copepods of the species *Acartia tonsa* were used as the first live feed organism in the Copepod/*Artemia* and Copepod/Cirripedia treatments, respectively. Naupliar stages from n1-n6 were fed according to the fish larval age (see Figure 2.2). The copepods were supplied by C-FEED AS (Vanvikan, Norway). Copepod eggs were received in bottles containing information about the amount of hatchable eggs in each bottle, and the proportion of eggs taken out from each bottle was dimensioned accordingly to reach the desired naupliar densities. *A. tonsa* eggs were hatched onsite in 100 L conical tanks containing seawater with a temperature of 22 °C. After addition of the eggs, aeration was applied to keep the eggs in suspension, and the eggs were kept in the tank for 24 hours to allow for hatching. After this time period, 10 L of *R. baltica* (approximately 1,5 cells ml^{-1}) was fed to the newly hatched nauplii, aeration was turned off and debris and empty egg shells were allowed to settle to the tank bottom for approximately 15 minutes. After this, the tank was drained of the bottom 5 liters of culture containing the settled wastes, and the remaining copepods were either

transferred to a copepod reservoir or fed directly to the fish larvae. Copepods of naupliar stages n4-n6 were supplied directly from C-FEED AS.

Rotifers

Rotifers from the species complex *Brachionus plicatilis* were cultivated at the SINTEF Plankton Centre in 100 L conical tanks containing heavily aerated brackish water with a temperature of 20-22 °C. The rotifers were cultured semi-continuously by removing a volume of the culture every day and providing constant feed via peristaltic pumps. This feed consisted of a 3:1 mixture of fresh baker's yeast (REMA 1000, Norway) and Rotifer Diet (Reed Mariculture, USA). Rotifers were enriched in a separate tank with the dry formula enrichment diet LARVIVA Multigain (BioMar), using 0.15 g Multigain million^{-1} rotifers at 24 °C for two hours. After enrichment, the rotifers were rinsed and either fed directly to the fish larvae or chilled stored at 4 °C for a maximum of 16 hours for use at later feedings.

Artemia

Metal ion coated *Artemia* cysts of the species *Artemia salina* (EG SepArt 225, INVE, Belgium) were hatched to reach a density of 500 individuals mL^{-1} in 60 L conical tanks containing heavily aerated seawater that was heated to approximately 25-28 °C over a period of 24 hours. Subsequently, the cyst shells were separated from the newly hatched *Artemia* nauplii by use of a magnetic separator(EG SepArt 225, INVE, Belgium), a specialized tube containing a row of magnets that gently filtered the metal coated cysts from the water containing *Artemia* nauplii. Rinsed nauplii were then transferred to an enrichment tank, also containing approximately 60 L of 25-28 °C heavily aerated water. The density of nauplii in the tank was counted, and 1 g LARVIVA Multigain (BioMar) million^{-1} *Artemia* nauplii was added as an enrichment feed for the nauplii. After 18 hours, another dose was added and after another 6 hours the *Artemia* nauplii were rinsed, concentrated and either fed directly to the fish larvae or chilled and stored at 4 °C for a maximum of 16 hours for use at later feedings.

Cirripeds

Two different experimental cirriped diets were fed to the ballan wrasse larvae assigned the Cirriped/Cirriped dietary group, and consisted of a combination of 50 % *Balanus crenatus* (n1-n2) and 50 % *Semibalanus balanoides* (n1-n2) as the first live feed offered (small experimental

cirripeds) and solely *Semibalanus balanoides* (n1-n2) as the second live feed offered (large experimental cirripeds). Both experimental diets were prepared in the following manner: Frozen pellets of cryopreserved cirripeds were thawed and rinsed in sea water before being allowed to revitalize in a tank containing aerated sea water with a temperature of 4 °C for approximately 6 hours. The invigorated cirripeds were then transferred to small conical reservoirs holding volumes of 5 L adjacent to the larval rearing tanks they were to be fed to, and were pumped into the fish tanks by use of peristaltic pumps (KRONOS 50 Peristaltic dosing pump, SEKO S.p.A.) in a time period of 4 hours on: 4 hours pause. The reservoirs were refilled with cirripeds 3 times day^{-1}.

2.2 Larval sampling and fixation

Sampling of ballan wrasse larvae for standard length and dry weight measurements was done by Arne Malzahn (SINTEF Ocean) before commencement of start feeding, in the middle of each feeding period and before each new feed was introduced as well as on the last day of the experiments. All samples were taken in the mornings before the first feeding of the day. Ballan wrasse larvae from each treatment and tank replicate were sampled on 4, 12, 18, 23, 32, 37, 42 and 48 dph (n=10 $sample^{-1}$). Larvae were sampled randomly from the fish tank and immediately euthanized in an overdose of 500 mg L^{-1} MS-222 (Finquel®, Agent Chemical Laboratories Inc., USA) before being frozen at -20 °C for preservation until further analysis.

Larvae used for histological analyses were similarly sampled from each tank replicate in all treatments (n=5 $sample^{-1}$, giving n=15 dietary $group^{-1}$), immediately euthanized in an overdose of 500 mg L^{-1} MS-222 (Finquel®, Agent Chemical Laboratories Inc., USA) before being fixated in 4 % paraformaldehyde (PFA) in phosphate buffered saline (pH 7.4, Apotekproduksjon AS; Norway), and stored at 4 °C before further processing. Larval samples for histological analyses were taken on the same days and time points as the growth parameter samples, but only samples from ages 4; 18; 32; and 48 dph were selected for analysis due to COVID limitations on laboratory work and following time constraints.

2.3 Growth, survival and histological analysis

2.3.1 Growth measurements

Frozen sampled larvae were thawed and photographed using a calibrated stereomicroscope (SZX10 Research Stereomicroscope System, Olympus, Japan), and subsequently measured for standard length using the image software Infinity Analyze (Teledyne Lumenera, Ottawa, Canada). Standard length was measured on the larvae from the tip of the snout to the end of the notochord in younger larvae or the caudal peduncle in older larvae.
Larvae that had been measured for length were then placed in pre-weighed tin capsules and dried at 60 °C for 48 hours before dry weight was measured using a microbalance. (UMX2 Ultra-microbalance, Mettler-Toledo)

The dry weight data was used to calculate the specific growth rate (SGR) for each dietary group by use of equation 2.1:

$$SGR = \frac{lnW_2 - lnW_1}{t_2 - t_1}$$

Equation 2.1

The values for SGR were further used to calculate the percentage of daily weight increase (DWI) by use of equation 2.2:

$$DWI = (e^{SGR} - 1) \times 100\%$$

Equation 2.2

2.3.2 Mortality coefficient estimation

The mortality of ballan wrasse larvae assigned different dietary groups in the experiment was estimated following the approach of Kotani et al. (2011), which calculates the mortality coefficient for a given experiment by assuming a constant mortality over time but allowing for correction for sampled larvae. The equation for the mortality coefficient is given as follows:

$$N_{\mathrm{t}} = e^{-mt}\left(N_0 - \sum N_{\mathrm{Sn}} e^{m d\mathrm{n}}\right)$$

Equation 2.3

Where t is the larval rearing period (days), N_0 the initial number of larvae, N_t the number of larvae at time t, m the mortality coefficient, N_{Sn} the number of larvae sampled at the nth sampling and dn the larval rearing period until removal of the nth sample.
From this equation the mortality coefficient for each dietary group was acquired by using the initial and final stocking densities in the larval rearing tanks, total number of days in the experiment, and number of sampled larvae as inputs.

2.3.3 Histological analysis

Ballan wrasse larvae (n=5) from each treatment and sampling age were randomly sampled for histological analysis. The fixated larvae were first rinsed in PBS, then photographed for standard length measurements (Figure 2.3) using a Leica MZ75 stereomicroscope equipped with a Zeiss Axiocam camera (Leica MZ75, Leica Microsystems, Germany; Zeiss Axiocam ERc 5s, Zeiss Inc., Germany) and morphological observations (magnifications: 0.63x for larger larvae and 1.00 for smaller larvae) before being embedded in Technovit® 7100 (Kulzer, Germany, Appendix 1). This particular embedment system was selected due to the high morphological resolutions achievable. After completed hardening, semi-thin transverse sections (2 µm) were cut from the blocks containing fish larvae using an ultramicrotome (Reichert Ultracut S, Leica Microsystems, Germany). The sections were taken out at the same region in each larva, and were selected on the basis of where both a large volume of liver and gut tissue could be observed and analyzed (Figure 2.3, complete list of selection criteria and

reference points for extracted sections at different ages given in table 2.1). For larvae of ages 32 and 48 dph this could be achieved by extracting sections from the same area, while in the youngest larvae of age 4 and 18 dph, sections were taken from two separate regions as the liver and gut tissue did not completely overlap. Sections were stained with Toluidine Blue (TB) 0.05% after the standard procedure at NTNU SeaLab (Appendix 2) and mounted (Neo-Mount™, Merck, Germany) before being scanned using a digital slide scanner (NanoZoomer SQ, Hamamatsu, Japan). Sections were scanned at a magnification of 40x and resolution of 0.23 µm pixel^{-1} in three layers of 0.5 µm, and visually inspected using an image viewing software (NDP.view2, Hamamatsu, Japan). Sections for analysis were imported to the image analysis software QuPath (v 0.2.3, Bankhead, P. et al. 2017).

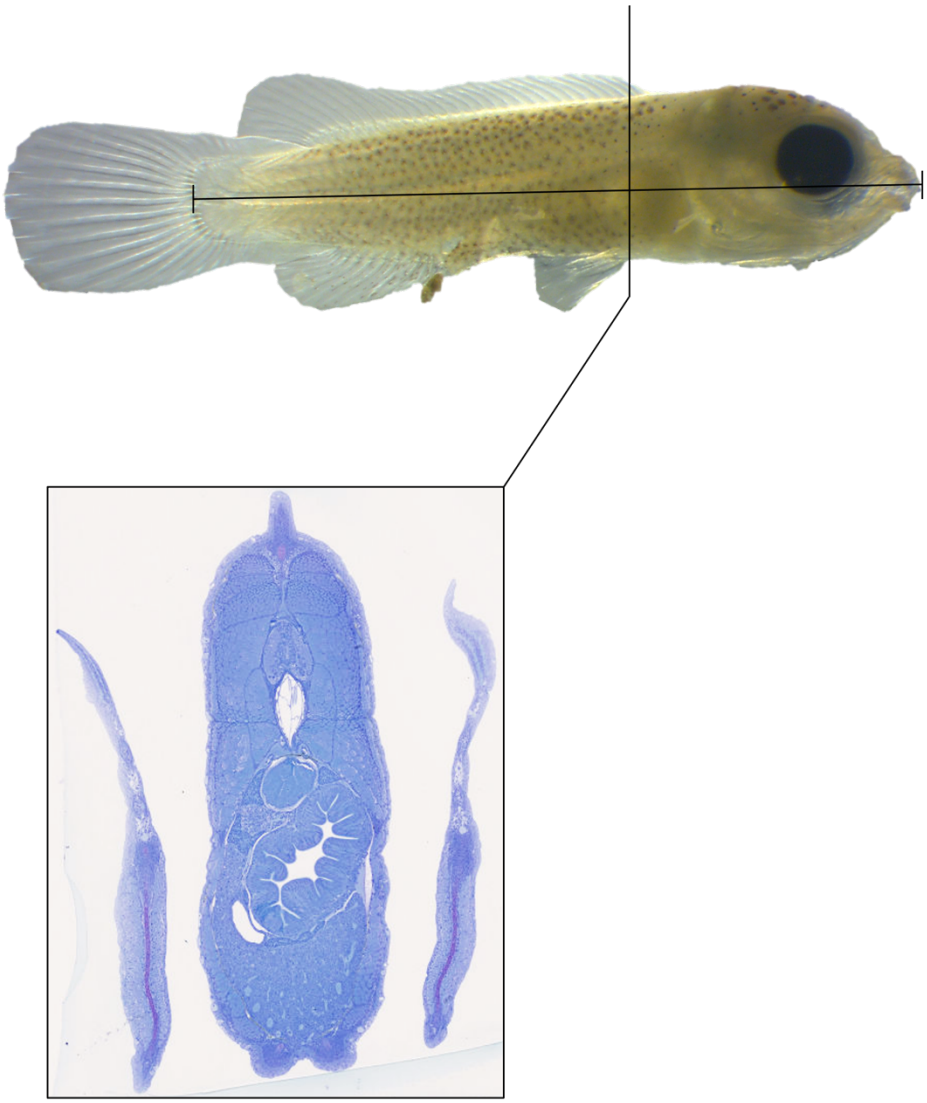


Figure 2.3: Section placement in ballan wrasse larvae. Example larva is from the Copepod/Artemia dietary group at 32 dph. Horizontal line shows the measurement of SL from snout to caudal peduncle. Vertical line shows the placement of transverse histological section for analysis of gut and liver tissue.

Table 2.1: Reference points for selection of histological sections for gut and liver analysis

Tissue	Reference point
Liver	A large proportion of the abdominal cavity contains liver tissue. In larvae at 4 and 18, the posterior part of the oesophagus is still present. At 32 and 48 dph, liver sections are analyzed right after the transition to foregut. Primary islet of Langerhans in the pancreas and gallbladder is visible in the two oldest larval age groups. For larvae at 4 dph, all sections containing liver tissue are selected for analysis to ensure enough measuring points. For larvae at 18 dph, a minimum of 3 sections are selected. For larvae at 32 and 48 dph, 1-2 sections are selected.
Gut	Striated muscle surrounding the oesophagus is no longer visible, indicating the completed transition to foregut. For the two youngest larval age groups, no liver tissue is visible. For larvae at 32 and 48 dph, the gut tissue occupies at least 25% of the abdominal cavity, with a large proportion of liver tissue concentrated below the gut. Primary islet of Langerhans in the pancreas and gallbladder is visible in the two oldest larval age groups. One gut section was selected per larva.

Liver analyses

Mean averages for hepatocyte cell area size, hepatocyte nucleus size, and surface area fractions of liver components were estimated from histological sections (Fig. 2.4.).

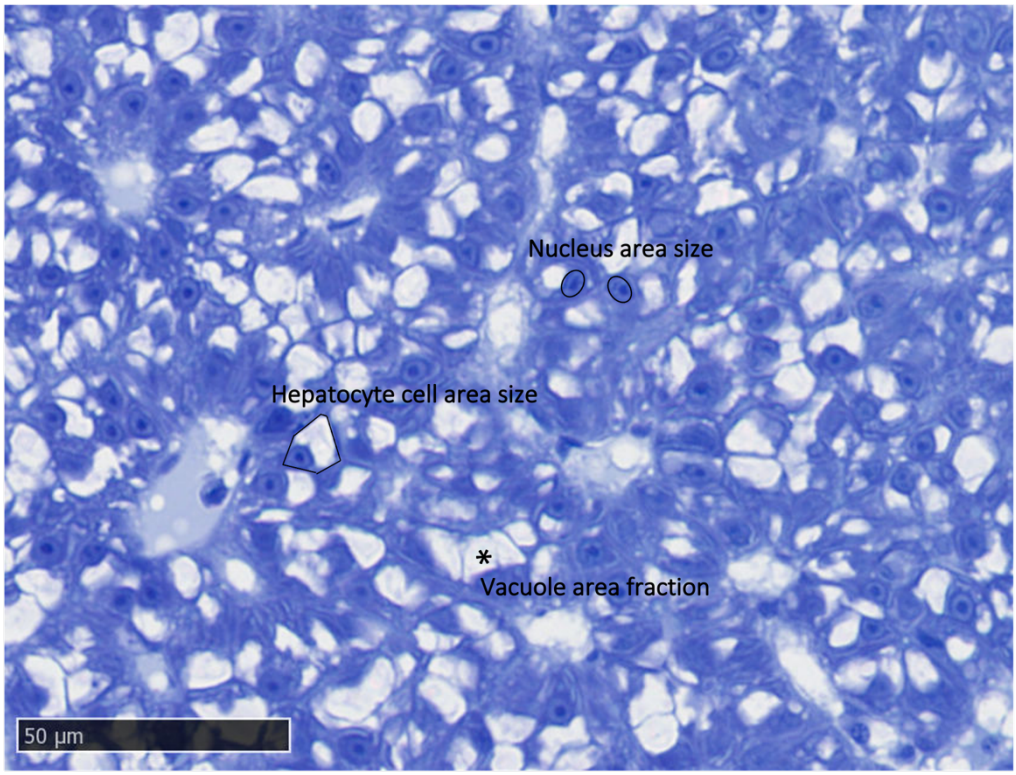


Figure 2.4. Analysis points of liver parameters in ballan wrasse larvae.

A liver surface area large enough to include a minimum of 200 hepatocyte nuclei per larva was analyzed (after Wold et al. 2009), thus encompassing a different number of sections for each age as the liver tissue increased in volume as the fish larvae grew. In larvae aged 48 dph, one section was usually enough to get an adequate liver area for analysis, while in the youngest larvae of age 4 dph the entire liver tissue was analyzed. An overview of the mean number of sections analyzed per larva and age is given in table 2.1, along with the reference points used to identify similar anatomical regions for analysis.

When a liver area consisting of exclusively liver tissue within a section had been identified for analysis, this region was first marked either using the rectangle or brush tool to get a measure of the total liver area analyzed (A_{liver}). Within this region, the polygon tool was then used to draw around individual nuclei, following the border of the nuclear envelope. Nuclei touching the lower and left edges of the region, missing a distinct nucleoli or nuclear envelope were not analyzed.

The total number of nuclei ($N_{nuclei)}$ analyzed was used along with the total liver area to estimate the hepatocyte cell area size by equation 2.4:

$$Hepatocyte\ cell\ area\ size = \frac{A_{liver}}{N_{nuclei}}$$

Equation 2.4

While the mean nuclear area size was calculated by equation 2.5:

$$Hepatocyte\ nucleus\ area\ size = \frac{A_{nuclei}}{N_{nuclei}}$$

Equation 2.5

Where A_{nuclei} denotes the total nuclear area.

The analyzed liver region was further imported into ImageJ for estimation of the surface area fraction of glycogen vacuoles in the liver tissue. A point grid was applied using the Grid tool, wherein the area per point was set to a measurement ensuring at least 200 points to hit the liver tissue (after Wold et al. 2009). Vacuoles were then marked using the Multi-point tool, and the number of vacuoles marked was then multiplied with the area per point value to get a measurement of the total vacuole area within the liver tissue ($A_{vacuoles}$). In the point grid

each point was represented by a cross, and vacuoles were only marked if they touched the upper and right hand of this cross.

To estimate the surface area fractions of liver components, A_{nuclei} and $A_{vacuoles}$ were both divided by A_{liver} to get the nuclear area fraction and vacuole area fraction, respectively. In addition, the area fraction of other liver components such as bile canaculi, sinusoids and cytoplasma were estimated by subtracting the nuclear area fraction and the vacuole area fraction from 1. This was done as the liver consisted of a relatively high fraction of "other" liver components, whereas the nuclear and vacuole area fraction made up a relatively small area in the liver tissue. To identify content of liver vacuoles, PAS-staining was performed on select larvae displaying either a high or low amount of vacuoles at 18 and 48 dph, as this stain may indicate presence of polysaccharides.

Gut analyses

Mean gut villus height, epithelial height and microvillus height were estimated in by applying the QuPath line tool. The villus height was measured on distinctly formed villi, up to 5 $larva^{-1}$. The line was drawn through the middle of the villi, starting at the lamina propria and ending at the apical border between the microvilli and intestinal lumen (Fig.2.5.). At 48 dph, there a high amount of shrinkage was registered in the gut tissue, thus villi bordering or containing regions of shrinkage were not measured to avoid inaccuracy and confounding.
The gut epithelial height was measured from the basal lamina of the enterocytes to the border between the enterocyte microvilli and intestinal lumen (Fig.2.5.). The line was drawn parallel to the enterocyte walls and through the enterocyte nuclei. Gut microvilli were measured from the apical membrane to the border toward the intestinal lumen (Fig.2.5.).
For epithelial height and microvillus height, 5 measurements were performed both dorsally and ventrally to determine the effect of location factor by pairwise comparisons. No effects of location were however observed in either epithelial or microvillus height, thus the data was pooled for statistical analysis.

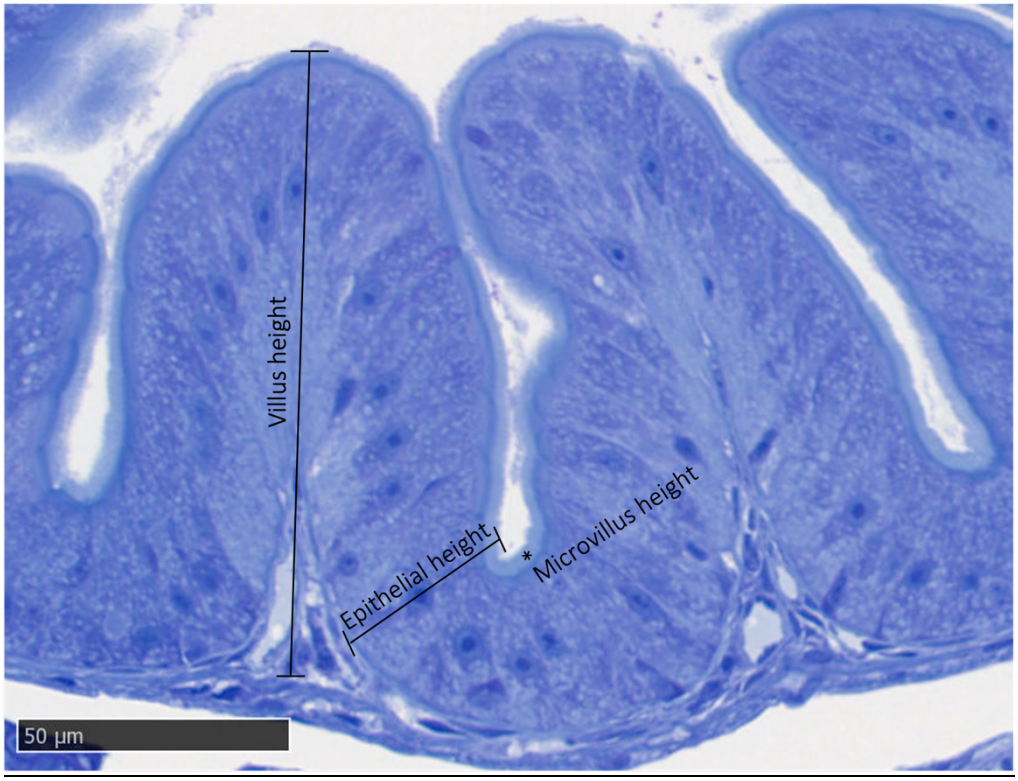


Figure 2.5. Analysis points of gut parameters in ballan wrasse larvae. Villus height was measured from lamina propria to apical end the enterocytes bordering the intestinal lumen. Epithelial height was measured from the basal lamina to the apical end bordering the intestinal lumen. Microvilli were measured from the apical membrane of the enterocyte to the border toward the lumen.

2.4 Statistical methods and data visualization

All statistical testing and data visualization was performed in R: A language and environment for statistical computing (version 3.6.3, R Core Team (2020). R Foundation for Statistical Computing, Vienna, Austria) equipped with the software RStudio (Version 1.2.5042, RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA).

