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Ontogeny of the digestive system in lumpfish (*Cyclopterus lumpus* L.) larvae in relation to growth and start feeding diet

A histological and stereological approach

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Marine Coastal Development
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Abstract

Global aquaculture production of Atlantic salmon (*Salmo salar*) has stabilized since 2012 due to high prevalence of the ectoparasitic sea lice (*Lepeophtheirus salmonis*). Deployment of lice-eating cleaner fish is considered one of the most effective and sustainable methods to control sea lice infestations in salmon cages, of which lumpfish (*Cyclopterus lumpus*) is the most used and commercially produced species in Norway. The Norwegian aquaculture lumpfish production has escalated greatly since 2012 but is currently characterized by variable growth and survival during the larval stage. Little is also known regarding nutritional requirements and development of the digestive system in lumpfish larvae, the latter of which is determining for larvae's capacity to assimilate nutrients required for all metabolic processes.

The present study aimed to describe digestive system ontogeny in lumpfish from hatching to 50 days post hatch (dph) based on histological and stereological methods and to evaluate effects of different start feeding regimes on growth, survival and development of the liver, stomach and gut. Ontogeny and dietary effects were investigated by conducting a start feeding experiment in which three triplicate groups received different diets during the start feeding period (2-22 dph). Larvae from one group were fed enriched *Artemia* while two groups were offered *Acartia tonsa*, either for the full start feeding period or with an early weaning to formulated diet 6-8 dph. Larvae from all groups were weaned 20-22 dph and fed the same inert diet until the experiment ended 51 dph.

The alimentary canal of lumpfish was divided into the esophagus, primordial stomach, midgut and hindgut by hatching, at which time it was coiled and open at both ends. Newly hatched larvae had a differentiated liver containing large, irregularly shaped glycogen-like vacuoles and an exocrine pancreas with numerous zymogen granules. The stomach had developed gastric glands 10 dph (6.6 – 6.8 mm standard length) and was divided into the cardia, pylorus and fundus by 34 dph. At this time the stomach lumen volume had increased significantly (8.0 – 9.1 mm standard length) and pyloric caeca formed in the anterior midgut. Feeding with *Artemia* greatly improved larval growth rates, survival after yolk resorption 21-34 dph and resulted in more rapid stomach development compared to both groups initially fed *A. tonsa*. Larvae showed lower hepatocyte cell and nucleus area sizes, absence of hepatic vacuoles and reduced midgut epithelium height during the period they were fed *A. tonsa*. These effects were pronounced on the liver of larvae from both groups fed *A. tonsa* by 10 dph and were reversed after weaning to dry feed, indicating that the liver accurately reflected larval nutritional status at the time of sampling. On the other hand, reduced midgut epithelium height was not observed until 21 dph, suggesting that midgut epithelium height might be a good indicator for starvation over longer periods of time in lumpfish.

Sammendrag

Global akvakulturproduksjon av Atlantisk laks (*Salmo salar*) har stabilisert seg siden 2012 grunnet høy prevalens av den ektoparasittiske lakselusen (*Lepeophtheirus salmonis*). Utsett av lusespisende rensefisk er ansett som en av de mest effektive og bærekraftige tiltakene for å kontrollere utbrudd av lakselus i laksemerder, hvorav rognkjeks (*Cyclopterus lumpus*) er den mest anvendte og kommersielt produserte arten i Norge. Den norske akvakulturproduksjonen av rognkjeks har eksaltert i stor grad siden 2012 men er for tiden karakterisert ved variabel vekst og overlevelse under yngelstadiet. Man vet også lite om ernæringskrav og fordøyelsessystem-utvikling hos rognkjekslarver, hvorav sistnevnte er avgjørende for fiskelarvers kapasitet til å assimilere næringsstoffer som er nødvendige for alle metabolske prosesser.

Dette studiet hadde som formål å beskrive utvikling av rognkjeksens fordøyelsessystem fra klekking frem til dag 50 etter klekking (dph) basert på histologiske og stereologiske metoder, samt å evaluere effekter av ulike startfôringsregimer på vekst, overlevelse og utvikling av lever, magesekk og tarm. Utvikling og dietteffekter ble undersøkt ved å gjennomføre et startfôrings-forsøk hvor tre parallelle grupper fikk ulike dietter under startfôringsperioden (2-22 dph). Larver fra en gruppe fikk anriket *Artemia* mens to grupper ble gitt *Acartia tonsa*, enten gjennom hele startfôringsperioden eller med en tidlig overgang til tørrfôr med en tilvenningsperiode fra 6-8 dph. Alle gruppene hadde en tilvenningsperiode til det samme tørrfôret fra 20-22 dph og ble gitt dette fôret frem til forsøkets slutt 51 dph.

Fordøyelseskanalen til rognkjeks var inndelt i spiserør, primordial magesekk, midttarm og baktarm ved klekking, ved hvilket tidspunkt kanalen var oppkveilet og åpen i begge ender. Nyklekte larver hadde en differensiert lever med store, irregulære glykogenlignende vakuoler og en eksokrin pankreas med tallrike zymogengranuler. Magesekken hadde utviklet gastriske kjertler 10 dph (6.6 – 6.8 mm standardlengde) og var inndelt i tre regioner (carida, pylorus og fundus) ved 34 dph. Ved dette tidspunktet var også magens lumenvolum signifikant større (8.0 – 9.1 mm standardlengde) og pylorusblindsekker var utviklet fremst i midttarmen. Fôring med *Artemia* hadde positiv effekt på larvenes vekstrater, overlevelse etter plommeresorpsjon 21-34 dph og resulterte i raskere mageutvikling sammenlignet med larver gitt *A. tonsa*. Larvene hadde mindre hepatocytter og hepatocyttkjerner, få hepatiske vakuoler og redusert midttarmepitel-høyde under perioden de ble fôret *A. tonsa*. Effektene på leveren var tydelige 10 dph men ble reversert etter overgang til tørrfôr, som indikerte at leveren nøyaktig reflekterte ernæringsstatus hos larvene ved prøvetaking. Redusert midttarmepitelhøyde ble derimot ikke observert før 21 dph, hvilket tyder på at epitelhøyde i denne tarmregionen muligens er en god indikator for sult over lengre perioder hos rognkjeks.

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Abbreviations

ARA	Arachidonic acid (20:4*n*-6). Polyunsaturated omega-6 fatty acid essential for marine fish larvae.
Artemia larva	Lumpfish larva fed *Artemia* during the treatment period (2-22 dph) in the present study.
Copepod larva	Lumpfish larva fed *Acartia tonsa* during the treatment period (2-22 dph) in the present study.
DHA	Docosahexaenoic acid (22:6*n*-3). Polyunsaturated omega-3 fatty acid essential for marine fish larvae.
Dph	Days post hatch. Number of days since a fish larva emerged from its egg.
DW	Dry weight. Body mass of an individual fish larva dried at 60 °C for a minimum of 24 hours.
DWI	Daily weight increase. Percentage daily increase in dry weight over a time interval.
d°	Degree-days. Days post hatch multiplied with temperature (°C).
EPA	Eicosapentaenoic acid (20:5*n*-3). Polyunsaturated omega-3 fatty acid essential for marine fish larvae.
FA	Fatty acid. Carboxylic acid with a carbon chain that is either saturated or unsaturated (one or more double bonds).
FAA	Free amino acid. Amino acid that is not linked to any other amino acid.
FD larva	Lumpfish larva fed *Acartia tonsa* and Gemma micro 150 (Skretting AS, Norway) during the treatment period (2-22 dph) in the present study.
HCl	Hydrochloric acid. An acid produced and secreted by oxyntopeptic cells in the stomach to denature dietary proteins.
Hg section	Hindgut section. Cross section used for histological analysis of the hindgut in lumpfish larvae from 0-50 dph in the present study.
HUFA	Highly unsaturated fatty acid. Fatty acid containing two or more double bonds and at least 20 carbon atoms in the carbon chain.
L section	Liver section. Cross section used for histological analysis of the liver in lumpfish larvae from 0-50 dph in the present study.
Mg section	Midgut section. Cross section used for histological analysis of the midgut in lumpfish larvae from 0-10 dph in the present study.

NL	Neutral lipid. Lipid insoluble in water solutions and containing no charged groups.
PAS	Periodic acid Schiff. Staining method used to detect polysaccharides in tissues by oxidation of sugars to aldehydes that gives a purple-magenta color with the Schiff reagent.
PBS	Phosphate buffered saline. Buffer solution maintaining constant pH in cells and tissues. Commonly used in biological research.
PFA	Paraformaldehyde. Polymer that can be depolymerized into formaldehyde in a solution to fixate biological material.
PL	Phospholipid. Lipid consisting of two fatty acids and one phosphate group. Important constituents in cell membranes.
SGR	Specific growth rate. Fractional daily increase in dry weight over a time interval.
SL	Standard length. Distance from the tip of the snout to the notochord end in a fish larva.
S_i	Stomach developmental stage. Categorical grouping of lumpfish larvae based on ontogenetic characteristics (stage S_1, S_2 or S_3) in the present study.
S section	Stomach section. Cross section used for histological analysis of the stomach and midgut in lumpfish larvae from 21-50 dph and the stomach (not midgut) in lumpfish larvae from 0-10 dph in the present study.
TAG	Triacylglycerol. Neutral lipid consisting of three fatty acids. Important metabolic energy source stored in cells.
TB	Toluidine blue. Basic dye with high affinity for acidic components that gives tissues a deep blue or purple color. Used to stain histological slides.
WW	Wet weight. Body mass of an individual fish larva.
Yolk sac larva	Lumpfish larva prior to introduction of feed 2 dph in the present study.

1 Introduction

1.1 Lumpfish in aquaculture

1.1.1 Lumpfish as a cleaner fish

Global production of Atlantic salmon (*Salmo salar*) has escalated rapidly since the 1970s and reached 2.1 million tonnes in 2012, of which 1.2 million tonnes were produced in Norway alone (FAO, 2018). However, the annual production has since stabilized due to high prevalence of the ectoparasitic sea lice (*Lepeophtheirus salmonis*) (Costello, 2006; Svåsand *et al.*, 2016). Sea lice infestations increases production costs and have negative impacts on both farmed and wild salmon populations (Pike, 1989; Costello, 2009a,b; Iversen *et al.*, 2015). Infected salmon are often treated with chemical therapeutants (Burridge *et al.*, 2010; Svåsand *et al.*, 2016), but because of increased resistance in sea lice and detrimental effects on non-targeted species (Egidius & Møster, 1987; Salte *et al.*, 1987; Bjordal *et al.*, 1988; Aaen *et al.*, 2015), alternative delousing methods are needed. This includes the use of cleaner fish, species of fish that partly feeds on parasites of another fish species (Feder, 1966), which is more cost-effective than chemical treatment and improves the welfare of farmed fish (Treasurer, 2002; Liu & vanhauwaer Bjelland, 2014). Commercial production of cleaner fish is considered one of the most important measures to control sea lice infestations (Marine Harvest, 2015).

Cleaner fish has been deployed in Norwegian Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) sea cages for more than 20 years, but the use has increased greatly since 2008. A total of 37.4 million wild-caught and farmed individuals were utilized to combat sea lice in 2016 (Figure 1.1A; Norwegian Directorate of Fisheries, 2017a), of which lumpfish (*Cyclopterus lumpus*) was the single most important species. Other species used are ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*) and corkwing wrasse (*Symphodus melops*). Wrasses have been used to control sea lice for almost three decades (Bjordal, 1991), but lumpfish as a cleaner fish holds several advantages over these species. In contrast to wrasses, lumpfish remain active and feed at low temperatures down to 4 °C (Sayer & Reader, 1996; Kelly *et al.*, 2014; Nytrø *et al.*, 2014), and results from recent studies suggests that lumpfish is a more suitable cleaner fish in cold-water environments (Imsland *et al*., 2014a,b). The production cycle of lumpfish varies from 4-7 months (Towers, 2013; Nytrø *et al.*, 2014; Powell *et al*., 2017), which is considerably shorter than the 1.5 years it typically requires to produce ballan wrasse ready for deployment (Helland *et al.*, 2014). Additionally, lumpfish is easier to culture, it can be stocked at higher densities (10-15 % of salmon density versus 4 % in ballan wrasse) and it is less susceptible to *Vibrio* infections (Towers, 2013; Imsland *et al*., 2014a). These differences are reflected in recent changes in the Norwegian cleaner fish industry (Figure 1.1B). The number of farmed ballan wrasse has been low and stable from

2012-2016 and reached a maximum of 1.3 million individuals in 2015 (Norwegian Directorate of Fisheries, 2017b). On the other hand, production of lumpfish juveniles increased from 0.4 million individuals in 2012 to 15.9 million in 2016 and was expected to reach 30 million in 2017 (Nodland, 2017; Norwegian Directorate of Fisheries, 2017b). Regardless of the rapid growth in commercial lumpfish production, knowledge is still scarce in several areas of the production cycle.

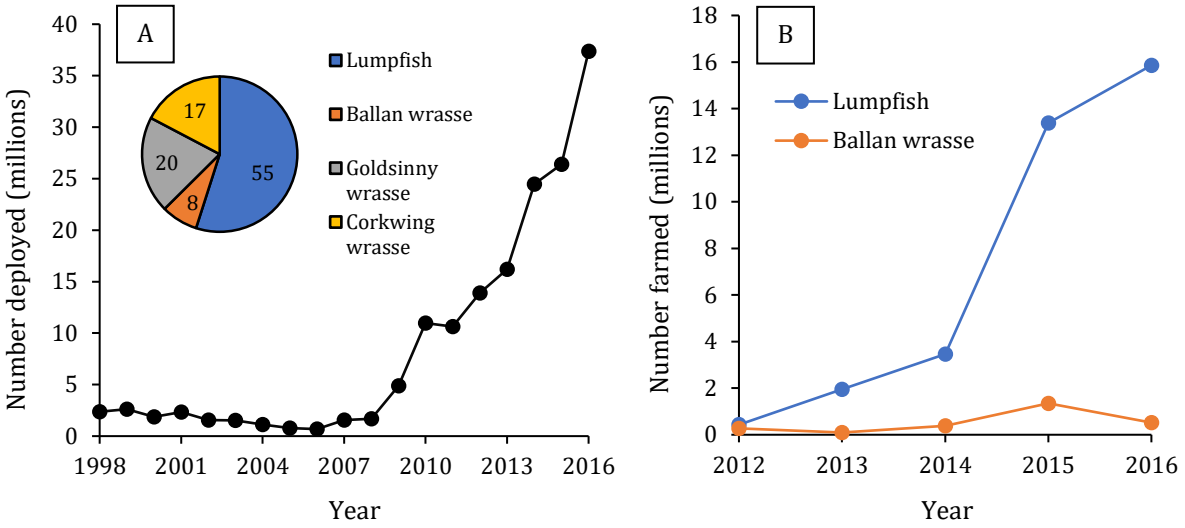

Figure 1.1. Deployed and farmed cleaner fish in Norwegian Atlantic salmon and rainbow trout sea cages. A) Millions wild-caught and farmed cleaner fish from 1998-2016. The circle chart displays species distribution (% of total number) in 2016. B) Millions farmed lumpfish and ballan wrasse from 2012-2016. Source: Norwegian Directorate of Fisheries (2017a,b).

1.1.2 Knowledge gaps in lumpfish aquaculture

A recent review by Powell and colleagues (2017) provides a description of the production cycle of lumpfish and related challenges, ranging from the reliance on a wild-caught brood stock to welfare issues and survival after deployment in sea cages. Larviculture, the farming of fish larvae from hatching to ongrowing, is one of the key areas in the production cycle of lumpfish where more information must be obtained. Rearing of lumpfish larvae is currently characterized by variable growth and survival, and little is known about their biology in captivity and nutritional requirements. As lumpfish is classified as 'near threatened' by IUCNs Red List because of reduction in several natural stocks (Lorance *et al.*, 2015), it must be produced entirely in captivity if the demanded delousing rates are to be met in a sustainable way. To achieve a sustainable and reliable production of high-quality lumpfish that can effectively control sea lice infestations in salmon aquaculture, it is essential to overcome the abovementioned knowledge gaps. Some of these are described in more detail in the following paragraphs.

Larval survival is a major problems in lumpfish larviculture. Powell *et al.* (2017) published data from the Centre of Sustainable Aquatic Research (Swansea, UK) showing that high mortality is observed 25-30 days post hatch (dph) when lumpfish larvae are reared at 10 °C. They pointed out that this coincided with potential stress during weaning from *Artemia* to formulated diets. Average survival after this period is approximately 78 %, although high variability is observed among families. A significant drop in survival around 300 degree-days (d°) is known to also occur in at least one Norwegian lumpfish farm (MoreFish AS, Tjeldbergodden, Norway, pers. comm., 2018). It is thus important to understand potential factors influencing survival during the larval stages of lumpfish. Survival in fish larvae of several species, including Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) and ballan wrasse, is influenced by the diet (Næss *et al.*, 1995; Dahle *et al*., 2014; Øie *et al.*, 2015).

Aspects related to feeding and nutrition are poorly understood in lumpfish larvae. In nature, they feed primarily on harpacticoid copepods and amphipods in floating seaweed (Daborn & Gregory, 1983; Ingólfsson & Kristjánsson, 2002), whereas in captivity both live and formulated diets can be used. Brown (1986) was among the first to conduct a start feeding trial with lumpfish, in which *Artemia salina* was offered to one group and formulated diet to another. After given respective diets for one month, larvae from the group fed *A. salina* and formulated diet had respective mean lengths of 12 and 7 mm. More recent studies have also shown increased larval growth when lumpfish are fed *Artemia* rather than inert diets (Nytrø *et al.*, 2014; Belova, 2015), and it appears that the copepod *Acartia tonsa* has the potential to improve growth and survival of lumpfish larvae as well (Dahle *et al*., 2017). Still, many commercial producers of lumpfish feed their larvae exclusively with formulated diets. Besides influencing growth and survival, nutritional condition during larval stages can also have future impacts. For example, reduced delousing efficiency and welfare in deployed lumpfish due to eye cataract can perhaps be explained by dietary deficiencies during the larval phase (Powell *et al*., 2017).

Nutritional requirements must also be met to promote proper larval development (Steffens, 1989; Sargent *et al.*, 1999a,b), but little is currently known about functional development in lumpfish. The digestive system is an important aspect of larval development, as the developmental, or ontogenetic, status of digestive organs is decisive for digestive capacity (Govoni *et al.*, 1986). This will in turn influence a fish larva's ability to assimilate dietary nutrients required for all metabolic processes, ranging from cellular respiration to organ growth and development. Knowledge about digestive system ontogeny in lumpfish in relation to different start feeding regimes will prove valuable when optimizing larval rearing protocols in this species, which is important to secure an effective and high-quality production of juveniles.

1.2 Digestive system development in fish larvae

The digestive system in teleost fish (Teleostei) comprises the gastrointestinal tract, which in juveniles and adults is divided into the buccopharynx, esophagus, stomach, intestine and anus. The liver, gallbladder and exocrine pancreas are also part of the digestive system. All these organs function in coordination to break down ingested feed into smaller units that are absorbed and distributed to peripheral tissues (Figure 1.2). Digestive system ontogeny in teleosts is commonly divided into three phases (Buddington, 1985; Bisbal & Bengtson, 1995): (1) the lecithotrophic phase when larvae fully rely on endogenous yolk nutrients; (2) exogenous feeding phase with limited proteolytic capacity due to an immature digestive system; (3) exogenous feeding phase with full capacity to digest dietary protein due a developed and functional stomach. The first and second phases may overlap in a period of mixed endogenous and exogenous feeding, as most fish larvae capture and ingest food items before exhaustion of yolk reserves (Kjørsvik *et al.*, 1991; Bisbal & Bengtson, 1995). Despite the similarity in developmental pattern, the duration of each stage is interspecific (Luizi *et al.*, 1999; Zaiss *et al.*, 2006), and knowledge about one species can therefore not be extrapolated to other species. Understand digestive system ontogeny in lumpfish is important so that commercial feeding regimes can be synchronized according to its developmental status.

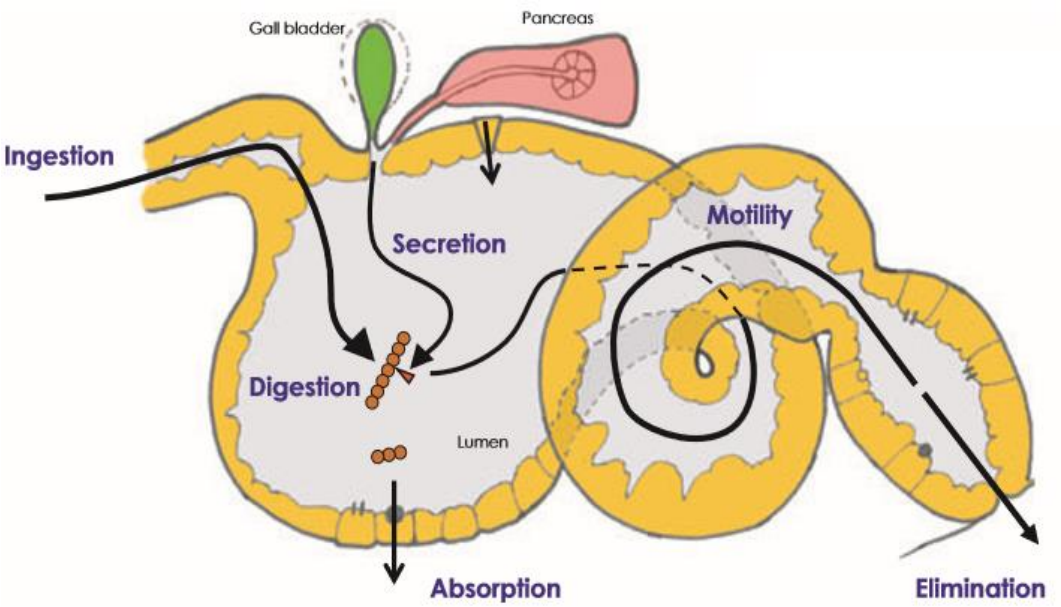

Figure 1.2. Processes in the teleostean digestive system. Ingested feed is exposed to digestive enzymes secreted by gastric gland cells in the stomach (not shown) and the pancreas. Bile is synthesized in the liver (not shown) and secreted by the gallbladder to facilitate lipid digestion. Digestion is assisted by motility along the gastrointestinal tract by a contracting muscular layer. Digested feed is absorbed by enterocytes lining the intestinal mucosa. Undigested feed is eliminated through the anus. Obtained and modified from Rønnestad *et al.* (2013).

1.2.1 Development of the liver and exocrine pancreas

The liver develops embryologically as a glandular outgrowth of the primitive gut (Young *et al.*, 2006) and is differentiated before or after hatching depending on life history traits of the species (Hoehne-Reitan & Kjørsvik, 2004). Atlantic cod and Atlantic wolffish (*Anarhichas lupus*) are examples of species with a fully differentiated liver at hatching (Morrison, 1993; Hoehne-Reitan & Kjørsvik, 2004), whereas in gilthead seabream (*Sparus aurata*) and Atlantic halibut the liver develops during the endogenous feeding phase (Sarasquete *et al.*, 1995; Hoehne-Reitan & Kjørsvik, 2004). A functional liver consists of numerous parenchymal hepatocyte cells separated by vascular channels called sinusoids. The sinusoids have both an arterial and venous blood supply that transport oxygen and nutrients, respectively (Young *et al*., 2006). Nutrients entering the liver originates from digested feed absorbed in the gastrointestinal tract and are stored primarily as glycogen granules and lipids vacuoles residing in the hepatocyte cytoplasm (Lazo *et al.*, 2011). The timing and amount of hepatic nutrient accumulation is interspecific (Hoehne-Reitan & Kjørsvik, 2004). As an example, lipid vacuoles are present in the liver of rainbow trout from hatching (Vernier & Sire, 1977) but are not stored in Atlantic cod until after metamorphosis (Lie *et al.*, 1986). Apart from maintaining nutritional homeostasis by storing and mobilizing nutrients, the liver synthesizes bile to emulsify dietary lipids in the midgut and facilitate digestion by lipolytic enzymes such as bile-dependent lipase (Hoehne-Reitan *et al.*, 2001). Lipases and other digestive enzymes are produced in the exocrine pancreas before or shortly after hatching to condition intake of exogenous feed (Segner *et al.*, 1993; Hoehne-Reitan & Kjørsvik, 2004).

The exocrine pancreas is a glandular organ of secretory cells grouped into acinar structures with excretory ducts transporting digestive zymogens, inactive enzyme precursors, into the intestinal lumen (Slack, 1995). In fish larvae, the exocrine pancreas generally appears as a differentiated organ during the lecithotrophic phase, as seen for instance in European sea bass (*Dicentrarchus labrax*) (Beccaria *et al.*, 1991). This species follows a typical developmental pattern of the pancreas found in teleostean larvae. At hatching, the organ is distinct and situated dorsally to the alimentary canal before it develops fully differentiated exocrine cells, excretory ducts and blood vessels before mouth opening (Beccaria *et al*., 1991). After fully differentiated, the exocrine pancreas increases in size and constitutes a gradually more diffuse organ towards the end of the larval stage (Kurokawa & Suzuki, 1996). Various digestive enzymes are secreted from the exocrine pancreas in fish larvae, including trypsin and chymotrypsin for protein digestion and lipase and phospholipase A_2 for lipid digestion (see review by Hoehne-Reitan & Kjørsvik, 2004). Secretion of digestive enzymes from the exocrine pancreas and bile salts from the gallbladder is regulated by cholecystokinin-like peptide hormones produced by enteroendocrine cells in the gastrointestinal tract of teleosts (Honkanen *et al.*, 1988; Smith, 1989; Einarsson & Davies, 1997).

1.2.2 Development of the gastrointestinal tract

Teleosts are categorized as either precocial or altricial based on degree of differentiation and functionality of the alimentary canal at onset of first feeding (Kjørsvik *et al*., 2004). A few species such as Atlantic salmon and Atlantic wolffish are precocial with a fully differentiated and functional digestive system when exogenous feeding commences (Gorodilov, 1996; Falk-Petersen & Hansen, 2001). But in general, pelagic fish larvae are altricial with their digestive canal being an undifferentiated tube closed at both ends at hatching (Kjørsvik *et al*., 1991; Ortiz-Delgado *et al.*, 2003). The mouth and anus open towards the end of lecithotrophic phase, and the digestive tract form the foregut, midgut and hindgut (Govoni *et al.*, 1986). The fore- and midgut is most active in digestion and absorption of lipids, while the hindgut absorb proteins through pinocytosis. This has been found for instance in Dover sole (*Solea solea*) and white seabream (*Diplodus sargus*) (Boulhic & Gabaudan, 1992; Ortiz-Delgado *et al.*, 2003). Absorptive capacity is greatly increased during the exogenous feeding phase through longitudinal folding of the intestinal mucosa, elongation and coiling of the gut and increased microvilli surface area (Boulhic & Gabaudan, 1992; Bisbal & Bengtson, 1995; Wold *et al.*, 2008). Towards the end of the larval stage, the foregut differentiates further into the esophagus and stomach, whereas the midgut and hindgut form the anterior and posterior intestine, respectively (Govoni *et al*., 1986).

The esophagus is the most anterior part of the alimentary canal and connects the posterior pharynx to the anterior region of the primordial stomach in gastric fish species (see review by Lazo *et al*., 2011). A differentiated stomach can be divided into a cardiac, fundic and pyloric region, of which the cardia and pylorus are separated from the esophagus and intestine by muscular sphincters that form during ontogeny (Chen *et al.*, 2006). Compartmentalization of the stomach allows it store ingested feed, a function that can be obtained before the proteolytic stomach activity is established (Rønnestad *et al*., 2000b). This proteolytic function is mediated through secretion of hydrochloric acid (HCl) and pepsinogen from oxyntopeptic cells (Garrido *et al.*, 1993) that denature and depolymerize dietary proteins into smaller peptide chains. As in higher vertebrates, these cells are grouped into tubular gastric glands in lamina propria and open into the lumen via gastric pits (Luizi *et al.*, 1999; Zaiss *et al.*, 2006). Appearance of the first gastric glands indicate metamorphosis from the larval to juvenile stage accompanied with increased digestive capacity (Tanaka, 1971). However, the glands are not necessarily functional directly after they develop (Mähr *et al*.,1983; Huang *et al.*, 1998; Darias *et al.*, 2005). Age at formation of gastric glands occurs already by 9 dph in shi drum (*Umbrina cirrosa*) but not until 60 dph in Atlantic halibut (Luizi *et al*., 1999; Zaiss *et al.*, 2006). As the duration from hatching to development of optimal proteolytic capacity is species specific, it is crucial to study stomach ontogeny in novel aquaculture species such as the lumpfish.

Until the gastric function is developed, proteolysis is restricted to pancreatic proteases as well as intestinal pinocytosis and intracellular digestion (Hoehne-Reitan & Kjørsvik, 2004; Kjørsvik *et al*., 2004). The intestinal mucosa is composed of different cell types, of which columnar enterocytes responsible for nutrient absorption are most abundant (Lazo *et al*., 2011). During maturation, enterocytes increase in height and form a brush border of microvilli with enzymes such as aminopeptidase, maltase and alkaline phosphatase that hydrolyze nutrients into absorbable monomers (Zambonino-Infante & Cahu, 2001). Goblet and enteroendocrine cells develop in the intestinal mucosa before or during the exogenous feeding phase (Sarasquete *et al.*, 1995; Ortiz-Delgado *et al.*, 2003). Goblet cells secrete acidic and neutral mucosubstances to create a protective barrier between the mucosa and gut lumen which may also facilitate pinocytosis (Kapoor *et al.*, 1975; Domeneghini *et al.*, 1998). Enteroendocrine cells receive signals from ingested feed and secrete peptide hormones to control peristaltic movement of the intestinal muscularis (Kamisaka *et al*., 2001), as well as secretory activities of the exocrine pancreas and gallbladder (Honkanen *et al.*, 1988; Smith, 1989; Einarsson & Davies, 1997). Pancreatic zymogens are activated in the gut lumen by the pancreatic protease trypsin, which itself is activated by the enterocytic brush border enzyme enterokinase and active trypsin (Brannon, 1990). Pancreatic enzymes have maximum catalytic activity in alkaline pH, which is obtained from bicarbonate (HCO_3^-) secreted from the exocrine pancreas and bile salts from the gallbladder (Hoehne-Reitan & Kjørsvik, 2004). In summary, digestion in teleosts depends on several processes and require that all involved organs are fully developed to reach optimum capacity (Govoni *et al.*, 1986).

1.2.3 Histological biomarkers and nutritional status

Some digestive organs exhibit different cellular mechanisms in response to diet quantity and quality, and the nutritional condition of fish larvae can thus be assessed by histological biomarkers (Table 1.1). Two such biomarkers are nutrient content and hepatocyte structure in the liver. Lipids and glycogen are rapidly mobilized during starvation to maintain nutritional homeostasis, hence the quantity of hepatic energy stores can be used to assess nutritional status (Weis, 1972; Leatherland, 1984; Segner & Möller, 1984). Fish larvae with a high energy content usually have large hepatocytes with their nucleus displaced in a peripheral position by lipid vacuoles, whereas food-deprived larvae have smaller hepatocytes with a central nucleus (Gisbert *et al.*, 2008). Nutritional status is also reflected in hepatocyte nucleus size, as a large nucleus with increased surface area allows for more rapid transfer of molecules between the nucleus and cytoplasm, indicating high metabolic activity (Ghadially, 1997). In contrast, reduced hepatonuclear size is associated with malnourishment and low growth rates (Segner *et al.*, 1988; Wold *et al*., 2009). There are several other biomarkers in the liver indicative of nutritional status as well. In the case starvation, histopathological changes include swelling and deformation of mitochondria, dilation of sinusoids, large intercellular spaces and hypertrophy of bile canaliculi (Gisbert *et al*., 2008).

Histological organization of the intestine is also sensitive to malnutrition (Table 1.1). Fish larvae of several species have shown weakened absorptive capacity during periods of starvation and suboptimal feeding, for example by reduced enterocyte height, degeneration of microvilli and fewer and smaller intestinal folds. For example, a reduction in gut epithelium height have been observed in starved larvae of both Atlantic herring (*Clupea harengus*) and European plaice (*Pleuronectes platessa*) (Ehrlich *et al.*, 1976). Kjørsvik *et al.* (1991) studied early ontogeny of the digestive tract in Atlantic cod under controlled conditions. They found that larvae starved beyond 9 dph had highly restricted absorptive capacity due to degeneration of microvilli. A pilot study with lumpfish larvae showed that the number and size of intestinal villi might be a sensitive biomarker in this species, as the villi seemed smaller and less abundant in larvae fed formulated diet rather than copepods (Dahle *et al.*, 2017). Lipid and protein inclusions in enterocytes are also useful parameters when assessing nutritional condition. Diets characterized by a high dietary lipid content can result in intestinal steatosis, which may reduce digestion and absorption through cellular abrasion, necrosis and inflammatory reactions (Deplano *et al.*, 1989; Deplano *et al.*, 1991; Segner *et al.*, 1993). Accumulation of acidophilic protein inclusions in the hindgut following pinocytosis usually decline after the gastric proteolytic function is attained (Ortiz-Delgado *et al.*, 2003). Variations in the presence of these inclusions may therefore reflect nutritional physiology in fish larvae such as proteolytic capacity.

Table 1.1. Histological biomarkers reflecting nutritional status in teleost larvae. Cellular criteria in the liver hepatocytes and gut epithelium used to categorize nutritional condition: degraded (grade 1), average (grade 2) and healthy (grade 3). Obtained and modified from Lazo *et al*. (2011) and sources therein.

Tissue	**Condition (grade)**		
	Degraded (1)	Average (2)	Healthy (3)
Liver hepatocytes	Nearly all nuclei pyknotic (shrunk) and with dark clumped chromatin. The cytoplasm lacks texture and intracellular vacuoles are absent. The cells are small and indistinct.	At least 50 % of all nuclei with dark granules and a central position. Nearly 50 % of cytoplasm is granular and vacuoles are reduced or absent. Boundaries of most hepatocytes are visible.	Nuclei distinct and often displaced laterally. The cytoplasm is lightly stained with abundant intracellular vacuoles containing lipids and glycogen. Hepatocyte boundaries are prominent.
Gut epithelium	Mucosal cell height reduced by >50 %, with some loss of striations in the bordering microvilli. Supranuclear vacuoles are reduced or absent.	Mucosal cell height reduced by 25-50 %, with some loss of striations in the bordering microvilli. Supranuclear vacuoles are reduced or absent.	Mucosa is deeply convoluted and mosaic. Mucosal cells are compact, pronounced in height and with distinct nuclei. Prominent supra-nuclear acidophilic inclusions and vacuoles.

1.3 Diets used in rearing of lumpfish larvae

1.3.1 Formulated diets

Feed companies like BioMar and Skretting supply fish farmers all over the world with different types of formulated diets, which are often preferred over live feeds as they are cheaper and less labor intensive (Tandler, 1985; Hamre *et al.*, 2013). Microparticulate diets can be used already from hatching and early larval stages in some species. This is frequently practiced in lumpfish farming but is undesirable due to variable larval growth and survival and as it stimulates bacterial growth (Dahle *et al.*, 2017). Larval diets also have a large surface/volume ratio and are susceptible to nutrient leaching upon rehydration. Free amino acids (FAA) are particularly unstable, with up to 95 % lost shortly after water immersion of some diets (López-Alvarado *et al.*, 1994; Baskerville-Bridges & Kling, 2000; Nordgreen *et al.*, 2009). Proteins make up 60-80 % of the dry weight in fish larvae and are quantitatively important due to a progressive increase in muscle mass associated with rapid growth (Kjørsvik *et al.*, 2004). Meeting dietary protein demands may thus prove challenging due to leaching, and even more so for slow feeders like Atlantic halibut larvae (Kvåle *et al.*, 2007). Lumpfish larvae attach to surfaces with their suction disc to feed passively (Killen *et al.*, 2007b), and are potentially more prone to dietary deficiencies induced by leaching as well.

Ingestion and digestion are other factors determining suitability of inert diets. To be ingested, the diet must first be identified as food items. Most fish larvae detect food items primarily through vision (Hunter, 1981), and lumpfish have functional eyes already from hatching (Brown, 1986). However, movement of inert diets after settlement on the tank floor is restricted to those induced by water currents (D'Abramo, 2002), hence predatory behaviour is not necessarily stimulated to the same extent as with live prey. Digestibility of formulated diets may also be limited due to polymerization of proteins to prevent leaching (Nordgreen *et al.*, 2008). This can potentially make it more difficult for marine fish larvae to meet dietary protein demands as most species lack a functional stomach during larval stages (Govoni *et al.*, 1986). It is possible that the variable growth observed in commercial lumpfish production is partly due to limited capacity to digest proteins in inert diets, but as previously mentioned little is known about lumpfish stomach development.

1.3.2 Copepods

Copepods are planktonic crustaceans constituting the most important prey organisms of marine fish larvae (Pauly & Christensen, 1995), and species in the order Harpacticoida are heavily preyed upon by lumpfish after yolk resorption (Ingólfsson & Kristjánsson, 2002). Fish larvae of several species achieve better growth, survival and stress tolerance compared to larvae fed traditional live prey such as *Artemia* (Næss *et al*., 1995; Dahle *et al*., 2014; Karlsen *et al.*, 2015; Øie *et al.*, 2015). These effects are obtained due to a high level of nutrients in copepods required by fish larvae (Mæland *et al.*, 2000; Hamre & Harboe, 2008; van der Meeren *et al.*, 2008; NRC, 2011).

Proteins make up more than half of the total dry mass in copepods (Olsen, 2004), of which free amino acids (FAA) constitutes a significant fraction of total amino acid content (van der Meeren *et al*., 2008). In contrast to amino acids bound in peptide chains, FAAs are easily absorbed by enterocytes in the gut of marine fish larvae (Rønnestad *et al.*, 2000a; Rønnestad *et al.*, 2003). An exogenous supply of FAAs is vital after yolk resorption as it is important in cellular energy generation and synthesis of muscle proteins to facilitate larval growth (Fyhn, 1989; Rønnestad *et al.*, 1999; Kjørsvik *et al.*, 2004). Some amino acids cannot be synthesized by fish *de novo* and are required for normal growth (Jobling, 2004), all of which are present in copepods (Helland *et al.*, 2003). However, the quantity of dietary protein is more important as amino acid composition varies little between organism groups. Apart from dietary protein content, composition of dietary lipids is another crucial aspect of larval nutrition (Rainuzzo *et al.*, 1997).

Lipids are composed of fatty acids (FA), some of which fish larvae are unable to synthesize *de novo* and must be supplied through the diet. This includes the *n*-3 highly unsaturated fatty acids (HUFA) docosahexaenoic acid (22:6*n*-3; DHA) and eicosapentaenoic acid (20:5*n*-3; EPA) and the *n*-6 HUFA arachidonic acid (20:4*n*-6; ARA), which are important cell membrane constituents and considered the most essential FAs in fish larvae (Izquierdo, 1996; Sargent *et al.*, 1999a). Copepods have a high *n*-3 HUFA content (Shields *et al*., 1999; van der Meeren *et al*., 2008), with a typical DHA/EPA ratio of 2:1 and EPA/ARA ratio > 16:1 (Conceição *et al.*, 2010). The *n*-3 HUFAs in copepods are primarily located in their phospholipids (PL) (Albers *et al.*, 1996) and are more effectively utilized for larval growth and development compared to *n*-3 HUFAs in neutral lipids (NL) (Gisbert *et al.*, 2005; Wold *et al.*, 2007; Kjørsvik *et al*., 2009; Wold *et al*., 2009). PLs are also required due to insufficient larval synthesis (Sargent *et al.*, 1993) and are important components in cell membranes and nervous tissue (Sargent *et al.*, 1999a). Additionally, PLs are emulsifying compounds and may thus improve absorption of dietary NLs and lipoprotein synthesis for export of NLs assimilated in enterocytes (Coutteau *et al.*, 1997; Tocher *et al.*, 2008).

1.3.3 *Artemia*

Encysted *Artemia* eggs are found in salt lakes worldwide and have been harvested since the 1960s as a source of live prey to marine fish larvae (Sorgeloos, 1980; Bengtson *et al.*, 1991; Dhont & van Stappen, 2003). As these cysts are cheap and hatch into easily mass cultured nauplii when incubated in seawater (Sorgeloos, 1980), *Artemia* has traditionally been used in larval production of several marine species (Skiftesvik & Bjelland, 2003; Olsen *et al*., 2004). *Artemia* is currently also used to feed lumpfish larvae at the Centre of Sustainable Aquatic Research (Swansea, UK) for two to three weeks after hatching before they are weaned to inert diets (Powell *et al*., 2017). Despite its popular use, the nutritional demands of marine fish larvae are often not met when fed *Artemia* (Næss *et al.*, 1995; Shields *et al*., 1999; Payne & Rippingale, 2000; Hamre *et al.*, 2002).

Artemia may contain sufficient levels of protein required by fish larvae (NRC, 2011), of which around 50 % of the total protein is water-soluble (Hamre *et al*., 2002). Water-soluble proteins are believed to be more easily digested by fish larvae than insoluble proteins often found in inert diets (Carvalho *et al.*, 2003). However, FAA content is much lower in *Artemia* than in copepods (van der Meeren *et al*., 2008) and may limit growth since this is the dietary protein source most readily assimilated by fish larvae (Rønnestad *et al*., 2000a; Rønnestad *et al.*, 2003). For instance, it has been found that larval growth rates in turbot (*Scophthalmus maximus*) fed *Artemia* is lower compared to those fed copepods due to insufficient levels of free methionine (Conceição *et al*., 1997). In the same study, the researchers also showed that uptake of dietary FAAs exceeded the total larval FAA pool by a factor of ten on a daily basis, thus highlighting the necessity for FAAs during larval stages. *Artemia* also have a low content of lipids and FAs crucial for marine fish larvae, as seen for instance in Atlantic halibut (Hamre *et al*. 2002; Evjemo *et al.*, 2003).

The relative content of PLs in *Artemia* rarely exceeds 30 % of their total lipids, whereas NLs such as triacylglycerols (TAG) makes up approximately 70 % of their total lipid content (Hamre *et al.*, 2002; van der Meeren *et al*., 2008). A low level of dietary PLs might have a negative impact on larval growth (Cahu *et al.*, 2003). Neither do *Artemia* fulfil the larval requirements for *n*-3 HUFAs as they contain no DHA and low amounts of EPA (Hamre *et al*., 2013). It is therefore necessary to enrich *Artemia* with lipid emulsions to increase dietary DHA and EPA content. However, *n*-3 HUFAs in enriched *Artemia* are primarily located in the NL rather than PL fraction (Conceição *et al.*, 2010). This makes *n*-3 HUFAs less available for larval growth and development, as shown in studies with Atlantic cod and European sea bass (Gisbert *et al*., 2005; Wold *et al.*, 2007; Kjørsvik *et al*., 2009; Wold *et al*., 2009). As for lumpfish, little is known regarding larval utilization capability of *n*-3 HUFAs incorporated in NLs. Obtaining a desirable DHA/EPA ratio during enrichment is also difficult as *Artemia* selectively metabolize DHA (Navarro *et al.*, 1999). Low DHA content in *Artemia* have shown negative effects on pigmentation and eye migration success in Atlantic halibut larvae (Hamre *et al*., 2002; Evjemo *et al*., 2003). Moreover, increased dietary NL/PL ratio in enriched *Artemia* may reduce lipid and FA utilization in fish larvae due to deficiency in PLs to promote lipoprotein formation (Liu *et al.*, 2002).

1.4 Aims and hypotheses

As commercial lumpfish production is escalating, the present study was primarily motivated by the fact that little is known about functional development of lumpfish larvae. The study aims were:

Aim 1: Provide a description of digestive system development in lumpfish from hatching to 50 dph based on histological and stereological methods.

Aim 2: Evaluate dietary effects on growth, survival and development of the liver, stomach and gut in lumpfish larvae fed different diets during the start feeding period (2-22 dph).

The aims were approached based on data from a start feeding experiment with lumpfish lasting from hatching to 50 dph, in which three larval diets were compared during the start feeding period from 2-22 dph: (1) *Artemia*, a traditional live feed in larviculture shown to benefit growth in lumpfish larvae (Brown, 1986). (2) *Acartia tonsa*, the natural live prey for most fish larvae that is advantageous for growth and development in several species (Næss *et al*., 1995; Evjemo *et al*., 2003; Dahle *et al*., 2014; Karlsen *et al*., 2015; Øie *et al.*, 2015). (3) Formulated diet, frequently used in lumpfish production, after a short term feeding with *A. tonsa* (2-8 dph). The diets were chosen in hope of attaining best possible growth so that ontogeny would be representable for larvae reared under an optimized production protocol. The following was hypothesized:

Hypothesis 1: Feeding lumpfish larvae with either *Artemia* or *A. tonsa* during the entire start feeding period (2-22 dph) will improve larval growth and survival compared to larvae weaned earlier (6-8 dph) from *A. tonsa* to formulated diet.

Hypothesis 2: All three start feeding diets will promote proper digestive system development in lumpfish larvae.

Parameters used to investigate growth effects of different diets were dry weight (DW), daily weight increase (DWI), wet weight (WW) and standard length (SL). The following morphometric parameters were considered to evaluate development and dietary effects of digestive organs: (a) Nucleus and cell area sizes of liver hepatocytes; (b) Area fractions of hepatocyte nuclei and hepatic vacuoles; (c) Epithelium height of the stomach, midgut and hindgut mucosa; (d) Number of gastric glands in the stomach mucosa; (e) Microvillus and villus height in the midgut and hindgut. Application of stereological methods provided the tools necessary to quantify the morphometric parameters of interest (a – e). Measurements were performed on digitalized transverse sections: one through the liver (*L* section), one or two comprising the stomach and midgut (S/Mg section) and one through the hindgut (*Hg* section). In addition to the transverse sections, longitudinal sections were observed histologically to study development of digestive organs.

2 Materials and methods

2.1 Start feeding of lumpfish larvae

2.1.1 Larval rearing

A total of 1.3 kg lumpfish eggs was provided from MoreFish AS at Tjeldbergodden, Norway. The eggs had been kept at 7 °C for approximately 200 d° and was disinfected with Buffodine before shipped to the larval rearing lab at NTNU Centre of Fisheries and Aquaculture (SeaLab) in Trondheim, Norway. After arrival, the eggs were incubated in two demersal egg incubators (FT family hatcher, Fish Tech AS, Norway) with continuous water flow (34 ppt salinity) and complete darkness (0:24 light/dark). Temperature during incubation was steadily increased from 7 °C up to 10 °C over the course of ten days.

After hatching occurred at approximately 300 d°, the fish were transferred from the hatching tank and distributed to nine cylindroconical tanks at an obtained density of 55-77 larvae L^{-1}. Each tank contained 100 L of seawater (34 ppt salinity) that had previously been pumped from 70 m depth in Trondheimsfjorden, filtered through a sand filter and a 1 µm filter, before microbially matured based on Skjermo *et al.* (1997). During maturation, the water was continuously degassed to prevent supersaturation of dissolved gasses before entering the larval rearing tanks with a temperature of 10 °C. Water exchange rate was increased stepwise from 5 tank volumes day^{-1} to 25 tank volumes day^{-1} 35 dph (Table 2.1). The tank water was ventilated by aeration tubes positioned near the water outlet in each tank. Oxygen saturation was measured every third to fourth day (ProODO Optical Dissolved Oxygen Instrument, YSI, USA) and was never below 80 %, while temperature was measured daily (2000T Digital Thermometer, Digitron, England). Each tank was illuminated with two fluorescent tubes (Sylvania Luxline Plus, 36W/830) from 03.00 AM-11.00 PM, maintaining a 20:4 light/dark regime during the whole experimental period. Two or three grey silicon mats (ca. 15 x 20 cm) were installed in each tank to provide additional surface area for the larvae to attach to.

Dead fish, debris and excess feed was removed from the tank bottom and walls once per day during the live feed period. After weaning to formulated diet, the tanks were supplied with protein skimmers to prevent accumulation of organic compounds in the water surface and cleaning was conducted twice a day. The sieves covering the water outlet, situated in the middle of the tanks, were exchanged and cleaned once every second or third day. These had a mesh size of 700 µm to allow live prey to be flushed out over time.

2.1.2 Larval feeding regimes

Three different feeding regimes were used during the start feeding experiment, each differing in type of feed given from 2-22 dph (Table 2.1). The first group received *Artemia* during this time interval (from now on referred to as the Artemia group). Newly hatched nauplii (instar I) were used for feeding the first three days (2-4 dph), equal fractions of newly hatched and enriched *Artemia* the following two days (5-6 dph), and only enriched *Artemia* from 7-22 dph (Table 2.2). *Acartia tonsa* copepodites (stages CI-CIII) were used as live feed for the two remaining groups. One group received copepods for the full period (from now on referred to as the Copepod group), whereas the other was weaned to a formulated diet (Gemma micro 150, Skretting AS, Norway) from 6-8 dph (from now on referred to as the FD group). Larvae from all groups were weaned from respective diets to the same formulated diet (Gemma micro 300, Skretting AS, Norway) from 20-22 dph, which they were fed until the experiment was terminated 51 dph. Three replicate start feeding tanks were used for each treatment, with a total of nine tanks.

The *Artemia* strain used in this experiment (EG ® INVE Aquaculture, Belgium) are approximately 900 µm in length when hatched (instar I) and grows to 1.2-1.3 mm (instar II-III) when enriched over 24 hours, obtaining an individual dry weight of 2.2-2.5 µg (Jan Ove Evjemo, SINTEF Ocean AS, pers. comm., 2018). Copepodites (CI-CIII) of *A. tonsa* have a cephalothorax length of 410-580 µm and a dry weight of 0.56-1.40 µg individual^{-1} ('On growing of copepods', user's manual from C-Feed AS). Live prey was transferred from their reservoirs to respective start feeding tanks through silicon hoses (diameter: 12 mm external, 6 mm internal) by peristaltic pumps (Watson-Marlow 520U with 313X 3 Roller Extension Pumphead, Watson-Marlow, USA) six times a day during the light period (feeding cycle: 02.00 PM, 06.00 PM, 10.00 PM, 03.00 AM, 06.30 AM and 10.00 AM) (Figure 2.1A). Prey organisms were distributed to obtain the following densities in each tank per feeding: 1000 L^{-1} 2 and 3 dph, 1500 L^{-1} 4 and 5 dph, 2000 L^{-1} 6 dph, and finally 3000 L^{-1} for the remaining live feed period. Prey density was identical regardless of feeding regime.

When starting the weaning period 6 dph for the FD group and 20 dph for the Artemia and Copepod groups, a feeding automat (Sterner 905, Fish Tech AS, Norway) was installed above each individual rearing tank (Figure 2.1B). These were programmed with an online management system (Normatic WebServer, Normatic AS, Norway) to deliver the following doses of formulated feed based on feeding tables from Skretting AS (Martin Davidsen, Skretting AS, pers. comm., 2018): 12 g day^{-1} from 6-8 dph, 24.4 g day^{-1} from 8-35 dph, 37.8 g day^{-1} from 36-40 dph, 44.1 g day^{-1} from 41-44 dph, and finally 50.4 g day^{-1} from 45-51 dph. The dry feed was distributed to the tanks 30-40 times every day during the light period. Before entering the tanks, the feed was mixed with intake water in a funnel located beneath each feeding automat.

Table 2.1. Experimental setup for start feeding of *C. lumpus*. Three different feeding regimes were used, each treatment having a triplicate number of tanks (n = 3). Days with overlapping feed types indicates weaning periods. The experiment was conducted in 100 L cylindroconical tanks at a constant temperature of 10 °C and 20:4 light/dark cycle.

Date	May 2017																							June 2017																				
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9-12	13	14	15	16	17	18	19	20	21-29
Dph	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31-34	35	36	37	38	39	40	41	42	43-51
d°	300	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600	610-640	650	660	670	680	690	700	710	720	730-810
Water exchange	5 tank volumes day^{-1}						10 tank volumes day^{-1}																	15 tank volumes day^{-1}									25 tank volumes day^{-1}								
Artemia (n = 3)							*Artemia* (Multigain)																																				
																								Formulated feed (Gemma micro 300)																				
Copepod (n = 3)							*Acartia tonsa* (*Rhodomonas baltica*)																																				
																								Formulated feed (Gemma micro 300)																				
Formulated diet (n = 3)			*A. tonsa* (*R. baltica*)																																								
							Formulated feed (Gemma micro 150)																																						
																								Formulated feed (Gemma micro 300)																				

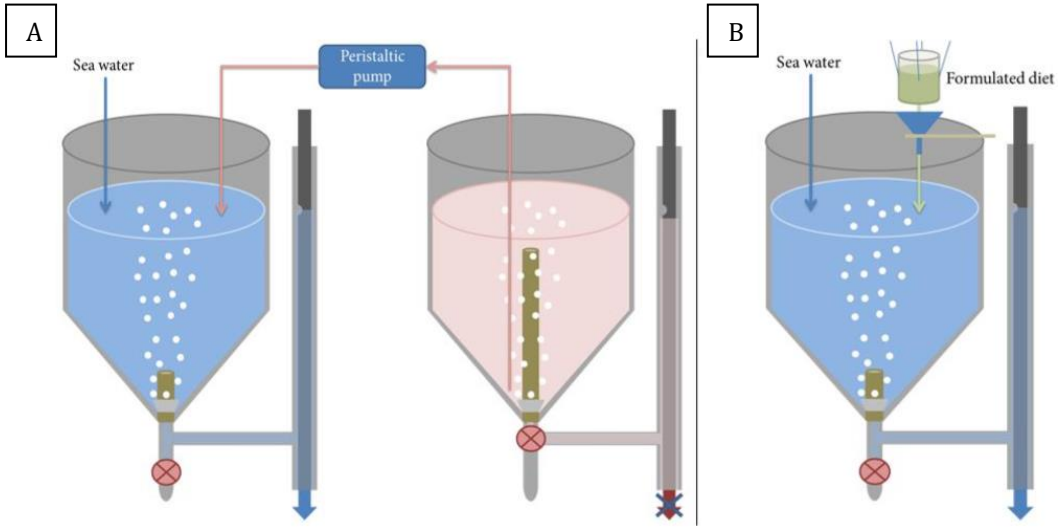

Figure 2.1. Feed distribution to larval rearing tanks. A) Larval rearing tank (left) and live feed tank (right) used during the live feed period. A peristaltic pump was used to transfer water with live feed to the larval rearing tanks. B) A feed automat located above each larval rearing tank was used to distribute formulated diet. The feed was mixed with in-take water in a funnel before entering the tanks. Illustration: Jon van Mil (Wageningen University, Netherlands).

Table 2.2. Percentage of *Artemia* stages fed per day to the Artemia group. Newly hatched *Artemia* (instar I) were harvested 24 hours after the cysts had been put up for hatching. Enriched *Artemia* were fed Multigain twice in 24 hours before harvested.

Dph	Artemia quality	
	Newly hatched (%)	Enriched (%)
2-4	100	0
5-6	50	50
7-22	0	100

2.2 Live feed production

2.2.1 Microalgae

The microalgae *Rhodomonas baltica*, derived from stock cultures at NTNU (clone NIVA 5/91, Cryptophycea: Pyrenomonadales), was cultured semi-continuously and used as feed for the *A. tonsa* copepodites. Production was conducted in two cylindrical 200 L polycarbonate plastic cylinders with a diameter of 40 cm. Prior to inoculation, 1 mL Conwy medium L^{-1} seawater was added as a source for inorganic nutrients (Walne, 1979). Each culture was continuously illuminated by six fluorescent tubes (GE Polylux XLR™, 58W/830) pointing towards the cylinder center from three different sides and aerated with 1-2% CO_2. Every day, 40-50 L of each culture was removed and replaced with new seawater and culture medium. The added seawater had previously been filtered through a sand filter and a 1 µm mesh, before disinfected by chlorination. Sodium hypochlorite (NaClO, 10-15 %) at a concentration of 0.25 mL L^{-1} was used to chlorinate the water for at least five hours before it was dechlorinated with 0.03 g L^{-1} sodium thiosulphate (Na_2SO_3) (Hoff & Snell, 1987). Dechlorination lasted for a minimum of five hours before the water was ready for use with a temperature of 20 °C.