All mean values for measurements with corresponding standard errors, specific growth rates and percentage daily weight increase as well as survival was calculated in Microsoft Excel (version 16.54). Tables were also made in Microsoft Excel.
A significance level of α = 0.05 used was used for difference in group means and correlations.

Before testing for significant differences, data sets were first tested for normal distribution using Shapiro-Wilk's test for normality, and then for homogeneity of variance between groups

using Levene's test. In addition, the data was visually inspected for normality using a Q-Q plot. Percentage/proportion data was arcsine transformed when no negative values were present before further statistical testing.

In the case of normally distributed data with homoscedastic variance, a One-Way ANOVA was conducted followed by the Post hoc test Tukey Honest Significant Difference when significant differences were detected by the ANOVA. When data was normally distributed but variance was heteroscedastic, Welch's ANOVA was applied followed by post hoc Dunnett's T3 test. In the case of non-normal data, Kruskal-Wallis followed by Dunn's test was used.

When comparing only two group means, e.g. in the case of testing for significance of location factor in epithelial and microvillus height measurements, a paired Student's t-test was used when the data was normally distributed while the non-parametric paired Wilcoxon's rank sum test was applied in the case of non-normal data. The same tests were also used to test for significant differences within dietary groups on different age levels.

Correlations were tested by use of Pearson's product-moment correlation. For significant correlations, linear regression was performed. Furthermore, when significant correlations were present, an ANCOVA was performed to identify any differences in slopes between treatments. For correlation plots, only lines between significant correlations were included.

As the effect of diet could not be assumed at 4 dph, it was decided to pool data for this sampling date for statistical analysis.

3. Results

3.1 Growth and survival

General observations

Upon arrival at the experimental facility, the newly hatched ballan wrasse larvae had a straight notochord and large head relative to the body size. The larval body was narrow and almost completely transparent, but with some pigmentation visible along the middle of the notochord. A yolk sac was attached ventrally to the abdomen of the larvae. The larvae displayed small bursts of swimming movements, but were mostly floating in the water column of the tanks.

At 4 dph, the mouth and anus of the larvae were open. The bodies of the larvae were still largely transparent, but slightly more pigmented along the head and notochord. The eyes had also gained more pigments and were large and black. The head of the larvae pointed straight ahead. The notochord was straight, and the entire larval body apart from the frontal part of the head, from otic capsule dorsally to the snout anteriorly and further down around the jaws ventrally, was surrounded by an undifferentiated fin fold. In addition, a vent in the fin fold was present by the anus. The yolk sac was distinguishable ventrally to the larval gut and was filled with yellow-tinted yolk. The gut was visible as a straight tube with beginning indications of a wider diameter in the anterior gut and narrowing to an intestinal valve toward the posterior gut (Fig. 3.1. A).

At 18 dph, the fin fold appeared slightly more differentiated into median and preanal fin fold, which now surrounded the larval body apart from the entire head region as well as the oesophagus and posterior part of the alimentary canal. The notochord was straight, and appeared to be surrounded by a thicker layer of muscle both dorsally and ventrally from the head to the caudal end of the notochord. The head, especially eyes and jaws, appeared more developed and large relative to the body. Both the head and larval body was covered in an increased amount of pigmentation, but no pigments were visible on the fin fold or the caudal part of the notochord. The intestine was visible in the now semi-transparent larvae, where a

thicker and wider gut tissue was apparent, especially in the anterior end. No yolk reserves were visible as the yolk sac was depleted by this stage (Fig. 3.1. B).

By 32 dph, most of the fin fold had differentiated into fins. Dorsal, pectoral, pelvic, anal and caudal fins were all distinguishable, though some undifferentiated fin fold tissue connected the dorsal, caudal and anal fins. The head was large and more developed, however the body volume had also increased so that the head was no longer as large in relation. The larval body was now almost completely pigmented, except for all fins but the anal. Operculae also appeared to be developing at this stage, and flexion had occurred in most larvae with the urostyle seen pointing upward. In addition, the intestine no longer appeared as long compared to the body length as on the two previous sampling dates, though a thickening and widening of the tissue was again apparent. Although not as readily visible in the less transparent larvae, indications of a looped intestine could be seen (Fig. 3.1. C).

At 48 dph, the now post-flexion larvae had a body and head well developed compared to earlier larval stages. The larval body was entirely pigmented except for the fins, and the fish appeared bluish yellow in color with brown pigment spots. The fins had completely differentiated, and operculae were fully formed. The intestine could be seen as a coiled tissue beneath the skin, and appeared large (Fig. 3.1 D).

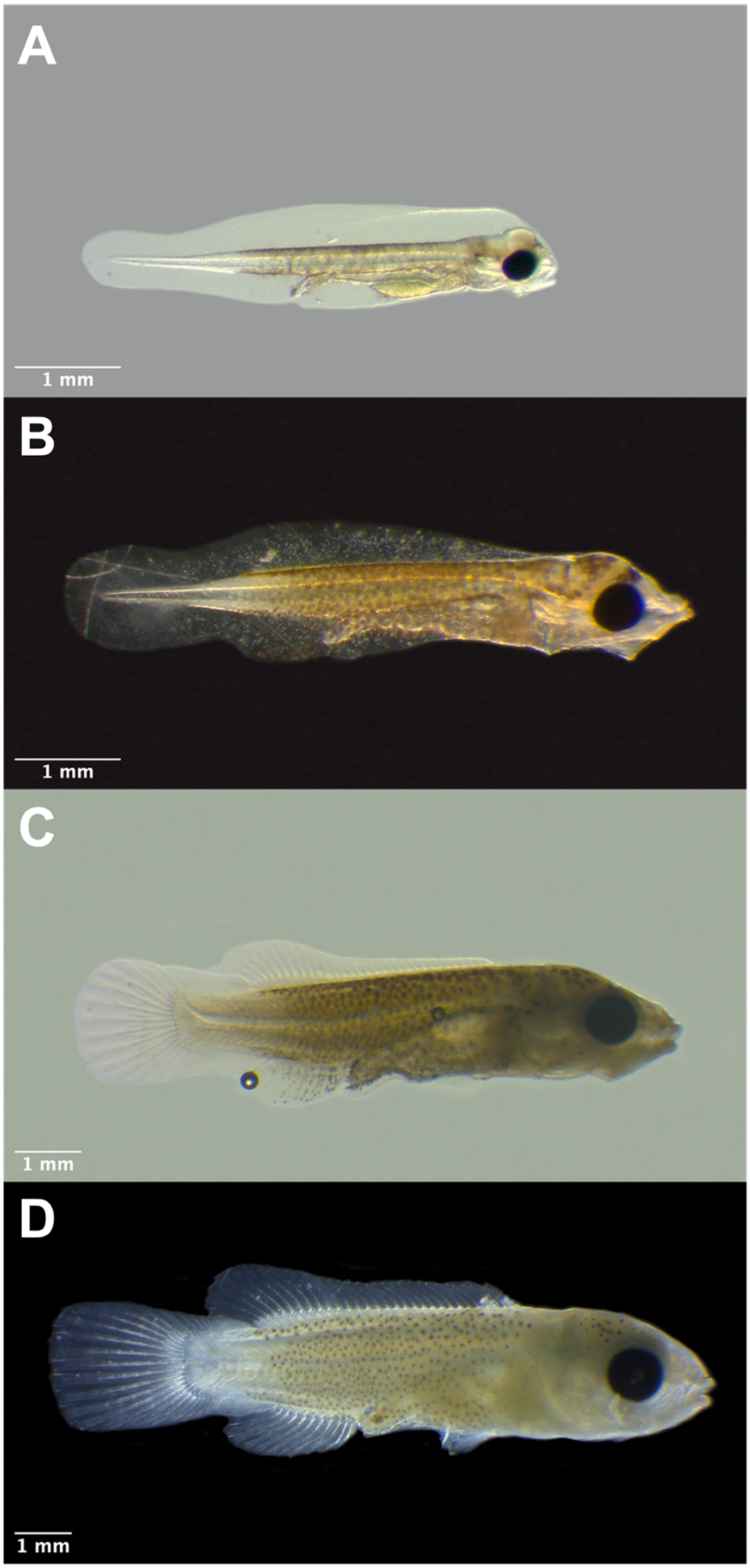


Figure 3.1: The development of ballan wrasse from A) 4 dph, yolk-sac larval stage; B) 18 dph, preflexion larval stage; C) 32 dph, flexion larval stage to D) 48 dph, postflexion larval stage. All larvae are from the

Copepod/Artemia treatment.

Dry weight

The starting mean dry weight of all larvae at 4 dph was 34.1 ± 0.7 µg (n=39, Fig 3.2.). For all treatments, except larvae from the Copepod/Cirriped treatment, a drop in dry weight was observed at approximately 12 dph due to depletion of yolk reserves (Fig 3.2.). After this, growth was similar in all treatments apart from larvae fed from the experimental Cirriped treatment which had a significantly lower weight than the other treatments. In this group, no mean weight gain was seen during the first live feed period, where the DW was similar to that of yolk sac larvae at 18 dph. After this, the larvae in this treatment gained weight but always had the lowest mean DW of all the treatments. For all treatments, the increase in dry weight appeared to be exponential, but with a lower slope for larvae fed exp. Cirripeds (Fig.3.2.). At 42 dph, which was the last sampling date with dry weight data from all treatments, the mean DW in the exp. Cirriped, Copepod/*Artemia*, Rotifer/*Artemia* and Copepod/Cirriped treatments were 641.7 ± 297.9 µg (n=6), 1406.6 ± 90.3 µg (n=30), 1582.4 ± 281.8 µg (n=5) and 1078.6 ± 99.7 µg (n=30), respectively. At 48 dph the DW had increased to 2086.1 ± 191.8 µg (n=29) and 2357.0 ± 238.2 µg (n=29) in the Copepod/*Artemia* and Copepod/Cirriped treatments, respectively.

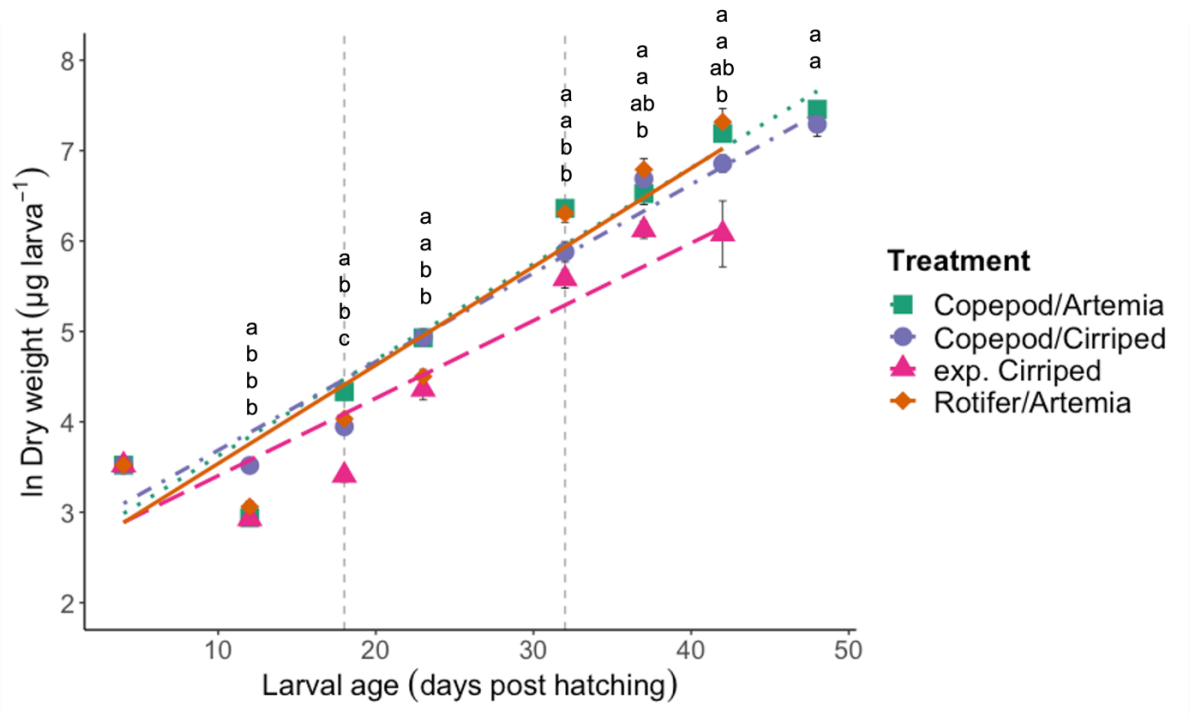


Figure 3.2: Mean dry weight (µg) over time (larval age, days post hatching) in ballan wrasse larvae fed different start feeding diets. Dashed vertical lines denote a change in feeding period. Error bars indicate ± SE for each

*mean value (n=5-30). Significant differences (p < 0.05) are denoted by unequal superscripts. Measurements are missing for exp. Cirriped and Rotifer/*Artemia *treatments on 48 dph due to high mortalities and therefore no available samples this day. Y-values are given in logarithmic scale.*

Standard length

The development of standard length (SL) was similar between all treatments during the first live feed period, where growth was relatively slow (Fig. 3.3.). The mean starting length at 4 dph was 4.3 ± 0.05 mm for all larvae (n=40). After the first live feed period, larvae fed experimental Cirripeds had a significantly lower growth than larvae from the other treatments, approximately from the middle of the second live feeding period and until the end of the experiment (Fig 3.3.). The length development followed a linear curve for all treatments, but the slope was steeper for the copepod and rotifer fed larvae than larvae fed experimental cirripeds. The post weaning mean SL at 42 dph was 6.7 ± 0.4 mm (n=6), 8.3 ± 0.1 mm (n=30), 8.5 ± 0.3 mm (n=5) and 7.98 ± 0.2 (n=30) mm for larvae fed exp. Cirripeds, Copepods/*Artemia*, Rotifers/*Artemia* and Copepods/Cirripeds, respectively.

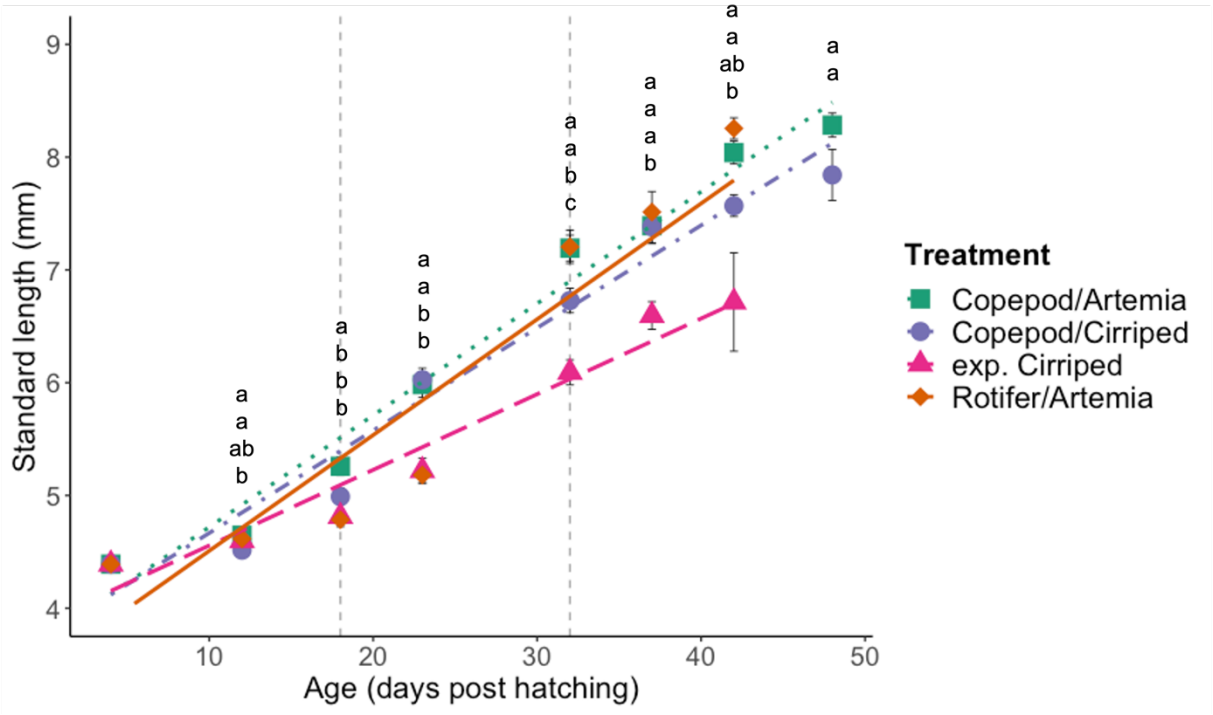


Figure 3.3: Mean standard length (mm) over time (larval age, days post hatching) in ballan wrasse larvae fed different start feeding diets. Dashed vertical lines denote a change in feeding period. Error bars indicate ± SE for

*each mean value (n=5-30). Significant differences (p < 0.05) are denoted by unequal superscripts. Measurements are missing for exp. Cirriped and Rotifer/*Artemia *treatments on 48 dph due to high mortalities and therefore no available samples this day.*

Length-weight relationship

Correlating standard length (mm) to dry weight (mg) for the pooled data set of all larvae revealed a squared polynomial relationship which was highly significant ($p < 0.05$, Pearson's correlation test). The regression could be described by the equation $DW=106.26*SL^2-1005.4*SL+2394.5$ for DW and was not significantly different between treatments. Figure 3.4 illustrates this relationship. At approximately 6 mm, flexion occurred and the growth rate increased steeply.

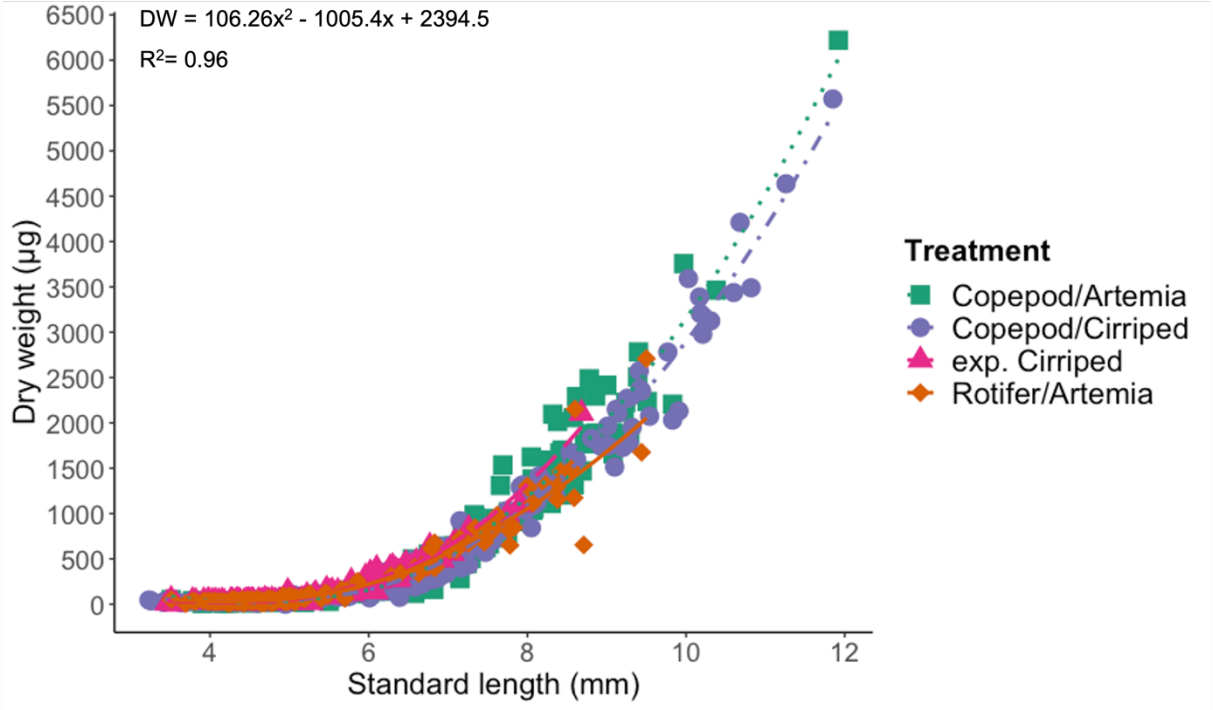


Figure 3.4: Relationship between dry weight (µg) and standard length (mm) in ballan wrasse larvae from ages 4-48 dph. The relationship between dry weight and standard length was squared polynomial, could be described by the equation $DW= 106.26SL^2 – 1005.4SL + 2394.5$ ($R^2=0.96$) and did not differ significantly between treatments ($p < 0.01$).

Daily weight increase

The percentage of daily weight increase (DWI) differed between groups both from the start of the experiment to the end of the first live feed period (4-18 dph); the end of the second live feed period (4-32); and completed weaning (4-42 dph) (Fig. 3.5.). For all treatments, the growth rate increased significantly from the end of the first live feed period to the end of the second, before remaining stable until completed weaning (Fig. 3.5.). Larvae from both the Copepod/*Artemia* and Rotifer/*Artemia* treatments had the highest mean growth rates throughout the experiment, which was always significantly higher than the mean growth rate of larvae from the exp. Cirriped treatment (Fig. 3.5.). Additionally, the latter treatment was the only one that exhibited a negative growth rate, which was seen in the time interval 4-18 dph, i.e. from before start-feeding to after completing the first live feed period. Larvae fed Copepods/Cirripeds had mean growth rates in the middle of the highest and lowest for all sampling points, and had significantly lower growth rates than the treatments with the highest growth rate on both 4-18 and 4-42 dph (Fig. 3.5.). At the same sampling points, the growth rate in the Copepod/Cirriped treatment was significantly larger than for the exp. Cirriped treatment.

After the first live feed period, the growth rate of both treatments fed copepods were different (Fig. 3.5.), which could not be explained by feed quality due to the treatments receiving feed from the same reservoir. Additionally, neither temperature, pH or oxygen parameters were different in these groups. It should be noted that the larval density was not counted during the experiment apart from at the start (4 dph) and at the end (48 dph).