2.2.2 Copepods

Cultures of *A. tonsa* (clone DFH.AT1) copepodites (stages CI-CIII) were supplied in 25 L plastic containers from C-Feed AS at Vanvikan, Norway. A total of three deliveries were received: the first for feeding 2-6 dph, the second for feeding 7-14 dph and the third for feeding 15-22 dph. After arrival at the start feeding facility (SeaLab), the density of each culture was estimated by adding 2-3 drops of Lugol's iodine solution (recipe: 20 mL acetic acid (99-100 %), 200 g potassium iodide and 10 g iodine in 200 mL distilled water) to a 20 mL sample and counting the number of individuals. The cultures were then transferred to tanks identical to those used for the fish larvae, and each tank contained enough copepods for one day of feeding (Table 2.3). Total volume in each feed reservoir was 100 L for feeding 2-8 dph since both the Copepod and FD groups received *A. tonsa* during this interval (n = 6 tanks). As only the Copepod group was fed *A. tonsa* from 9-22 dph (n = 3 tanks), each feed reservoir used for feeding on these days contained only 50 L. Half the volume of each tank was drained daily before starting a new feeding cycle at 02.00 PM. This was replaced with fresh 10 °C seawater and 5-6 L of *R. baltica* culture that had been harvested during its stationary phase when the culture had a dark red or brown color. The exact quantity of added microalgae was not estimated because of low feed intake and slow growth of *A. tonsa* at low temperatures (Miller *et al.*, 1977; White & Roman, 1992). Aeration tubes were situated near the water outlet in each tank, covered by bottom sieves with a 100 µm mesh to prevent copepods from escaping. The copepods were exposed to a light/dark cycle and illumination source identical to that of the fish larvae.

Table 2.3. Number of *A. tonsa* used per day to feed the copepod and FD groups. The number per fish tank (N tank^{-1}) was obtained by multiplying feed concentration (N L^{-1} feeding^{-1}) with fish tank volume (100 L) and number of feedings per day (6). As only the Copepod group got *A. tonsa* from 9-22 dph, copepod tank volume was halved during this period.

Dph	Concentration (N L^{-1} feeding^{-1})	Number per fish tank (N tank^{-1})	Fish tanks	Total number (N)	Copepod tank volume (L)
2-3	1000	600 000	6	3 600 000	100
4-5	1500	900 000	6	5 400 000	100
6	2000	1 200 000	6	7 200 000	100
7-8	3000	1 800 000	6	10 800 000	100
9-22	3000	1 800 000	3	5 400 000	50

2.2.3 *Artemia*

Artemia cysts (EG ® INVE Aquaculture, Belgium) were weighed (w_1) and hydrated in 20-25 °C fresh water for one hour. These were decapsulated according to FAO's manual for *Artemia* production (Sorgeloos *et al.*, 1986), originally described by Sorgeloos and colleagues (1977). The decapsulated cysts were weighed again (w_2) before stored at 4 °C for up to one week. Every day the needed quantity of cysts was put up for hatching in a 60 L cylindroconical tank. The amount was calculated from an assumed hatching efficiency of 200 000 nauplii g^{-1} dry weight of cysts (determined from previous hatchings) and the obtained weight factor w_2/w_1 (gram wet weight per gram dry weight). Heavily aerated seawater with a temperature of 25-28 °C was used to optimize hatching conditions (Sorgeloos *et al.*, 1986). After 24 hours, newly hatched *Artemia* were separated from unhatched cysts and hatching debris before transferred to an enrichment tank at a maximum density of 300 nauplii mL^{-1}. The culture was enriched with 10 g Multigain (BioMar AS, Norway) per 60 L of seawater twice during the next 24 hours, before being washed and transferred to the feed reservoir. The reservoir contained 50 L stagnant seawater with a temperature of 10 °C and was aerated from the bottom center in the tank. Illumination source and light regime was the same as for the fish larvae. Culture density was determined prior to each new feeding cycle.

2.3 Larval sampling and fixation

Before any further treatment, all sampled larvae were sedated using tricaine methanesulfonate (MS-222 Finquel®, Argent Chemical Laboratories Inc., USA) with a concentration of 2 g L^{-1}, diluted approximately 1:1 with seawater from the fish rearing tanks. Number of larvae sampled on given days post hatch are given in Table 2.4. It was not distinguished between treatments 0 and 2 dph as feed had not yet been introduced at the time of sampling, hence these larvae were sampled from the hatching tank. Larvae sampled for histological analyses were fixated in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) with pH 7.4 (Apotekproduksjon AS, Norway). Fixated larvae were stored in vials at 4 °C until processed further.

Table 2.4. Number of larvae sampled and analyzed for histology and growth. Larvae on day 0 and 2 post hatch were sampled from the hatching tank, and the numbers display total sample size (black). From 6-51 dph, sample sizes are shown per tank for growth (light grey) and per treatment for histology (dark grey).

Dph	0	2	6	10	13	17	21	24	29	34	38	42	45	50	51
Growth															
Sampled	16	16	5	5	5	5	5	5	5	5	5	10	10	10	16
Analyzed	16	16	5	5	5	5	5	5	5	5	5	10	10	10	16
Histology															
Sampled	15	15	10	10	10	10	10	10	10	10	10	10	10	10	0
Analyzed	5	0	0	5	0	0	5	0	0	5	0	5	0	5	0

2.4 Larval growth and survival

2.4.1 Growth

Standard length (SL), dry weight (DW) and wet weight (WW) were measured on larvae at 13 different times during the experiment: 2 dph (n = 16), 6, 10, 13, 17, 21, 24, 29, 34 and 38 dph (n = 5 per tank), and 42, 45 and 50 dph (n = 10 per tank). Additionally, samples were collected for DW only at the day of hatching (n = 16) and before terminating the experiment 51 dph (n = 16 per tank). SL was measured from the tip of the snout to the end of the notochord. When the notochord was no longer visible, the caudal peduncle was used as the posterior measuring point. The measurements were performed using the image processing software ImageJ (Schneider *et al.*, 2012) on pictures taken in a stereo microscope (Leica MZ7$_5$, Leica Microsystems, Germany; Zeiss Axiocam ERc 5s, Zeiss Inc., Germany) at magnifications 0.63, 0.80 or 1.00. An accuracy of ± 0.1 mm was used for all measurements. The images were also used to study external morphology of the larvae. After rinsing the larvae in distilled water, they were placed individually in pre-weighed tin capsules and their WW was determined using a micro balance (UMX2 Ultra-microbalance, Mettler-Toledo, USA). The samples were then dried at 60 °C for a minimum of 24 hours before weighed again to obtain the DW. Specific growth rates (SGR) were calculated for specific sampling intervals according to the following equation (Houde & Schekter, 1981):

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

where W_2 and W_1 is the individual larval dry weight at time t_2 and t_1, respectively. The obtained SGR values were used to determine percentage daily weight increase (DWI) (Houde & Schekter, 1981):

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

2.4.2 Survival

When the experiment was terminated 51 dph, all remaining larvae were euthanized with 2 g L^{-1} MS-222 and preserved in 70 % ethanol (VWR ®, USA). These were counted, and together with registered samplings and mortalities, the total number of larvae in each tank was estimated. Survival (S_t) throughout the experiment was calculated for each tank using the following equation:

$$S_t = \frac{N_t}{N_0} \cdot 100\%$$

where N_t is the number of larvae alive at time t (dph) and N_0 is the total number of larvae at the start of the experiment. When performing the calculations, it was assumed that all sampled larvae (ca. 10 % of the total larvae in each tank) would be alive 51 dph. Survival across treatments was compared after the two weaning periods, 8 and 23 dph, as well as on day 34, 42 and 51 post hatch.

2.5 Histological analyses

2.5.1 Mapping of larval digestive system

The larval digestive system was first mapped using one larva from the Artemia group aged 50 dph as a reference. This larva was dehydrated (Leica TP1020, Leica Biosystems, Germany) and embedded in paraffin (Tissue-Tek® III Embedding wax, Sakura, UK), before cut into 4 µm thick longitudinal sections parallel to the sagittal plane (Leica RM2255, Leica Biosystems, Germany). One section every 100-200 µm was stained with Hematoxylin (Mayer's hemalum solution, Merck, Germany) and Eosin (Eosin Y-solution 0.5 % aqueous, Merck, Germany). Based on visual examination of these slides (Zeiss Axioskop 2 plus, Zeiss Inc., Germany), transverse sectioning of the digestive system was outlined (Figure 2.2): one section for the liver (*L* section), one or two sections for the stomach and midgut (*S*/*Mg* section) depending on larval age, and one section for the hindgut (*Hg* section). Reference points used during sectioning are listed in Table 2.5.

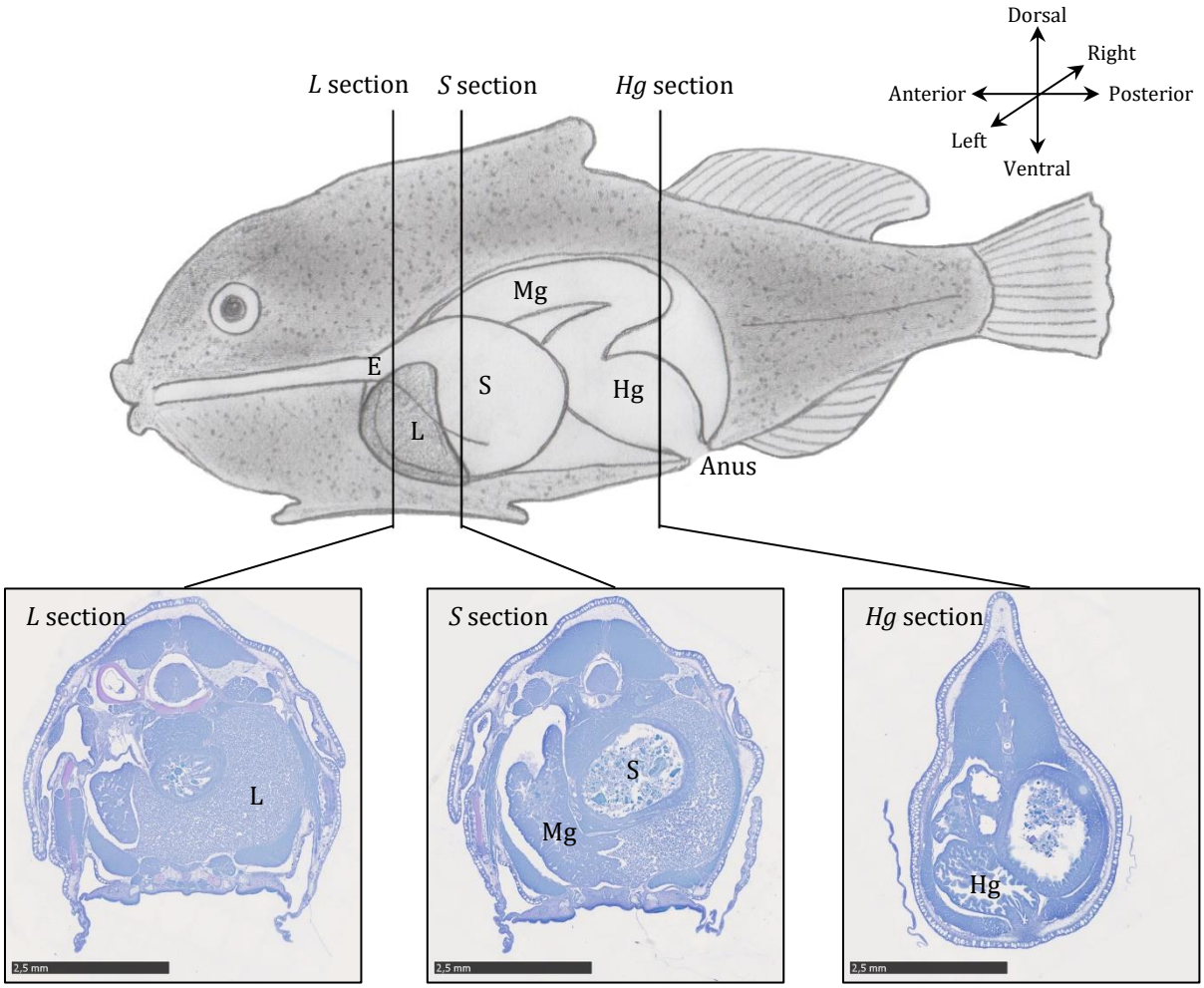

Figure 2.2. Overview of transverse sectioning. Schematic drawing of *C. lumpus* larva 50 dph showing approximate location for the liver (*L* section), stomach/midgut (*S*/*Mg* section) and hindgut (*Hg* section) transverse sections (vertical lines). Body axes are displayed in the upper right corner and an example is shown for each section category. All pictures are transverse sections from an Artemia larva (50 dph) stained with TB 0.05 %. Scale bars: 2.5 mm. Abbreviations: E = esophagus, Hg = hindgut, *Hg* = hindgut section, L = liver, *L* section = liver section, Mg = midgut, S = stomach, *S* section = stomach and midgut section.

Table 2.5. Reference points for transverse sectioning. The reference points were used during sectioning to decide when to cut the liver (*L* section), stomach/midgut (*S*/*Mg* section) and hindgut (*Hg* section) transverse sections.

Transverse section	Reference points
Liver (*L* section)	1. The liver constitutes a major proportion of the abdominal cavity's total area fraction. 2. The esophagus is still present and no transition to the stomach (or developing stomach tissue) can be seen.
Stomach and midgut (*S*/*Mg* section)	1. The first section that includes pancreatic tissue and comprises both the stomach and the midgut (*S* section). 2. The liver is still present but covers a significantly reduced area. 3. For the youngest larvae (0 and 10 dph), one separate section is made for the midgut alone (*Mg* section), taken directly after transitioning from the stomach (or developing stomach tissue) to the gut.
Hindgut (*Hg* section)	1. The abdominal cavity and total muscle tissue covers an approximately equivalent sized area. 2. The serosa surrounding the hindgut is ventrally connected to the abdominal wall, in proximity of the anus (or the anus can be seen).

2.5.2 Sample processing for histological analyses

Histological analyses of the digestive system were performed on transverse sections of the larvae 0 dph (n = 5), 10, 21, 34, 42 and 50 dph (n = 5 per treatment). Additionally, longitudinal sectioning was performed on one larva from the Artemia group for each of the selected age groups. SL was measured on each larva (Section 2.4.1), before being dehydrated and embedded in plastic (Technovit® 7100, Kulzer, Germany; Appendix 1). This was chosen as embedding material rather than paraffin due to improved morphological resolution (Nusbickel & Swartz, 1979; Sasso-Cerri *et al.*, 2001). The embedded larvae were then cut into 2 µm sections by using a microtome (Jung Autocut 2055, Leica Biosystems, Germany) with carbide metal blades (Leica TC65 Microtome Blade, Leica Biosystems, Germany). The sections were stained with 0.05 % toluidine blue (TB) (Honeywell Riedel-de-Haën™, Germany), a basic dye with high affinity for acidic components that gives tissues a deep blue or purple color (Sridharan & Shankar, 2012). This staining method was selected after performing small scale tests on liver and gut tissue (Appendix 2) as it was simple, fast and resulted in high-resolution sections (Appendix 3). Each stained section was then mounted (Neo-Mount®, Merck, Germany) and scanned in three 0.5 µm layers at 40x magnification using a digital slide scanner (NanoZoomer SQ, Hamamatsu Photonics, Japan). An image acquisition software (NDP.view2, Hamamatsu Photonics, Japan) was used to study development of digestive system organs, focusing primarily on the liver, stomach and gut.

2.5.3 Liver analyses

Hepatocyte nucleus area size, hepatocyte cell area size and liver area fractions (hepatocyte nuclei, lipid vacuoles and 'other'), were estimated on images exported from NDP.view2 to ImageJ. One image with an area size of 175 266.3 µm^2 was exported per liver section (L section) and larva, encompassing the whole liver (0 and 10 dph) or a randomly selected area consisting exclusively of liver tissue (21-50 dph). The freehand selection tool in ImageJ was used to measure the area size of individual hepatocyte nuclei (see also Pantic *et al.*, 2016), on average 257 per image. Nuclei touching the edges of the image or missing a distinct nucleolus were not measured. This data was used to estimate two parameters: Hepatocyte cell size, calculated as analyzed liver area divided by the number of nuclei within the frame ($A_{\text{liver}}/N_{\text{nuclei}}$), and nuclear area fraction, calculated as the total nuclear area divided by analyzed liver area ($A_{\text{nuclei}}/A_{\text{liver}}$). A point grid was applied to each image to estimate the area fraction constituted by vacuoles in the hepatocytes ($A_{\text{vacuoles}}/A_{\text{liver}}$; Gundersen *et al.*, 1988), using ImageJ's Cell Counter plugin as a counting tool. The point grid had a density ensuring 200 points or more to hit the liver tissue (after Wold *et al.*, 2009). Finally, the sum of nuclear and vesicular area fractions was used to derive the total area fraction of 'other' liver components ($A_{\text{other}}/A_{\text{liver}}$), such as bile canaliculi, hepatocyte organelles, sinusoids and more.

2.5.4 Stomach analyses

Stomach epithelium height was measured, development categorized, and total number of gastric glands in the stomach mucosa counted on each stomach section (*S* section) and larva using NDP.view2. Epithelium height was defined as the distance from the basal lamina to the apical membrane and was measured at five random points in each section with an accuracy of ± 0.1 µm (Figure 2.3A). Development was categorized into three different ontogenetic stages (S_i) using the following criteria: the primordial stomach appears as a slight enlargement of the esophagus (S_1); tubular gastric glands are present in the stomach mucosa (S_2); formation of the fundic stomach and greatly increased lumen volume (S_3, Figure 2.3B-D). Based on these categories, intervals (*a* – *b*) were determined to obtain a size range at transition to S_2 and S_3 from S_1 and S_2, respectively. The smallest number in the interval (*a*) represents the SL of the smallest larva in the respective developmental stage ($S_{2/3}$), whereas the largest number (*b*) denotes the SL of largest larva that had not yet reached this stage ($S_{1/2}$).

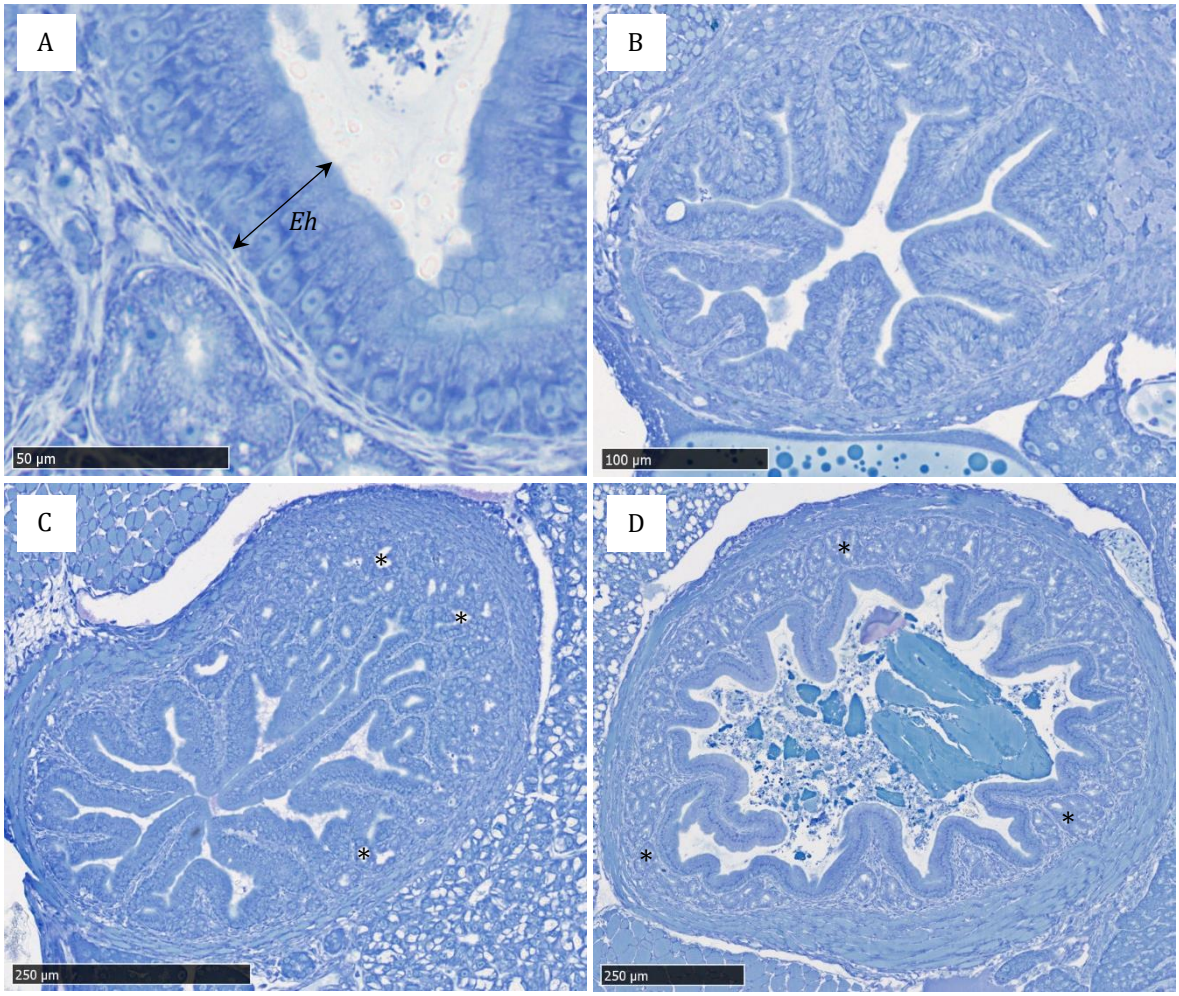

Figure 2.3. Measurements and stomach developmental stages in *C. lumpus*. A) Epithelium height (*Eh*) was measured from the basal lamina to the apical membrane (50 dph). Scale bar: 50 µm. B) S_1: The stomach is an enlargement of the esophagus (10 dph). Scale bar: 100 µm. C) S_2: Gastric glands (*) can be seen in the stomach mucosa (21 dph). Scale bar: 250 µm. D) S_3: Greatly increased lumen volume and formation of the fundic stomach region (50 dph). Scale bar: 250 µm. All pictures are transverse sections of larvae from Artemia treatment stained with TB 0.05 %.

2.5.5 Gut analyses

Epithelium, microvillus and villus height were measured on the midgut (*S/Mg*) and hindgut (*Hg*) sections of each larva using NDP.view2. Epithelium height was defined as the distance from the basal lamina to the borderline between the microvilli and gut lumen, whereas microvillus height was measured from the apical membrane of the enterocytes to the borderline between the microvilli and gut lumen (Figure 2.4A). The epithelium and microvillus height variables were measured both dorsally and ventrally at five random points in the gut of each section with accuracies of ± 0.1 µm. Paired sample t-tests and Wilcoxon's signed-rank tests were used on normally and non-normally distributed data, respectively, to test whether the position factor (dorsal versus ventral) had a statistically significant effect within each treatment 10 and 50 dph. One test was run per variable and section category (*S/Mg* and *Hg* section). As only two out of 24 tests returned a significant result ($p < 0.05$), the position factor was neglected to obtain ten measurements per variable and section of all larvae. Villus height was also defined as the distance between the basal lamina and the borderline between the microvilli and lumen but was only measured at places where the mucosa was folded to form distinct villi (Figure 2.4B). Up to five villi were measured per section with an accuracy of ± 1 µm.

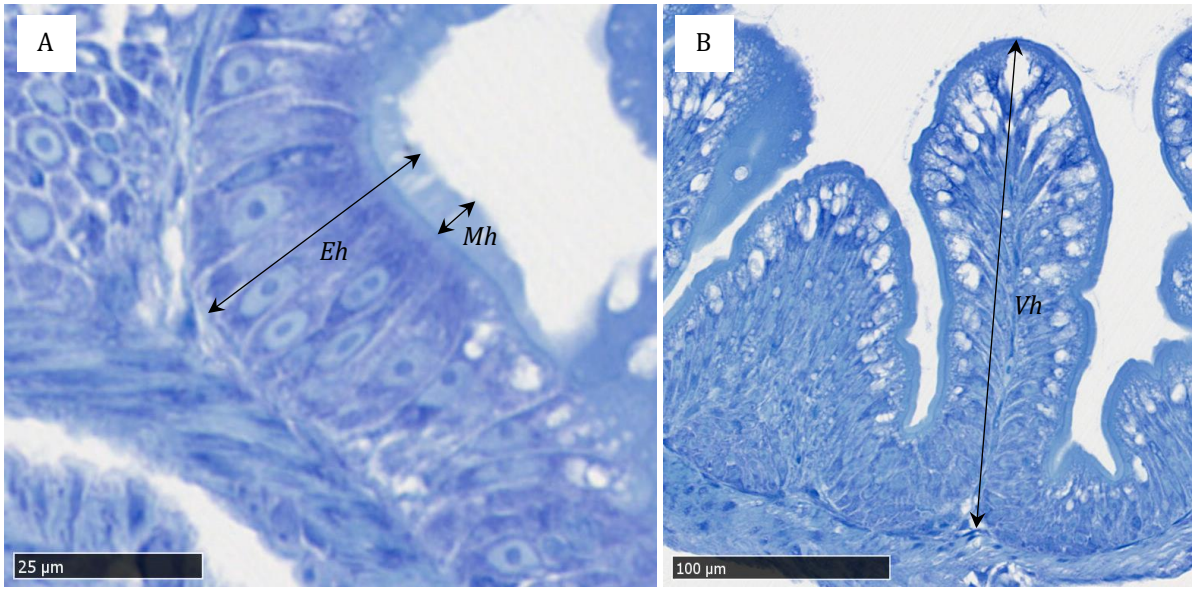

Figure 2.4. Measurements in the gut of *C. lumpus*. A) Epithelium height (*Eh*) was measured from the basal lamina to the borderline between the microvilli and gut lumen, whereas microvillus height (*Mh*) was measured from the apical membrane to the borderline between the microvilli and gut lumen. Scale bar: 25 µm. B) Villus height (*Vh*) was measured from the basal lamina to the borderline between the microvilli and gut lumen in areas where the mucosa was folded to form villi. Scale bar: 100 µm. All pictures are transversal sections of larvae from the Artemia treatment 50 dph stained with TB 0.05 %.

2.6 Statistical analyses

All statistical analyses were performed in R 3.4.3 (R Core Team, 2017) with RStudio (RStudio Team, 2016). In addition to default R packages, the following packages were installed to conduct statistical testing: 'car' (Fox *et al.*, 2017), 'FSA' (Ogle, 2017), 'mutoss' (Team MC *et al.*, 2012) and 'PMCMRplus' (Pohlert, 2018). All applied R functions and in which package they were found are listed in Appendix 4. A significance level of $\alpha = 0.05$ was used for all statistical tests except for correlations and regressions, where $\alpha = 0.01$ was used. Differences were thus considered statistically significant when $p < 0.05$ and $p < 0.01$, respectively. In the result section (Section 3), an asterisk (*) following a *p*-value denotes tests that were evaluated with $\alpha = 0.01$ to distinguish the two significance levels. Data consisting of percentages and area fractions were transformed to arcsine values before applying statistical tests. Graphs and tables were made in Microsoft Office Excel 2016, and all mean values are presented with corresponding standard errors (± SE).