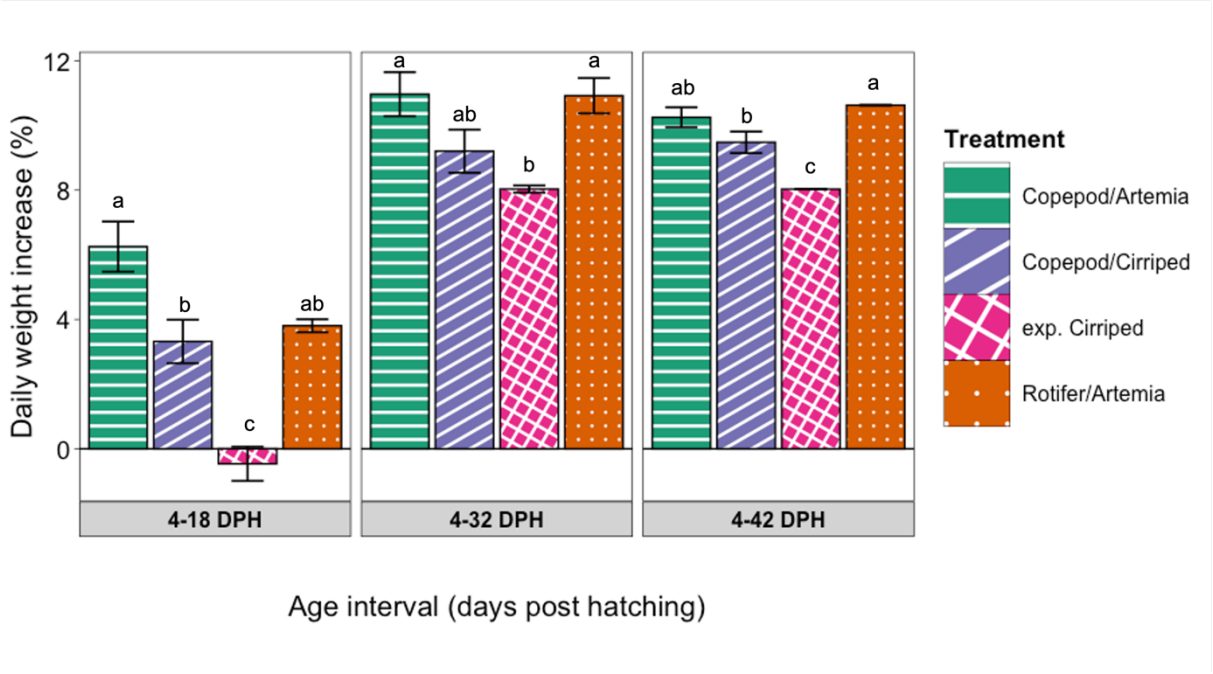


*Figure 3.5: Mean percentage of daily weight increase in ballan wrasse larvae fed different start feeding diets at different age intervals (days post hatching, dph): 4-18 dph (yolk sac larvae to completed first live feed period); 4-32 dph (yolk sac larvae to completed second live feed period); and 4-42 dph (yolk sac larvae to completed weaning onto formulated feed). Error bars indicate ± SE for each mean value (n=3). Significant differences ($p < 0.05$) are denoted by unequal superscripts. Measurements from 48 dph were omitted as data are missing for exp. Cirriped and Rotifer/*Artemia *treatments at this sampling date due to high mortalities.*

Mortality

Mortality during the experiment was similar between both groups of larvae fed copepods as their first live feed diet, and between larvae fed either rotifers or exp. cirripeds as their first diet. Larvae given the Copepod/*Artemia* and Copepod/Cirriped diets had significantly lower mean mortality coefficients at 0.05 ± 0.00 and 0.06 ± 0.01 than larvae receiving either the Rotifer/*Artemia* diet with a mortality coefficient of 0.11 ± 0.01 or exp. Cirriped diet where the mortality coefficient was also 0.11 ± 0.01 (Fig. 3.6. A).

The starting number of larvae was estimated to be 19 716 tank^{-1}, with 452-920 larvae sampled tank^{-1} over the course of the experiment. When finishing the experiment at 48 dph, Copepod/*Artemia* and Copepod/Cirriped fed larvae had 3791 and 2863 larvae left in the tank, respectively, while the groups fed the experimental Cirriped diet and the Rotifer/*Artemia* diet had 136 and 134 surviving larvae, respectively. The survival over the entire experiment based on the mortality coefficients is modeled in Figure 3.6. B, however there were periods of

higher mortality that are not reflected in the plotted curve. The calculated survival for the entire experiment, based on the mortality coefficient, was 0.7 ± 0.4%, 8.1 ± 0.9%, 0.7 ± 0.3% and 6.6 ± 2.3% for the exp. Cirriped, Copepod/*Artemia*, Rotifer/*Artemia* and Copepod/Cirriped diets, respectively. Peak mortality was seen around 14 dph, corresponding to the depletion of yolk reserves, and this was especially apparent in the tanks assigned the Rotifer/*Artemia* and exp. Cirriped diets. After this, there were few remaining larvae in these treatments compared to the groups started on either copepod diet. Another period of increased mortality was seen during weaning from live feed to formulated feed, before the mortality rate was stabilized until the end of the experiment.

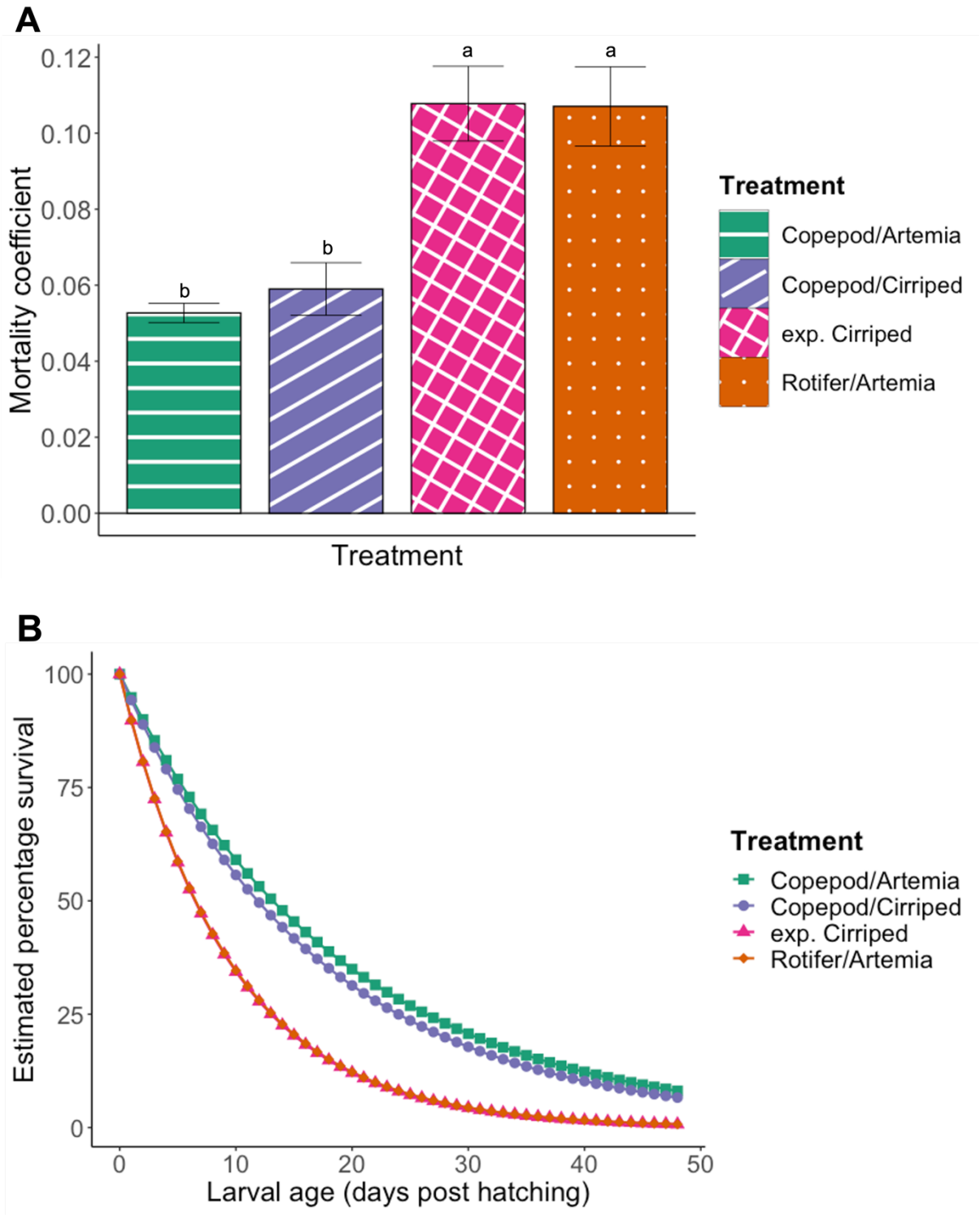


Figure 3.6 A) Mean mortality coefficients for larvae fed different start feeding diets. Error bars indicate ± SE for each mean value (n=3). Significant differences ($p < 0.05$) are denoted by unequal superscripts B) Modelled survival of ballan wrasse larvae fed different start feeding diets. The modelled survival values are based on the mortality coefficients.

3.2 Liver histology

Morphology of liver tissue

Prior to onset of start-feeding, at 4 dph, the liver in the sectioned larvae was small and located below the oesophagus. No liver tissue could be detected after the transition from esophagus to anterior intestine. The tissue consisted mostly of basophilic parenchymal cells, and was loosely arranged, with polygonal hepatocyte cells interconnected to each other and arranged in a cord-like asymmetrical network with large intercellular spaces (Figure 3.7 A). No blood cells could be detected in these spaces, thus the sinusoids were not yet fully developed. The hepatocyte cells were small with indistinct borders, while the highly basophilic hepatocyte nuclei appeared large and spherical/oval in shape (Figure 3.7 A, B). Small vacuoles varying in shape could be seen in the cells, especially in larvae that still had large yolk reserves (Figure 3.7 B). A later PAS-staining test of similar looking vacuoles in older larvae revealed that the vacuoles likely contained glycogen (Figure 3.10 E, F).

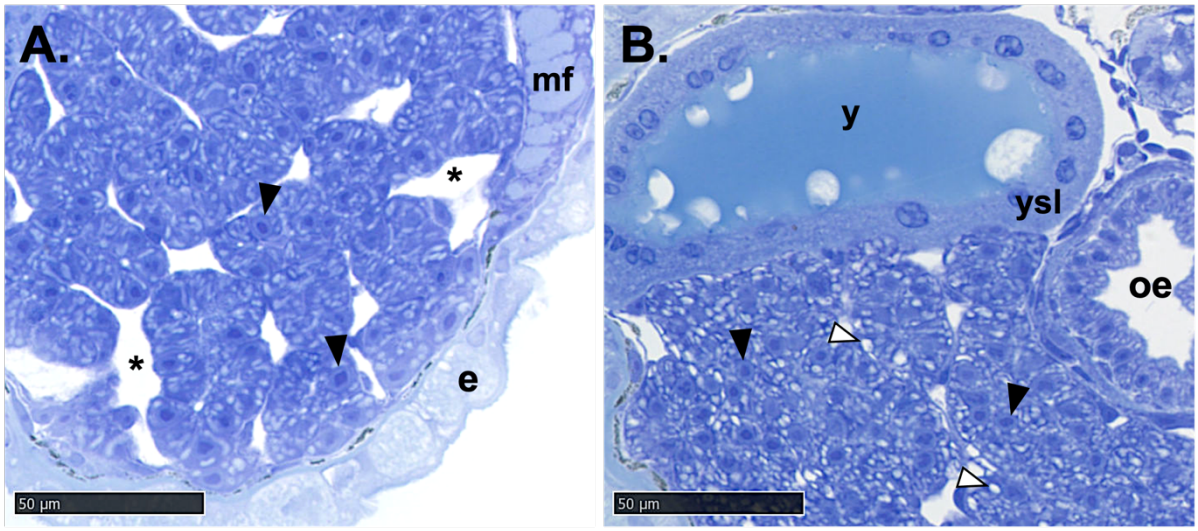


Figure 3.7 Liver tissue of ballan wrasse larvae at 4 dph (40x magnification). The liver was small and located beneath the oesophagus (oe) and above the yolk sac (y, ysl). The liver tissue consisted of small, indistinct polyhedral hepatocytes arranged in a cord-like manner with large intracellular spaces (asterisk) in between. Hepatocyte nuclei (black arrowheads) were large with distinct nucleoli. In some larvae, small vacuoles (white arrowheads) could be seen in the hepatocytes.

Black arrowheads: hepatocyte nuclei; white arrowheads with black borders: vacuoles; asterisks: intracellular spaces; e: epithelial tissue; mf: muscle fibers; oe: oesophagus; y: yolk; ysl: yolk syncytial layer

At 18 dph, at the end of the first live feed period, the liver had increased in size, and the tissue now consisted of both hepatocytes and sinusoids (Figure 3.8 A, B, C, D). Although much of the liver still was located ventral to the oesophagus, some liver tissue could also be detected after the transition to anterior gut. For larvae from all treatments, the liver tissue appeared more cohesive with larger hepatocyte cells that had anastomosed with smaller intracellular spaces. This was especially true for copepod fed larvae (Figure 3.8 A, B). In all groups, sinusoids with blood cells had appeared. In larvae fed rotifers or exp. Cirripeds, the sinusoids appeared to be dilated (Figure 3.8 C, D), while in copepod fed larvae, they appeared more compacted with hepatocytes forming a more distinct pattern around the vessels (Figure 3.8 A, B). The nuclei of hepatocytes in different treatments appeared slightly different, where copepod or exp. Cirriped fed larvae had nuclei with defined edges and a spherical shape compared to larvae fed rotifers where the nuclei had less distinct edges and a more uneven shape. In addition, the hepatocytes appeared larger with more distinct borders in copepod fed groups than in other larvae. All groups had one or several larvae with small amounts of vacuoles within the hepatocytes at this stage (Figure 3.8 A).

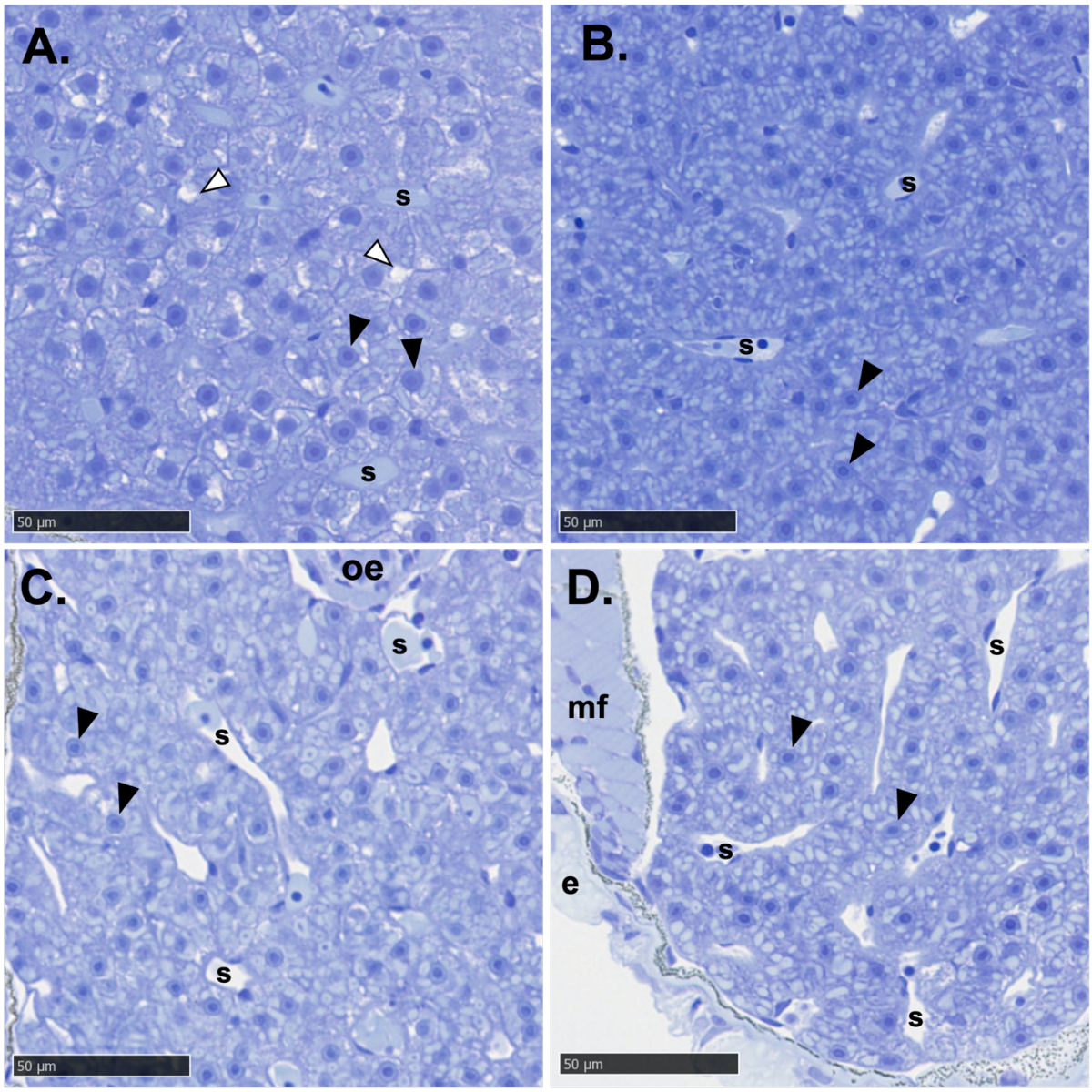


*Figure 3.8 Liver tissue in ballan wrasse larvae at 18 dph (40x magnification). A) Copepod/Cirriped larvae had a cohesive liver tissue, where the hepatocytes had distinct borders and large and spherical nuclei (black arrowheads) with distinct nucleoli, arranged around oval sinusoids (s) containing erythrocytes. Small vacuoles could be seen in the tissue (white arrowheads). B) Copepod/*Artemia *larva with similar liver tissue to Copepod/Cirriped larva C) Rotifer/*Artemia *larvae had a smaller amount of vacuoles and less distinct hepatocyte nuclei (black arrowheads), with sinusoids (s) appearing narrow and long compared to larvae fed copepods D) exp. Cirriped larva with similar liver tissue to Rotifer/*Artemia *larva.*
Black arrowheads: hepatocyte nuclei; white arrowheads with black borders: vacuoles; e: epithelial tissue; mf: muscle fibers; oe: oesophagus; s: sinusoids

At 32 dph, after the second live feed period and prior to weaning, the liver had increased in size. The liver tissue had expanded below the esophagus to dorsally around the alimentary canal and caudally taking up more space beneath the anterior intestine. Larvae from all

groups now had strongly vascularized livers with numerous sinusoids (Fig. 3.9. A, B, C, D), though the shape of the vessels still differed between larvae fed the Rotifer/*Artemia* diet and all others, where sinusoids of the latter group still appeared to show signs of dilation (Fig. 3.9. C). In addition, in this group, what appeared to be lipid droplets had accumulated in the liver tissue (Fig 3.9. C). Some irregularly shaped vacuoles could also be seen in this group, and nuclei appeared less defined than in other groups. Larvae fed either of the copepod diets appeared to have more vacuoles than the other groups, however, and larvae fed exp. Cirripeds had very few to no vacuoles. The hepatocytes of larvae fed copepods also had more distinct borders, while larvae fed the Rotifer/*Artemia* or exp. Cirriped diet had less distinct hepatocyte borders.

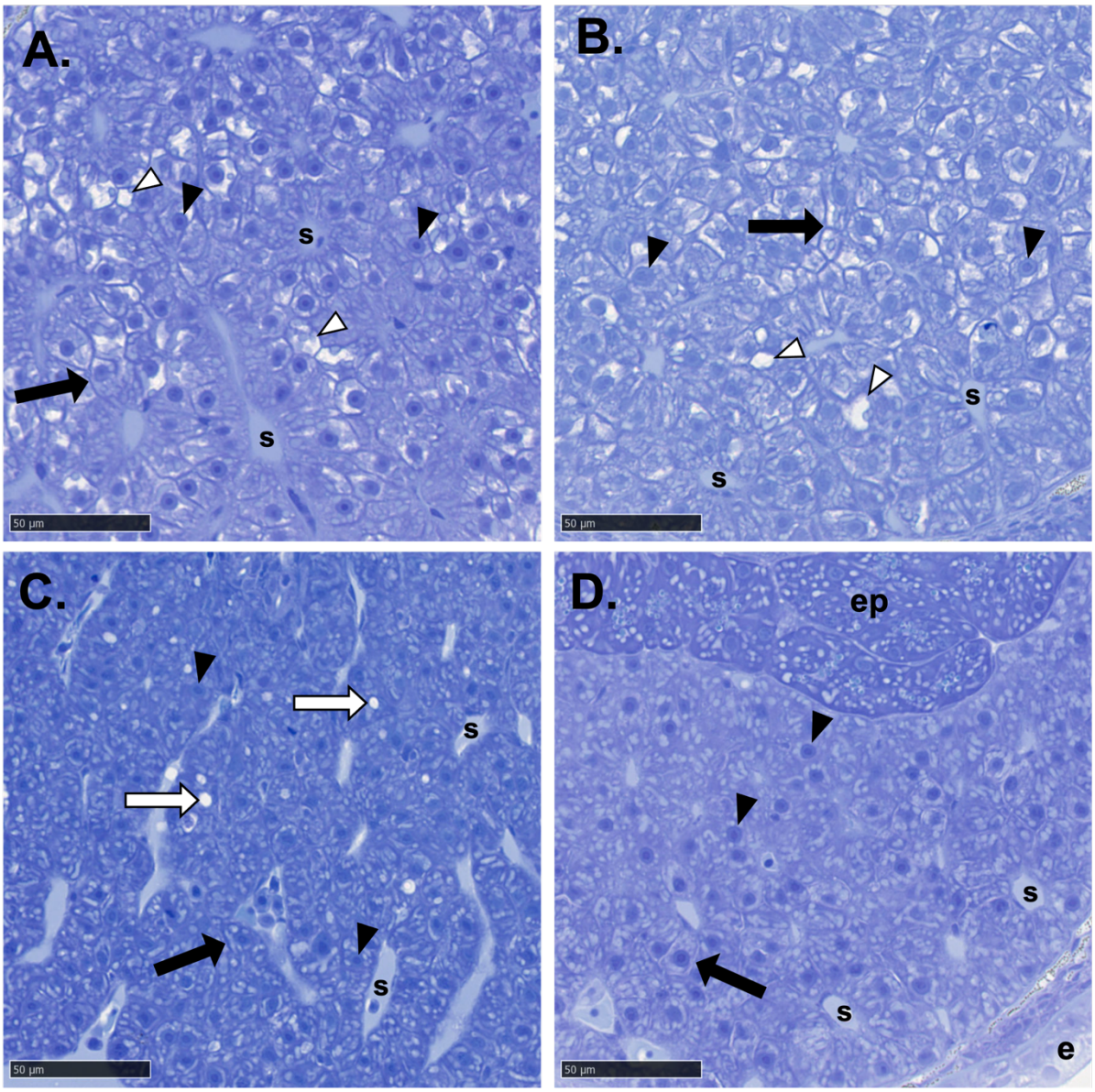


*Figure 3.9 Liver tissue in ballan wrasse larvae at 32 dph (40x magnification). A) Copepod/Cirriped larvae had hepatocytes with distinct borders (black arrow) and nuclei (black arrowheads), a well-developed sinusoidal network (s) and several vacuoles (white arrowheads) B) Copepod/*Artemia *larva with similar liver tissue to Copepod/Cirriped larva C) Rotifer/*Artemia *larvae had small-looking hepatocytes with indistinct borders (black arrow) and nuclei (black arrowheads). Small, spherical droplets were spread throughout the tissue (white arrows with black borders). Sinusoids were narrow and long (s) D) exp. Cirriped larvae had hepatocytes with indistinct borders (black arrow) and very indistinct nuclei (black arrowheads) Sinusoids (s) appeared well developed. No vacuoles could be seen.*

Black arrowheads: hepatocyte nuclei; white arrowheads with black borders: vacuoles; black arrows: hepatocyte borders; white arrows with black borders: lipid droplets; e: epithelial tissue; ep: exocrine pancreas; s: sinusoids

After weaning to formulated feed, at 48 dph, the liver in the larvae had grown large, surrounding the alimentary canal dorsoventrally from oesophagus to anterior midgut, and

ventrally toward the posterior midgut. Larvae from all treatments had accumulated more vacuoles in their hepatocytes than at any previous stage. This was especially apparent in larvae fed the Copepod/Cirriped diet, where the vacuoles were large, increasing the hepatocyte cell size and displacing the hepatocyte nuclei to the edges of the cells (Figure 3.10 A). Larvae fed either the Copepod/*Artemia* diet or exp. Cirriped diet had smaller, but still large vacuoles displacing the hepatocyte nuclei peripherally (Figure 3.10 B, D. Larvae fed the Rotifer/*Artemia* diet had the least amount of vacuoles and small hepatocytes (Figure 3.10 C). PAS-stained liver sections of larvae from the Rotifer/*Artemia* and Copepod/Cirriped treatments indicated the presence of polysaccharides in both groups, but a low amount in in the former and high amount in the latter group (Figure 3.10 E, F). In addition, signs of sinusoidal dilation were still present in the Rotifer/*Artemia* group (Figure 3.10 C, E).

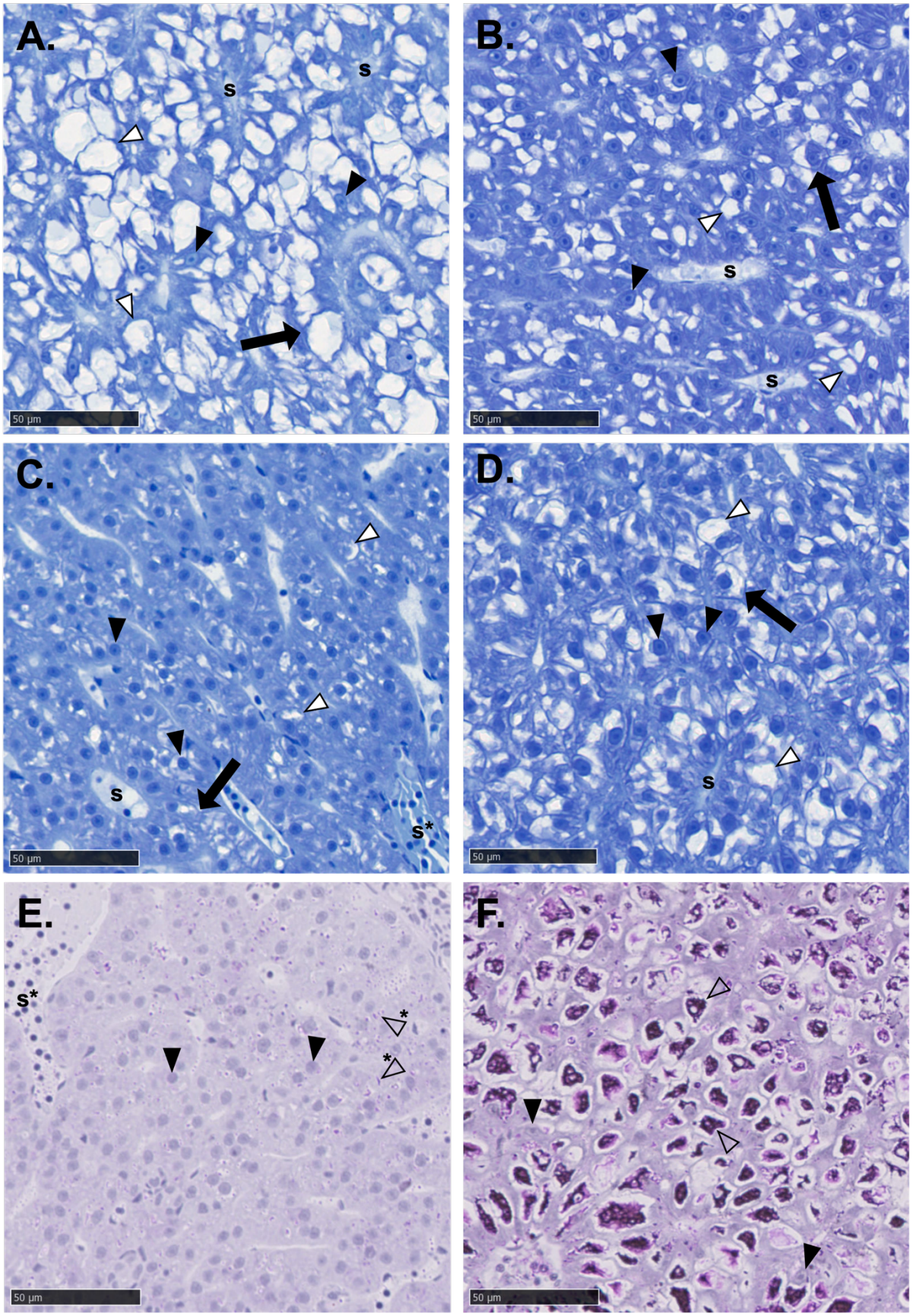


Figure 3.10 Liver tissue in ballan wrasse larvae at 48 dph. A) Copepod/Cirriped larvae had large, irregularly shaped vacuoles (white arrowheads) within the hepatocytes displacing the nuclei (black arrowheads) to the edges

*of the cell. Hepatocyte nuclei appeared less spherical in shape than at earlier stages. Hepatocyte borders (black arrow) are distinct. B) Copepod/*Artemia *larvae with medium sized irregularly shaped vacuoles (white arrowheads) displacing the hepatocyte nuclei (black arrowheads) slightly. Hepatocyte borders (black arrow) are distinct. C) Rotifer/*Artemia *larvae had small hepatocytes with indistinct borders (black arrow). Hepatocyte nuclei (black arrowheads) were numerous and distinct. Small vacuoles (white arrowheads) were spread throughout the tissue. Some sinusoids displayed signs of dilation (s*). D) exp. Cirriped larvae with medium sized irregularly shaped vacuoles (white arrowheads) displacing the hepatocyte nuclei (black arrowheads) slightly. Hepatocyte borders (black arrow) are distinct. E) PAS-stained Rotifer/*Artemia *larva displaying small amounts of glycogen storage (transparent arrowheads with asterisk). F) PAS-stained Copepod/Cirriped larva displaying large amounts of glycogen storage (transparent arrowheads).*
Black arrowheads: hepatocyte nuclei; white arrowheads with black borders: vacuoles; black arrows: cell walls; transparent arrowheads with a black border: strongly positive PAS-reaction; transparent arrowheads with black border and asterisk: weakly positive PAS-reaction; s: sinusoids; s: dilated sinusoid*

Hepatocyte parameters

The size of hepatocyte cells in the liver of ballan wrasse larvae increased throughout the course of the experiment in all treatments (Fig. 3.11. A.), but no differences in cell size were seen until 48 dph, after weaning. The mean starting cell size for all larvae at 4 dph was 196.9 ± 5.0 μm^2 (Table 3.11.). For larvae fed Rotifers/*Artemia*, the cell size post weaning was similar to that of yolk-sac larvae, although the decrease in cell size from after the second live feed was not significant (Fig. 3.11. A.). The hepatocyte cells of larvae from the Copepod/Cirriped treatment grew significantly from each sampling point to the next, and were larger than both larvae from the Copepod/*Artemia* and Rotifer/*Artemia* treatments at 48 dph (Fig 3.11 A., Table 3.1). The latter treatment had the smallest mean hepatocyte cells, which were also smaller than those of the larvae fed exp. Cirripeds post weaning (Fig. 3.11. A., Table 3.1.).

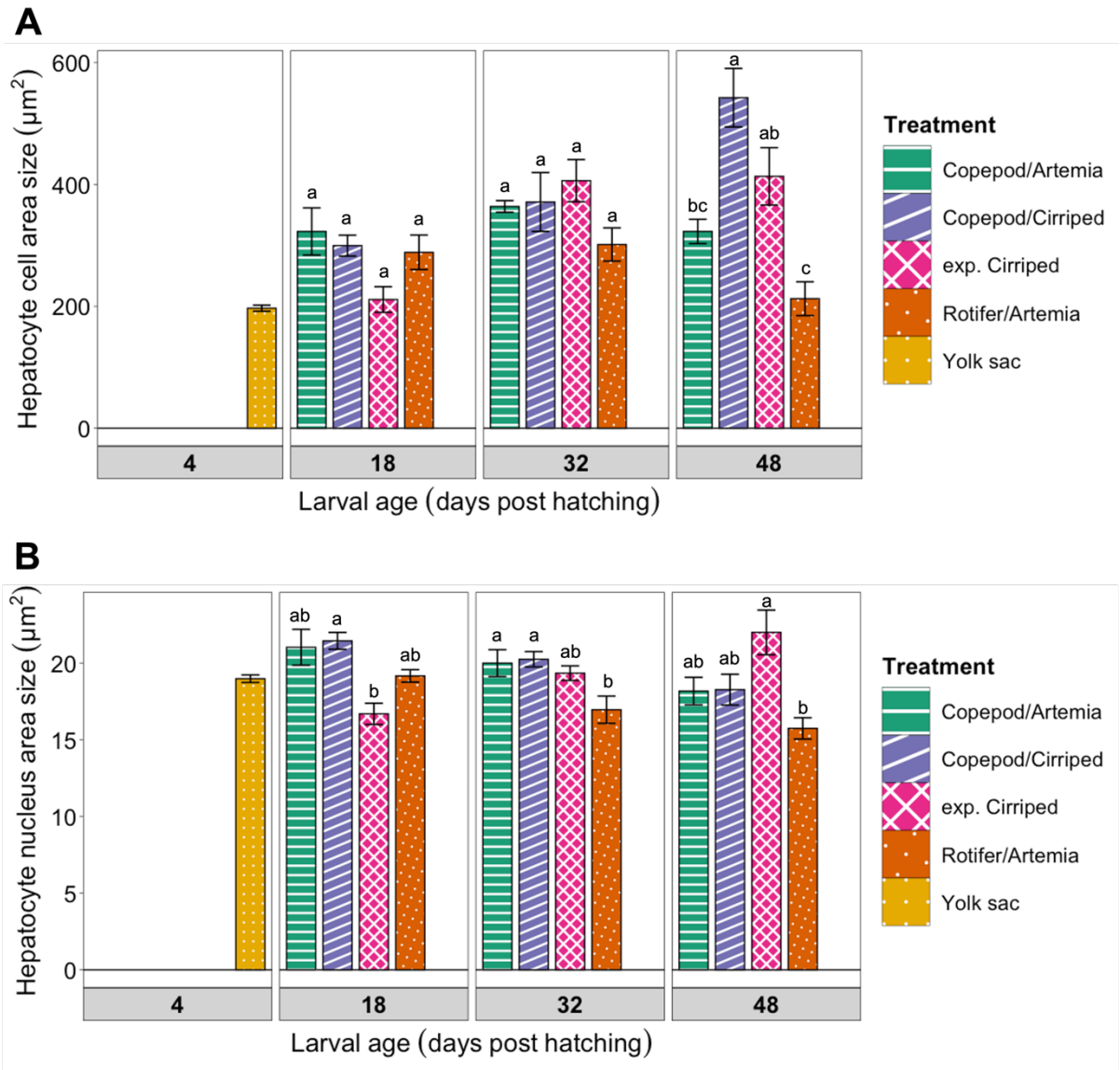


Figure 3.11. A) Mean cell area size (µm²) and B) mean nucleus area size (µm²) of hepatocytes over time (days post hatching, dph) in ballan wrasse larvae fed different start feeding diets. 4; 18; 32 and 48 dph correspond to the different feeding periods of the larvae (yolk sac; first live feed; second live feed; and formulated feed, respectively). Error bars indicate ± SE for each mean value (n=5). Significant differences ($p < 0.05$) are denoted by unequal superscripts.

The hepatocyte nuclei, on the other hand, varied in size over time with several significant differences between treatments appearing at different sampling points (Fig. 3.11. B.). The mean nucleus size for all larvae at 4 dph was 19.0 ± 0.2 (Table 3.1.). After this, the hepatocyte nuclei of Copepod/Artemia fed larvae were similar in size for the rest of the experimental period (Table 3.1.); larvae from the Copepod/Cirriped treatment experienced an increase in nucleus size after the first live feed period before it decreased again to be the same size as for

yolk sac larvae post weaning (Table 3.1.); larvae fed Rotifers/*Artemia* decreased in nucleus size from after the first live feed period and remained smaller than for yolk sac larvae throughout the remainder of the experiment (Table 3.1.), while larvae fed exp. Cirripeds had a decrease in nucleus size after the first live feed period before increasing significantly after the second live feed period and onward (Table 3.1.). These different patterns resulted in the mean nucleus size being larger in the Copepod/Cirriped treatment than the exp. Cirriped treatment at 18 dph (Fig. 3.11. B.), larvae from both copepod treatments having larger nuclei than larvae from the Rotifer/*Artemia* treatment at 32 dph (Fig. 3.11. B.), and the latter group having smaller nuclei than the treatment with the largest nuclei, which was the exp. Cirriped treatment, at 48 dph (Fig. 3.11. B.).

In all treatments, the amount of vacuoles increased over the course of the experiment with post-weaning larvae having significantly larger amounts of vacuoles than yolk-sac larvae ($p<0.05$) (Table 3.1.). Here, the only difference between different treatments was found, where larvae fed Copepods/Cirripeds had a significantly larger mean vacuole area fraction than larvae fed Rotifers/*Artemia* (Table 3.1.). Apart from this, the vacuole content varied little both within and between treatments, except for a reduction in vacuoles from the first sampling in yolk sac larvae to larvae that had completed both the first and second live feed period in the exp. Cirriped treatment, before the amount of vacuoles increased again post weaning (Table 3.1.).

Table 3.1: Surface area fraction of hepatic vacuoles, hepatocyte cell area size (µm^2) and hepatocyte nucleus area size (µm^2) in ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5 larvae sampled diet^{-1} age^{-1}). Measurements are presented as mean values ± SE. Significant differences (p < 0.05) between treatments at different dph are denoted by unequal superscripts.

Age (dph)	Treatment	Hepatic vacuole area fraction (%)	Hepatocyte cell area size (µm^2)	Hepatocyte nucleus area size (µm^2)
4	Yolk sac	5.8 ± 0.8	196.9 ± 5.0	19.0 ± 0.2
18	Copepod/*Artemia*	3.1 ± 1.1^{a}	322.8 ± 38.8^{a}	21.0 ± 1.2^{ab}
	Copepod/Cirriped	8.1 ± 2.3^{a}	299.6 ± 17.2^{a}	21.5 ± 0.5^{a}
	Exp. Cirriped	2.5 ± 0.6^{a}	211.1 ± 21.0^{a}	16.7 ± 0.7^{b}
	Rotifer/*Artemia*	4.8 ± 1.3^{a}	288.7 ± 28.4^{a}	19.2 ± 0.4^{ab}
32	Copepod/*Artemia*	4.8 ± 3.8^{a}	363.9 ± 9.5^{a}	20.0 ± 0.9^{a}
	Copepod/Cirriped	3.2 ± 2.9^{a}	371.2 ± 48.3^{a}	20.3 ± 0.5^{a}
	Exp. Cirriped	0.3 ± 0.3^{a}	406.3 ± 34.6^{a}	19.4 ± 0.5^{ab}
	Rotifer/*Artemia*	2.2 ± 1.5^{a}	301.4 ± 27.2^{a}	17.0 ± 0.9^{b}
48	Copepod/*Artemia*	26.4 ± 5.6^{ab}	322.9 ± 20.0^{bc}	18.2 ± 0.9^{ab}
	Copepod/Cirriped	46.6 ± 0.6^{a}	542.4 ± 48.0^{a}	18.3 ± 1.0^{ab}
	Exp. Cirriped	31.5 ± 5.5^{ab}	413.3 ± 47.1^{ab}	22.0 ± 1.5^{a}
	Rotifer/*Artemia*	18.9 ± 6.9^{b}	212.5 ± 27.8^{c}	15.8 ± 0.7^{b}

Both the hepatocyte cell area size ($r = 0.67$, $p < 0.05$) and vacuole area fraction ($r = 0.51$, $p < 0.05$) were positively correlated to standard length (Fig. 3.12. A., B.). The relationship between hepatocyte size and SL was affected by diet, where larvae fed exp. Cirripeds had the steepest slope ($R^2 = 0.7$) followed by larvae fed the Copepod/Cirriped diet ($R^2 = 0.85$). These groups had slopes of 73.6 and 53.1 respectively, meaning that for each mm increase in SL, an increase of 73.6 µm^2 in hepatocyte cell size was expected for larvae fed exp. Cirripeds, and an increase of 53.1 µm^2 in cell size was expected for larvae fed the Copepod/Cirriped diet. Larvae fed the Copepod/*Artemia* had a significantly lower slope ($R^2 = 0.47$), where the slope of 26.9 predicted an increase of 26.9 µm^2 in hepatocyte size mm^{-1} increase in SL.

The hepatic vacuole area fraction was also expected to increase with SL, where treatment affected the relationships and the slopes of exp. Cirriped and Copepod/Cirriped fed larvae

was higher than Copepod/Artemia fed larvae also here. For these correlations, the slopes predicted an increase of 5.8, 5.4 and 2.7% for the exp. Cirriped (R^2 = 0.19) Copepod/Cirriped (R^2 = 0.53) and Copepod/*Artemia* (R^2 =0.19) treatments, respectively.

For both the detected relationships between liver parameters and SL, the slope for the Rotifer/*Artemia* fed larvae was not significantly different from 0. In addition, testing the correlation between hepatocyte nucleus size and SL revealed no significant relationship. No correlations were found between DWI and any of the measured liver parameters.

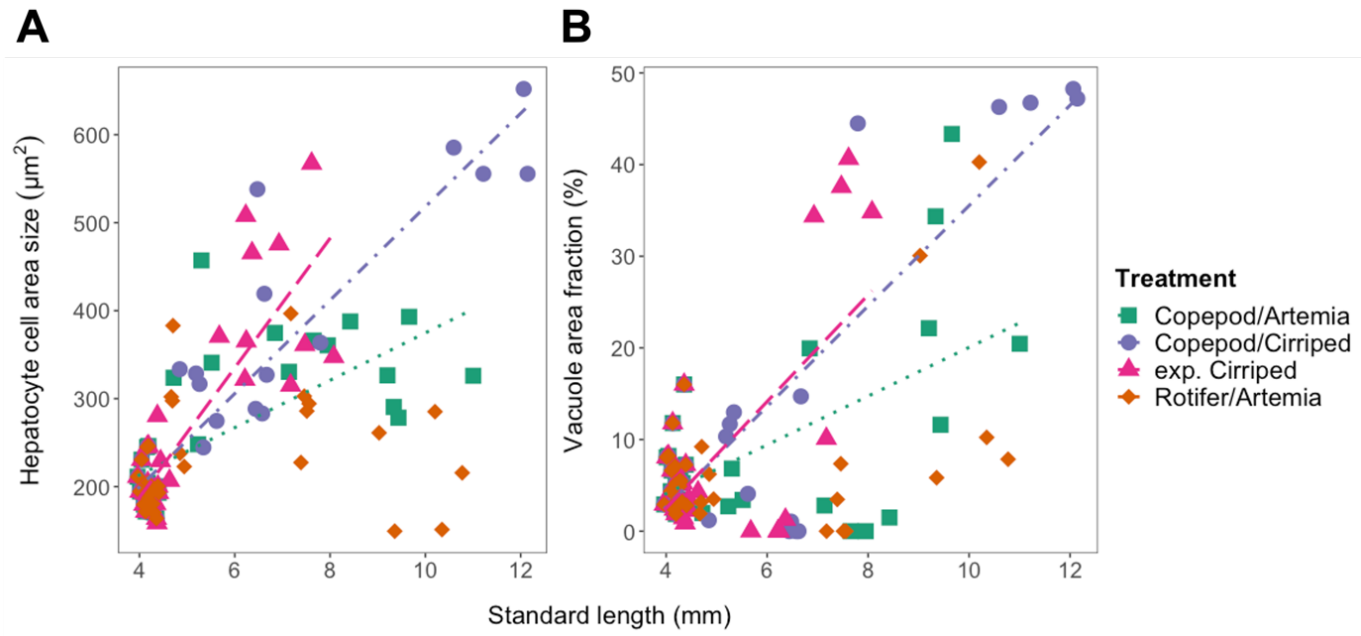


Figure 3.12 Relationship between standard length and the A) hepatic vacuole area fraction (%) and B) hepatocyte cell area size (µm²) in ballan wrasse larvae fed different start feeding diets. For both relationships, all correlations were significant except for in the Rotifer/Artemia treatment.

A significant correlation was found between hepatocyte cell area size and both hepatocyte nucleus area size (Fig. 3.13., r = 0.31, $p < 0.05$) and vacuole area fraction (r=0.34, $p < 0.05$) in the liver, where the fraction of vacuoles in the liver tissue was expected to increase with 4.7% for each µm increase in hepatocyte size (R^2=0.26) and the hepatocyte cell size was expected to increase 16.8 µm² for each µm² increase in nuclear area size (R^2 = 0.09). Diet did not affect the relationship between vacuole area fraction and hepatocyte cell size, however, and none of the groups except for Copepod/Cirriped had a slope significantly different from 0. For the relationship between hepatocyte cell size and nucleus size (Fig. 3.13.), including treatment effects into the regression model gave a significant effect, however only larvae from the exp.

Cirriped treatment had a slope significantly different from 0. No correlation was found between hepatocyte nucleus size and vacuole area fraction.

3.3 Gut histology

Morphological observations

The alimentary canal of the ballan wrasse yolk sac larva, at 4, dph, was a straight tube with luminal constrictions between the foregut and oesophagus rostrally, and midgut and hindgut caudally. The intestinal mucosa was basophilic and stained blue, and consisted of tall columnar epithelial enterocytes lined by a prominent brush border apically toward the intestinal lumen, and lamina propria basally (Fig 3.13 A, B.). The enterocytes contained highly basophilic basally to medially placed oval nuclei with distinct nucleoli. Surrounding the mucosa were thin concentric cell layers that could not be distinguished into submucosa, muscularis or externa (Fig 3.13 B). Nascent folding of the gut epithelium could be seen, but no distinct villi had formed yet (Fig 3.13 A).