Normality was tested using a Shapiro-Wilk test. Additionally, due to small sample sizes, quantile-quantile (Q-Q) plots were investigates visually to evaluate normality. Levene's test was then applied to test homogeneity of variance between treatments. Differences in group means of normally distributed data were compared using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls' post hoc test. When the data was normally distributed but variance between treatments heteroscedastic, Welch's ANOVA was used to compare group means, and pairwise comparisons were conducted using Dunnett's T3 test post hoc test. The non-parametric Kruskal-Wallis was applied to non-normal data, followed by Dunn's post hoc test with Holm's correction for adjustment of *p*-values (Holm, 1979). Paired sample t-tests and Wilcoxon's signed-rank tests were used on normally and non-normally distributed data, respectively, to compare mean values for different age groups within the same treatment. Linear correlation between continuous variables was tested with a two-tailed Pearson's correlation test. When significant correlations were found, linear regression was conducted to define the relationship between the two variables of interest and to obtain the coefficient of determination (r^2). Analysis of covariance (ANCOVA) was used to investigate if the linear equations differed significantly between the treatments.

3 Results

3.1 Larval development and general observations

3.1.1 Larval development

External and internal characteristics of lumpfish larvae and their development from hatching to 34 dph are provided in Table 3.1, which should be read while reviewing Figure 3.1 and 3.2. The descriptions are based on specimens from the Artemia group, as larvae from this treatment exhibited best growth (Section 3.2). Few apparent morphological changes were observed after 34 dph, and more detailed descriptions the digestive organs and how each of them developed throughout the experiment are covered in the following sections (Section 3.3-3.6).

Table 3.1. Larval development of *C. lumpus* 0-34 dph. Morphology of live and sectioned larvae from the Artemia group 0, 10, 21 and 34 dph. The descriptions are based on Figure 3.1 and 3.2.

Dph	Description
0	**Live larvae.** The body shape resembled that of a tadpole (Figure 3.1A), with a large head and trunk (region between the head and anus) and laterally flattened tail (region behind the anus). The eyes, head and trunk were well pigmented by yellow (head and trunk) and black (eyes, head and trunk) pigment cells. The tail region was transparent and surrounded by a continuous fin fold. Fin rays were visible in the pectoral and caudal fins. The suction disc was differentiated and functional, situated on the ventral side of the larval trunk. Red blood cells were observed circulating in the heart, gills and some major blood vessels. Each gill was hidden by a gill cover.
	Sectioned larvae. The yolk sac occupied most of the abdominal cavity and all digestive organs had a dorsal position (Figure 3.2A). The liver was small and situated to the left in the anterior abdomen. The alimentary canal had coiled to form a loop, was separated into an anterior and posterior half by an intestinal valve (not shown) and was open at both ends. The exocrine pancreas was attached dorsally to the gut. The stomach anlage appeared as a dilatation posterior to the esophagus (not shown).
10	**Live larvae.** A pair of transparent external nostrils were observed above the mouth and light reflecting pigment cells had appeared on each side of the head region, reaching from the mouth to the end of the gill cover (Figure 3.1B). The tail region was more muscular compared to that of newly hatched larvae and was covered with yellow and black epidermal pigment cells. All fins had well developed fin rays, and the first dorsal fin was partly overgrown by epidermal tissue. The fin fold was almost fully resorbed, with only some remnants surrounding the individual fins along the tail. The notochord end was flexing upwards above the caudal fin (flexion stage).

Table 3.1. Larval development of *C. lumpus* 0-34 dph (continued). Morphology of live and sectioned larvae from the Artemia group 0, 10, 21 and 34 dph. The descriptions are based on Figure 3.1 and 3.2.

Dph	Description
10	**Sectioned larvae.** The yolk sac was reduced in size and restricted to the right half of the abdominal cavity, surrounded by digestive organs that had increased in volume since hatching (Figure 3.2B). The liver had grown to cover most of the anterior abdomen's left half, whereas the gut had elongated and coiled further. The pancreas had increased in size, with the exocrine part enveloping the endocrine tissue. Gastric glands had appeared in the stomach region (not shown).
21	**Live larvae.** The whole larval body was fully pigmented with primarily yellow and black epidermal pigment cells (Figure 3.1C). The fin rays of the first dorsal fin were no longer visible as this fin was entirely overgrown by the epidermis. The second dorsal, caudal and anal fins appeared to be fully developed, and the fin fold was completely resorbed.
	Sectioned larvae. Further reduced yolk sac volume was accompanied by an increased volume of liver and gut tissue (Figure 3.2C). The liver constituted the major organ in the left half of the anterior abdomen, whereas the gut occupied most of the remaining abdominal space. The pancreas was no longer a compact organ but had branches reaching out to different areas along the gut (not shown).
34	**Live larvae.** The body shape was less slender compared to younger larvae due to seemingly larger body height (vertical distance from ventral to dorsal side at a given point along the body) relative to standard length (Figure 3.1D). Body pigmentation appeared more variable among individuals regardless of treatment, ranging from yellow to red and brown. Several fins (e.g. second dorsal and caudal fins) had attained some but very faint pigmentation.
	Sectioned larvae. The yolk sac was completely resorbed, and the space previously occupied by the yolk sac had been replaced by digestive organs. Morphogenesis of the stomach was complete as it was differentiated into the cardia, fundus and pylorus (Figure 3.2D). The stomach had greatly increased lumen volume and occupied a significant share of the dorsal and central parts of the abdomen. A distinct cardiac and pyloric sphincter separated the stomach from the esophagus and intestine, respectively. Pyloric caeca were present at the gastrointestinal junction in the anterior end of the midgut.

3.1.2 General observations

Most larvae were constantly swimming around after transfer to their respective tanks on the day of hatching. After introducing feed 2 dph, most individuals were instead attached to either tank surfaces or the installed silicon mats. This behavior was most common in the Artemia tanks, whereas a comparably larger fraction of the Copepod and FD larvae kept moving around in the water column during the start feeding period (2-22 dph). Larvae from the Artemia group were more difficult to catch during sampling than larvae from the two other groups.

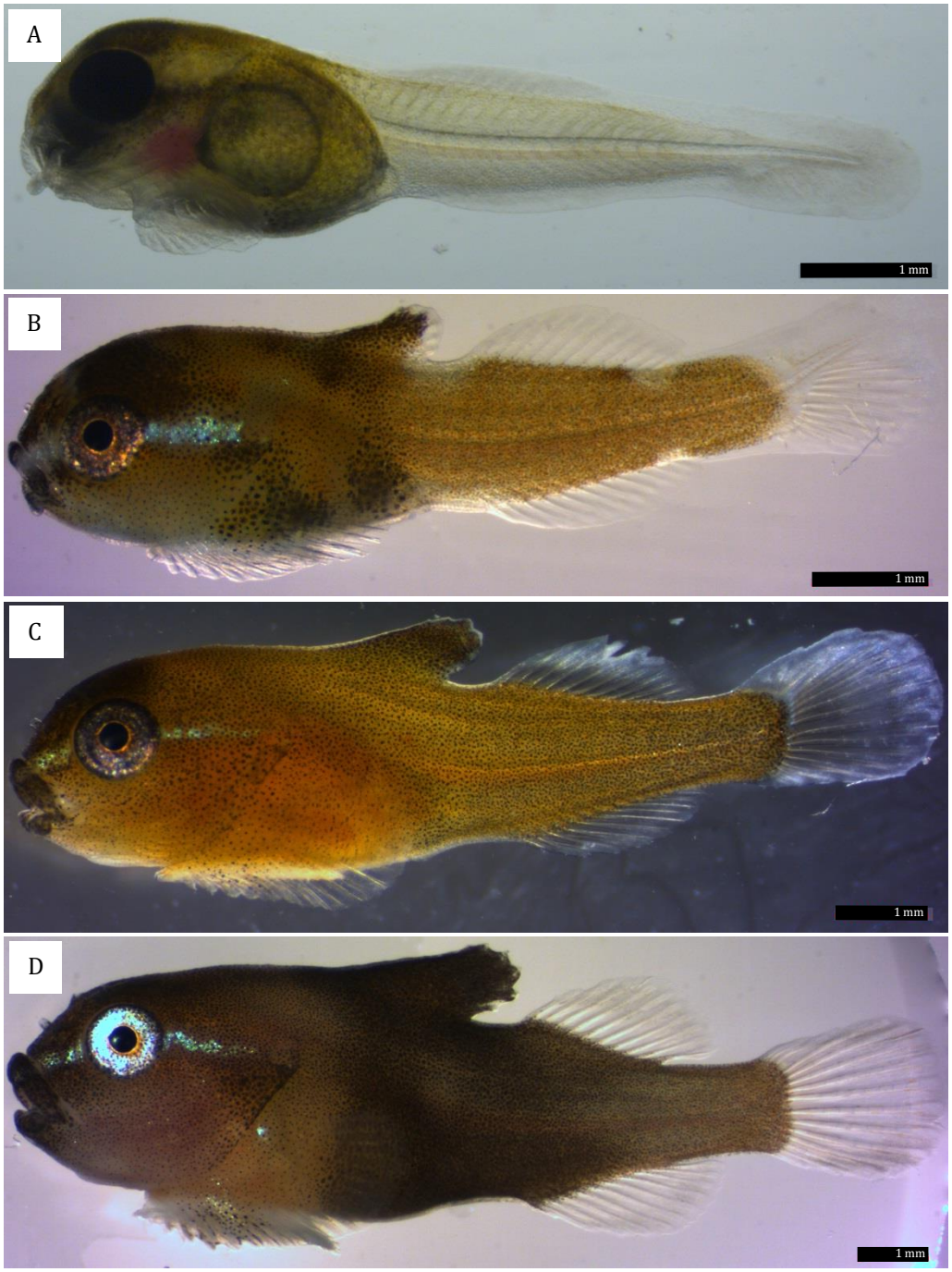

Figure 3.1. External morphology of *C. lumpus* 0-34 dph. Each picture represents a larva from the Artemia treatment on different days: A) newly hatched (0 dph), B) 10 dph, C) 21 dph and D) 34 dph. Photos taken by Job van Mil (Wageningen University, Netherlands). Scale bar: 1 mm.

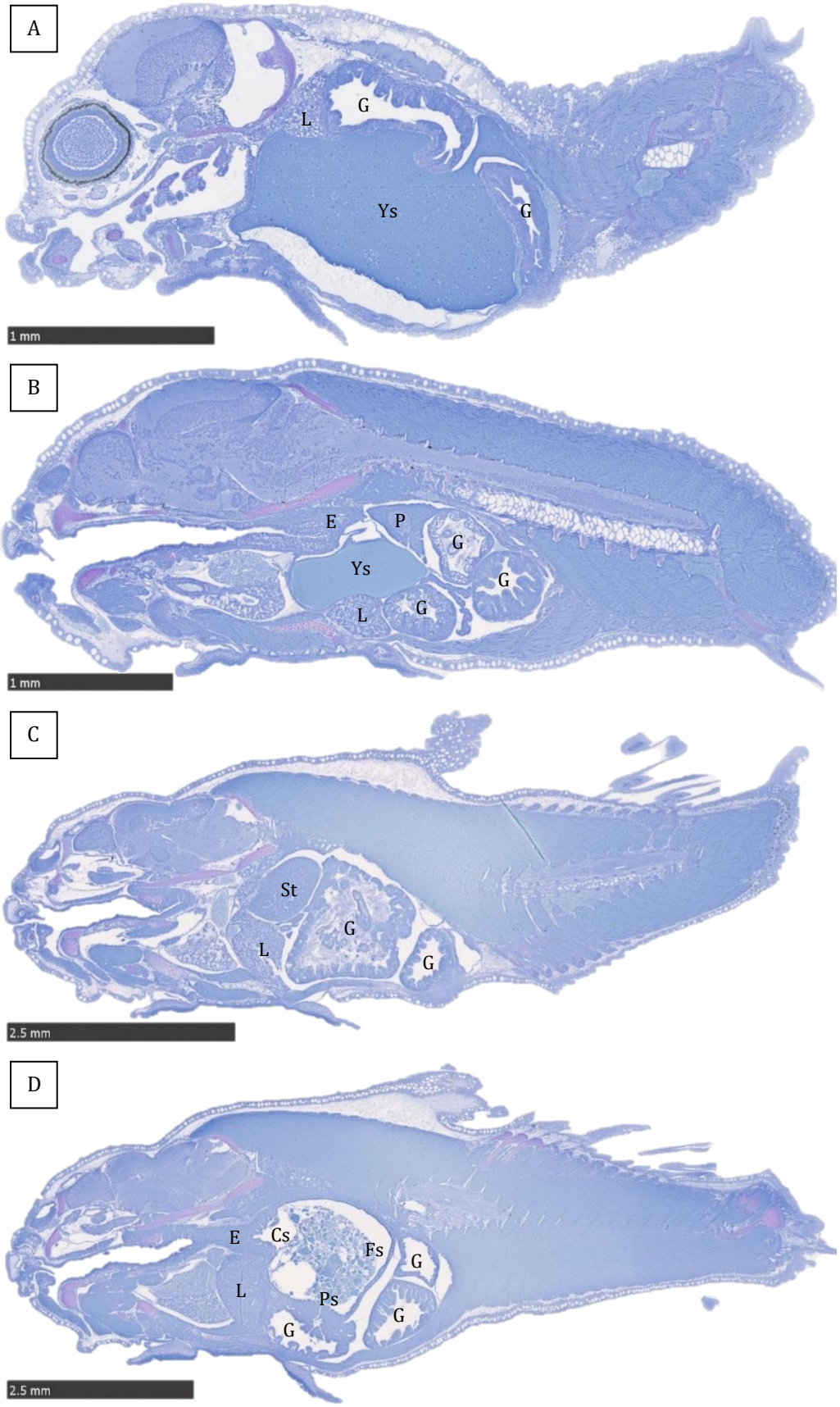

Figure 3.2. Development of the digestive system in *C. lumpus* 0-34 dph. Each picture represents a larva from the Artemia treatment on different days: A) newly hatched (0 dph), B) 10 dph, C) 21 dph and D) 34 dph. All pictures are longitudinal sections stained with TB 0.05 %. Scale bar: 1 mm (A and B) and 2.5 mm (C and E). Abbreviations: Cs = cardiac stomach, E = esophagus, Fs = fundic stomach, G = gut, L = liver, P = pancreas, Ps = pyloric stomach, St = stomach, Ys = yolk sac.

3.2 Larval growth and survival

3.2.1 Dry and wet weight

Larval dry weight (DW) was on average 1.07 ± 0.01 mg before introducing feed 2 dph (Figure 3.3, Appendix 5). Larvae initially fed *Artemia* grew exponentially during the whole experiment and were significantly larger than Copepod and FD larvae already by 6 dph. On the other hand, limited larval growth was observed in the Copepod and FD groups until 34 and 29 dph, respectively. Copepod larvae were larger than FD larvae 13-21 dph, coinciding with the period when *A. tons*a was supplied exclusively to the Copepod group. These differences were statistically significant 13 and 17 dph. After weaning to Gemma micro 300, the highest mean DW amongst Copepod and FD larvae was mostly observed in the FD treatment, with the largest difference 29 dph (p = 0.08). However, no significant differences were found between these two groups for the remainder of the experiment. When terminating the experiment 51 dph, Artemia larvae had attained a DW that was approximately the double of that of Copepod and FD larvae.

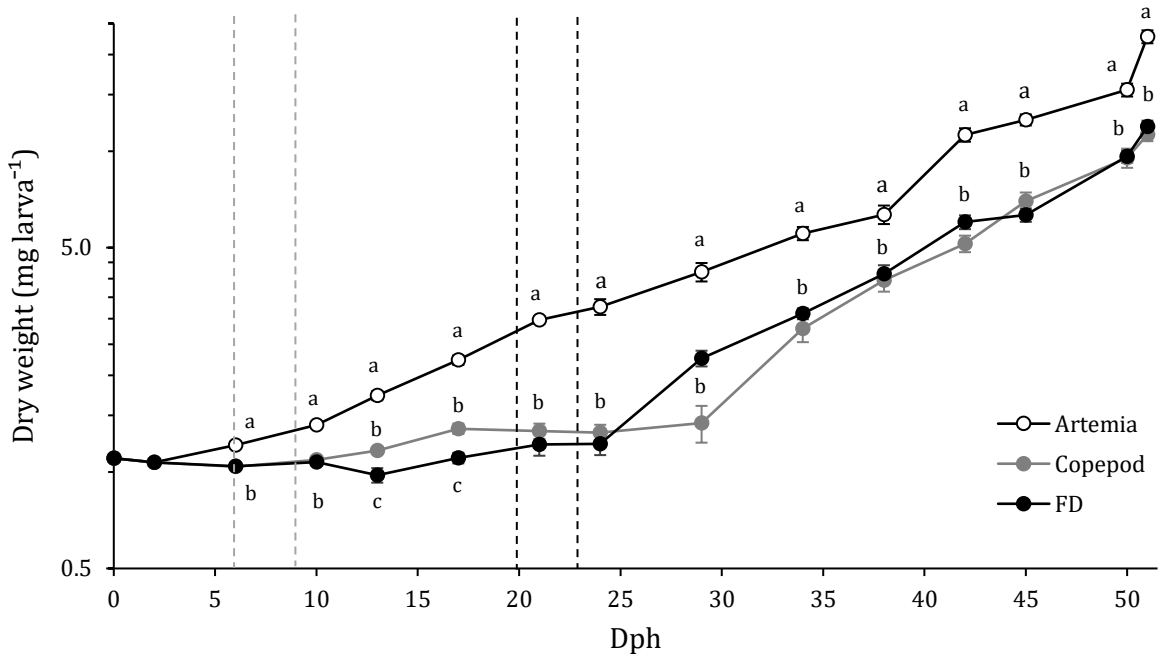

Figure 3.3. Mean dry weight in *C. lumpus* 0-51 dph. The averages (mg larva^{-1}) are based on an increasing sample size: n = 16 (0 and 2 dph), n = 15 per treatment (6-38 dph), n = 30 per treatment (42-50 dph) and n = 39-48 per treatment (51 dph). The grey dashed lines denote weaning from *A. tonsa* to Gemma micro 150 in the FD group, whereas the black dashed lines denote weaning from respective feeds to Gemma micro 300 in all groups. Significant differences (p < 0.05) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE). The vertical axis is in logarithmic scale.

A similar pattern was observed larval wet weight (WW; Appendix 6), and there was a strong positive linear correlation between WW and DW (all groups: $r > 0.99$, $p < 0.01$*). The estimated slope coefficients expressing the linear relationship in the Artemia, Copepod and FD groups were 6.88, 6.57 and 6.62 mg WW per mg DW, respectively. No significant difference regarding this relationship was found between the Copepod and FD treatments ($p = 0.4$*), whereas the slope coefficient in the Artemia group was significantly larger compared to both other groups (p-values < 0.01*). It was assumed that this difference was due to increased variability in the WW/DW relation for the largest individuals in the Artemia treatment sampled towards the end of the experiment (45 and 50 dph), and that very few larvae from the Copepod and FD groups grew to a similar size. Thus, a regression model for the pooled data set was made (Figure 3.4). In this model, variation in DW explained 99.2 % of the total observed variance in WW ($r^2 = 0.992$) and the estimated slope coefficient was 6.79 mg WW per mg DW ($p < 0.01$*).

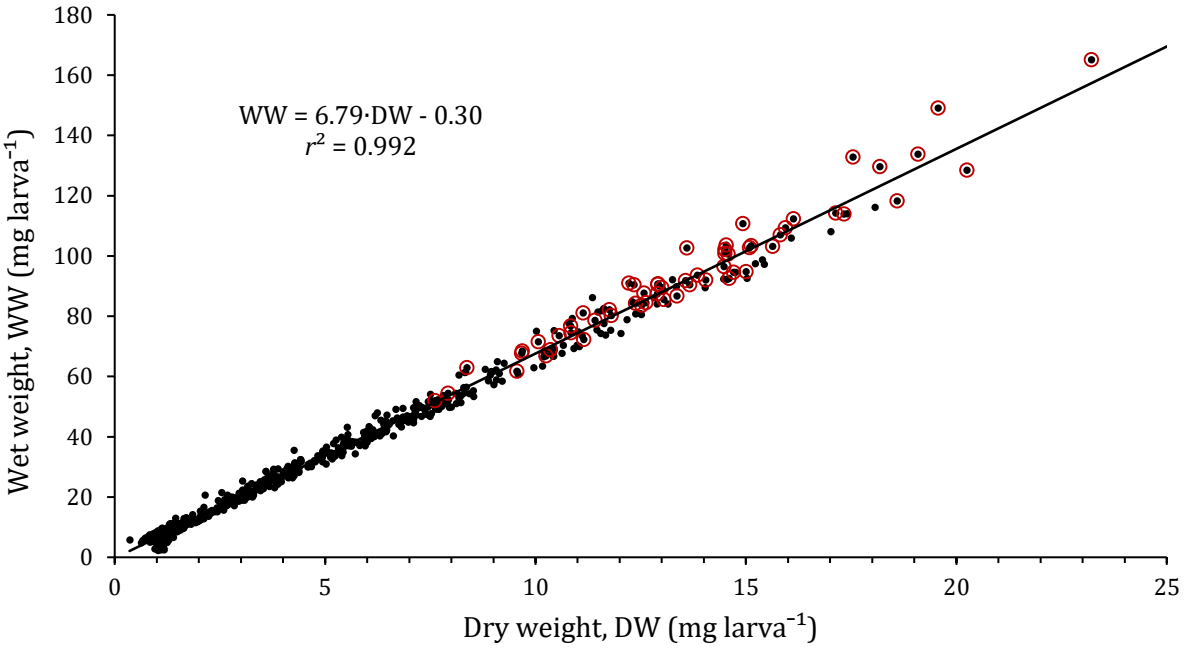

Figure 3.4. Wet weight as a function of dry weight in *C. lumpus* 2-50 dph. Each point represents the dry weight (mg larva^{-1}) and corresponding wet weight (mg larva^{-1}) of an individual larva, with a total of $n = 691$ larvae from 2-50 dph. Data from the different treatments are pooled. The encircled data points (dark red) represent larvae from the Artemia group 45 and 50 dph. Pearson correlation: $r = 0.996$ ($p < 0.01$*).

3.2.2 Daily weight increase

A negative change in DW was observed from hatching prior to addition of live feed 2 dph due to metabolism of yolk reserves (Figure 3.5, Appendix 7). From 2-10 dph, larvae fed *Artemia* had a positive daily weight increase (DWI) that was significantly larger than both the Copepod and FD groups, where hardly any growth occurred. During the following period from 10-21 dph, Artemia larvae grew at a significantly faster rate than observed within this treatment during the preceding

time interval (2-10 dph). DWI had also increased in the Copepod and FD groups from 10-21 dph, but this was not of statistical significance. No significant difference in DWI was detected between Artemia and FD larvae from 10-21 dph due to large variation between FD tanks. Over the course of the whole treatment period (2-22 dph), DW increased by an average of 5.53 ± 0.13 % day^{-1} in the Artemia treatment. This was significantly larger compared to DWI in the Copepod and FD groups, with respective means of 1.15 ± 0.39 % and 0.57 ± 0.62 % day^{-1}.

A significant increase in DWI was observed within the FD treatment in the 21-34 dph interval compared to preceding periods, whereas no significant changes were found within the Artemia and Copepod treatments. Although not statistically significant, the FD group also exhibited a larger DWI from 21-34 dph compared to the two other groups. During this period, Artemia and Copepod larvae were adjusting to the formulated diet after weaning from their respective live feeds, whereas FD larvae were simply given larger particles of the same diet (Gemma micro 300 instead of 150). No significant differences were observed between treatments during any of the three periods following weaning (21-34, 34-42 and 42-50 dph), and DWI was also highly variable within each treatment. The Artemia, Copepod and FD groups had respective growth rates of 6.35 ± 0.24, 4.88 ± 0.19 and 5.04 ± 0.09 % day^{-1} over the course of the experiment (2-51 dph), none of them which differed significantly from each other ($p > 0.05$).

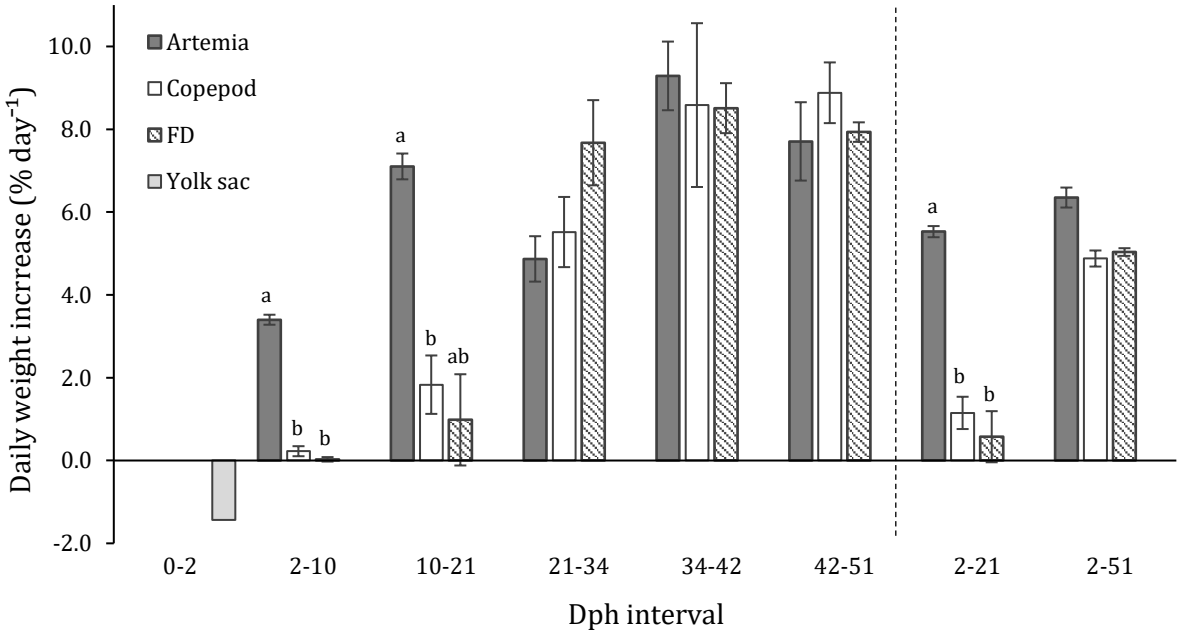

Figure 3.5. Mean daily weight increase in *C. lumpus* 0-51 dph. Average daily weight increase (% day^{-1}) in dry weight (mg larva^{-1}) in each of the three treatments (n = 3) during specific time intervals. The interval 0-2 dph represents endogenous feeding prior to addition of live feed. The dashed line distinguishes successive intervals from the treatment period (2-21 dph) and the whole experimental period (2-51 dph). Significant differences ($p < 0.05$) between treatments at a given interval are denoted by different letters. Errors bars indicate ± standard error (SE).

3.2.3 Standard length

Standard length (SL) of yolk sac larvae aged 2 dph was 6.4 ± 0.1 mm (Figure 3.6, Appendix 8). Larvae from the Artemia group grew linearly throughout the experiment and were significantly larger than Copepod and FD larvae on all days except for 10 dph, when no difference was found when compared to the Copepod group. As for Copepod and FD larvae, no significant growth was observed between 17-34 dph and 10-24 dph, respectively. Copepod larvae had a significantly larger SL than FD larvae from 13-21 dph, corresponding to the period when *A. tonsa* was fed exclusively to the Copepod group. On day 21 post hatch, during weaning to Gemma micro 300, Artemia larvae had a mean SL of 8.4 ± 0.1 mm. Similar sizes were not observed in the Copepod and FD groups until 34 dph, when respective means of 8.2 ± 0.2 and 8.6 ± 0.1 mm had been attained. From 29 dph, larvae from all groups exhibited a linear growth pattern for the remainder of the experiment. No differences were observed between Copepod and FD larvae after weaning until 50 dph, at which time the size of larvae from all treatments differed significantly from each other. Artemia larvae had the largest size, whereas Copepod larvae were smallest.