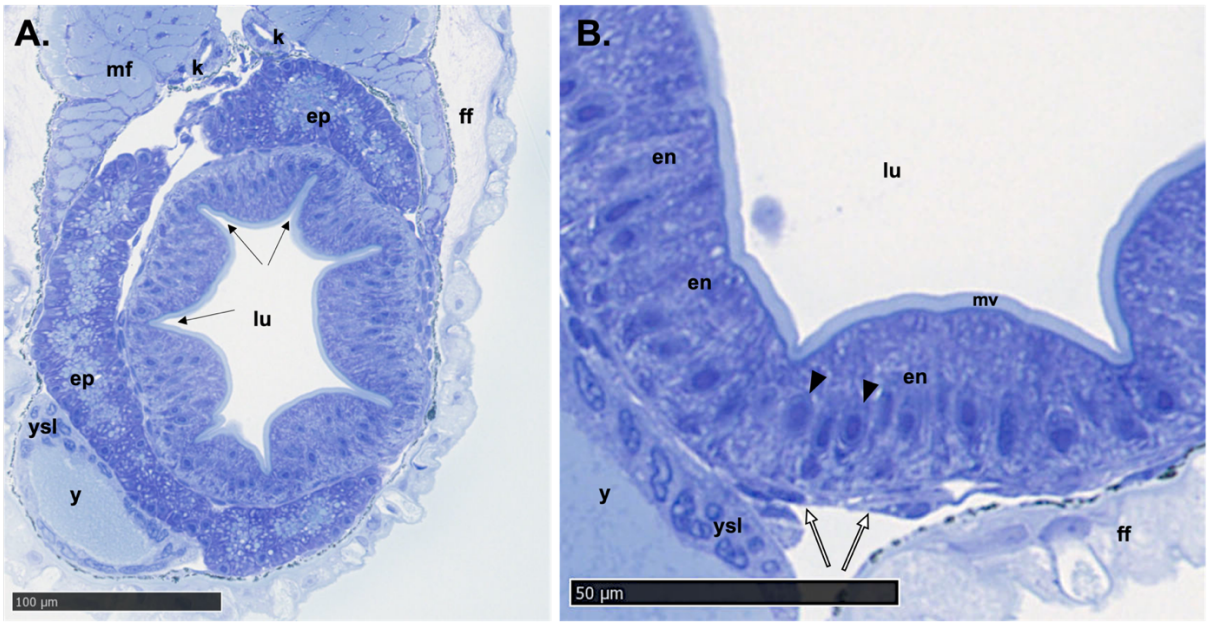


Figure 3.13 Sections of proximal intestine in ballan wrasse larvae at 4 dph, at 40x (A) and 60x (B) magnification. e=enterocyte; ep=exocrine pancreas; ff=fin fold; k=kidney; lu=lumen; mf=muscle fiber; mv=microvilli; y=yolk; ysl=yolk syncytial layer; black arrows=intestinal mucosal folds; white arrows with black borders=lamina propria with surrounding thin layers of submucosa and muscularis externa ; arrow heads=enterocyte nuclei

By the end of the first live feed period, at 18 dph, the now pre-flexion larvae had what appeared to be a more mature gut tissue where the mucosa was folded into distinct villi that ranged in shape from leaf-like to more finger-like. (Fig 3.14.). The mucosa appeared slightly thicker and more folded in larvae fed copepods than larvae fed rotifers or experimental Cirripeds (Fig 3.14). At the basal end of the enterocytes, the thin lamina propria consisting of loose connective tissues interwoven with blood capillaries could be seen, surrounded by a now thicker layer of submucosa, muscularis and serosa which was more readily distinguished into distinct regions. Some goblet cells, distinguished from enterocytes by the purple stain caused by mucins, could be seen in the gut of larvae fed copepods (Fig 3.14. A.). In addition, spherical droplets could be seen in the apical region of enterocytes of some larvae, independent of diet (Fig 3.14. A, D). The brush border appeared slightly denser.

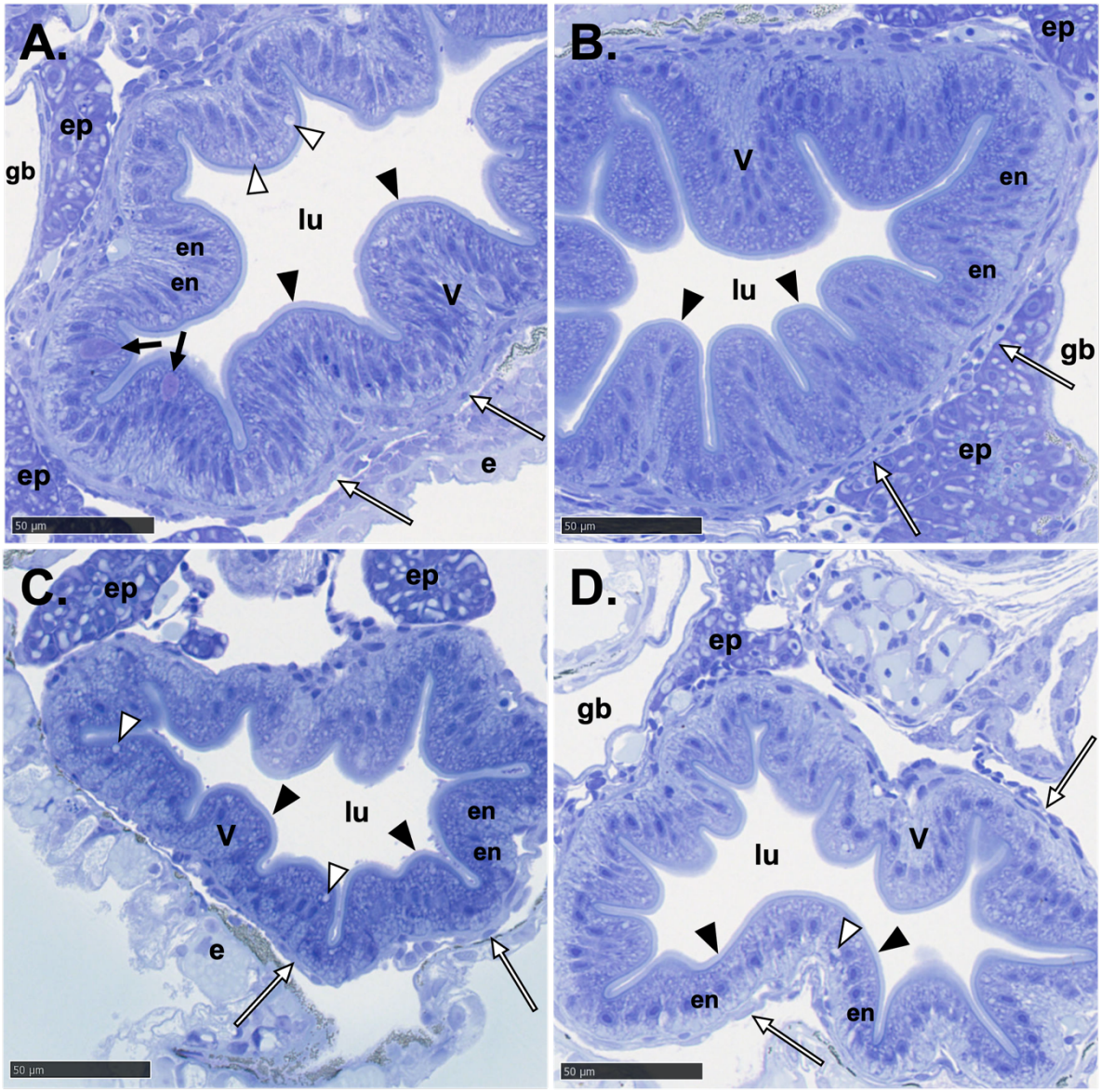


*Figure 3.14 Proximal intestine in ballan wrasse larvae at 18 dph (40x magnification) A) Copepod/Cirriped; B) Copepod/*Artemia*; C) exp. Cirriped D) Rotifer/*Artemia*.*

e=epidermis; en=enterocyte; ep=exocrine pancreas; gb=gall bladder; lu=lumen; v=villus; black arrowhead=brush border; white arrowhead=goblet cell; white arrow with black border=submucosa,muscularis and serosa

After switching to the second live feed, the intestinal mucosa of the 32 dph flexion-larvae had increased in amount and length of folds, however this was most apparent in larvae given the Copepod/*Artemia* and Copepod/Cirriped diets (Fig 3.15. A, B). The mucosa of larvae fed the Rotifer/*Artemia* diet was also folded, but the villi appeared shorter (Fig 3.15. D). Larvae fed exp. Cirripeds had what seemed to be a thinner gut epithelium with fewer folds than larvae from any other diet (Fig 3.15. C). Goblet cells were now seen in all groups (Fig. 3.15. B). The brush border was prominent and appeared slightly denser and longer, especially at the base

of villi and in larvae from the Copepod/*Artemia* group. (Fig 3.15. B). Partially digested feed particles could be seen in the gut of several larvae, independent of diet (Fig. 3.15. A, C, D).

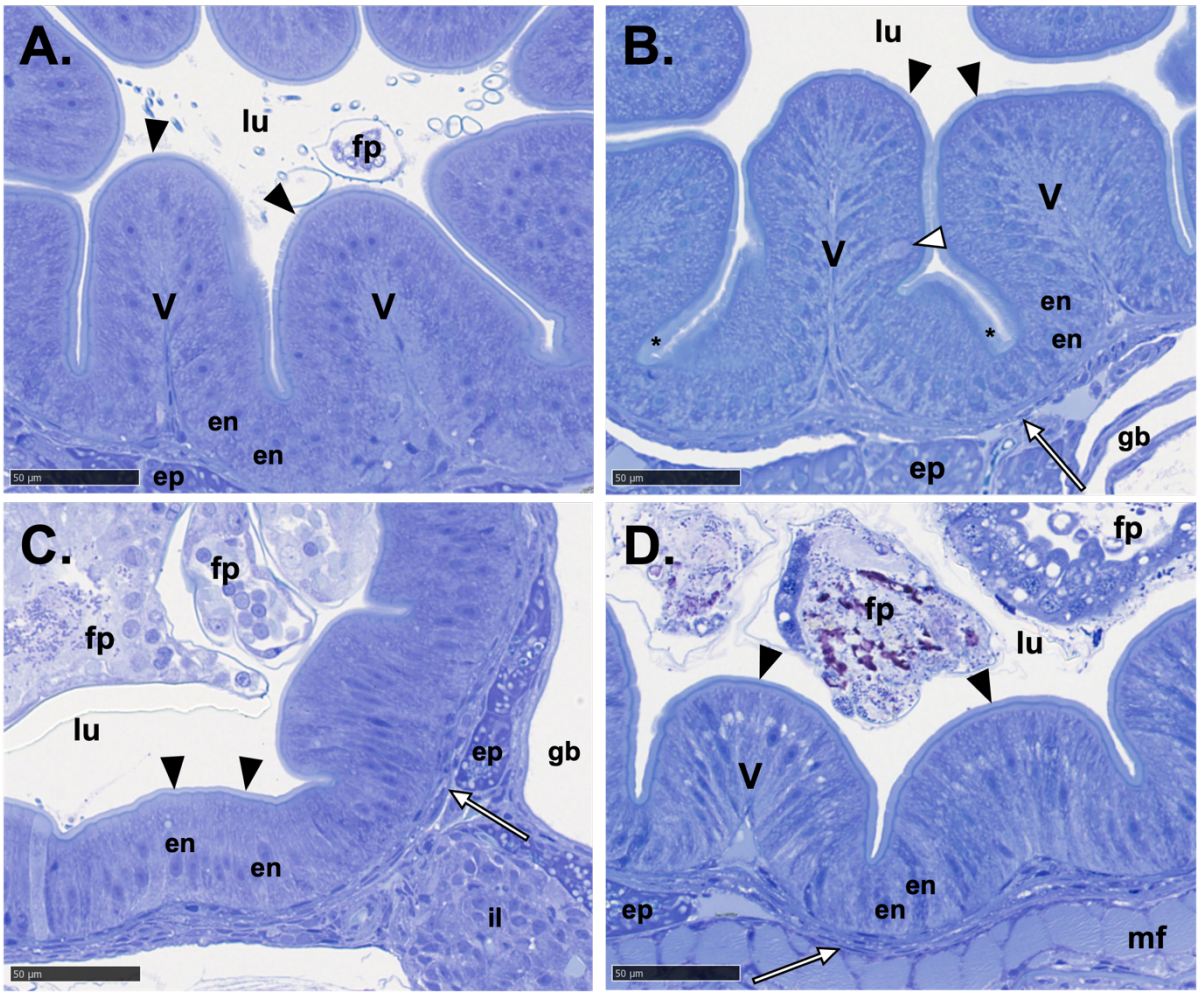


*Figure 3.15 Proximal intestine in ballan wrasse larvae at 32 dph (40x magnification). A) Copepod/Cirriped; B) Copepod/*Artemia*; C) exp. Cirriped D) Rotifer/*Artemia

en=enterocyte; ep=exocrine pancreas; fp=feed particle; gb=gall bladder; il=islet of Langerhans; lu=lumen; mf=muscle fiber; v=villus; black arrowhead=brush border; white arrowhead=goblet cell; white arrow with black border=submucosa,muscularis and serosa; asterisk=dense brush border

Post weaning, at 48 dph, the intestinal mucosa was deeply convoluted and folded into long finger-like villi in all treatments but larvae fed exp. Cirripeds, where folds were considerably shorter (Fig 3.17. A, B). Fixation artefacts in the form of shrinkage was present in all groups, especially in larvae appearing to have a large gut volume (Fig 3.17 A, D). Sloughing of cells into the lumen could also be seen (Fig. 3.17. C). Lipid droplets were present in enterocytes of

some Rotifer/*Artemia* larvae, and could be seen both apically, medially and basally (Fig 3.17. C). This was not registered in any other groups.

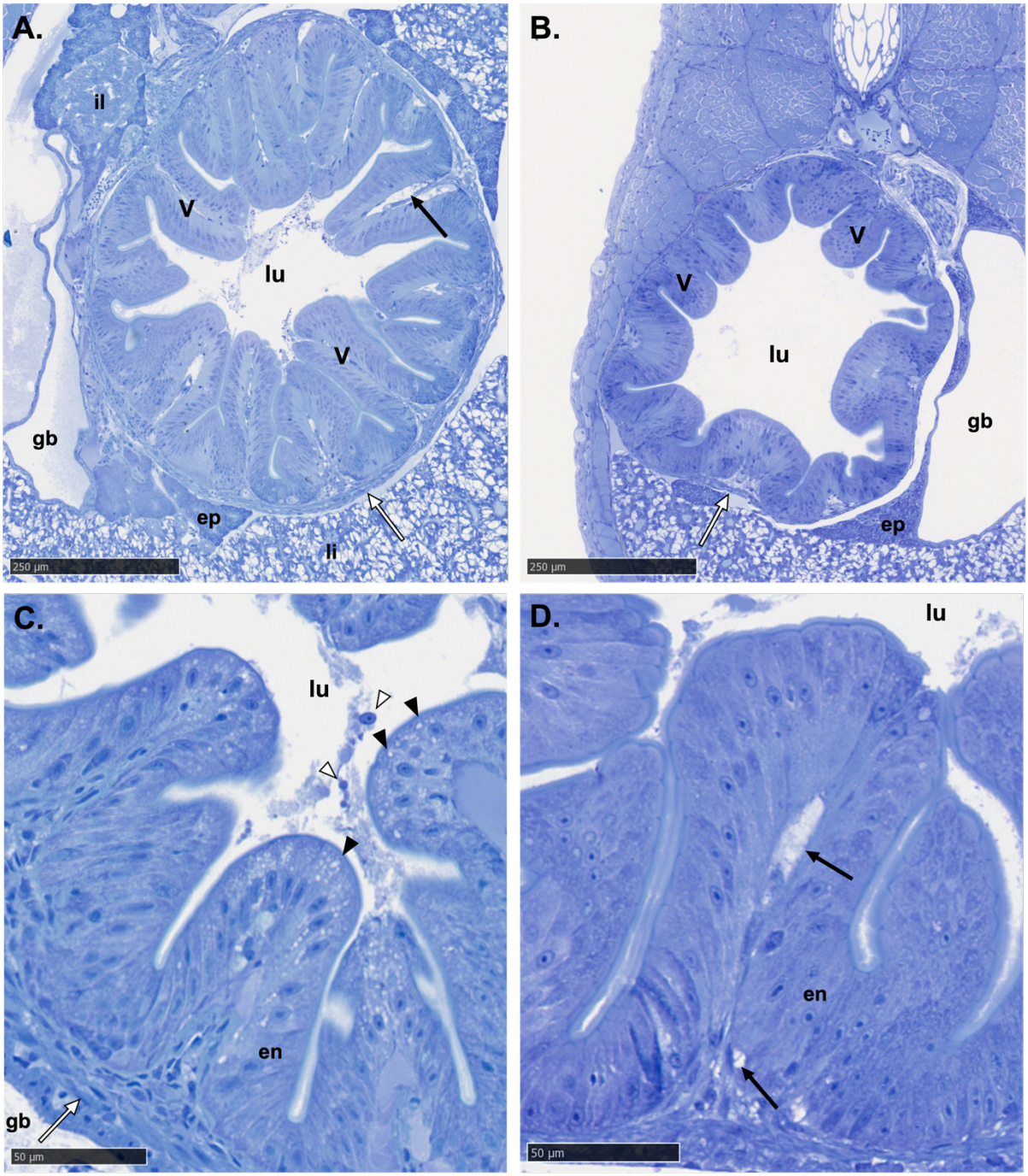


*Figure 3.16 Proximal intestine in ballan wrasse larvae at 48 dph. A) and B) 10x magnification, C) and D) 40x magnification. A=Copepod/Cirriped treatment, B=exp. Cirriped treatment, C=Rotifer/*Artemia *and D) Copepod/*Artemia

en=enterocyte; ep=exocrine pancreas; gb=gall bladder; il=islet of Langerhans; lu=lumen; v=villus; black arrowhead=brush border; white arrowhead with black border=sloughed enterocyte nucleus; white arrow with black border=submucosa,muscularis and serosa; black arrow=shrinkage; black arrowhead=lipid droplet

Gut surface area parameters

Over the course of the start-feeding experiment, a significant lengthening of intestinal villi was seen in all treatments. For all sampling points, larvae fed exp. Cirripeds had the shortest mean villi, while one of the groups offered copepods as the first live feed had the longest. At the end of the first live feed period, at 18 dph, larvae assigned the Copepod/Cirriped diet had larger mean intestinal villi at 68.7 ± 4.6 µm than larvae fed exp. Cirripeds at 52.0 ± 3.1 µm ($p < 0.05$). After completion of the second live feed period, larvae fed exp. Cirripeds had a mean villus height of 90.9 ± 5.0 µm, while larvae fed the Copepod/*Artemia* diet had significantly longer mean villi at 120.1 ± 6.3 µm ($p < 0.05$). By the end of the experiment, after completed weaning to formulated feed, larvae reared on the Copepod/Cirriped diet had the largest mean villi of all groups at 198.5 ± 21.1 µm, which was significantly higher than the mean villus height found in larvae from both the exp. Cirriped at 118.2 ± 4.4 µm ($p < 0.05$) and Rotifer/*Artemia* at 137.9 ± 12.7 ($p < 0.05$) treatments.

The height of the gut epithelium was variable both between larvae from different treatments and larvae from the same groups at different sampling points. At 4 dph, the mean gut epithelial height was 28.5 ± 0.9 µm for all larvae. In general larvae, fed copepods as the first live feed increased in gut epithelial height over the course of the experimental period with significantly taller epithelium after completed weaning, at 33.3 ± 1.2 µm and 39.4 ± 1.5 µm for larvae fed the Copepod/*Artemia* and Copepod/Cirriped diet respectively, than before onset of the first feeding period. This could not be seen in larvae fed either exp. Cirripeds or the Rotifer/*Artemia* diet, even though both groups had significant changes in cell height at different sampling points during the experiment. By the end of the first live feed period at 18 dph, the mean enterocyte height of larvae fed either copepods or rotifers had not had any significant change, but the enterocyte height of larvae fed exp. Cirripeds had decreased ($p < 0.05$). At this sampling point, both groups fed copepods had significantly taller mean enterocytes at 27.7 ± 0.5 µm and 29.0 ± 0.8 µm for larvae assigned the Copepod/*Artemia* diet and Copepod/Cirriped diet, respectively, than larvae fed either exp. Cirripeds at 24.1 ± 0.2 µm ($p < 0.05$, Student's t-test) or rotifers at 23.2 ± 1.4 µm ($p < 0.05$, Student's t-test). For three out of four treatments, the switch to the second live feed was followed by a significant increase in mean enterocyte height. This happened in larvae fed both Copepods/*Artemia*, exp. Cirripeds and Rotifers/*Artemia*, where intestinal epithelial height had increased to 35.6 ±

1.3 µm ($p < 0.05$), 36.4 ± 0.8 µm ($p < 0.05$) and 31.9 ± 1.1 µm ($p < 0.05$), respectively. No change in epithelial had occurred in larvae fed Copepods/Cirripeds. The latter group had significantly shorter mean enterocytes at 30.6 ± 1.0 ($p < 0.05$) than the mean heights found in both the Copepod/*Artemia* and exp. Cirriped treatments. These two groups also had significantly taller mean enterocytes than larvae fed Rotifers/*Artemia* ($p < 0.05$). After completed weaning, the mean gut epithelial height of larvae fed Copepods/*Artemia* and Rotifers/*Artemia* remained the same as after the second live feed period, while larvae fed exp. Cirripeds experienced a reduction in cell height to 32.6 ± 1.0 µm ($p < 0.05$) and larvae given the Copepod/Cirriped diet had an increase in cell height ($p < 0.05$) ending up at a mean enterocyte height of 39.4 ± 1.5 µm which was significantly larger than all other groups ($p < 0.05$).

Before onset of start-feeding, the mean gut microvillus length of all larvae was 1.9 ± 0.04 µm. Changes in the length of the intestinal brush border were however seen after all three feeding periods, where the microvilli underwent both lengthening and degradation according to diet. Larvae fed copepods increased in mean microvillus length during the first feeding period, but only significantly so in the Copepod/*Artemia* treatment, where the mean brush border length was significantly larger at 2.2 ± 0.1 µm than larvae fed either rotifers with a mean length of 1.8. ± 0.1. µm ($p < 0.05$) or exp. Cirripeds with a length of 1.8 ± 0.04 µm ($p < 0.05$) at this time point. After switching to the second live feed, the mean microvillus height of larvae fed Copepods/*Artemia* again increased to be significantly larger than that of all other treatments at 2.8 ± 0.1 µm ($p < 0.05$), but after completed weaning the brush border had decreased significantly in length to 2.2 ± 0.1 µm ($p < 0.05$). Larvae fed Copepods/Cirripeds, on the other hand, did not have a significant lengthening of microvilli until post weaning, where the mean microvillus height had increased to be significantly larger than all other treatments at 2.7 ± 0.2 µm ($p < 0.05$). In larvae fed exp. Cirripeds, no significant change was seen over the course of the experiment ($p < 0.05$), leading to this group always having significantly shorter mean microvilli than at least one treatment after onset of startfeeding. The microvillus length development of larvae fed the Rotifer/*Artemia* diet showed a similar pattern, with the exception of a lengthening of microvilli to 2.3 ± 0.1 µm after switching to the second live feed period ($p < 0.05$). Here, the mean microvillus length was significantly larger than for exp. Cirripeds at 1.9 ± 0.1 µm ($p < 0.05$) but smaller than for Copepod/*Artemia* ($p < 0.05$). After

this, the brush border length was significantly reduced again to a mean length of 2.0 ± 0.03 µm post weaning ($p < 0.05$).