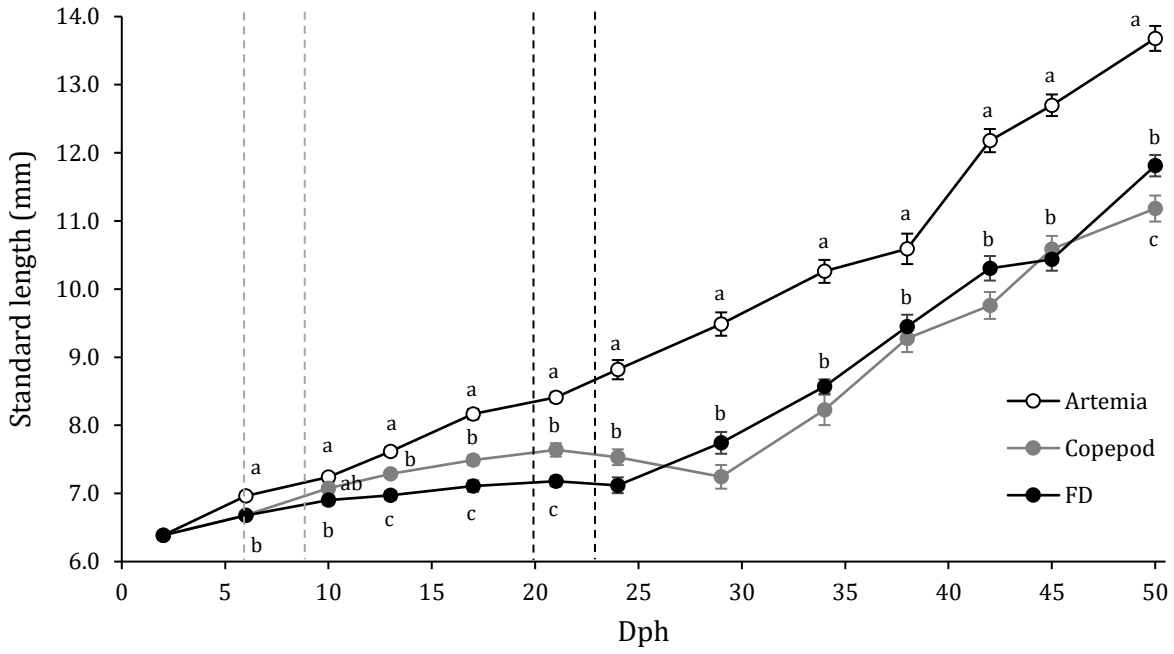

Figure 3.6. Mean standard length in *C. lumpus* 2-50 dph. The averages (mm) are based on an increasing sample size: n = 16 (2 dph), n = 15 per treatment (6-38 dph) and n = 30 per treatment (42-50 dph). The grey dashed lines denote weaning from *A. tonsa* to Gemma micro 150 in the FD group, whereas the black dashed lines denote weaning from respective feeds to Gemma micro 300 in all groups. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters. Error bars indicate ± standard error (SE).

There was a strong positive linear correlation between SL and DW (r = 0.959, p < 0.01*) and SL and WW (r = 0.962, p < 0.01*) for the pooled data. Transforming the weight values to natural logarithms increased the linearity of both correlations (DW: r = 0.974, p < 0.01* and WW: r = 0.969, p < 0.01*), suggesting an exponential relationship. The highest correlation coefficient was found for the DW/SL relation with natural log transformed DW values (r = 0.974), in which both slope coefficients and intercepts expressing the linear relationship between these two variables differed significantly between all treatments (p < 0.01*). Larvae from the Copepod group had the largest slope coefficient while larvae from the Artemia group had the largest intercept (Figure 3.7). In all three models, SL explained > 94 % of the total observed variation in DW (r^2 = 0.949 – 0.968).

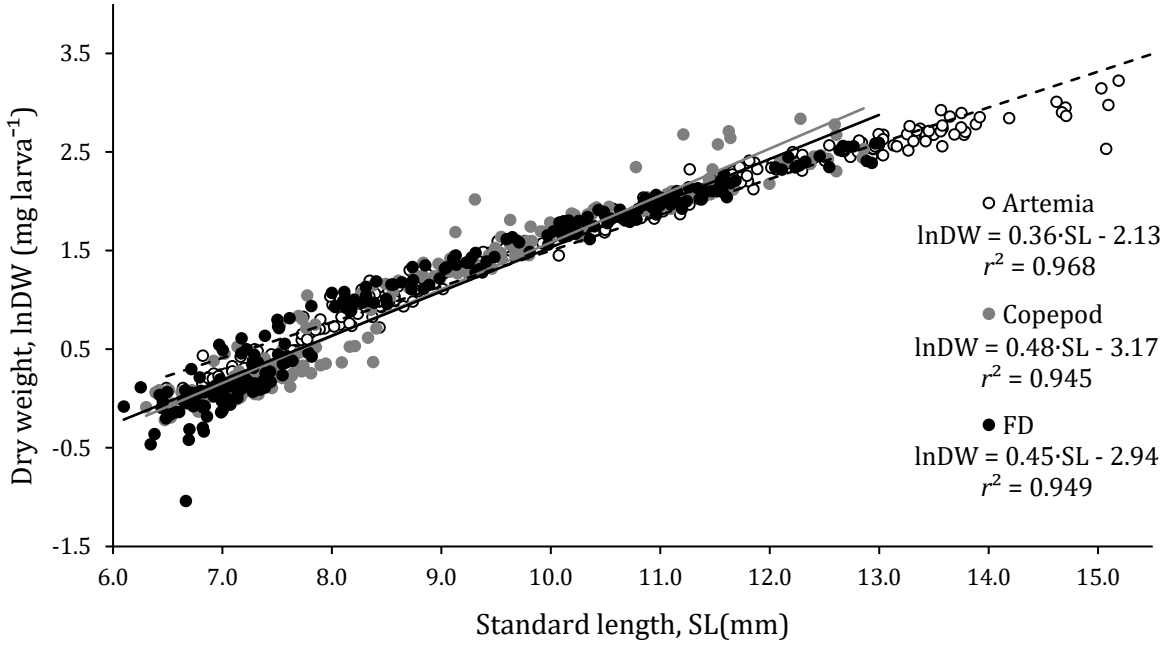

Figure 3.7. Larval dry weight as a function of standard length in *C. lumpus* 6-50 dph. Each point represents the standard length (mm) and corresponding natural log transformed dry weight (*ln* mg larva^{-1}) of an individual larva, with a total of n = 224-225 larvae per treatment from 6-50 dph. Data from the different treatments are separated into individual regressions. Pearson correlation: r = 0.984 (Artemia, p < 0.01*), 0.972 (Copepod, p < 0.01*) and 0.974 (FD, p < 0.01*).

3.2.4 Survival

Nearly no mortality was observed in any treatment for the first half of the experiment. This trend changed after weaning to Gemma micro 300, which was accompanied by increased bacterial growth seen as filamentous aggregates floating in the water and attached to the tank walls. Filament formation first occurred around 26 dph in some FD and Copepod tanks (tank 4, 5 and 10), but was seen in all tanks during the experiment. Dead larvae were frequently observed trapped inside these aggregates, but many larvae were also found dead on the tank bottoms. Although bacterial growth was less prominent in the Artemia tanks, entangled larvae were rarely found, and it seemed as if this did not cause any larval mortality in this treatment. On day 34 post

hatch, survival was significantly higher in the Artemia group compared to the Copepod and FD groups (Table 3.2). This was after a period of massive mortality, where particularly tank 10 had large numbers of dead larvae laying on the tank floor (Figure 3.8). Survival stabilized in most tanks after this period, except for an incidence in one FD replicate (tank 4) 48 dph. By the end of the experiment, average survival in the Artemia, Copepod and FD treatments were 97 ± 0, 79 ± 6 and 83 ± 4 %, respectively. Despite Welch's ANOVA test returning a significant result ($p < 0.05$), no difference of statistical significance was found between any group 51 dph when using Dunnett's T3 post hoc test for pairwise comparisons (p-values > 0.09). Number of larvae alive on each day of the experiment is shown for individual tanks in Appendix 9.

Table 3.2. Mean survival of *C. lumpus* 8-51 dph. The averages (% ± SE) are presented for each treatment ($n = 3$) on selected days. 9 and 23 dph represents the first day after the two weaning periods. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters.

Dph	Mean survival (%)		
	Artemia	Copepod	FD
9	100 ± 0	100 ± 0	100 ± 0
23	100 ± 0	100 ± 0	100 ± 0
34	99 ± 0[a]	83 ± 7[b]	89 ± 2[b]
42	99 ± 0[a]	80 ± 7[b]	87 ± 2[b]
51	97 ± 0	79 ± 6	83 ± 4

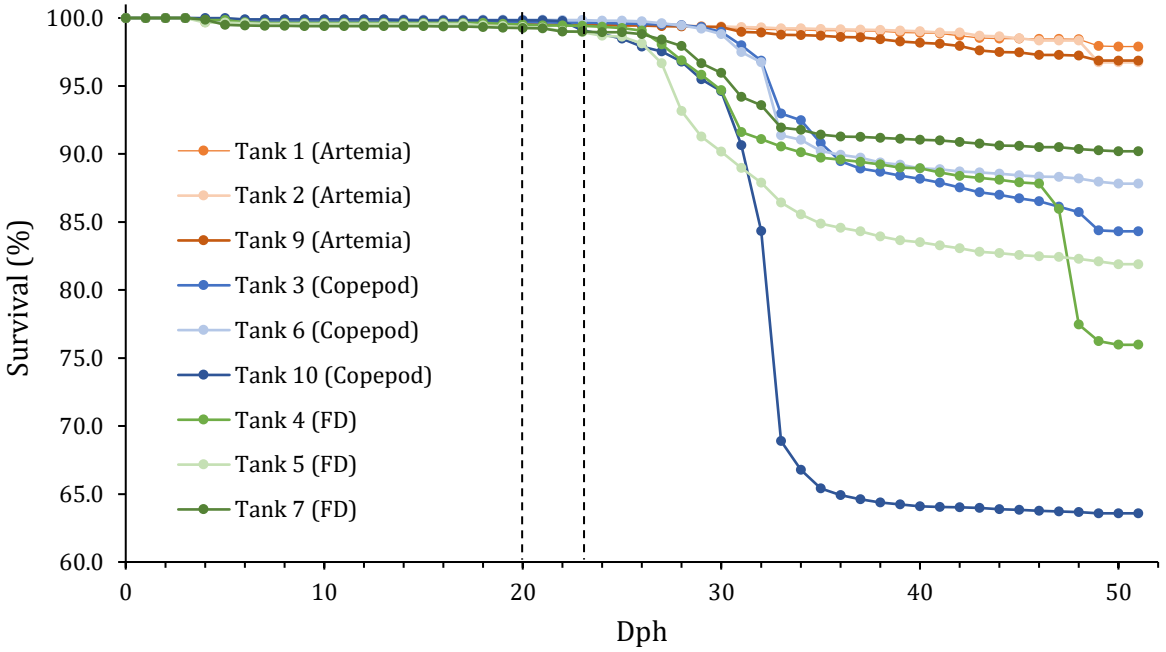

Figure 3.8. Survival of *C. lumpus* 0-51 dph. Survival (%) is shown for each treatment replicate ($n = 3$) to accentuate differences between individual tanks. The values at a given day are calculated as the number of larvae alive at that day divided by the total number of larvae, estimated as the sum of dead, sampled and living larvae per tank 51 dph. The black dashed lines denote weaning from respective feeds to Gemma micro 300 in all groups. Note that the vertical axis starts at 60 %.

3.3 Liver histology

3.3.1 Liver morphology

At hatching the liver constituted a small organ situated dorsally to the yolk sac in the left half of the anterior abdomen (Figure 3.9A) and was enveloped by a serous membrane that attached it to the abdominal wall and the gallbladder serosa. The liver parenchyma was fully differentiated with a homogenous structure of polyhedral hepatocytes arranged around a central vein and infused with sinusoids and bile canaliculi. The hepatocytes appeared voluminous due to a high content of glycogen-like aggregates residing in vacuolated cytoplasm (Figure 3.9B). Each vacuole contained single or multiple such aggregates with a faint brown or orange color and an irregular shape, the latter of which was reflected in the uneven vacuolar surface. These vacuoles displaced the nuclei peripherally within individual hepatocytes. The hepatocyte nuclei were mostly spherical and had very distinct nucleoli and boundaries that clearly distinguished them from erythrocytes.

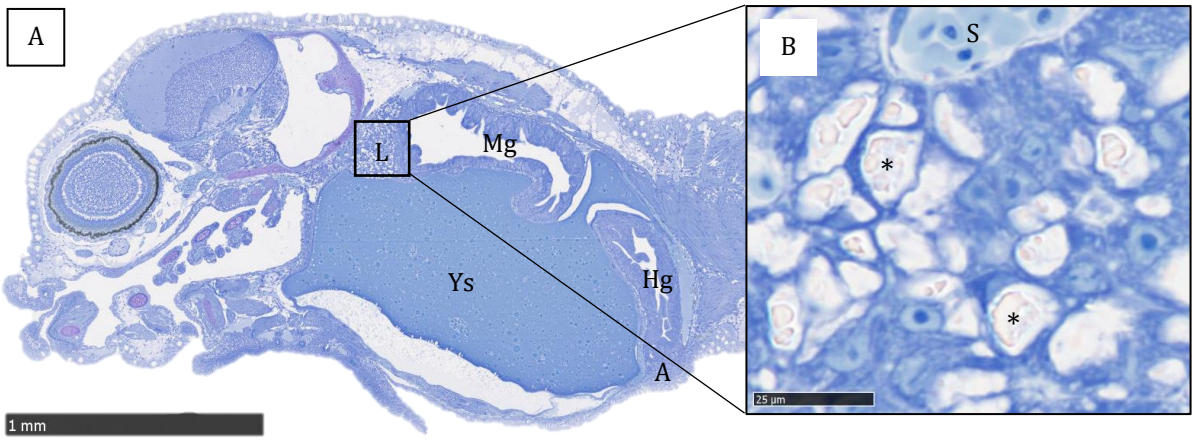

Figure 3.9. Longitudinal section of newly hatched *C. lumpus.* A) The liver had a dorsal position and was located to the left in the most anterior part of the abdominal cavity. Scale bar: 1 mm. B) The liver parenchyma appeared to be fully differentiated and the hepatocytes contained large, irregular glycogen-like vacuoles (*). Scale bar: 25 µm. The section is stained with TB 0.05 %. Abbreviations: A = anus, L = liver, Hg = hindgut, Mg = midgut, S = sinusoid, Ys = yolk sac.

Vacuole content differed greatly between larvae during the treatment period (2-22 dph). All Artemia larvae had hepatocytes with a high degree of cytoplasmic vacuolization (Figure 3.10A), while Copepod and FD larvae had almost no vacuoles in their liver. In addition to deprivation of hepatic vacuoles, some Copepod and FD larvae had seemingly smaller livers, showed signs of sinusoidal dilation, had less distinct hepatocyte boundaries and hepatocyte nuclei in a central position (Figure 3.10B). Abovementioned characteristics applied to both Copepod and FD larvae 10 dph, but only Copepod larvae 21 dph. Larvae from all treatments had a high vacuole content by 34 dph, but most of these vacuoles were spherical rather than irregular and contained lipids that had been lost during tissue processing (Figure 3.11A). Polysaccharide content such as glycogen was indicated by positive period acid Schiff (PAS) reactions on the liver an Artemia larva aged 50 dph (Figure 3.11B; Appendix 3, Figure A1.C-F).

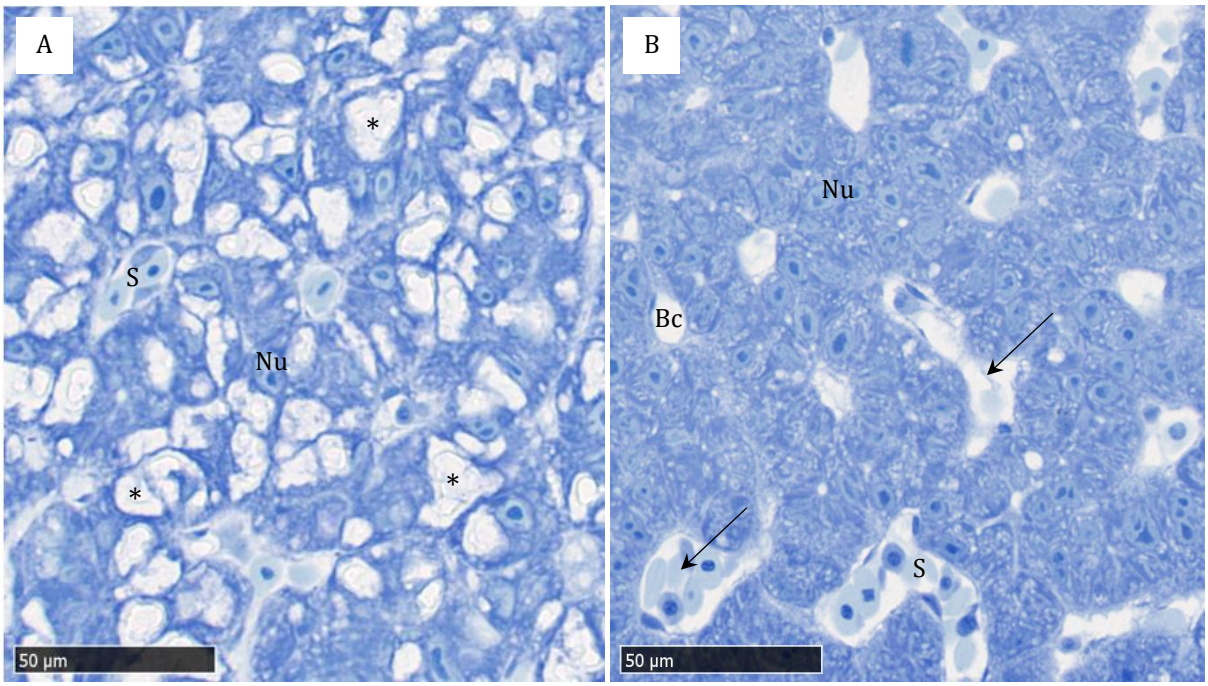

Figure 3.10. Liver structure in *C. lumpus* 10 dph. A) High content of glycogen-like vacuoles (*) in the liver of an Artemia larva with hepatocyte nuclei displaced peripherally. B) Absence of vacuoles in the liver of a FD larva showing signs of sinusoidal dilation (black arrows) and central hepatocyte nuclei. Scale bar: 50 µm. Both pictures are *L* sections stained with TB 0.05 %. Abbreviations: Bc = bile canaliculus, Nu = nucleus, S = sinusoid.

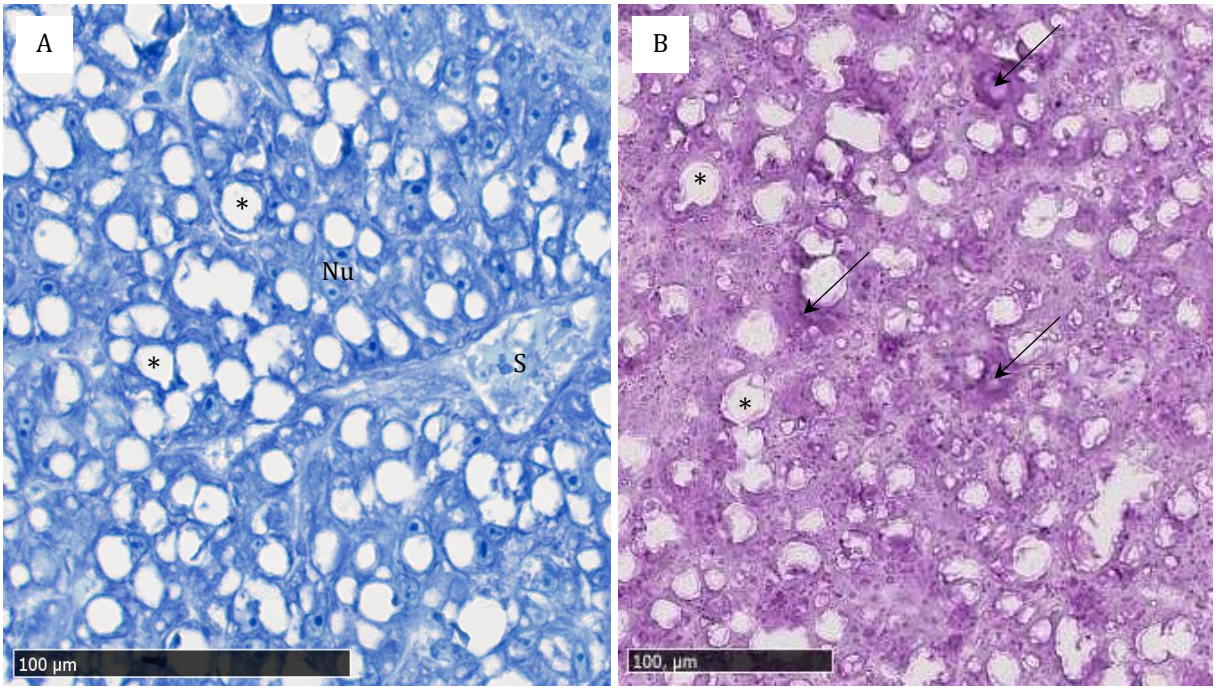

Figure 3.11. Hepatic nutrients in *C. lumpus* 50 dph. A) Vacuoles (*) located in the hepatocytes had a spherical shape and had been deprived of their lipid content during tissue processing. B) Polysaccharide content (e.g. glycogen) indicated by positive periodic acid Schiff (PAS) reactions (black arrows). Scale bar: 100 µm. Both pictures are *L* sections from an Artemia larva stained with (A) TB 0.05 % and (B) Alcian blue with periodic acid Schiff (AB-PAS). Abbreviations: Nu = nucleus, S = sinusoid.

3.3.2 Hepatocyte nucleus area size

Hepatocyte nucleus area size was on average 35.1 ± 1.5 μm² on the day of hatching (Figure 3.12; Appendix 10, Table A7). An increased nucleus size was observed in hepatocytes of *Artemia*-fed larvae 10 dph, as opposed to Copepod and FD larvae nucleus size was reduced and significantly smaller compared to larvae from the Artemia group. On day 21 post hatch, an increased but also more variable nuclear area size was observed in FD larvae, and nucleus size differed significantly between Artemia and Copepod larvae. None of the mean values observed within treatments 10 and 21 dph differed significantly from that of yolk sac larvae, but on day 34 post hatch larvae from all groups had a mean nuclear area size that was significantly larger than that of yolk sac larvae. Larvae from the Artemia group had a nucleus size that was significantly larger compared to Copepod larvae 34 dph. These differences diminished over the remainder of the experiment, and on day 50 post hatch mean hepatocyte nucleus size was almost identical regardless of treatment.

Nucleus area size was positively correlated to SL for the pooled data set ($r = 0.592$, $p < 0.01$*), with an estimated increase of 1.4 μm² per mm increase in SL ($p < 0.01$*). However, only 35.1 % of the observed variation in nucleus size could be attributed to variation in SL ($r^2 = 0.351$), suggesting a non-linear relationship. Including the effect of different treatments had no significant effect on this relationship ($p = 0.05$*).

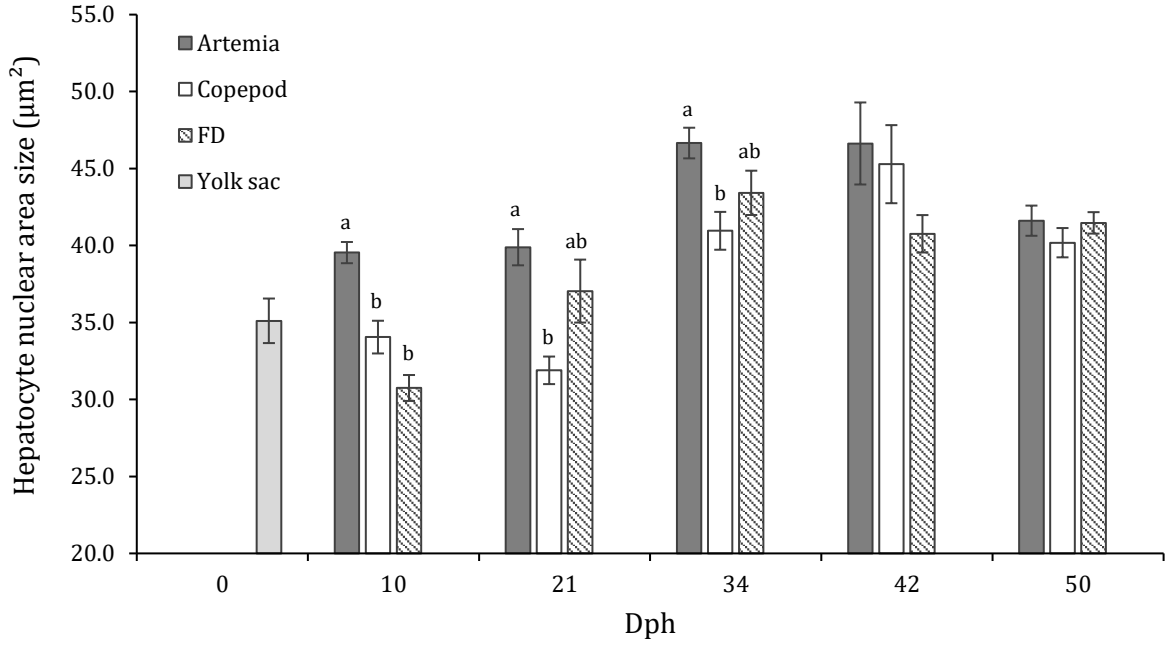

Figure 3.12. Mean hepatocyte nuclear area size in *C. lumpus* 0-50 dph. The values (μm²) are based on mean nucleus size in $n = 5$ larvae (0 dph) and $n = 5$ larvae per treatment (10-50 dph), with an average of 257 nuclei measured per *L* section and larva. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

3.3.3 Hepatocyte area size

Yolk sac larvae had an estimated hepatocyte area size of 558.0 ± 8.0 μm² (Figure 3.13; Appendix 10, Table A7). Hepatocytes of Artemia larvae showed significant hypertrophy from 0-10 dph and were significantly larger compared to hepatocytes of FD larvae 10 dph, which were significantly reduced from hatching. No significant differences were found between Artemia and FD larvae 21 dph. However, larvae from both these groups had an estimated cell size significantly larger than Copepod larvae, where cell size had not changed since hatching. On day 34 post hatch, larvae from all treatments exhibited a mean cell area size that was significantly larger compared to that of yolk sac larvae, but no differences were observed between groups. Further hepatocyte hypertrophy was observed in Artemia and Copepod larvae from 34-42 dph, and mean cell area size was significantly larger in Artemia larvae compared to FD larvae 42 dph. From 34-50 dph, hepatocyte cell area size had increased significantly in larvae from the FD treatment as well, and no differences of statistical significance were found between the groups 50 dph.

Hepatocyte area size was positively correlated to SL (r = 0.755, p < 0.01*) and nuclear area size (r = 0.778, p < 0.01*) for the pooled data, the latter indicating that hepatocyte hypertrophy was accompanied by an increase in nucleus size. With a slope coefficient of 31.15 (p < 0.01*), 60.5 % of the variation in cell area size could be explained by variation in nuclear area size (r^2 = 0.605). Treatment effects were not of statistical significance regarding this linear relationship (p = 0.3*).

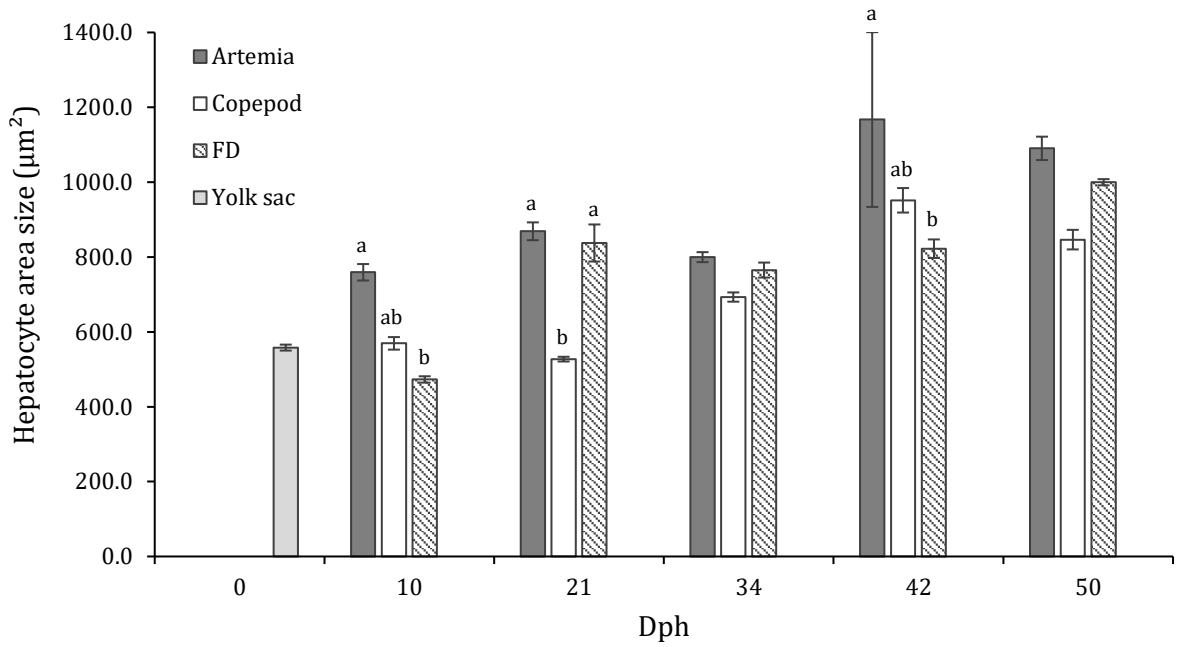

Figure 3.13. Mean hepatocyte area size in *C. lumpus* 0-50 dph. The values (μm²) are based on mean cell size in n = 5 larvae (0 dph) and n = 5 larvae per treatment (10-50 dph), estimated as analyzed liver area in the L section divided by number of nuclei. Significant differences (p < 0.05) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

3.3.4 Area fractions of liver components

Hepatocyte nuclei constituted between 3.3 and 7.3 % of the total liver tissue over the course of the experiment, and generally decreased from hatching (Table 3.3). Artemia larvae exhibited a significant reduction in nuclear area fraction from hatching to 10 dph, when the mean value of this group was significantly lower compared to the FD group. Both Copepod and FD larvae aged 10 dph had a nuclear area fraction similar to that of yolk sac larvae, but this fraction was significantly reduced in FD larvae from 10-21 dph. Hepatocyte nuclei constituted a significantly lower fraction of the total liver area in Artemia larvae compared to Copepod larvae 21 dph, both of which had mean values that did not differ significantly from those observed in respective groups 10 dph. An overall increase was observed across groups from 21-34 dph, although only Artemia larvae exhibited a significant change. On day 42 post hatch, Artemia larvae had a mean nuclear area fraction that was significantly lower compared to both Copepod and FD larvae. Larvae from all groups showed a significant reduction in nuclear area fraction from 34-50 dph, and no significant differences could be detected between larvae from the different groups 50 dph.

Hepatic vacuoles accounted for an average of 18.4 ± 2.3 % of the total liver tissue in yolk sac larvae (Table 3.3), and the only differences of statistical significance were observed 10 and 21 dph. Artemia larvae showed a mean vacuolar area fraction similar to that of yolk sac larvae 10 dph, whereas vacuole content was significantly lower and almost absent in both Copepod and FD larvae on day 10 post hatch. Larvae from the FD group had accumulated a significant quantity of hepatic vacuoles from 10-21 dph, while Copepod larvae still showed a very low vacuole content that was significantly lower compared to both Artemia and FD larvae 21 dph. Differences between the groups disappeared after weaning 20-22 dph, and from 34-50 dph all examined larvae had a vacuole content that did not differ significantly from each other or from that of yolk sac larvae.

A negative correlation was found between nuclear area fraction and SL ($r = -0.680$, $p < 0.01$*) for the pooled data, implying that liver components other than nuclei constituted a relatively larger fraction of the total liver area in larger compared to smaller larvae. It was estimated that nuclear area fraction was reduced by 0.3 % per mm increase in SL and that variation in SL attributed to 46.3 % of observed variation in nuclear area fraction ($r^2 = 0.463$). This linear relationship was not significantly influenced by the treatments ($p = 0.2$*). As for hepatic vacuole content, no significant relationship was found when correlating this variable to SL ($p = 0.07$*). However, the area fraction of hepatic vacuoles was positively correlated to nucleus area size ($r = 0.397$, $p < 0.01$*). It was estimated that the vacuole area fraction increased by 0.6 % per μm^2 increase in nucleus area size for the pooled data, but this relationship was clearly non-linear ($r^2 = 0.158$). Extending the linear model to account for differences between treatments had a significant effect on the relationship between vacuole content and nucleus size ($p < 0.01$*). In this extended model, the slope coefficient

for the Artemia group was not significantly different from zero (p = 0.06*), indicating no relationship between vacuole content and nucleus size in Artemia larvae. As for larvae from the Copepod and FD groups, it was estimated that the area fraction of vacuoles increased by 0.8 % and 1.0 % per μm² increase in nucleus area size, respectively. Still, the slope coefficients for Copepod and FD larvae did not differ significantly from each other (p = 0.7*).

Table 3.3. Mean hepatocyte nuclear and vacuole area fractions in *C. lumpus* liver 0-50 dph. The averages (% ± SE) are based on estimates in the *L* section from n = 5 larvae (0 dph) and n = 5 larvae per treatment (10-50 dph). Nuclear area fractions are estimated as the sum of hepatocyte nuclear sizes divided by analyzed liver area and vacuole content from a point grid. The remaining area fractions are categorized as other (e.g. hepatocyte organelles). Significant differences (p < 0.05) between groups are denoted by different letters.

Dph	Treatment	Mean surface area fraction (%)		
		Nuclei	Vacuoles	Other
0	Yolk sac	6.3 ± 0.1	18.4 ± 2.3	75.3 ± 2.1
10	Artemia	5.3 ± 0.1^{a}	22.2 ± 1.4^{a}	72.5 ± 1.3^{a}
	Copepod	6.1 ± 0.1^{ab}	2.1 ± 1.4^{b}	91.9 ± 1.2^{b}
	FD	6.5 ± 0.1^{b}	0.5 ± 0.2^{b}	92.9 ± 0.3^{b}
21	Artemia	4.6 ± 0.1^{a}	25.2 ± 2.2^{a}	70.2 ± 2.3^{a}
	Copepod	6.1 ± 0.0^{b}	2.5 ± 0.7^{b}	91.4 ± 0.7^{b}
	FD	4.7 ± 0.2^{ab}	20.8 ± 2.5^{a}	74.5 ± 2.1^{a}
34	Artemia	5.9 ± 0.1	14.4 ± 2.1	79.7 ± 2.0
	Copepod	6.0 ± 0.1	16.3 ± 2.5	77.8 ± 2.3
	FD	5.8 ± 0.2	16.5 ± 3.4	77.8 ± 3.3
42	Artemia	4.0 ± 0.0^{a}	16.8 ± 0.6	79.2 ± 0.6
	Copepod	4.8 ± 0.1^{b}	15.3 ± 1.5	79.9 ± 1.6
	FD	5.0 ± 0.1^{b}	16.9 ± 1.8	78.1 ± 1.8
50	Artemia	3.9 ± 0.1	15.3 ± 1.5	80.8 ± 1.4
	Copepod	4.8 ± 0.1	14.0 ± 2.0	81.1 ± 1.7
	FD	4.2 ± 0.0	17.7 ± 1.4	78.1 ± 1.5

3.4 Stomach histology

3.4.1 Stomach morphology

The stomach anlage of newly hatched larvae appeared as a slight dilated elongation posterior to the esophagus, with an anterior cardiac and posterior pyloric region (Figure 3.14A). The pyloric stomach was separated from the midgut by a preliminary pyloric sphincter, a constriction of the surrounding muscularis with an inner circular and outer longitudinal layer of striated muscle fibers. Cubical cells deficient of microvilli and with central nuclei lined the cardiac and pyloric mucosa. Gastric glands were observed in the lamina propria by 10 dph (Figure 3.14B). These

tubular glands consisted of cuboidal secretory cells and were connected to the stomach lumen via gastric pits that had formed through invagination of the mucosa. Relatively few gastric glands were observed 10 dph and these were mostly situated dorsally in the cardiac stomach. Additional gastric glands were observed with increasing age, were continuously distributed in the cardiac mucosa by 34 dph (Figure 3.14C). By 34 dph, the stomach of most larvae also had significantly increased lumen volume and comprised a distinct fundic region in addition to the cardiac and pyloric regions. At this point, the stomach was curved rather than elongated, with the fundus defined by the posterior end of the dorsal curvature and the pyloric sphincter situated on the ventral side of the stomach (Figure 3.15). Feed particles were usually more abundant in the lumen of larvae with a fundic stomach (≥ 34 dph) compared to larvae without a fundus (≤ 34 dph).

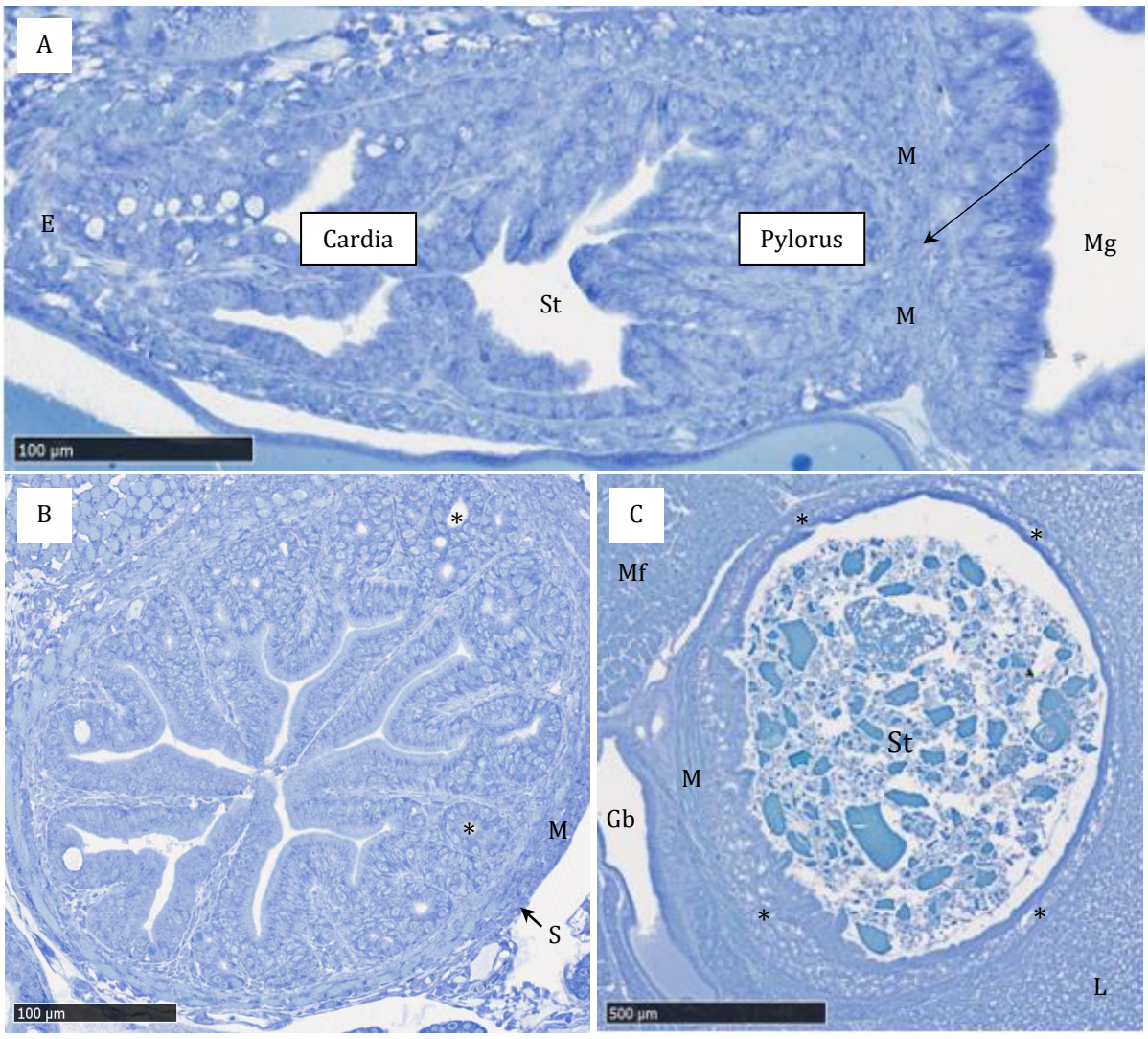

Figure 3.14. Stomach development in *C. lumpus* 0-34 dph. A) The stomach anlage at hatching appeared as a slight enlargement of the esophagus, with an anterior cardiac and posterior pyloric region separated from the midgut by a muscular sphincter (black arrow). Longitudinal section. Scale bar: 100 µm. B) Transverse section of the stomach 10 dph displaying the first gastric glands (*) in lamina propria. Scale bar: 100 µm. C) Transverse section of the stomach 34 dph with greatly increased lumen volume and a continuous distribution of gastric glands in the mucosa. Scale bar: 500 µm. All pictures are sections from Artemia larvae stained with TB 0.05 %. Abbreviations: E = esophagus, Gb = gallbladder, L = liver, M = muscularis, Mf = muscle fibers, Mg = midgut, S = serosa, St = stomach.

Gastric glands and pits were numerous in both the cardiac and fundic regions of larvae with a histologically differentiated stomach but were, as in larvae from 10-21 dph, absent in the more muscular pyloric region. Thickened and constricted muscularis was also observed at the cardiac sphincter that separated the posterior end of the esophagus from the anterior cardiac stomach. Epithelial cells lining the stomach mucosa in larvae from 34-50 dph were columnar rather than cubical (Figure 3.16). The oval nucleus of these cells was situated towards the basal lamina, while secretory vesicles and other cellular components were located in the apical end.

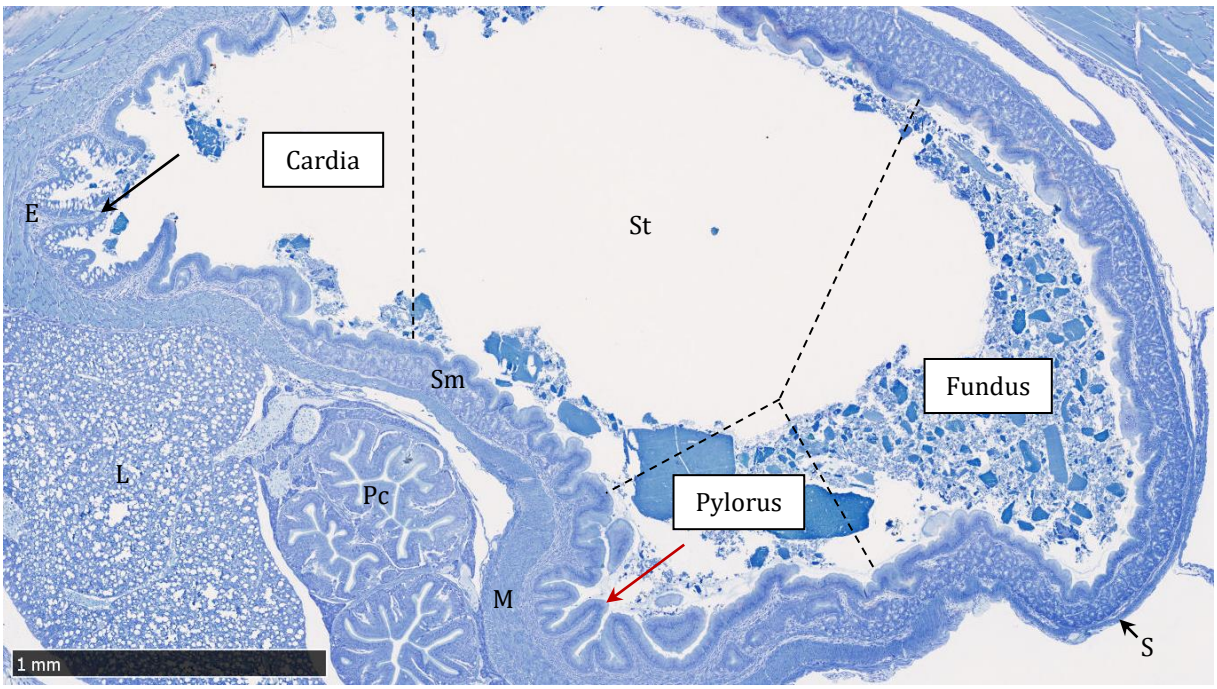

Figure 3.15. Stomach regions in *C. lumpus* 50 dph. The stomach was curved and consisted of the cardiac, fundic and pyloric regions. The cardiac sphincter (black arrow) separated the esophagus from the cardiac stomach, whereas the pyloric sphincter (red arrow) separated the pyloric stomach from the midgut. The picture is a longitudinal section from an Artemia larva stained with TB 0.05 %. Scale bar: 1 mm. Abbreviations: E = esophagus, L = liver, M = muscularis, Pc = pyloric caeca, S = serosa, Sm = stomach mucosa, St = stomach.

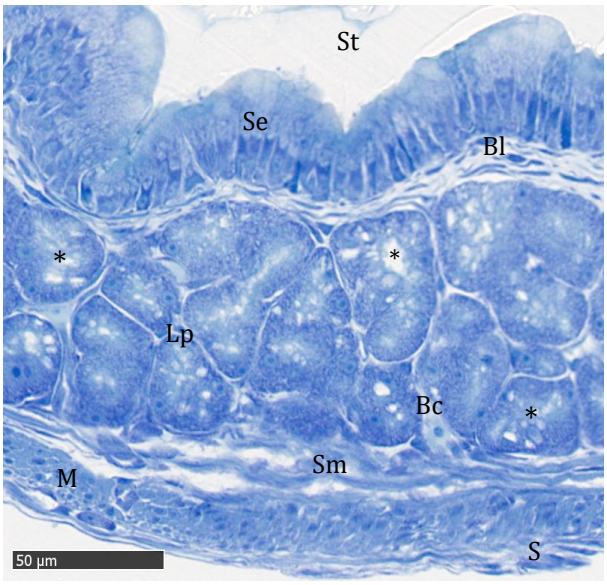

Figure 3.16. Fundic stomach in *C. lumpus* 50 dph. The mucosal layer was invaginated and lined with columnar epithelial cells covered with mucus (not shown). Gastric glands (*) were situated in the lamina propria and surrounded by connective tissue and blood capillaries. The picture is a longitudinal section from an Artemia larva stained with TB 0.05 %. Scale bar: 50 µm. Abbreviations: Bc = blood capillary, Bl = basal lamina, Lp = lamina propria, M = muscularis, S = serosa, Se = stomach epithelium, Sm = submucosa, St = stomach.

3.4.2 Stomach developmental stages

All examined yolk sac larvae were assigned to the S_1 stage, with the stomach anlage appearing as a dilatation between the esophagus and gut (Table 3.4). One out of five (1/5) and 2/5 larvae in the Copepod and FD treatments were still in S_1 10 dph, respectively. Rest of the larvae in these two groups, including all specimens in the Artemia group, had gastric glands formed in their cardiac stomach mucosa and were assigned to the S_2 stage. No morphological changes were observed 21 dph, and all larvae were categorized as S_2. All Artemia larvae, 3/5 Copepod larvae and 3/5 FD larvae had reached the S_3 stage by 34 dph, with greatly increased lumen volume and formation of the fundic stomach. All larvae had stomachs characteristic for the S_3 stage 42 and 50 dph.

Table 3.4. Stomach developmental stages in *C. lumpus* 0-50 dph. Number of larvae assigned to each stomach developmental stage, S_i (S_1: enlargement of esophagus, S_2: presence of gastric glands and S_3: enlargement of lumen volume and formation of the fundic stomach), and mean standard length (mm ± SE) for each treatment on the following days post hatch: 0 dph (n = 5), 10, 21, 34, 42 and 50 dph (n = 5 per treatment). Significant differences ($p < 0.05$) in standard length between treatments at a given day are denoted by different letters.

Dph	Treatment	Mean SL (mm)	Stomach developmental stage, S_i		
			S_1	S_2	S_3
0	Yolk sac	5.9 ± 0.0	5	-	-
10	Artemia	7.2 ± 0.1^{a}	0	5	-
	Copepod	6.8 ± 0.1^{ab}	1	4	-
	FD	6.7 ± 0.1^{b}	2	3	-
21	Artemia	8.4 ± 0.2^{a}	-	5	-
	Copepod	7.7 ± 0.2^{ab}	-	5	-
	FD	7.2 ± 0.1^{b}	-	5	-
34	Artemia	10.2 ± 0.3^{a}	-	0	5
	Copepod	8.3 ± 0.1^{b}	-	2	3
	FD	8.6 ± 0.3^{b}	-	2	3
42	Artemia	12.1 ± 0.5^{a}	-	-	5
	Copepod	10.3 ± 0.2^{b}	-	-	5
	FD	10.6 ± 0.2^{b}	-	-	5
50	Artemia	14.1 ± 0.5^{a}	-	-	5
	Copepod	11.3 ± 0.6^{b}	-	-	5
	FD	11.9 ± 0.2^{b}	-	-	5

Standard length (SL) of the smallest larva that had reached S_2 was 6.6 mm, while the largest larva that had not yet reached S_2 was 6.8 mm long, both of which were aged 10 dph and belonged to the FD group. The transition window from S_1 to S_2 was thus 6.6 – 6.8 mm (Figure 3.17). Transition from S_2 to S_3 was more variable, with an observed size range of 8.0 – 9.1 mm. The smallest larva in S_3 was from the Copepod group and aged 34 dph, while the largest larva still in S_2 was from the Artemia group and aged 21 dph.

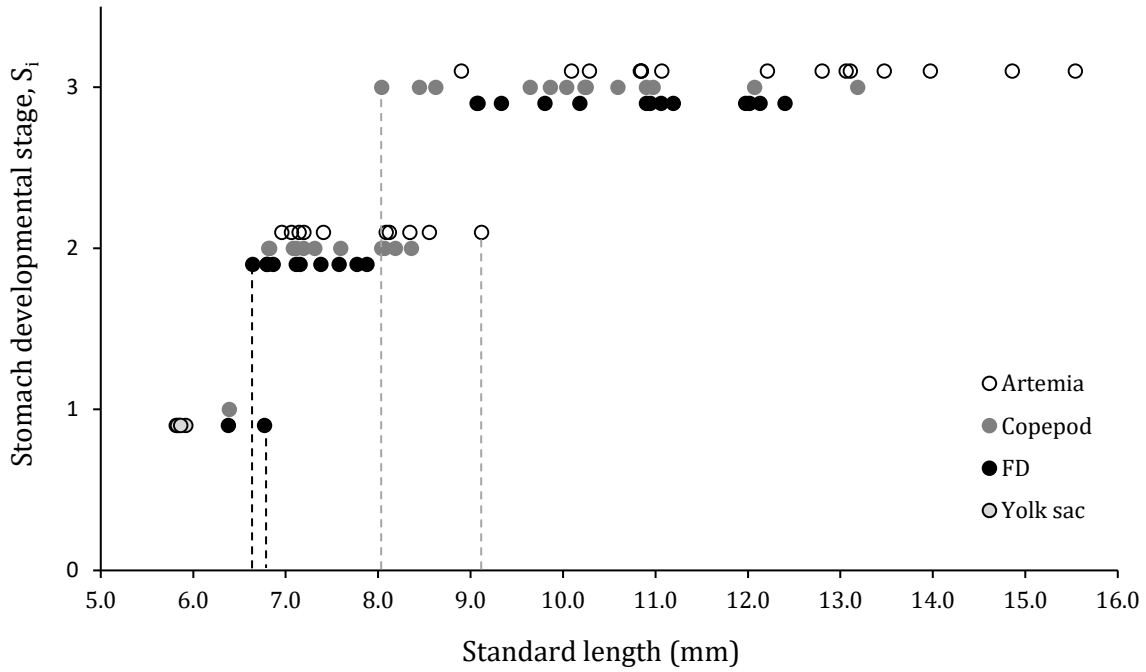

Figure 3.17. Stomach developmental stage and standard length in *C. lumpus*. Each point represents developmental stage, S_i (S_1: enlargement of esophagus, S_2: presence of gastric glands or S_3: enlargement of lumen volume and formation of the fundic stomach), and corresponding standard length (mm) of individual larvae, with a total of n = 5 larvae on 0 dph and n = 25 larvae per treatment from 10-50 dph. The black dashed lines denote size range at transition from S_1 to S_2 and the grey dashed lines denote size range at transition from S_2 to S_3.

3.4.3 Number of gastric glands

Average total number of gastric glands observed in the stomach section from 10-50 dph is given in Table 3.5 below. Artemia larvae had most gastric glands in all examined age groups, but the only difference of statistical significance was found 34 dph. The number of gastric glands increased significantly between successive age groups in the Artemia treatment from 10-42 dph, and from 10-34 dph within the Copepod and FD treatments. No significant changes were observed from 42-50 dph. A positive correlation was found between the number of gastric glands and SL for the pooled data (r = 0.854, p < 0.01*), with an estimated increase of 24 glands per mm increase in SL (r^2 = 0.730, p < 0.01*). This relationship was not affected by the treatments (p = 0.6*).

Table 3.5. Total number of gastric glands in the *S* section of *C. lumpus* 10-50 dph. The averages (N_{glands} ± SE) are based on n = 5 larvae per treatment for each age group 10-50 dph. Significant differences (p < 0.05) between treatments at a given age are denoted by different letters.

Dph	Total number of gastric glands (N_{glands})		
	Artemia	Copepod	FD
10	9 ± 2	4 ± 1	3 ± 1
21	31 ± 5	19 ± 3	18 ± 2
34	83 ± 10[a]	57 ± 7[b]	48 ± 5[b]
42	194 ± 13	121 ± 30	99 ± 22
50	169 ± 17	116 ± 24	116 ± 18

3.4.4 Stomach epithelium height

Mean stomach epithelium height from 10-50 dph is shown for each group in Figure 3.18 below (Appendix 10, Table A8). From 10-34 dph, no significant differences were observed within or between treatments. Both Artemia and Copepod larvae exhibited significantly increased stomach epithelium height from 34-42 dph, and Artemia larvae had a mean epithelium height that was significantly larger compared to FD larvae 42 dph. No significant changes were observed within the Copepod or FD groups from 42-50 dph, whereas Artemia larvae showed further significant growth of the stomach epithelium. The only significant increase observed within the FD treatment was from 34-50 dph. On day 50 post hatch, average epithelium height in larvae from the Artemia group was significantly larger than that of Copepod and FD larvae.

Stomach epithelium height was positively correlated to SL for the pooled data (r = 0.873, p < 0.01*). With an estimated slope coefficient of 1.8 (p < 0.01*), 76.2 % of the total observed variation in epithelium height could be attributed to variation in SL (r^2 = 0.762). Accounting for differences between treatments was not of statistical significance regarding this linear relationship (p = 0.8*). A positive correlation was also found between epithelium height and number of gastric glands for the combined data (r = 0.820, p < 0.01*), and it was estimated that epithelium height increased by 0.1 µm^2 for each additional gland (p < 0.01*). Variation gastric gland abundance accounted for 67.2 % of the total observed variation in epithelium height (r^2 = 0.672). No significant treatment effects were found regarding this linear relationship (p = 0.5*).