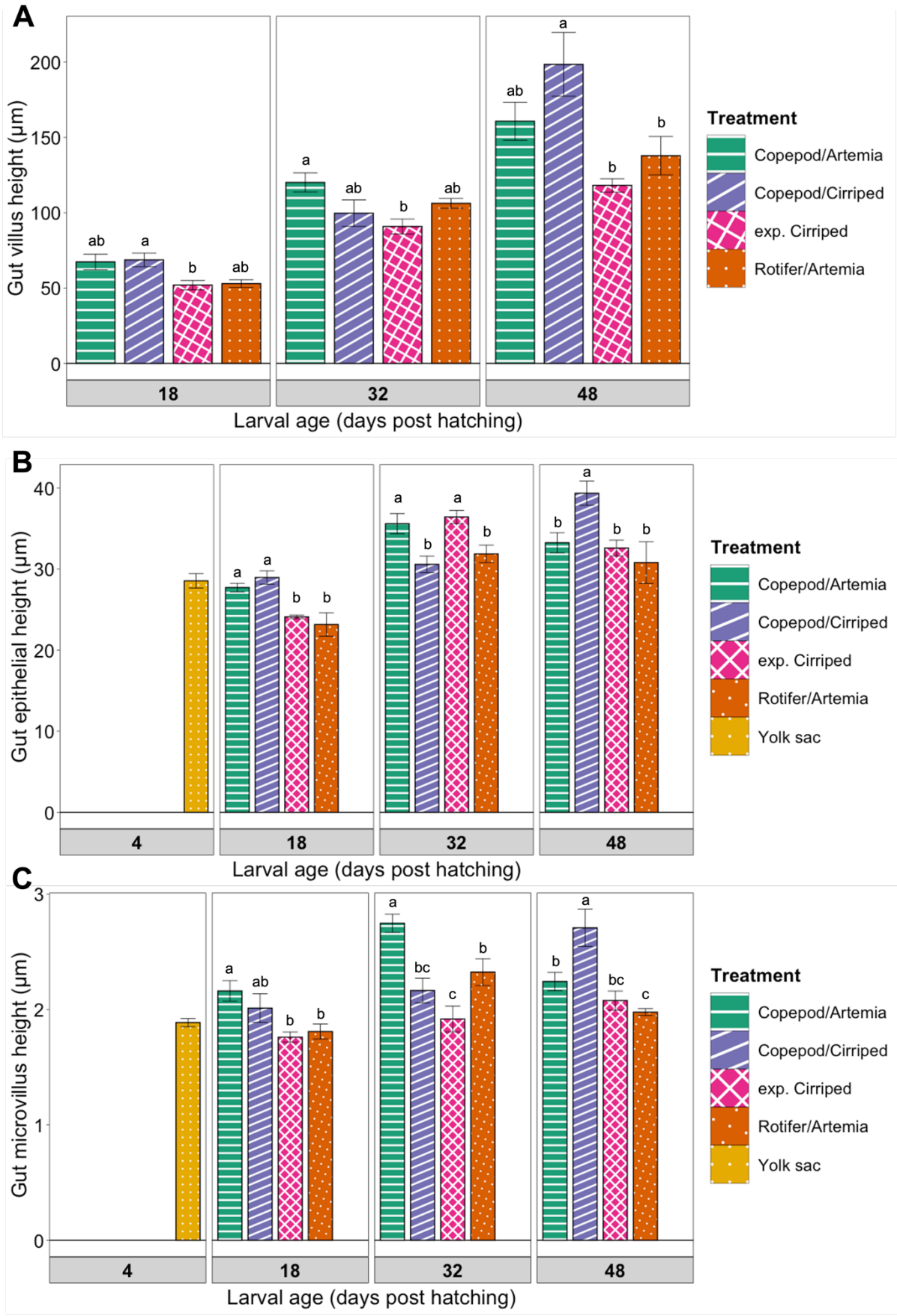


Figure 3.17. A) Mean gut villus height (µm), B) mean gut epithelial height (µm) and C) mean gut microvillus height (µm) over time (days post hatching, dph) in ballan wrasse larvae fed different start feeding diets. 4; 18; 32

and 48 dph correspond to the different feeding periods of the larvae (yolk sac; first live feed; second live feed; and formulated feed, respectively). Error bars indicate ± SE for each mean value (n=5-40). Significant differences (p < 0.05) are denoted by unequal superscripts. Distinctly formed gut villi of ballan wrasse larvae were only seen from 18 dph onward, therefor values for earlier sampling dates are not shown in A).

Both gut villus height, epithelial height and microvillus height were positively correlated to standard length in the ballan wrasse larvae (Fig. 3.18). For villus height, this relationship was highly significant with a correlation coefficient close to 1 (Fig. 3.18. A., $r = 0.94$, $p < 0.05$), and fitting a linear regression model taking dietary effects into account revealed significant differences in the slopes for Copepod/Cirriped fed larvae and Rotifer/*Artemia* fed larvae ($p < 0.05$). In the Copepod/Cirriped treatment, villus height was expected to increase with 24.8 µm mm^{-1} increase in SL ($R^2 = 0.96$) while in the Rotifer/*Artemia* group, the villus height increase was estimated at 16.6 µm mm^{-1} increase in SL ($R^2 = 0.87$). For the exp. Cirriped and Copepod/*Artemia* groups, an increase of 21.1 and 20.7 µm in villus height, respectively, mm^{-1} increase in SL was expected, and did not differ from any of the other groups.

The gut epithelial height ($r= 0.54$, $p < 0.05$) and microvillus height ($r = 0.58$, $p < 0.05$) were also expected to increase with increased SL, where for the relationship between epithelial height and SL (Fig. 3.18. B), a difference in slopes was found between larvae from the Rotifer/*Artemia* treatment and larvae from both the Copepod/Cirriped and exp. Cirriped treatments. The slopes of the two latter groups were steeper than the slope of the former, where epithelial height was predicted to increase by 2.1 and 1.6 µm mm^{-1} increase in SL for the exp. Cirriped ($R^2 = 0.29$) and Copepod/Cirriped larvae ($R^2 = 0.57$), respectively, while the epithelial height of Rotifer/*Artemia* larvae was expected to increase by 0.7 µm mm^{-1} increase in SL ($R^2 = 0.1$). The relationship between gut microvillus height and SL was also affected by treatment, where the slopes of larvae fed Copepods/*Artemia* and Copepods/Cirripeds was steeper than for larvae fed Rotifers/*Artemia*, where an increase of 0.1, 0.1 and 0.04 µm mm^{-1} increase in SL was expected for larvae from the Copepod/Artemia ($R^2 = 0.33$), Copepod/Cirriped ($R^2 = 0.58$) and Rotifer/*Artemia* ($R^2 = 0.03$) treatments, respectively (Fig. 3.18. C.). Larvae from the exp. Cirriped treatment had a slope that was not significantly different from 0.

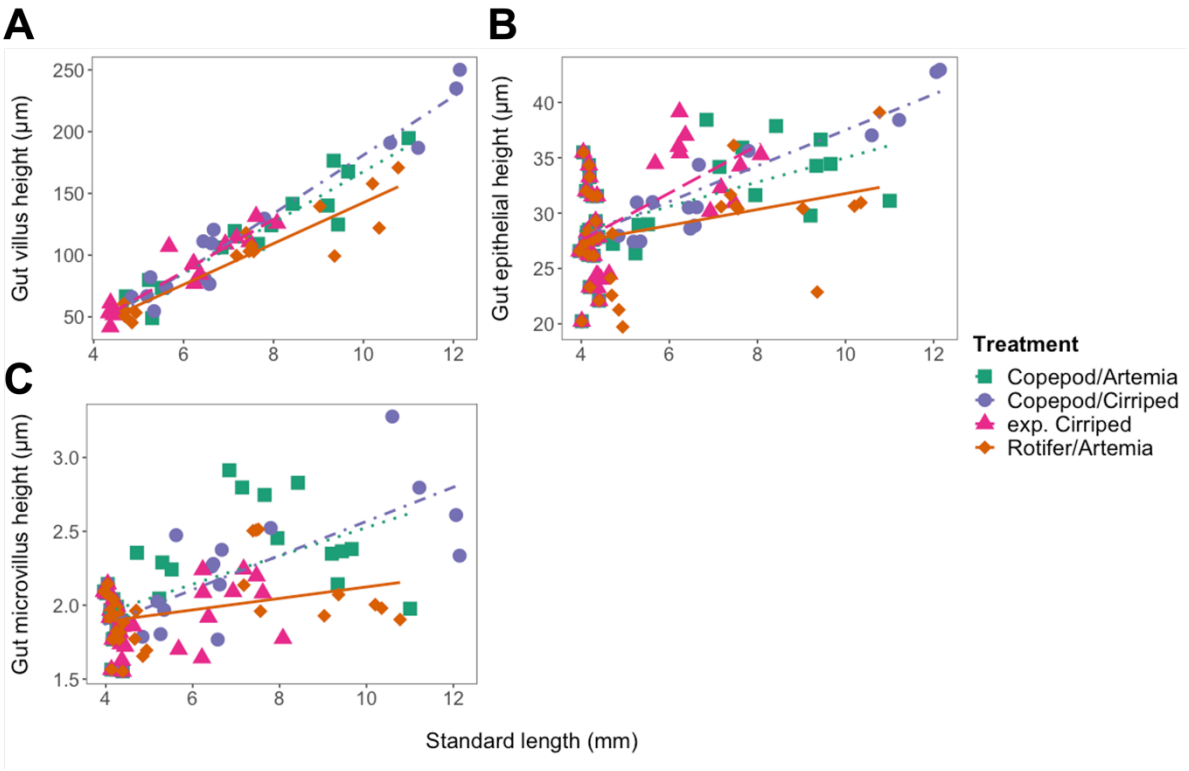


Figure 3.18 Relationship between standard length (mm) and A) gut villus height (µm); B) gut epithelial height (µm) and C) gut microvillus height in ballan wrasse larvae fed different start feeding diets. When correlating villus height and epithelial height to SL, significant correlations were found in all treatments. For the relationship between microvillus height and SL, exp. Cirriped had a slope not significantly different from 0.

Correlating villus height and epithelial height to DWI gave a significant result ($p < 0.05$), but when comparing the slopes none were significantly different from 0. Nonetheless, for the pooled data, both villus height and epithelial height were expected to increase with an increase in mean DWI.

All gut parameters were also correlated to each other (Fig 3.20.) indicating that a lengthening of either gut villus height, epithelial height or microvillus height was also followed by a lengthening of any other measured gut parameter. For all relationships, however, one or more treatments had a non-significant correlation or a slope not different from 0.

Correlating gut epithelial height to gut villus height (Fig 3.20. A., $r = 0.75$, $p < 0.05$) showed differences in slopes between the Copepod/Cirriped fed larvae and all other treatments ($p < 0.05$ for all comparisons): in larvae from the Copepod/Cirriped treatment, villus height was expected to increase by 11.7 µm μm^{-1} increase in epithelial height ($R^2 = 0.94$), while the other

groups had significantly lower slopes where larvae fed the Rotifer/*Artemia* and exp. Cirriped diets were expected to increase by 5.7 µm (R^2 = 0.62), and 4.1 µm (R^2 = 0.59) in villus height μm^{-1} increase in epithelial height, respectively (Fig. 3.20. A.). For the Copepod/*Artemia* treatment, the slope was not significantly different from 0. The relationship between gut villus height and microvillus height (r = 0.48, p < 0.05) was not affected by diet, where the villus height was predicted to increase by 63,2 µm μm^{-1} increase in microvillus height (R^2 = 0.23) but only the Copepod/Cirriped group had a slope coefficient significantly different from 0. Lastly, correlating gut epithelial height to gut microvillus height yielded a positive relationship (r = 0.56 p < 0.05) that was not affected by treatment, where the epithelial height was expected to increase by approximately 8.0 µm μm^{-1} increase in microvilli height (R^2 = 0.24, Fig 3.20. B). The slope of exp. Cirriped fed larvae was however not significantly different from 0.

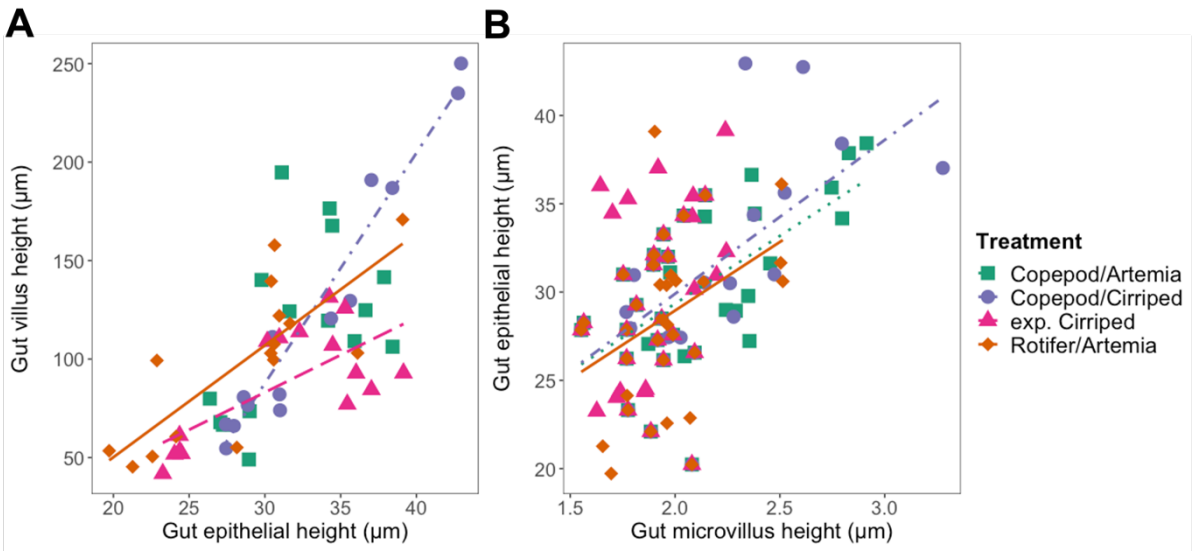


*Figure 3.19 Relationship between gut epithelial height (µm) and A) gut villus height (µm); B) gut microvillus height (µm). Relating villus height to epithelial height revealed a significant correlation in all treatments but Copepod/*Artemia *fed larvae. The relationship between gut epithelial height and microvillus height was only present in the Copepod/*Artemia *and Copepod/Cirriped treatments.*

Comparing the measured parameters of gut and liver showed several significant relationships. Gut villus height, epithelial height and microvillus height were all correlated to hepatocyte cell area size (Fig 3.21) as well as the area fraction of hepatic vacuoles (Fig 3. 22). Again, these correlations were not present in all treatments, as some slopes not significantly different 0 were found for all relationships.

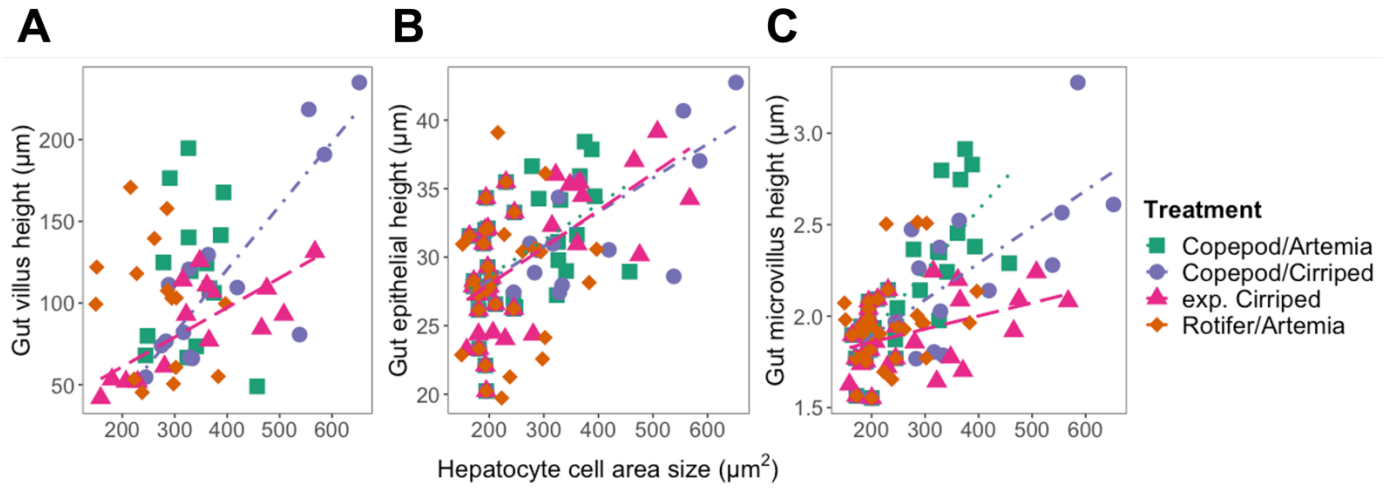


*Figure 3.20: The relationship between hepatocyte cell area size (µm²) and gut microvillus height (µm). The relationship was only significant in larvae fed either Copepods/*Artemia*, Copepods/Cirripeds or exp. Cirripeds.*

The gut villus height, epithelial height and microvillus height were all positively correlated to hepatocyte cell area size, where all relationships were influenced by diet (Fig 3.21.). When correlating hepatocyte cell size to villus height ($r = 0.49$, $p < 0.05$), larvae fed exp. Cirripeds and Copepods/Cirripeds were expected to increase by 3.1 (R^2=0.53) and 1.7 µm² (R^2=0.65) in hepatocyte cell size for each µm increase in villus height, respectively (Fig. 3.20. A.) Likewise, for each increase of 1 µm in gut epithelial height, the hepatocyte cell size of Copepod/Cirriped, exp. Cirriped and Copepod/*Artemia* fed larvae was expected to increase by 18.3 (R^2=0.44), 13.9 (R^2=0.36) and 9.0 (R^2=0.21) µm², respectively ($r=0.59$, $p < 0.05$) and the slope for the Copepod/*Artemia* treatment was lower than for those of the Copepod/Cirriped and exp. Cirriped treatments (Fig. 3.20. B.). For the correlation between hepatocyte size and microvillus height, the slopes of Copepod/Cirriped fed larvae and Copepod/*Artemia* differed, where the former had a steeper slope than the latter (Fig. 3.20. C.). Here, an increase of 293.2, 247.7 and 188.7 µm² in hepatocyte cell area size was predicted for each µm increase in microvillus height for the Copepod/Cirriped, exp. Cirriped and Copepod/*Artemia* treatments, respectively ($r=0.57$, $p < 0.05$). For all relationships, larvae fed Rotifers/*Artemia* had a slope that was not significantly different from 0. This also applied for the Copepod/*Artemia* in the case of the correlation between villus height and hepatocyte size.

The hepatic vacuole area fraction was predicted to increase with increasing height of all gut parameters. For the correlation between vacuole fraction and gut villus height, the slope of larvae fed exp. Cirripeds was larger with an increase of 0.38 % in vacuole fraction µm^{-1}

increase in villus height than that of the Copepod/Cirriped treatment where an increase of 0.28% μm^{-1} increase in villus height was predicted (Fig. 3.21.). Here, the slope of larvae fed Rotifers/*Artemia* was not significantly different from 0 (Fig 3.21.). Relating both gut epithelial height and gut microvillus height to vacuole area fraction revealed that only larvae fed Copepods/Cirripeds had slopes that were significantly different from 0. However, for the pooled data set, vacuole area fraction was expected to increase by 1.0 (R^2=0.36) and 15.0% (R^2=0.13) for each µm increase epithelial height (r=0.36, $p < 0.05$), and microvillus height (r=0.28, $p < 0.05$), respectively.

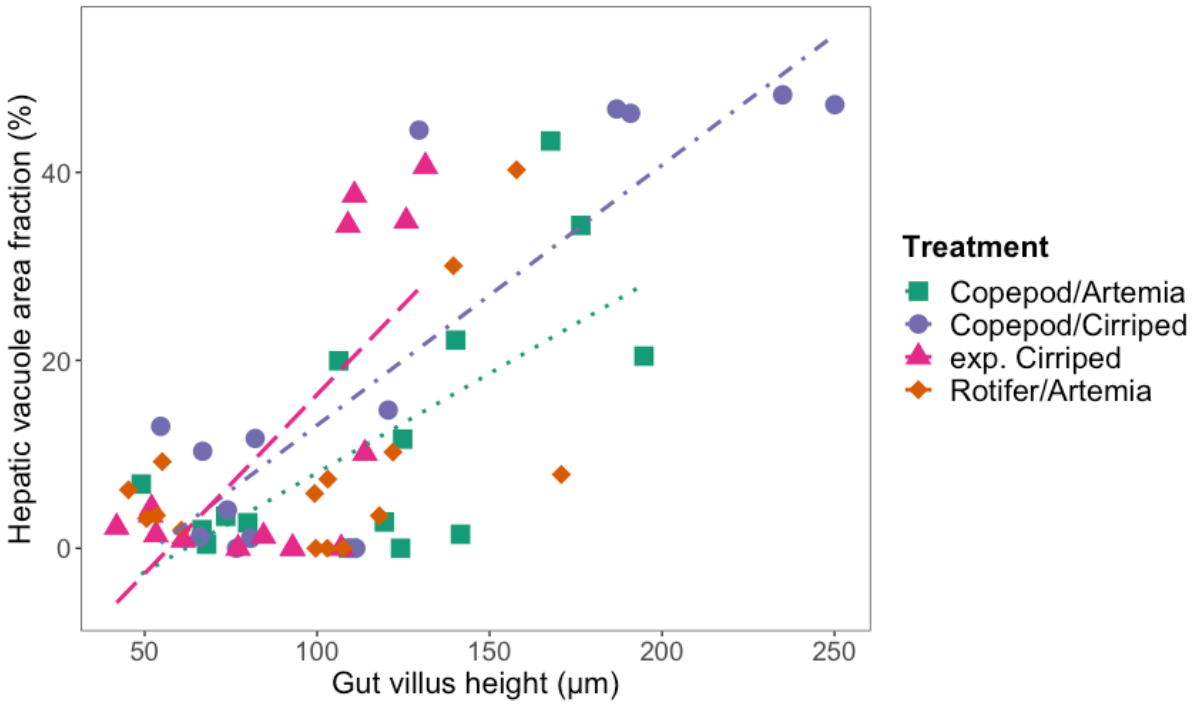


*Figure 3.21: The relationship between hepatic vacuole area fraction (%) and gut villus height (µm) in ballan wrasse larvae fed different start feeding diets. The slope for the exp. Cirriped treatment was significantly larger than for the Copepod/Cirriped treatment. For larvae fed Rotifers/*Artemia*, the slope was not significantly different from 0.*

4. Discussion

4.1 Growth and survival of ballan wrasse larvae

Three out of four dietary groups, namely those groups fed Rotifers/*Artemia*, Copepods/*Artemia* and Copepods/Cirripeds had a similar development of DW and SL, while the experimental Cirriped group had a significantly lower mean DW and SL from the start of the second live feed period and throughout the remainder of the experiment. Notably, a significantly lower DW was also observed in this group after the first live feed period, where the DW was similar to the yolk sac stage, and thus no mean weight had been gained from onset of startfeeding. This was also reflected in the DWI, where the growth rate was slightly negative in this treatment, whereas all other treatments had a positive growth rate where weight was gained during the course of the first live feed period. Another notable result for the DWI during this period was the significantly different growth rates of the two treatments fed copepods. As this difference could not be explained by prey quality or density, since the copepods were fed from the same reservoir, there may have been some abiotic factors at play. However, investigating tank-wise temperatures, pH and oxygen levels did not reveal any obvious differences in the abiotic parameters. Two possible explanations can be proposed: 1) the density of fish larvae was different in the tanks, as this was only counted at the start and end of the experiment and could have varied throughout, or 2) the microalgal amounts were higher in some larval rearing tanks, allowing the copepod nauplii to feed and survive for a longer time, thus giving a larger concentration of prey animals. The microalgae were also supplied from the same reservoir, but it is possible that e.g. the light conditions were better in some tanks, allowing for a slightly higher microalgal cell density. It is however not possible to conclusively say what caused the variation, as neither prey density or microalgal density was counted in the tanks at any time, and the larval density was only known at the start and end of the experimental period. Growth in our experiment was lower than seen in several other studies on ballan wrasse (Hansen et al., 2013; Piccinetti et al., 2017), but in these studies the temperature was higher during larval rearing which may have increased the growth rate (Jobling, 1997). In another study comparing the growth and survival of ballan wrasse larvae fed with copepods versus rotifers (Øie et al., 2015), higher differences in both DW and SL were observed, where feeding with copepods gave significantly larger larvae compared to

feeding with rotifers. This was not seen in our study, but, as mentioned, lower SL and DW were seen in larvae fed exp. Cirripeds. A possible explanation for the high DW and SL seen in Rotifer/Artemia fed larvae in our experiment compared to Øie et al. (2015), could be the high mortality of larvae fed rotifers during the first live feed period. Thus, only the largest larvae may have remained, giving a size-specific mortality where true effects of diet are masked due to disappearance of the small larvae (Perez-Dominguez & Dahm, 2011). However, as the dead larvae in our experiment were not measured or weighed, this cannot be known for sure. Growth rate during the present experiment differed from Øie et al. (2015), who found that feeding with copepods during the first live feed period resulted in larger mean DWI compared to feeding with rotifers. In our study, one of the groups fed copepods had a similar growth rate to larvae fed rotifers during the first live feeding period, and growth rates remained similar between the copepod fed and the rotifer fed larvae throughout the live feeding periods. In addition, while the DWI over the entire experiment was not significantly different between groups in the study of Øie et al. (2015), the larvae receiving Rotifers/Artemia in our study had a significantly higher mean DWI than both the exp. Cirriped and Copepod/Cirriped dietary groups. This may again have reflected selection for large individuals caused by mortality of small larvae in the former group, while the larvae fed Copepods/Cirripeds had a higher population with a wider size distribution and larvae fed exp. Cirripeds were smaller than all other larvae in general.