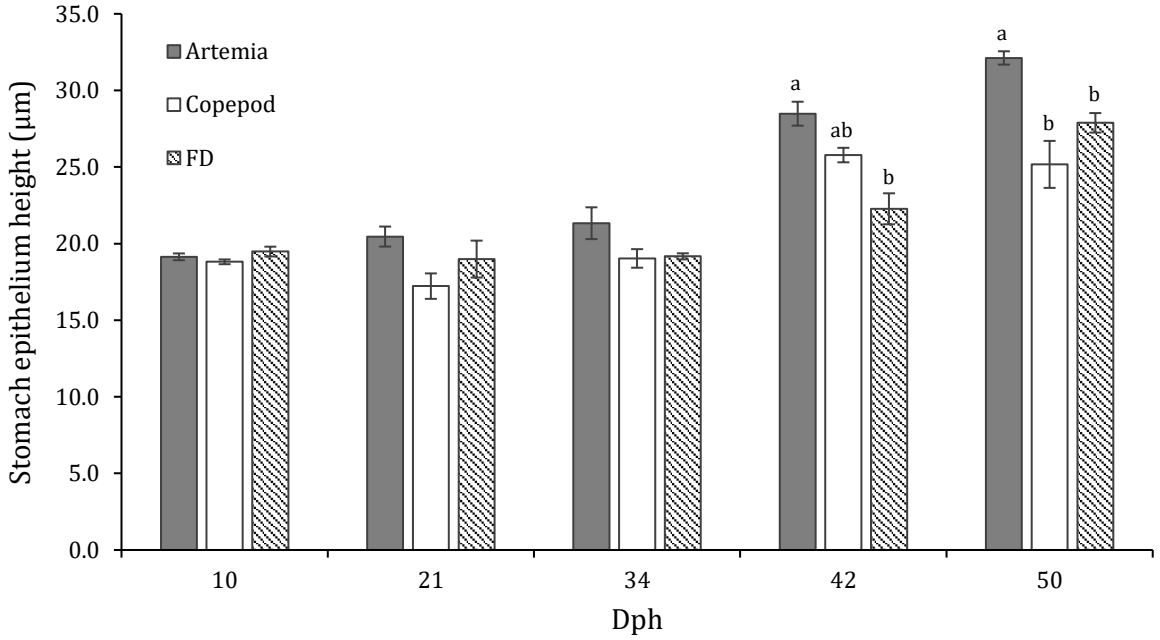

Figure 3.18. Mean stomach epithelium height in *C. lumpus* 10-50 dph. The values (µm) are based on mean stomach epithelium height in n = 5 larvae per treatment, with five measurements per *S* section and larva. Epithelium height was measured from the basal lamina to the apical membrane. Significant differences (p < 0.05) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

3.5 Gut histology

3.5.1 Gut morphology

The gut of newly hatched larvae was coiled and separated into an anterior and posterior part by an intestinal valve (Figure 3.19), observed as a constriction of the mucosa and thickening of the surrounding muscular layers. Villi were present throughout the length of the gut, observed as mucosal folds protruding into the lumen. At hatching, these folds were mostly situated dorsally in the anterior part of the gut, whereas the posterior part had villi equally distributed in both dorsal and ventral positions. The mucosa was lined with a single layer of columnar enterocytes with microvilli extending from the apical membrane. Two layers of smooth muscle fibers composed the muscularis, whereas the surrounding serosa consisted of simple flat epithelium.

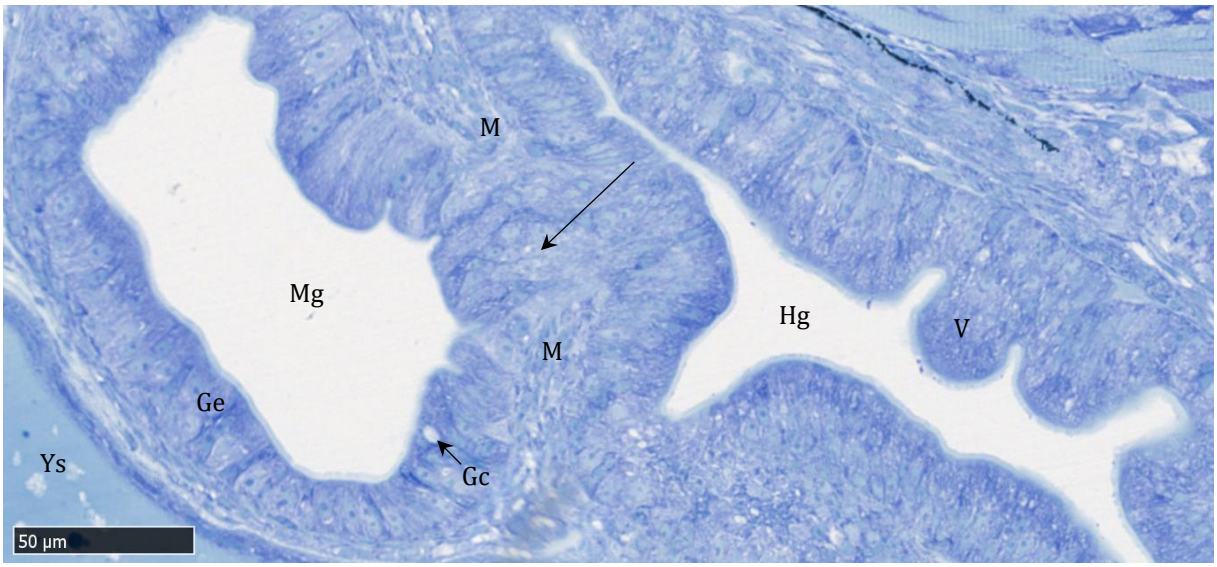

Figure 3.19. Intestinal valve in newly hatched *C. lumpus*. The mid- and hindgut were separated by an intestinal valve (black arrow), characterized by constricted intestinal mucosa and thickened muscularis. Scale bar: 50 µm. The picture is a longitudinal section stained with TB 0.05 %. Abbreviations: Gc = goblet cell, Ge = gut epithelium, Hg = hindgut, M = muscularis, Mg = midgut, V = villus, Ys = yolk sac.

The number of midgut and hindgut villi greatly increased both dorsally and ventrally as the larvae aged, accompanied by a thickening of the gut wall through elongation of the enterocytes. The midgut was particularly involved in lipid absorption, and numerous lipid droplets with a diameter > 5 µm were located in midgut enterocytes of *Artemia*-fed larvae 10 and 21 dph (Figure 3.20A). These lipid droplets were absent in enterocytes of both Copepod and FD larvae, and were not observed in larvae from any group beyond 21 dph. Ingested feed seemed less abundant in the midgut lumen of larvae from the Copepod and FD groups from 10-21 dph and 10 dph, respectively, whereas it was comparably more abundant in Artemia larvae during the same period. Larvae from the Artemia group also seemed to have thicker midgut mucosa with more villi relative to larvae from the Copepod and FD groups with same age. One of five examined FD larvae 21 dph had conspicuously thin midgut epithelium with very few villi (Figure 3.20B).

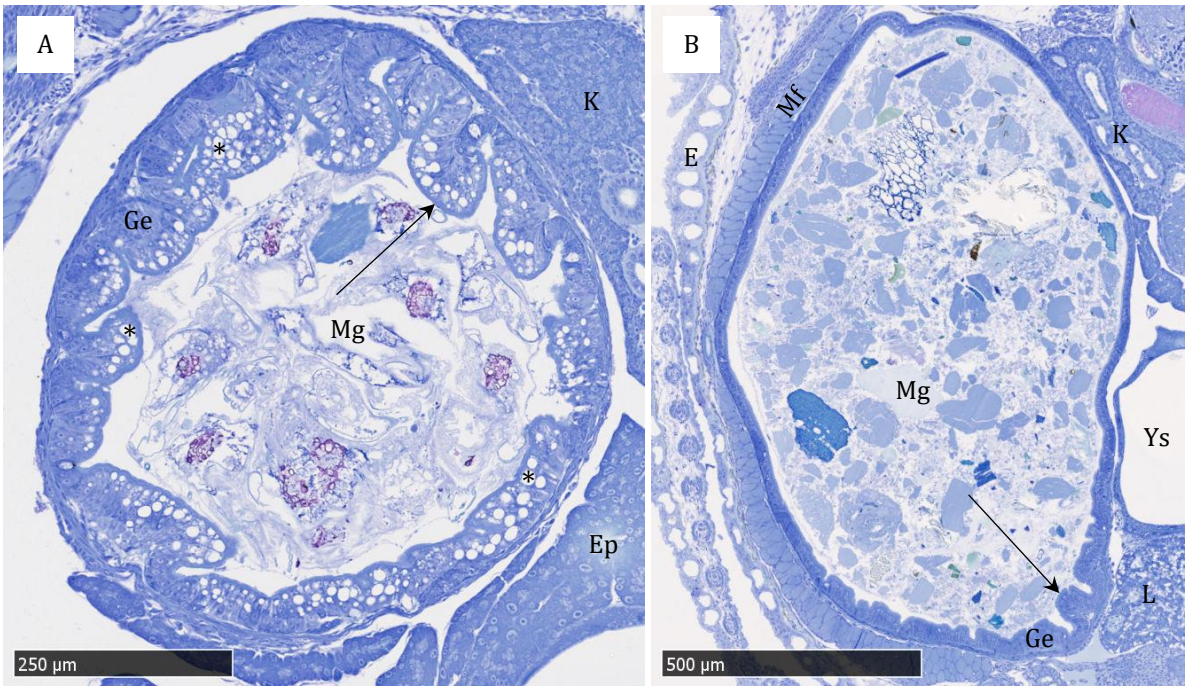

Figure 3.20. Midgut epithelium in *C. lumpus* 21 dph. A) Larva from the Artemia group displaying numerous longitudinal folds and villi (black arrow) in the mucosal layer. Lipid droplets (*) were accumulated in the enterocytes. Scale bar: 250 µm. B) Larva from the FD group with thin epithelium and very few villi. Scale bar: 500 µm. Both pictures are *S* sections stained with TB 0.05 %. Abbreviations: E = epidermis, Ep = exocrine pancreas, Ge = gut epithelium, K = kidney, L = liver, Mf = muscle fibers, Mg = midgut, Ys = yolk sac.

Hindgut enterocytes were characterized by the presence of supranuclear vesicles from 10-50 dph (Figure 3.21A). Numerous acidophilic compounds were integrated in these vesicles of FD larvae by 21 dph and were present in larvae from all groups from 34-50 dph (Figure 3.21B). Unlike the midgut, the hindgut did not seem to participate in lipid absorption and was also distinguishable from the midgut by an overall inferior abundance of villi.

Three different cell types were observed in the intestinal mucosa, of which enterocytes were most abundant. These cells had a basophilic cytoplasm with oval nuclei in a central or basal position within each cell. The enterocytic microvilli were short at hatching but increased in length with larval age and established a prominent basophilic brush border. Non-identifiable cellular content, such as vesicles and organelles, also increased with age in the apical end of both midgut and hindgut enterocytes. Goblet and enteroendocrine-like cells were the two other intestinal cell types found in lumpfish larvae (Figure 3.22), observed from hatching and 10 dph, respectively. These cells were interposed between enterocytes and constituted a minor fraction of the total mucosal cell population. Goblet cells contained acidophilic substances and they had a columnar shape with slightly increased diameter towards the lumen. Spherical and highly basophilic enteroendocrine-like cells were overall least numerous, most of which were situated in proximity of the basal lamina. Goblet cells were dispersed throughout the length of the gut, whereas enteroendocrine-like cells were exclusive to the midgut.

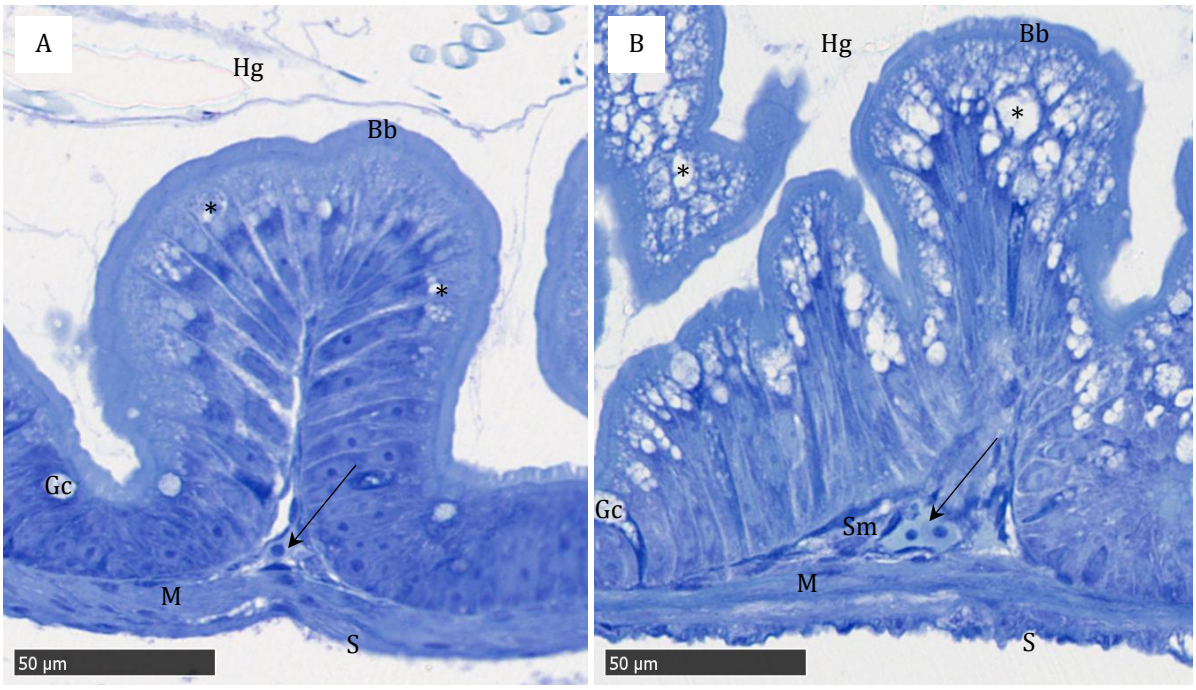

Figure 3.21. Hindgut epithelium in *C. lumpus* 21 and 42 dph. Supranuclear vesicles (*) in hindgut enterocytes 21 (A) and 42 dph (B). Erythrocytes (black arrow) were observed in submucosal blood vessels located at the base of each villus. Scale bar: 50 µm. Both pictures are Hg sections from an Artemia larva stained with TB 0.05 %. Abbreviations: Bb = brush border, Gc = goblet cell, Hg = hindgut, M = muscularis, S = serosa, Sm = submucosa.

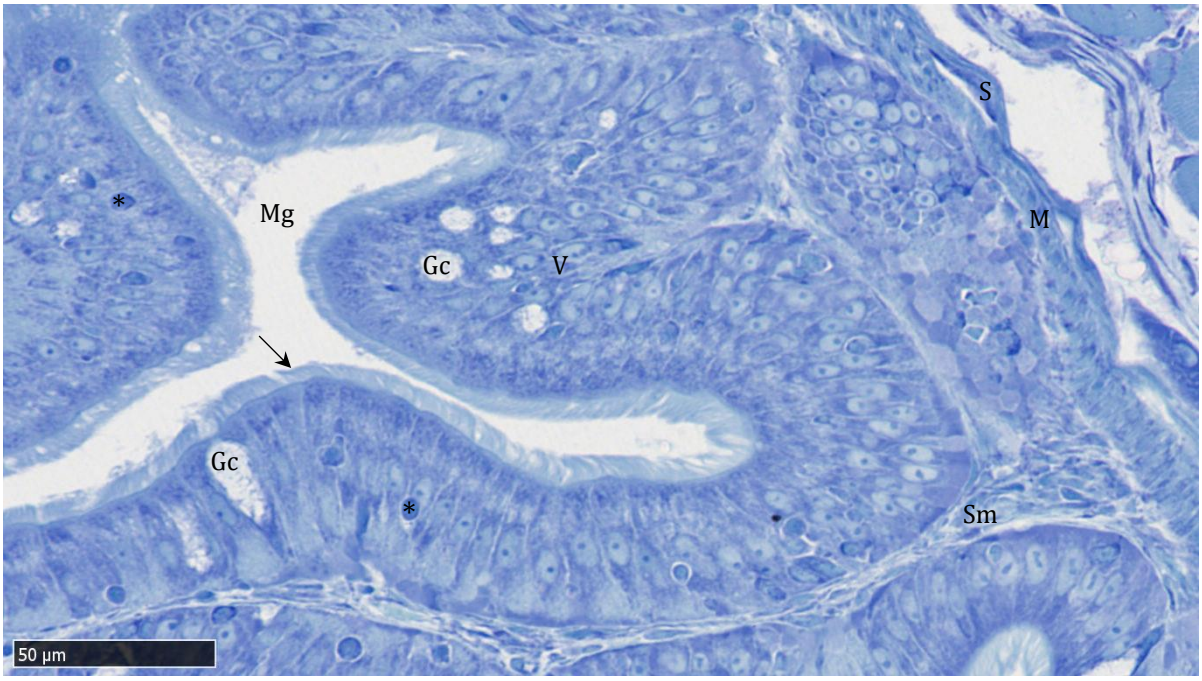

Figure 3.22. Intestinal cell types in the midgut mucosa of *C. lumpus*. Enterocytes developed a prominent brush border (black arrow) of microvilli protruding from their apical surface. Goblet cells and enteroendocrine-like cells (*) were interposed between the enterocytes but constituted a minor fraction of the total mucosal cell population in the midgut. Scale bar: 50 µm. The picture is a *S* section from an Artemia larva 50 dph stained with TB 0.05 %. Abbreviations: Gc = goblet cell, M = muscularis, Mg = midgut, S = serosa, SM = submucosa, V = villus.

3.5.2 Gut epithelium height

Yolk sac larvae had a mean midgut epithelium height of 29.9 ± 1.3 µm (Figure 3.23A; Appendix 10, Table A9). Midgut epithelium height differed significantly between larvae from all treatments 10 dph, of which only Artemia and Copepod larvae displayed a significant increase from hatching. From 10-21 dph, Copepod larvae showed a significant reduction, while no changes were observed within the Artemia and FD treatments. Artemia larvae had a mean midgut epithelium height that was significantly larger than both Copepod and FD larvae 21 dph. No significant differences were found between treatments or within the Artemia group from 34-50 dph. Copepod larvae showed a significant increase in midgut epithelium height between successive age groups from 21-42 dph, whereas the midgut epithelium in FD larvae grew significantly from 21-34 dph.

Hindgut epithelium height was generally lower than in the midgut section of the same larva, with a mean of 25.7 ± 0.7 µm at hatching (Figure 3.23B; Appendix 10, Table A9). Larvae from all groups showed significantly increased hindgut epithelium height from 0-10 dph, but no differences were found between the groups 10 dph. From 10-21 dph, the hindgut epithelium of Artemia larvae had increased significantly, while no change was observed within the Copepod and FD groups. Mean hindgut epithelium of Artemia larvae was significantly larger than both Copepod and FD larvae 21 dph. No significant differences were observed within the Artemia treatment beyond 21 dph, whereas larvae from the Copepod and FD treatments exhibited a significant increase from 21-42 and 21-34 dph, respectively. On day 50 post hatch, Artemia larvae had a mean hindgut epithelium height that was significantly larger compared to that of Copepod larvae.

Epithelium height was positively correlated to SL in both the midgut and hindgut sections, with correlation coefficients of 0.717 and 0.686 for the pooled data, respectively (p-values < 0.01*). Midgut epithelium height was significantly affected by the treatments (p < 0.01*), and was estimated to grow by 0.6, 1.7 and 2.8 µm per mm SL increase in Artemia, Copepod and FD larvae, respectively. The slope coefficients differed significantly between the Artemia and FD groups (p < 0.01*). Variation in SL attributed to 32.7 – 63.6 % of total observed variation in midgut epithelium height within the different groups (r^2 = 0.327 – 0.636). As for the hindgut section, no significant treatment effects were found regarding the linear relationship between epithelium height and SL (p = 0.3*). However, it was estimated that epithelium height in the hindgut increased by an average of 1.7 µm per mm increase in SL for the pooled data (p < 0.01*). Variation in SL explained 47.0 % of total observed variation in hindgut epithelium height (r^2 = 0.470).

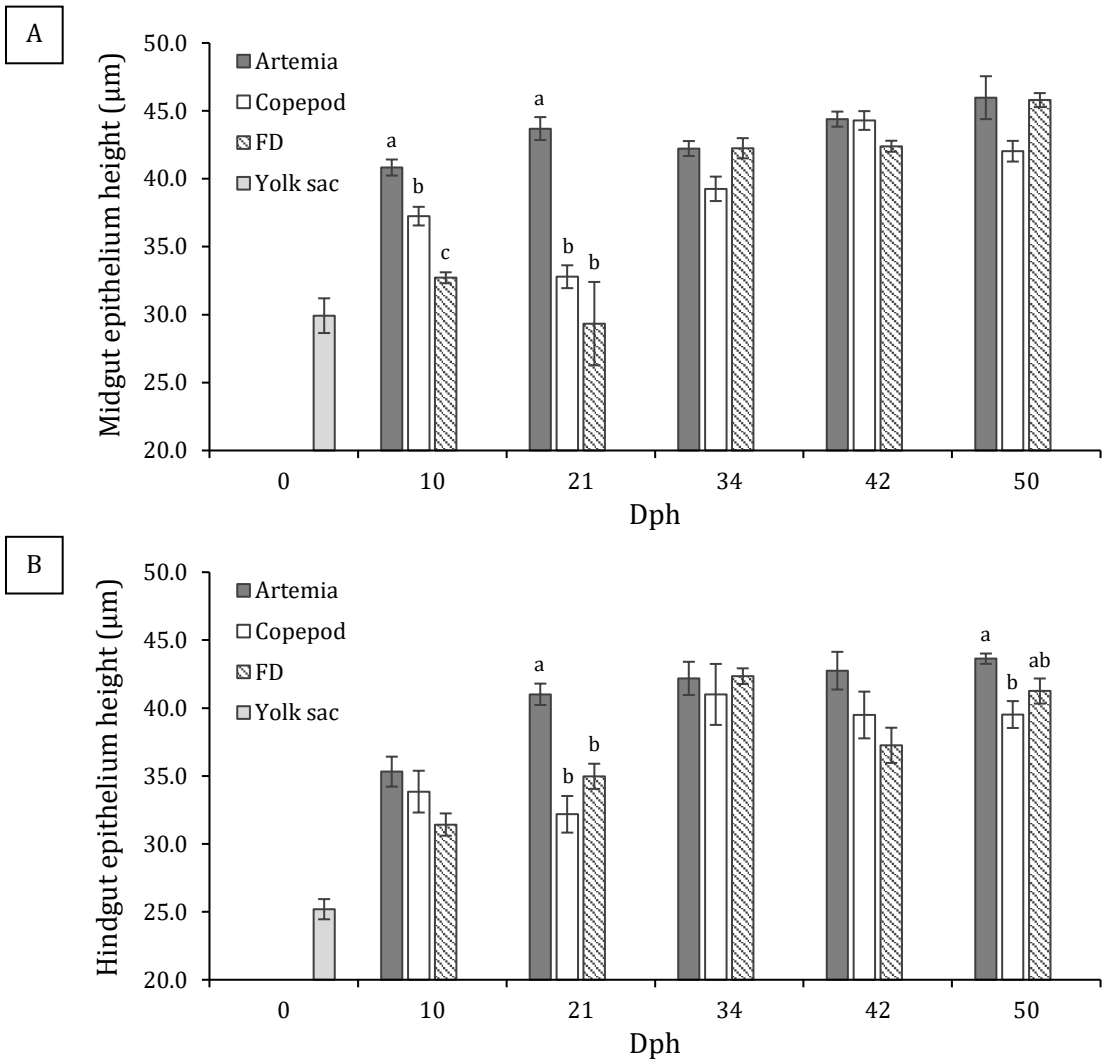

Figure 3.23. Mean gut epithelium height in *C. lumpus* 0-50 dph. The values (µm) are based on mean midgut (A) and hindgut (B) epithelium height in $n = 5$ larvae (0 dph) and $n = 5$ larvae per treatment (10-50 dph), with ten measurements per *S*/*Mg* and *Hg* section. Epithelium height was measured from the basal lamina to the borderline between the microvilli and gut lumen. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

3.5.3 Gut microvillus height

Microvillus height in the midgut section of newly hatched larvae was on average 1.5 ± 0.1 µm (Figure 3.24A; Appendix 10, Table A9). Larvae from all groups exhibited significantly increased midgut microvillus height from hatching to 10 dph, with Artemia larvae having a mean value that was significantly larger than FD larvae. The height of midgut microvilli continued to increase significantly between successive age groups within all treatments from 10-34 dph, while Copepod larvae also showed a significant increase from 34-42 dph. No changes of statistical significance were observed within any group from 42-50 dph, but midgut microvillus height had increased significantly in larvae from the Artemia treatment from 34-50 dph.

Yolk sac larvae had a mean hindgut microvillus height of 1.8 ± 0.1 µm (Figure 3.24B; Appendix 10, Table A9). On day 10 post hatch, larvae from all groups showed a mean hindgut microvillus height that was significantly larger compared to that of yolk sac larvae. Further significant growth was observed from 10-21 dph in larvae from both the Artemia and Copepod groups, whereas mean hindgut microvillus height in FD larvae 10 and 21 dph were statistically similar. From 21-34 dph, hindgut microvillus height had increased significantly only in Artemia larvae, while Copepod and FD larvae showed a significant increase from 21-42 dph. Apart from these changes, no significant differences were observed within or between treatments.

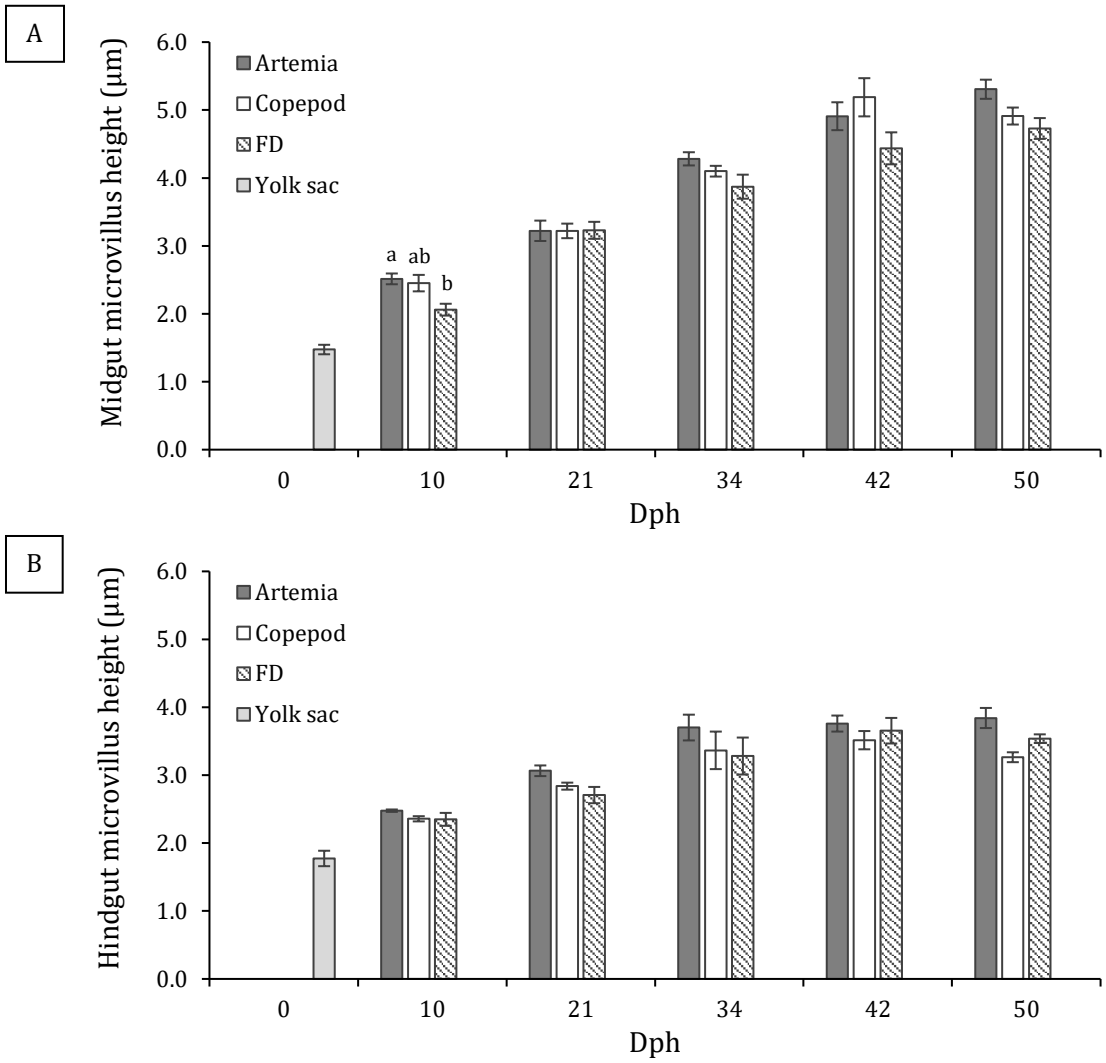

Figure 3.24. Mean gut microvillus height in *C. lumpus* 0-50 dph. The values (µm) are based on mean midgut (A) and hindgut (B) microvillus height in n = 5 larvae (0 dph) and n = 5 larvae per treatment (10-50 dph), with ten measurements per *S*/*Mg* and *Hg* section. Microvillus height was measured from the apical membrane of the enterocytes to the borderline between the microvilli and gut lumen. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

Both midgut and hindgut microvillus height were positively correlated to SL, with correlation coefficients of 0.866 and 0.767, respectively (p-values < 0.01*). It was estimated that midgut and hindgut microvillus height increased by an average of 0.4 and 0.2 µm per mm increase in SL, respectively (p-values < 0.01*). Of the total observed variation in midgut and hindgut microvillus height, 74.9 and 58.8 % could be attributed to variation in SL, respectively (r^2 = 0.749 and 0.588). No treatment effects were found for either of the linear relationships (p = 0.2* for the midgut and p = 0.9* for the hindgut).