Feeding with copepods however gave marked effects on survival compared to feeding with experimental Cirripeds or Rotifers/*Artemia*, where larvae fed copepods as their first diet had significantly higher survival compared to the other groups. For all groups, the largest mortality was seen during the transition from mixed endogenous and exogenous to solely exogenous feeding, at approximately 14 dph, whereafter there were few larvae left in the groups fed either rotifers or exp. Cirripeds as their first diet. The high mortality could be explained by several different factors, such as a mismatch between prey size and larval mouth, and thus less ingested feed, or a suboptimal nutritional composition of the offered prey, both leading to starvation and mortality (Conceição et al., 2010; Devries et al., 1998; Yúfera, 2011). In the case of exp. Cirripeds, the most likely explanation is that of the prey size, as the first live feed offered consisted of a mixture of 50% *Balanus crenatus* and 50% *Semibalanus balanoides* in terms of number of prey, where the latter species is larger than the first. Following suspicions of larval gape size vs prey size discrepancy in this diet, a small scale experiment was

conducted to measure length and width of cirriped nauplii offered in the start-feeding experiment. There, it was found that the larger nauplii had a mean length of 339 ± 21.8 µm and width of 194 ± 22.9 µm, while the smaller nauplii had a length and width of 267 ± 18.8 µm and 154 ± 26 µm, respectively. In contrast, nauplii of *Acartia tonsa* range from <125-179 µm in body length during n1-n3 stages and 180-279 µm during n4-n6 stages (Maren Ranheim Gagnat, CFEED AS, pers. comm., 2021). During the start-feeding experiment, the copepod fed larvae were offered n1-n3 stages from 4-11 dph and n4-n6 stages from 12-18 dph, which may have been a more appropriate size for the larvae. Larvae fed rotifers, on the other hand, likely received a prey that had a suboptimal nutritional value compared to the larval requirements (Conceição et al., 2010). It is well established that feeding with copepods may improve both growth and survival in marine fish larvae compared to feeding with rotifers (Betancor et al., 2019; Busch et al., 2010; Cassiano et al., 2011; Koedijk et al., 2010; Payne et al., 2001; Rajkumar & Vasagam, 2006; Støttrup et al., 1998). This has also been demonstrated in ballan wrasse larvae (Romundstad, 2015; Øie et al., 2015). The effect is mainly attributed to the beneficial nutritional composition of copepods, including high levels of both HUFA, PL, proteins and FAAs, including taurine, and important micronutrients (Bell et al., 2003; Hamre et al., 2008; Karlsen et al., 2015; van der Meeren et al., 2008), while rotifers need to be enriched to achieve the same levels (Lubzens & Zmora, 2003; Mæhre et al., 2013; Rainuzzo et al., 1997). The enrichment process may also lead to an excess of dietary lipids and suboptimal dietary protein content (Conceição et al., 2010), causing imbalance between the nutritional composition of the enriched rotifers and the larval requirements. Analysis for total lipid and fatty acid profiles revealed differences between the feed types (Malzahn et al., 2022), where although the total lipid amount was higher in both the supplied rotifers and *Artemia*, both copepods and exp. Cirripeds had higher proportions of unsaturated fatty acids. Copepods also contained the highest amount of omega-3 fatty acids, while cirripeds had the highest EPA/DHA ratio. A high amount of EPA in relation to DHA has been reported to result in poor viability and decreased growth performance of fish larvae in some studies (Rodriguez et al., 1997; Watanabe, 1993), and may have contributed to the high mortality and low growth seen in the exp. Cirriped group.

4.2 Effects of diet on liver and gut histology

In the present study, several effects were observed in the histological characteristics of the gut and liver in ballan wrasse larvae fed different start-feeding diets. Along with differing growth and survival between treatments, this indicated a difference in the quality as feed for these larvae. Although the highest survival was seen in both treatments fed copepods, and growth appeared mostly similar between these two groups, some differences were seen in their liver and gut morphology, especially after weaning to formulated feed. Furthermore, some of the histological findings in the gut and liver of larvae fed both exp. Cirripeds or Rotifers/Artemia, particularly at the later stages of the experiment, seemed to contradict the growth and survival patterns seen in these groups.

Larvae from both copepod treatments seemed to have had a faster development of the liver, where the tissue appeared more cohesive with hepatocytes forming a pattern around compacted sinusoids after the first live feed period, at 18 dph, compared to larvae fed exp. Cirripeds and Rotifers/Artemia where the tissue seemed less structured with sinusoids that appeared dilated. In addition, the hepatocyte nuclei of larvae from the exp. Cirriped treatment decreased significantly in size from the yolk sac stage until after the first live feed period. A reduced hepatonuclear size has been reported to be an indicator of malnutrition or starvation in several species, including cod, gilthead seabream and pejerrey (Power et al., 2000; Strussmann & Takashima, 1990; Wold et al., 2009), likely due to altered fatty acid metabolism resulting from an unbalanced diet (Gisbert et al., 2008). On the other hand, an increased hepatonuclear size was related to faster growth in common whitefish (Segner et al., 1988). In the latter study, this was suggested to be due to an enhanced hepatocellular metabolism. Relating hepatocyte nucleus area size to DWI did not give any significant results in the present study. However, it is notable that for the time interval 4-18 dph the exp. Cirriped treatment had a negative mean DWI, indicating a weight loss, in contrast to all the other treatments. A high mortality was also observed in both the exp. Cirriped treatment and Rotifer/Artemia treatments during the same time period. In addition, at this particular sampling, larvae fed exp. Cirripeds had a mean DW lower than all other treatments (Appendix 4). This suggests that larvae fed exp. Cirripeds were subject to starvation during the first live feed period, resulting in both high mortalities, low DW and DWI as well as reduced metabolic

capacity in the form of shrunken hepatocyte nuclei. After switching to the second live feed, the starvation effects seen in the liver of exp. Cirriped fed larvae seemed to diminish as both hepatocyte nuclei and hepatocyte cell size increased significantly. After weaning to formulated feed, larvae from this treatment had the largest mean hepatocyte nuclei of all treatments. This could suggest some beneficial effect of the second live feed offered in this treatment, where the effects of feeding this particular prey resulted in a higher metabolic capacity as well as energy storage, as these larvae also had a moderate to large amount of hepatic vacuoles post weaning. This is supported by the fact that larvae fed firstly on copepods followed by cirripeds showed the largest hepatocytes and amount of vacuoles of all treatments at the end of the experiment. Large hepatocytes and a high amount of vacuoles in the liver is associated with a high capacity for energy assimilation and storage, reflecting a good nutritional condition (Gisbert et al., 2008). However, it could also be indicative of an increased nutrient assimilation efficiency with age, or development of defense mechanisms to better withstand starvation in underfed larvae (Green & McCornick, 1999).

Previous studies on gut and liver histology of ballan wrasse fed rotifers or copepods as the first live feed (Gagnat, 2012; Romundstad, 2015) gave different results from this study in terms of vacuoles, where Gagnat (2012) found that the amount of vacuoles varied both within and between treatments and no clear correlation to either SL or diet was found. Romundstad (2015) on the other hand, saw the highest amount of vacuoles in larvae reared on rotifers, which contrasts with the findings in this study. Notably, both the aforementioned authors sampled for histological analysis up until 15 and 21 dph, and in the present study, significant differences in vacuole area fraction in the liver were not seen until after weaning to formulated feed. Romundstad (2015) linked the amount of vacuoles in rotifer fed larvae to the high carbohydrate content of rotifers compared to copepods, however in the present study the largest amounts of vacuoles irrespective of diet was seen at 48 dph where all larvae had been fed the exact same diet for 11 days. Thus, the results presented in this thesis rather suggest that the live feed periods had an effect on later energy storage ability of ballan wrasse larvae. It could appear that feeding with copepods as the first diet resulted in a better developed liver tissue during the first live feed period when comparing with the other treatments. This would then have allowed the second live prey diet of cirripeds to give its full advantages in the form of a well-structured liver containing large hepatocytes with high

amounts of energy reserves. It is, however, unknown what exactly caused this effect as we do not know the specific nutritional composition of neither of the cirriped species offered in the present study, only the total lipids and amount and type of FAs. Although feeding *Artemia* after copepods instead of cirripeds also resulted in what appeared to be healthy, normal liver tissue, none of the measured liver parameters differed significantly from either of the exp. Cirriped or Rotifer/Artemia diets, and visually, the liver morphology appeared similar to that of the exp. Cirriped larvae. In larvae fed Rotifers/Artemia, on the other hand, both sinusoidal dilation and lipid droplets were observed in the liver tissue both at the end of the second live feed period and after completed weaning, where both the hepatocyte nucleus size and hepatocyte cell size had decreased. Here, mean nucleus size was smaller than in larvae fed Copepods/Cirripeds and mean hepatocytes were smaller than for all other treatments. Additionally, the nuclei, nucleoli and hepatocyte borders in the liver tissue of Rotifer/Artemia larvae all appeared small and indistinct, and the surface area fraction of hepatic vacuoles was lowest in this group. These can all be considered to be indicators of poor nutritional condition (Gisbert et al., 2008), where sinusoidal dilation suggests biliary dysfunction (Diaz et al., 1998), reduced hepatonuclear size is associated with starvation (Strussmann & Takashima, 1990), reduced hepatocyte size and vacuole fraction suggests depletion of glycogen reserves (Gisbert et al., 2008), while the presence of lipid droplets in the liver can either indicate a good nutritional condition as they represent energy reserves (Gisbert et al., 2004; Sarasquete et al., 1995) or indicate a disturbance in hepatocellular lipid transfer and metabolism (Segner & Witt, 1990; Wold et al., 2009). As the aforementioned biomarkers of degraded or average liver condition were present in the Rotifer/*Artemia* fed larvae in this study, the most likely explanation for the lipid droplets in the liver was a poor ability of these larvae to metabolise lipids and thus an accumulation of lipid in the liver followed. The first lipid droplets were observed after the second live feed period, when *Artemia* had been fed. When comparing the total lipids in the different diets, it was found that both rotifers and *Artemia* contained a high amount of total lipids, out of which saturated fatty acids were most abundant. Copepods and cirripeds contained a lower quantitative amount of lipids, but a higher fraction of unsaturated fatty acids including DHA and EPA (Malzahn et al., 2022). Although no analysis was done on the relative proportion of EFAs bound in PL versus NL in this study, marine copepods contain a higher ratio of EFAs bound in PL compared to NL than do rotifers and *Artemia* (van der Meeren et al., 2008). Although enrichment most likely increased the amount of PL in rotifers

and Artemia in the current experiment, the high amount of total lipids may still have offset the beneficial effect of PL as a high total lipid:PL ratio is not optimal for developing fish larvae (Cahu et al., 2003). Although currently there is no scientific documentation on the lipid classes present in cirripeds, the commercial producer has claimed that it is rich in DHA and EPA contained in PLs (Tokle, 2021). Thus, it could be that in the present study, the fraction of NLs to PLs was suboptimal in the Rotifer/*Artemia* diet, leading to a reduced uptake and transport of lipids in larvae from this treatment. This has also been seen in other species such as cod, gilthead seabream and European sea bass (Gisbert et al., 2005; Salhi et al., 1999; Wold et al., 2009)

An accumulation of lipid droplets was observed in the enterocytes of larvae from the Rotifers/*Artemia* treatment after weaning to formulated diets, whereas no such observations were made in larvae from any other treatment. This further supports the possibility of a disturbed lipid metabolism and transport in this treatment, as seen for gilthead sea bream (Izquierdo et al., 2000; Salhi et al., 1999). In the liver, lipid deposition in the enterocytes may also be indicative of well-fed larvae (Gisbert et al., 2008), but when observed together with the other histological findings, it seems unlikely that the lipid droplets in the gut observed in this study indicates anything other than a suboptimal feed. Similarly to the liver, gut tissue appeared to have a better nutritional condition from start-feeding until completion of the first live feed period in larvae fed copepods compared to exp. Cirripeds or rotifers. Gut epithelial height was thicker in both groups fed copepods than the other treatments, in addition to longer and denser microvilli and longer villi in at least one copepod fed group compared to at least one of the other treatments. In addition, goblet cells appeared earlier in the copepod fed larvae, being present in the foregut at 18 dph while no goblet cells were seen in the gut of rotifer and exp. Cirriped fed larvae at the same age. The appearance of goblet cells may be due to recent feeding, but can also be size-dependent, appearing gradually in increasing numbers during ontogeny (Boulhic & Gabaudan, 1992; Di Pane et al., 2020; Gisbert et al., 2004). After the second live feed period, the switch to *Artemia* induced a large increase in surface area of the gut in larvae from the Copepod/*Artemia* treatment, where both villus height and epithelial height were larger than at least one other treatment. In addition, this group had longer microvilli than all other treatments. After weaning onto formulated feed, however, a degradation of microvilli was seen in this treatment. This was

also seen in larvae fed Rotifers/*Artemia*. Interestingly, larvae from the Copepod/Cirriped treatment did not display lengthening of any gut parameters from the first to the second live feed period, although weaning onto formulated feed resulted in both longer microvilli and thicker epithelium in this treatment compared to all other groups, while villus height was larger than in both the exp. Cirriped and Rotifer/*Artemia* treatments. For the exp. Cirriped, a thinning of the gut epithelial height was seen from 4 to 18 dph, concurrent with the liver parameters and low growth indicating starvation in this group. At this stage, the epithelium of the Rotifer/*Artemia* fed larvae had also thinned, although fewer effects were seen in this group regarding growth or any measured liver parameters, apart from the liver morphology appearing similar to that of larvae fed exp. Cirripeds. Some alterations of the gut histological characteristics as a response to starvation or suboptimal diets include reduced gut epithelial height as well as number and size of intestinal villi, in addition to degradation of microvilli (Gisbert et al., 2008). Reduced epithelial height could be linked to starvation or poor diet in at least one treatment in this study, as has also been seen in other species such as red porgy, California halibut, cinnamon clownfish and European eel (Diaz et al., 2013; Gisbert et al., 2004; Green & McCornick, 1999; Rodríguez et al., 2005). In addition, the degraded microvilli seen in the larvae fed *Artemia* after switching to inert diets may also have indicated suboptimal feeding or starvation in these groups, as has previously been observed in cod and turbot (Kjørsvik et al., 1991; McFadzen et al., 1994), suggesting that feeding *Artemia* gave unfavorable effects in the form of a restricted absorptive capacity. Even so, the brush border was still present and appeared dense in these treatments, and did not appear severely altered or degraded. Thus, the changes were likely not irreversible. The gut villus height seemed less indicative of degraded or average nutritional condition as it increased over time for all treatments. It could be that the villus height was more influenced by larval size, as evidenced by the strong correlation to SL. It should, however, be noted that villus height was only measured on distinctly formed villi, and there could be as few as 2 of these per larva in any treatment (Appendix 9), especially after the first live feed period, thus possibly giving a false image of the actual level of folding in the intestinal mucosa. In addition, at the last sampling point, i.e. after completed weaning, a large amount of shrinkage in the gut tissue was found in all treatments, likely affecting the measured gut parameters. Consequently, results of the gut histology at this particular sampling date should be interpreted with caution.

Although almost all measured parameters in the gut and liver were related to SL, with the exception of hepatocyte nucleus area size, large variations between treatments were present in several of the slopes and amount of variability explainable by the correlation, as determined by the R^2-value. In the liver, correlations between the hepatocyte cell area size and vacuole area fractions were significant. The relationships between measured liver parameters and SL seemed stronger in the two treatments including cirripeds, whereas the Copepod/*Artemia* treatment had slightly lower slopes. For both the significant correlations detected here, the Rotifer/*Artemia* fed larvae had slopes that were not significantly different from 0, indicating no real relationship. Thus, it seemed that an increase in larval size could predict a simultaneous increase in hepatocyte cell area size and vacuole area fraction in the liver, but only for larvae fed natural zooplankton. This could tie in with the ability to assimilate and store nutrients in the liver as discussed earlier, where this seemed to increase over time for all treatments, but particularly in those larvae fed cirripeds, and less so for larvae fed Rotifers/*Artemia*. No correlation was found between DWI and any measured liver parameter, thus the histological characteristics of the liver did not appear to be related to growth rate. Although correlating hepatocyte cell size to both vacuole fraction and hepatocyte nucleus size gave significant results, the slopes were only significant for one treatment per correlation. Therefore, these results are somewhat questionable, and deducing the true nature of them is difficult.

For gut parameters, the strongest relationship to SL was found for villus height, where all treatments had a slope significantly different from 0 and the variability in villus height was highly explainable by variation in SL, with R^2 ranging from 0.87 (lowest) to 0.96 (highest). For both the gut epithelial height and microvillus height, larvae fed Rotifers/*Artemia* had significantly lower slopes than larvae fed Copepods/*Cirripeds*, with less of the observed variability explainable by the correlation. For larvae fed exp. Cirripeds, the slope was not significantly different from 0 when correlating SL to microvillus height. Again, it could appear that well-fed larvae displayed a stronger relationship between size and gut surface area than those who had displayed signs of starvation and/or a suboptimal diet. Although both the villus and epithelial height were significantly correlated to DWI, none of the slopes were significantly different from 0. This makes it difficult to draw a line between these parameters. Not surprisingly, all gut parameters were also correlated to each other, where a lengthening

of one measured parameter often was followed by a lengthening of the two other, however only larvae from the Copepod/Cirriped treatment had slopes significantly different from 0 for all correlations, where all the other treatments seemed to have at least one gut parameter unrelated to another. Thus, a distinct pattern was not found here.

Relating the measured histological parameters of the gut and liver to each other revealed significant correlations between both the hepatocyte cell area size as well as vacuole area fraction to all gut parameters. Apparently, an increase in gut surface area was followed by an increased ability to store nutrients in the form of vacuoles and larger hepatocytes. Again, these relationships varied between treatments, but for all correlations, larvae fed Rotifers/*Artemia* had a slope that was not significantly different from 0. In addition, it seemed that the correlations were stronger in larvae fed cirripeds than larvae from the Copepod/*Artemia* group. However, the amount of variation explainable by the correlations varied and were sometimes low. Hence, although there seemed to be some interaction between surface area of the gut and nutrient storage capability in the liver, the nature of the relationships are still unclear.

4.3 Uncertainties and limitations of the study

The high amount of variation seen in gut and liver histology in the present study can at least partly be explained by small sample size, which for all treatments was n=5 after the yolk-sac stage. This makes it hard to interpret the observed differences with absolute certainty, although together with observed patterns in growth and survival an indication of the nutritional condition can be given. In addition, sample sizes of the DW and SL varied considerably toward the end of the experiment, where the high mortality only made it possible to sample 5-6 larvae in total from the exp. Cirriped and Rotifer/*Artemia* treatments at 42 dph. In contrast, 30 larvae in total were sampled for each of the Copepod/*Artemia* and Copepod/Cirriped treatments. Samples for DW and SL in the exp. Cirriped and Rotifer/*Artemia* treatments were also not available 48 dph due to the high mortality, thus we do not know the final weight in these treatments, which obviously also leaves room for uncertainty in the growth rate.

As mentioned, results for the measured gut parameters may also have been uncertain at the sampling point of 48 dph, due to the high amount of shrinkage in the gut seen at this stage. This shrinkage likely resulted from fixation artefacts, and altered the structure of the gut tissue. It should also be noted that the hepatocyte cell size was not completely accurate, due to the nature of the formula used for calculation, which only uses the amount of measured liver tissue and the number of measured hepatocyte nuclei together with the area fractions of hepatocyte nuclei and vacuoles to calculate the cell size. This formula does not take into account other components found in the liver, such as sinusoids and bile ducts, leading to an overestimation of the mean hepatocyte cell size.

Sampling bias may also have played a part in introducing uncertainty into the study, as it is possible that when sampling from the larval tank, the fastest and strongest larvae were able to escape, especially at later points of the experiment. In addition, although it would not have been consciously, some bias could have happened when sampling for histology as blinding the study was not possible.

4.4 Future recommendations

Future studies investigating gut and liver histology of ballan wrasse could be focused on a number of different parameters to further elucidate the effects of diet on nutritional condition. The large effects seen post weaning in the present study suggests that further growth and survival of the larvae could have been impacted, thus a feeding trial including the same live feeds but lasting to the juvenile stage or longer would be of interest to further examine the long-term effects of early nutrition in this species. In addition, focus should be on analyzing and publishing the exact nutritional composition, as this not known at present. Feeding cirripeds seemingly had an effect on the ability to both assimilate and store nutrients after weaning to formulated feed, indicating a better utilization of the inert diet than those larvae fed *Artemia*, and it could be of interest to identify the underlying factors behind this effect. The low growth and survival seen during the first feeding period in the exp. Cirriped treatment was suggested to be due to starvation, following a mixture of large and small

experimental cirriped species which likely lead to a high amount of prey that were too large for the ballan wrasse larvae to capture and ingest. Hence, if attempted again, another start-feeding experiment involving exp. Cirripeds as a live feed diet for ballan wrasse larvae should be focused on only administering exp. small cirripeds during the first live feeding stage.

5. Conclusion

The present study showed that feeding ballan wrasse larvae with different live prey organisms resulted in large differences in both growth, survival and histological characteristics of the gut and liver. Feeding with copepods resulted in the best growth, survival and nutritional condition of ballan wrasse larvae, and thus may be a viable option to replace rotifers in aquaculture of this species. The best results were obtained by feeding copepods followed by cirripeds, where a large surface area of the gut as well as high amount of energy reserves in the liver indicated a high metabolic capability and resistance to starvation, as well as a good utilization of the formulated feed. The nutritional condition of the larvae was reflected in the gut and liver histology, with several biomarkers indicating both healthy, well-fed larvae and larvae displaying signs of starvation or suboptimal diets. The live feed organisms offered in this study differed in their quality as live feed for ballan wrasse larvae, where feeding with copepods resulted in a high growth and survival and overall normal, healthy development of the gut and liver tissue, with a following high nutritional condition as displayed by histological biomarkers such as high amounts of glycogen vacuoles, large hepatocytes and a large surface area of the gut. Large differences were however seen between the two copepod fed groups after weaning onto formulated feed, where a shortening of microvilli occurred in those larvae fed Copepods/*Artemia* while larvae fed Copepods/Cirripeds showed a remarkable increase in all gut parameters in addition to larger hepatocytes and amounts of vacuoles in the liver. Feeding with rotifers followed by *Artemia* resulted in a high growth, but large mortalities. Additionally, histopathological signs of degradation were seen here in the form of sinusoidal dilation, small and indistinct hepatocyte cells and nuclei, a lower amount of glycogen vacuoles and lipid droplets in the liver, as well as shortened microvilli, with enterocytes containing accumulated lipid droplets in the gut. Larvae fed the exp. Cirriped diet had both low growth and survival, but this was assumed to be largely due to inadequate size distributions of live

prey organisms offered during the first live feed stage, as the gut and liver histology displayed several characteristics of a healthy tissue after completing the second live feed period.

Although all of the histological parameters except for hepatocyte nucleus area size were related to SL, the relation to DWI was more uncertain. In addition, the correlations between the parameters and SL varied, with several treatments having an apparently weaker relationship between the histology and growth. In general, it seemed that for well-fed larvae, several displayed biomarkers of a good nutritional condition followed along with growth of the larvae, but for larvae fed suboptimal diets, the connection between nutritional condition biomarkers of the gut and liver and size was less evident. However, due to small sample sizes, the nature and strength of the differences in histological parameters as well as the relationships between growth and gut and liver histology shown here should be interpreted with caution.

Future studies could be focused on determining the exact nutritional composition of cirripeds as well as reviewing the long term effects of the first live feed diet given to ballan wrasse larvae on late larval and juvenile growth as well as survival to adulthood.

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Appendices

Appendix 1: Dehydration and embedding procedure in Technovit 7100

Technovit 7100 components

Component 1:	Basic resin 500 ml (monomer)	hydroxyethylmethacrylat (HEMA)
Component 2:	Activator 5 pk. a 1 g powder	benzol peroxide
Component 3:	Hardener II 40 ml	

Dehydration

Dehydration was performed by immersing ballan wrasse larvae in a 3:1 solution of water + Basic Resin for 3 hours at 4 °C. Thereafter, the larvae were transferred to a 2:2 solution of water + Basic Resin, where it was kept for two hours at room temperature.

INFILTRATION FLUID:

50 ml Basic Resin + 0,5 g activator

The infiltration fluid was prepared by dissolving 0.5 g activator in 50 ml Basic Resin using a magnet stirrer. After dehydration, the larvae were immersed in the infiltration fluid and placed on a digital shaker (ROCKER 3D digital, IKA ®, USA) overnight.

POLYMERIZATION FLUID

750 µl unused infiltration fluid	+ 50 µl hardener II
1,5 ml unused innfiltreringsvæske	+ 100 µl hardener II

Either 800 ml or 1,6 ml polymerization fluid was prepared, according to the amount of larvae to be embedded. Disposable tubes and pipettes were used. The polymerization fluid was mixed for 3-5 minutes before being used immediately, as the hardening process was quick.

When embedding, a small amount of polymerization fluid was placed in a PTFE embedding mould before placing the larva into the mould to ensure proper orientation. The mould was then filled with polymerization fluid and covered with a strip of aclar film. The block was allowed to polymerize for 40-120 minutes at room temperature before being removed from the mould.

The blocks were sectioned by use of glass knives. Sections were floated out on water droplets placed on microscope slides, before being dried on a 60 °C hot plate at a minimum of 15 minutes to secure drying and fastening of the section to the slide.

Appendix 2: Staining Technovit 7100 sections with Toluidine Blue or Periodic acid Schiff

The staining protocols were developed at NTNU SeaLab's morphology lab. The described protocol was obtained from Marthinsen (2018) and modified to fit the present study:

A. Toluidine blue (TB)

1. Stain with toluidine blue in saturated borate buffer for 30 seconds.
2. Rinse with distilled water to remove excess stain.
3. Rinse with 70 % ethanol and distilled water.
4. Dry on hot plate (75 °C) for 20 minutes or more.

B. Periodic acid Schiff (PAS)

1. Immerse in periodic acid at 56 °C for 15 or 30 minutes.
2. Stain with Schiff's reagent for 15 minutes.
3. Rinse with running tap water for 2-3 minutes.
4. Stain with Hematoxylin for 5 minutes.
5. Quick immersion in acid alcohol (1% HCl in 70 % ethanol). Repeat 5 times.
6. Rinse with running tap water for 2-3 minutes and dip in distilled water.
7. Dry on hot plate (75 °C) for 20 minutes or more.

Appendix 3: Protocol for cultivation of *Rhodomonas baltica* at SINTEF Plankton Centre

Rhodomonas baltica cultures were cultivated semi-continuously in 100 L acrylic cylinders, using filtered water from Trondheimsfjorden that had been disinfected by chlorination and then neutralized by use of sodium thiosulfate. Conwy medium (Walne, 1974) containing essential macro- and micronutrients was added, and the culture was supplied with CO_2 enriched air (1% CO_2) and continuously illuminated by fluorescent light tubes (24:0, light intensity 150 µmol $m^{-2}sec^{-1}$ measured at cylinder surface) to provide the microalgae with the necessary factors for optimal growth. The concentration of microalgal cells in the culture was counted daily by use of particle counter (Multisizer™ 4e Coulter Counter®, Beckmann Coulter, USA) to measure density and growth of cells and identify when dilution was necessary.

Appendix 4: Dry weight

Table A4.1: Dry weight (µg) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5-39 larvae sampled treatment^{-1}). All values are given as mean DW ± SE.

Age (dph)	Treatment	Mean DW ± SE (µg larva^{-1})	Total n
4	Yolk sac	34.1 ± 0.7	39
12	Copepod/Artemia	19.8 ± 1.1	30
	Copepod/Cirriped	34.9 ± 1.5	28
	exp. Cirriped	18.9 ± 1.1	30
	Rotifer/Artemia	22.1 ± 1.5	30
18	Copepod/Artemia	80.0 ± 4.7	29
	Copepod/Cirriped	54.3 ± 3.1	30
	exp. Cirriped	32.3 ± 2.5	26
	Rotifer/Artemia	57.4 ± 2.0	29
23	Copepod/Artemia	155.6 ± 15.5	27
	Copepod/Cirriped	152.9 ± 12.4	29
	exp. Cirriped	88.5 ± 10.0	20
	Rotifer/Artemia	96.0 ± 7.3	23
32	Copepod/Artemia	636.3 ± 50.0	28
	Copepod/Cirriped	421.8 ± 48.2	27
	exp. Cirriped	298.0 ± 29.1	24
	Rotifer/Artemia	609.4 ± 56.1	25
37	Copepod/Artemia	824.8 ± 93.8	29
	Copepod/Cirriped	923.8 ± 89.7	30
	exp. Cirriped	502.1 ± 51.8	21
	Rotifer/Artemia	1004.2 ± 113.7	19
42	Copepod/Artemia	1406.6 ± 90.3	30
	Copepod/Cirriped	1078.6 ± 99.7	30
	exp. Cirriped	641.7 ± 297.9	6
	Rotifer/Artemia	1582.4 ± 281.8	5
48	Copepod/Artemia	2086.1 ± 191.8	29
	Copepod/Cirriped	2357 ± 238.2	29

Appendix 5: Standard length

Table A5.1: Standard length (mm) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5-40 larvae sampled treatment^{-1}). All values are given as mean SL ± SE.

Age (dph)	Treatment	Mean SL ± SE (mm larva^{-1})	Total n
4	Yolk sac	4.3 ± 0.0	40
12	Copepod/Artemia	4.6 ± 0.1	30
	Copepod/Cirriped	4.3 ± 0.1	29
	exp. Cirriped	4.5 ± 0.1	30
	Rotifer/Artemia	4.6 ± 0.1	30
18	Copepod/Artemia	5.3 ± 0.1	30
	Copepod/Cirriped	5.0 ± 0.1	30
	exp. Cirriped	4.8 ± 0.1	26
	Rotifer/Artemia	4.8 ± 0.1	29
23	Copepod/Artemia	6.0 ± 0.1	27
	Copepod/Cirriped	6.0 ± 0.1	30
	exp. Cirriped	5.2 ± 0.1	20
	Rotifer/Artemia	5.2 ± 0.1	23
32	Copepod/Artemia	7.2 ± 0.1	28
	Copepod/Cirriped	6.7 ± 0.1	27
	exp. Cirriped	6.1 ± 0.1	24
	Rotifer/Artemia	7.2 ± 0.1	26
37	Copepod/Artemia	7.5 ± 0.2	30
	Copepod/Cirriped	7.6 ± 0.2	30
	exp. Cirriped	6.6 ± 0.1	21
	Rotifer/Artemia	7.6 ± 0.2	19
42	Copepod/Artemia	8.2 ± 0.1	30
	Copepod/Cirriped	8.0 ± 0.2	30
	exp. Cirriped	6.7 ± 0.4	6
	Rotifer/Artemia	8.5 ± 0.3	5
48	Copepod/Artemia	8.7 ± 0.2	29
	Copepod/Cirriped	9.2 ± 0.3	29

Appendix 6: Hepatocyte cell area size

Table A6.1: Hepatocyte cell area size (μm^2) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5 larvae analyzed treatment^{-1}). All values are given as mean μm^2 ± SE.

Age (dph)	Treatment	Mean hepatocyte cell area size (μm^2) ± SE	$N_{larvae\ measured}$
4	Yolk sac	196.9 ± 5.0	5
18	Copepod/Artemia	322.8 ± 38.8	5
	Copepod/Cirriped	299.6 ± 17.2	5
	Exp. Cirriped	211.1 ± 21.0	5
	Rotifer/Artemia	288.7 ± 28.4	5
32	Copepod/Artemia	363.9 ± 9.5	5
	Copepod/Cirriped	371.2 ± 48.3	5
	Exp. Cirriped	406.3 ± 34.6	5
	Rotifer/Artemia	301.4 ± 27.2	5
48	Copepod/Artemia	322.9 ± 20.0	5
	Copepod/Cirriped	542.4 ± 48.0	5
	Exp. Cirriped	413.3 ± 47.1	5
	Rotifer/Artemia	212.5 ± 27.8	5

Appendix 7: Hepatocyte nucleus area size

Table A7.1: Hepatocyte nucleus area size (µm^2) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5 larvae analyzed treatment^{-1}). All values are given as mean µm^2 ± SE.

Age (dph)	Treatment	Mean nucleus area size (µm^2) ± SE	$N_{larvae\ measured}$	$N_{total\ nuclei\ measured}$
4	Yolk sac	19 ± 0.2	5	4422
18	Copepod/Artemia	21.04 ± 1.2	5	1088
	Copepod/Cirriped	21.5 ± 0.5	5	1077
	Exp. Cirriped	16.7 ± 0.7	5	1127
	Rotifer/Artemia	19.2 ± 0.4	5	1106
32	Copepod/Artemia	20.0 ± 0.9	5	1060
	Copepod/Cirriped	20.3 ± 0.5	5	1038
	Exp. Cirriped	19.4 ± 0.5	5	1104
	Rotifer/Artemia	17.0 ± 0.9	5	1022
48	Copepod/Artemia	18.2 ± 0.9	5	1117
	Copepod/Cirriped	18.3 ± 1.0	5	1076
	Exp. Cirriped	22.0 ± 1.5	5	1075
	Rotifer/Artemia	15.8 ± 0.7	5	1176

Appendix 8: Surface area fractions of liver components

Table A8.1: Surface area fractions of liver components in ballan wrasse larvae at 4, 18, 32 and 48 dph (n = 5 analyzed larvae treatment^{-1}). All values are given as mean percentages ± SE.

Age (dph)	Treatment	Vacuoles	Nuclei	Other
4	Yolk sac	5.8 ± 0.8	9.7 ± 0.4	84.5 ± 1.6
18	Copepod/Artemia	3.1 ± 1.1	6.7 ± 0.5	90.2 ± 0.6
	Copepod/Cirriped	8.1 ± 2.3	7.2 ± 0.4	84.7 ± 2.5
	Exp. Cirriped	2.5 ± 0.6	8.1 ± 0.5	89.4 ± 0.8
	Rotifer/Artemia	4.8 ± 1.3	7.2 ± 0.3	87.9 ± 1.4
32	Copepod/Artemia	4.8 ± 3.8	5.5 ± 0.2	89.7 ± 4.0
	Copepod/Cirriped	3.2 ± 2.9	5.8 ± 0.7	91.0 ± 3.1
	Exp. Cirriped	0.3 ± 0.3	4.9 ± 0.3	94.9 ± 0.3
	Rotifer/Artemia	2.2 ± 1.5	5.7 ± 0.3	92.1 ± 1.7
48	Copepod/Artemia	26.4 ± 5.6	5.7 ± 0.5	67.9 ± 5.2
	Copepod/Cirriped	46.6 ± 0.6	3.6 ± 0.6	49.8 ± 0.2
	Exp. Cirriped	31.5 ± 5.5	5.5 ± 0.4	63.0 ± 5.3
	Rotifer/Artemia	18.9 ± 6.9	7.8 ± 0.8	73.3 ± 6.2

Appendix 9: Gut villus, epithelial and microvillus height

Table A9.1 Gut villus height (µm) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n=5 treatment^{-1}). All values are given as mean µm ± SE

Age (dph)	Treatment	Mean villus height ± SE (µm)	$N_{larvae\ measured}$	$N_{total\ villi\ measured}$
18	Copepod/Artemia	67.4 ± 5.2	5	10
	Copepod/Cirriped	68.7 ± 4.6	5	10
	Exp. Cirriped	52.0 ± 3.1	5	10
	Rotifer/Artemia	53.0 ± 2.5	5	14
32	Copepod/Artemia	120.1 ± 6.3	5	12
	Copepod/Cirriped	99.7 ± 8.8	5	10
	Exp. Cirriped	90.9 ± 5.0	5	16
	Rotifer/Artemia	106.3 ± 3.2	5	16
48	Copepod/Artemia	160.8 ± 12.6	5	11
	Copepod/Cirriped	198.5 ± 21.1	5	13
	Exp. Cirriped	118.2 ± 4.4	5	14
	Rotifer/Artemia	137.9 ± 12.7	5	10

Table A9.2 Gut epithelial height (µm) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n=5-20 treatment^{-1}). All values are given as mean µm ± SE

Age (dph)	Treatment	Mean enterocyte height ± SE (µm)	$N_{larvae\ measured}$	$N_{total\ enterocytes\ measured}$
4	Yolk sac	28.5 ± 0.9	20	200
18	Copepod/Artemia	27.7 ± 0.5	5	50
	Copepod/Cirriped	29.0 ± 0.8	5	50
	Exp. Cirriped	24.1 ± 0.2	5	50
	Rotifer/Artemia	23.2 ± 1.4	5	50
32	Copepod/Artemia	35.6 ± 1.3	5	50
	Copepod/Cirriped	30.6 ± 1.0	5	50
	Exp. Cirriped	36.4 ± 0.8	5	50
	Rotifer/Artemia	31.9 ± 1.1	5	50

48	Copepod/Artemia	33.3 ± 1.2	5	50
	Copepod/Cirriped	39.4 ± 1.5	5	50
	Exp. Cirriped	32.6 ± 1.0	5	50
	Rotifer/Artemia	30.8 ± 2.6	5	50

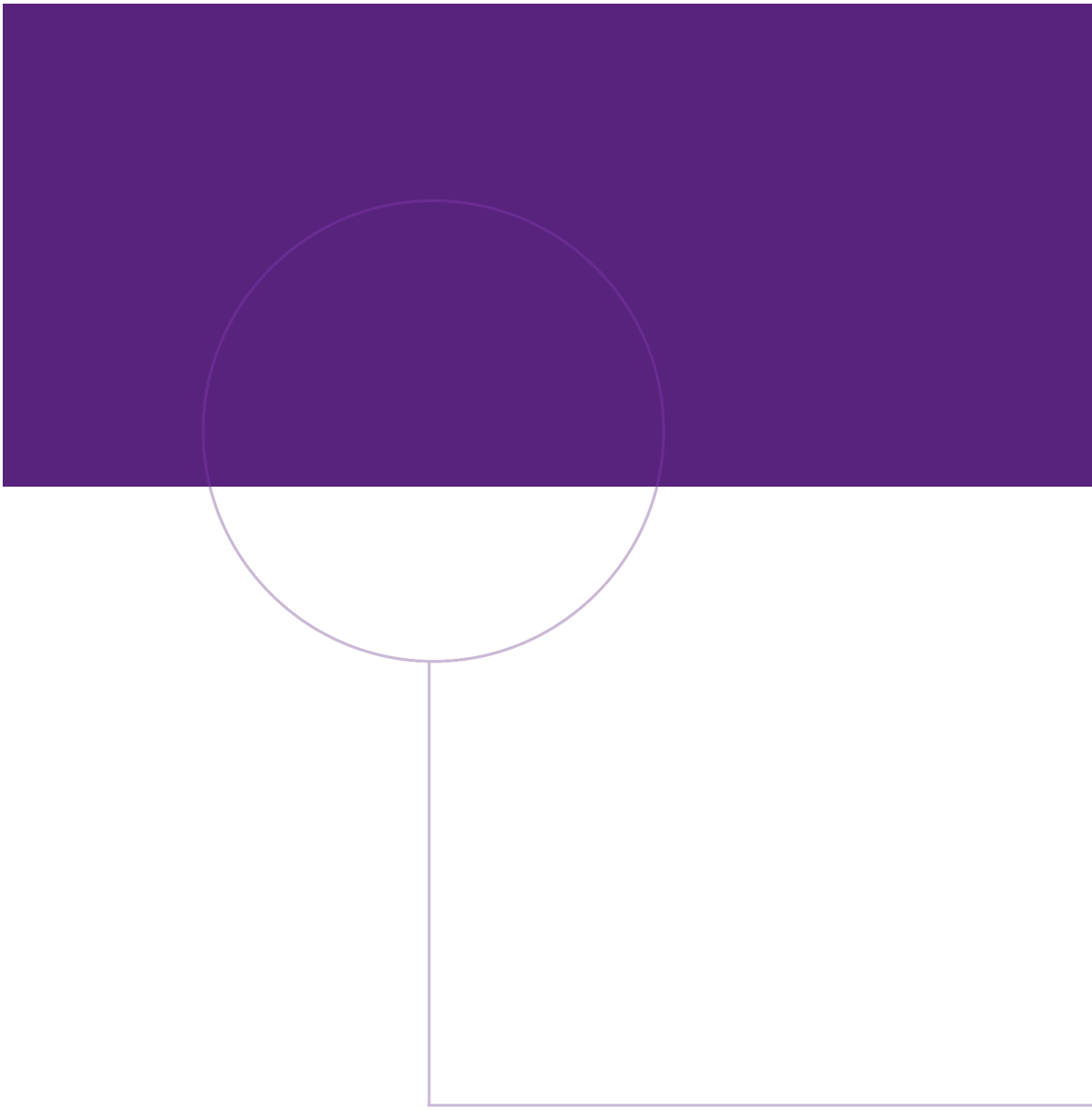
Table A9.3 Gut epithelial height (µm) of ballan wrasse larvae at 4, 18, 32 and 48 dph (n=5-20 treatment^{-1}). All values are given as mean µm ± SE

Age (dph)	Treatment	Mean enterocyte microvillus height ± SE (µm)	$N_{larvae\ measured}$	$N_{total\ enterocytes\ measured}$
4	Yolk sac	1.9 ± 0.04 µm	20	200
18	Copepod/Artemia	2.2 ± 0.1	5	50
	Copepod/Cirriped	2.0 ± 0.1	5	50
	Exp. Cirriped	1.8 ± 0.0	5	50
	Rotifer/Artemia	1.8. ± 0.1	5	50
32	Copepod/Artemia	2.8 ± 0.1	5	50
	Copepod/Cirriped	2.2 ± 0.1	5	50
	Exp. Cirriped	1.9 ± 0.1	5	50
	Rotifer/Artemia	2.3 ± 0.1	5	50
48	Copepod/Artemia	2.2 ± 0.1	5	50
	Copepod/Cirriped	2.7 ± 0.2	5	50
	Exp. Cirriped	2.1 ± 0.1	5	50
	Rotifer/Artemia	2.0 ± 0.0	5	50

Appendix 10: Standard length of larvae sampled for histology

Table A10.1: Standard length (mm) of ballan wrasse larvae analyzed for histology at 4, 18, 32 and 48 dph (n = 5-20 larvae analyzed treatment^{-1}). All values are given as mean mm ± SE.

Age	Treatment	SL (mm) ± SE
4 dph	Yolk sac	4.2 ± 0.0
18 dph	Copepod/Artemia	5.2 ± 0.2
	Copepod/Cirriped	5.3 ± 0.1
	exp. Cirriped	4.4 ± 0.1
	Rotifer/Artemia	4.8 ± 0.1
32 dph	Copepod/Artemia	7.6 ± 0.3
	Copepod/Cirriped	6.6 ± 0.0
	exp. Cirriped	6.1 ± 0.1
	Rotifer/Artemia	7.4 ± 0.1
48 dph	Copepod/Artemia	9.7 ± 0.3
	Copepod/Cirriped	10.8 ± 0.8
	exp. Cirriped	7.5 ± 0.2
	Rotifer/Artemia	9.9 ± 0.3



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