3.5.4 Gut villus height

Midgut villus height was on average 81 ± 5 µm in larvae on the day of hatching (Figure 3.25A; Appendix 10, Table A9). Larvae from the Artemia group showed a progressive and significant increase from hatching until 21 dph, whereas the mean values observed within the Copepod and FD groups 10 and 21 dph did not differ significantly from that of yolk sac larvae. No significant growth in villus height was observed in larvae from any group from 21-34 dph, but all groups exhibited a significantly increased midgut villus height from 34-50 dph. Additionally, Copepod and FD larvae showed a significant increase from 34-42 dph and 42-50 dph, respectively.

At hatching, hindgut villus height was on average 71 ± 1 µm (Figure 3.25B; Appendix 10, Table A9). Larvae from all groups aged 10 dph had a mean hindgut villus height that was significantly larger than that of yolk sac larvae. Additionally, hindgut villus height was significantly larger in Copepod larvae compared to both Artemia and FD larvae. Hindgut villus height had increased significantly in both Artemia and FD larvae from 10-21 dph, whereas mean hindgut villus height of Copepod larvae 21 dph did not differ significantly from that of yolk sac larvae. Relative to respective group means 21 dph, hindgut villi of FD larvae had grown significantly by 34 dph and in Artemia and Copepod larvae by 42 dph. No changes were observed within any group 42-50 dph, but Artemia larvae had a hindgut villus height that was significantly larger than FD larvae 50 dph.

Both midgut and hindgut villus height were positively correlated to SL for the pooled data, with corresponding correlation coefficients of 0.911 and 0.840 (p-values < 0.01*). Midgut villus height was estimated to increase by 15, 24 and 19 µm per mm SL increase in the Artemia, Copepod and FD groups, respectively. Variation in SL accounted for 75.1 to 89.2 % of the observed variation in midgut villus height (r^2 = 0.751 - 0.892). The slope coefficients and intercepts differed significantly between the Artemia and Copepod groups (p-values < 0.01*), whereas the slope coefficient and intercept for the FD group did not differ significantly from any other group (p-values > 0.09). No treatment effects were found regarding the relationship between hindgut villus height and SL (p = 0.3*), but hindgut villus height was estimated to increase by an average of 12 µm per mm SL increase (p < 0.01*). Variation in SL attributed to 70.6 % of the observed variation in hindgut villus height (r^2 = 0.706).

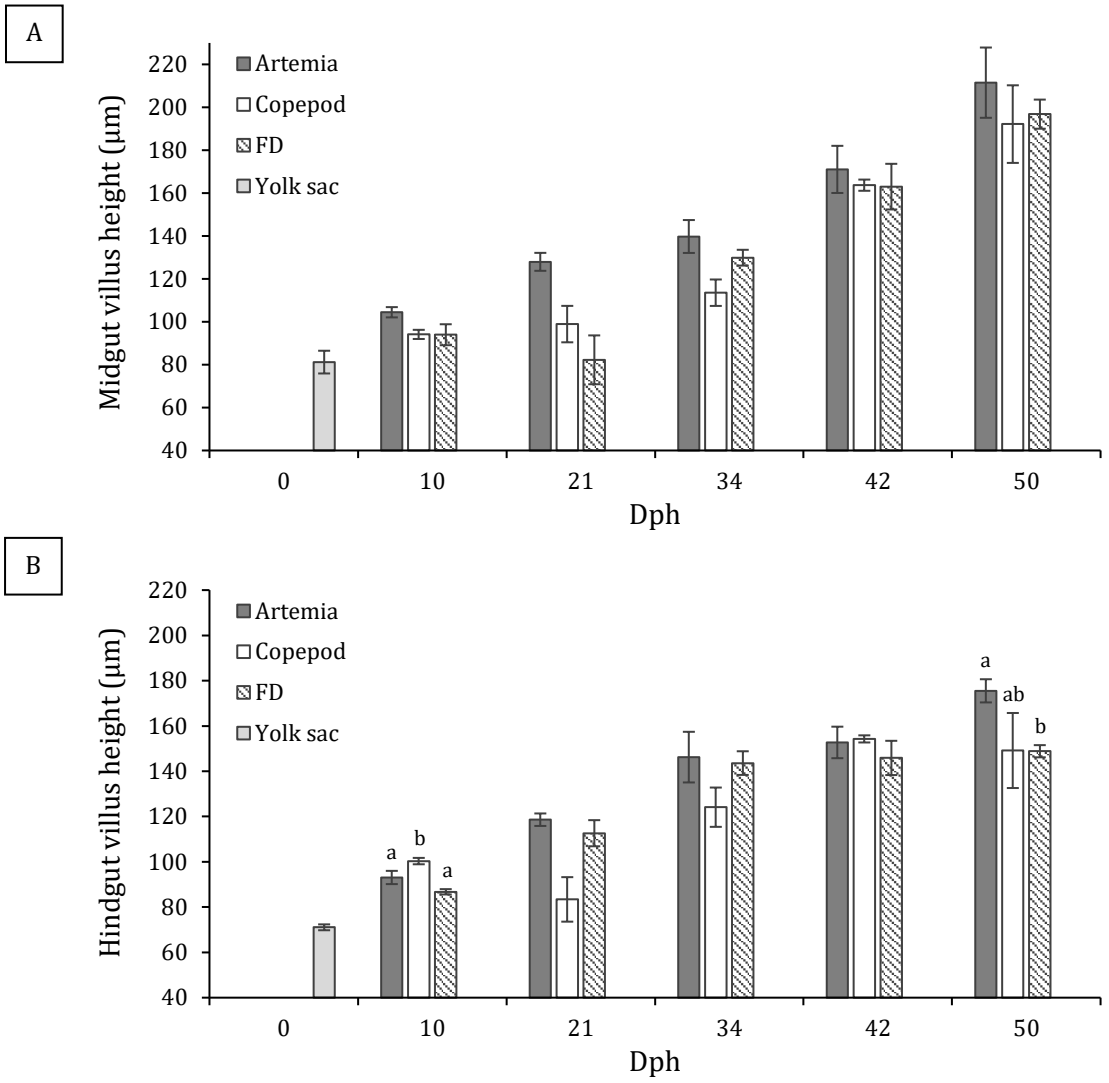

Figure 3.25. Mean gut villus height in *C. lumpus* 0-50 dph. The values (µm) are based on mean midgut (A) and hindgut (B) villus height in n = 4-5 larvae (0 dph) and n = 3-5 larvae per treatment (10-50 dph), with up to five measurements per *S*/*Mg* and *Hg* section. Villus height was measured from the basal lamina to the borderline between the microvilli and gut lumen in areas where the mucosa was folded to form villi. Significant differences ($p < 0.05$) between treatments at a given age are denoted by different letters. Errors bars indicate ± standard error (SE).

3.6 Histology of other digestive organs

3.6.1 Esophagus morphology

The esophagus connected the posterior pharynx to the stomach region and had a muscularis composed of striated muscle fibers arranged in an inner longitudinal and outer circular layer. The circular layer was observed from hatching and increased progressively in thickness as the larvae aged, whereas the much less defined longitudinal layer was observed by 21 dph. Longitudinal folding of the mucosa was observed from hatching, but the folds became more distinct and developed a comprehensive network of submucosal blood vessels as the larvae increased in size (Figure 3.26). The esophageal mucosa was initially lined with a single layer of squamous epithelial

cells but were columnar by 21 dph. Spherical or columnar shaped mucosal goblet cells were also present from hatching, and had short microvilli protruding into the lumen from their apical surface and a strongly basophilic base. There seemed to be a longitudinal gradient in the chemical nature of these cells. Most goblet cells in the anterior part of the esophagus contained acidic substances (Figure 3.27A), while goblet cells located in the middle and posterior parts had basic or neutral substances (Figure 3.27B). This gradient was observed from hatching but became more apparent as additional goblet cells formed with increasing larval age. The latter goblet cell type closely resembled those observed in the intestinal mucosa.

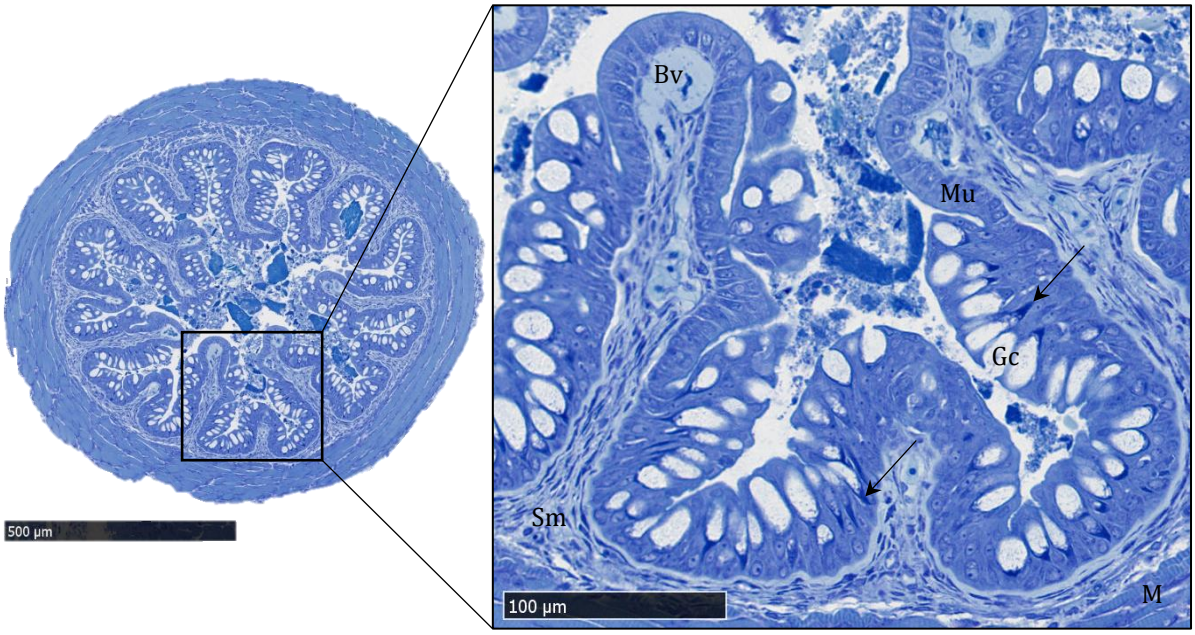

Figure 3.26. Esophagus in *C. lumpus* 50 dph. The mucosa was lined with columnar epithelium and interposed with numerous goblet cells with a basophilic base (black arrows). The submucosa contained a comprehensive network of blood vessels, particularly in the apex of the folds. Scale bar: 500 µm (left) and 100 µm (right). The picture is a transverse section from a Copepod larva stained with TB 0.05 %. Abbreviations: Bv = blood vessel, Gc = goblet cell, M = muscularis, Mu = mucosa, Sm = submucosa.

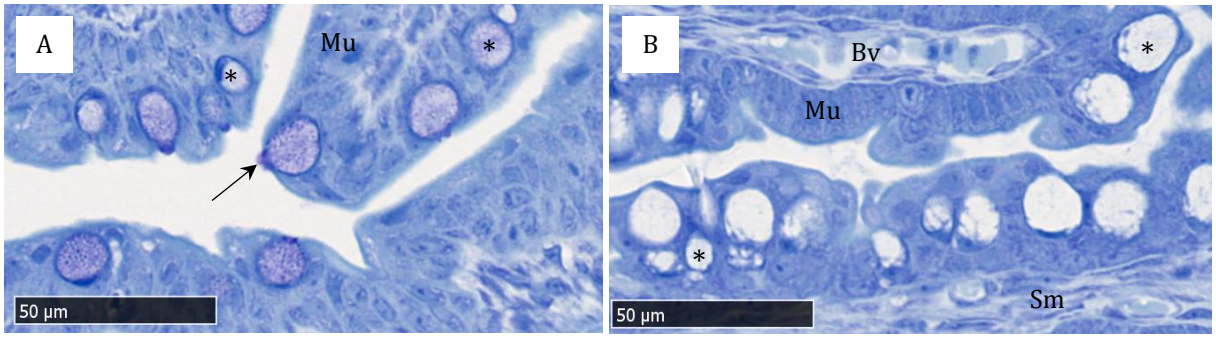

Figure 3.27. Esophageal goblet cells in *C. lumpus* 21 dph. Mucosal goblet cells (*) with short microvilli (black arrow) contained acidic substances in the anterior part of the esophagus (A) and neutral or basic substances in the middle and posterior parts (B). Scale bar: 50 µm. The pictures are transverse sections from an Artemia larva stained with TB 0.05 %. Abbreviations: Bv = blood vessel, Mu = mucosa, Sm = submucosa.

3.6.2 Pancreas morphology

The pancreas initially appeared as a distinct organ attached dorsally to the digestive tract in the anterior abdomen and consisted of exocrine cells, each with a basal nucleus and prominent nucleolus (Figure 3.28A). The cytoplasm of the exocrine cells was strongly basophilic and contained numerous acidophilic zymogen granules already from hatching. Islets of Langerhans constituted the endocrine pancreas and were enveloped by exocrine pancreatic tissue (Figure 3.28B). These islets were observed by 10 dph. The pancreas seemed to increase in size with larval age and composed a gradually less distinct organ, with branches of exocrine tissue reaching out to different parts of the intestine by 21 dph. The pancreas also developed additional associated blood vessels and excretory intralobular ducts, the latter of which were lined with simple cuboidal epithelium (Figure 3.28C).

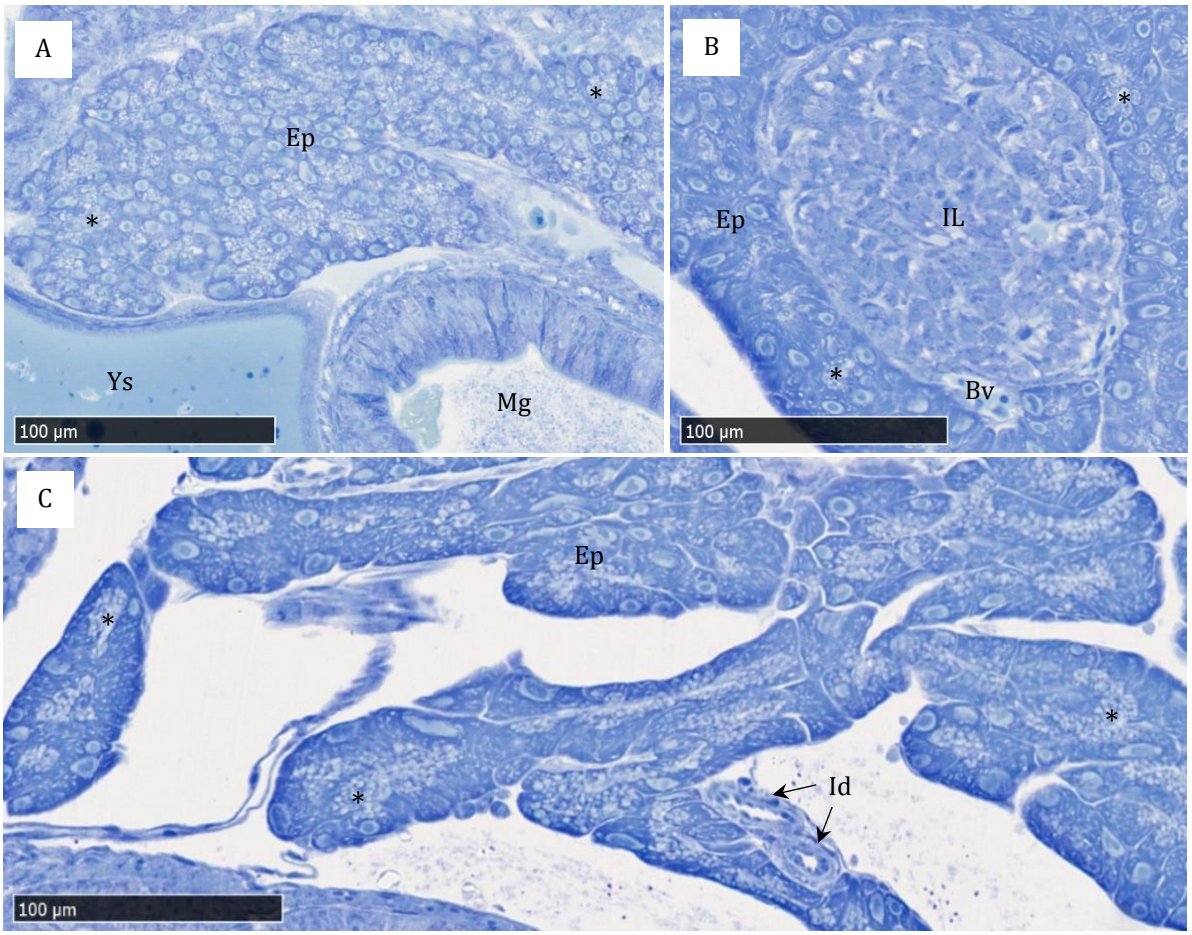

Figure 3.28. Pancreas development in *C. lumpus* 0-34 dph. A) The pancreas of newly hatched larvae appeared as a dorsal bud on the anterior part of the digestive canal. Numerous zymogen granules (*) were observed in the basophilic cytoplasm of the exocrine cells. B) Endocrine pancreatic cells constituted islets of Langerhans and were enveloped by exocrine tissue (10 dph). C) The pancreas developed clusters of exocrine cells and had branches of exocrine tissue that reached out to different parts of the digestive canal (34 dph). Scale bar: 100 µm. All pictures are longitudinal sections from an Artemia larva stained with TB 0.05 %. Abbreviations: Bv = blood vessel, Ep = exocrine pancreas, Id = Intralobular duct, IL = Islet of Langerhans, Mg = midgut, Ys = yolk sac.

3.6.3 Pyloric caeca morphology

Pyloric caeca were observed by 34 dph and formed intestinal appendages in the most anterior part of the intestine, at the intersection between the stomach and midgut (Figure 3.29). These projections comprised all the four layers characterizing the gastrointestinal tract (i.e. mucosa, submucosa, muscularis and serosa), with the mucosal and muscular layers closely resembling that of the intestine. The mucosa was lined with a single layer of columnar enterocytes that had developed a prominent brush border of microvilli protruding from their apical surface. Goblet cells and enteroendocrine-like cells resembling those observed in the intestinal mucosa were also integrated in the mucosa of pyloric caeca. The muscularis was composed of a longitudinal layer of smooth muscle fibers. Number of villi and caecum diameter seemed to increase accordingly, with up to nine villi observed in the largest caeca. All caeca were surrounded by branches of exocrine pancreatic tissue. As this organ was difficult to distinguish histologically from the intestine, the only evident pyloric caeca were observed in some longitudinal sections of Artemia larvae 34 and 50 dph. A maximum of seven caeca were observed per larva.

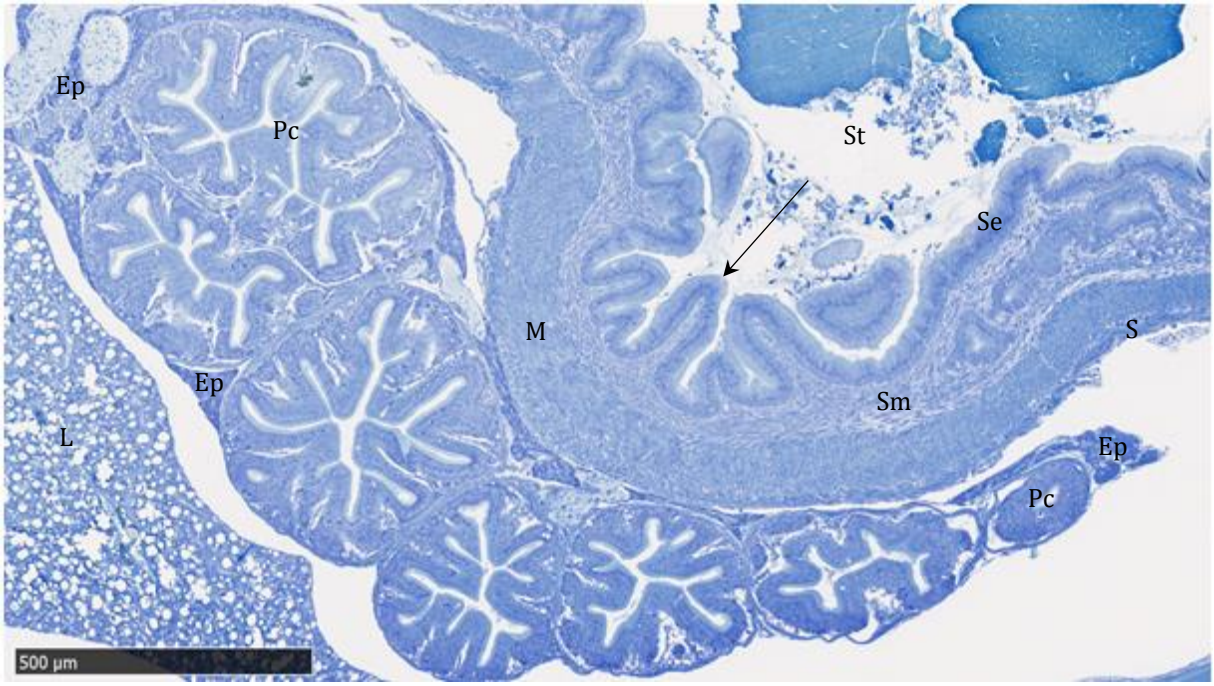

Figure 3.29. Pyloric caeca in *C. lumpus* 50 dph. Several pyloric caeca formed intestinal appendages at the intersection between the stomach and intestine (black arrow). Scale bar: 500 µm. The picture is a longitudinal section from an Artemia larva stained with TB 0.05 %. Abbreviations: Ep = exocrine pancreas, L = liver, M = muscularis, Pc = pyloric caeca, S = serosa, Se = stomach epithelium, Sm = submucosa, St = stomach.

3.7 Summary of digestive system development

Development of the digestive system in lumpfish larvae from hatching up to day 50 post hatch in relation to some external and internal markers as well as standard length (SL) is summarized in Figure 3.30 below. The illustration is based on morphological descriptions and mean SL values in Artemia larvae, as larvae from this treatment exhibited best growth throughout the start feeding experiment.

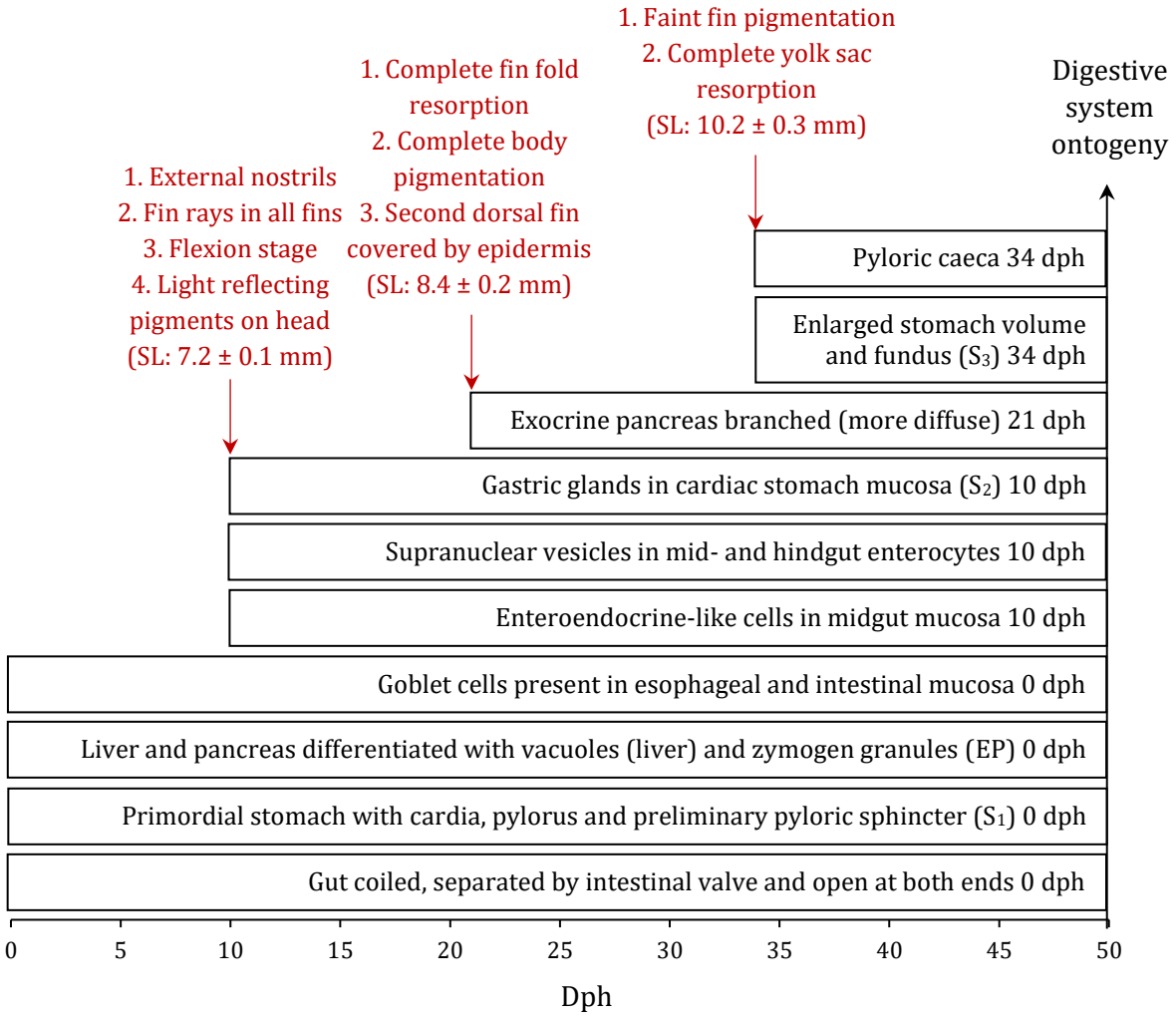

Figure 3.30. Summary of digestive system ontogeny in *C. lumpus* 0-50 dph. Major ontogenetic events in digestive organs (esophagus, stomach, midgut, hindgut, pyloric caeca, liver and exocrine pancreas) in relation to some external and internal features and mean standard length (dark red) 10, 21 and 34 dph (n = 5 per day). The descriptions and mean standard length values (± SE) are based on larvae from the Artemia group. Abbreviations: EP = exocrine pancreas, S_i = stomach developmental stage (1, 2 or 3).

4 Discussion

4.1 Lumpfish digestive system development

The present study demonstrated that all three larval diets promoted proper development of the digestive system in lumpfish, which followed the general pattern found in teleostean larvae (Luizi *et al*., 1999; Ortiz-Delgado *et al.*, 2003; Chen *et al*., 2006; Zaiss *et al.*, 2006; Faulk *et al.*, 2007). Already by hatching, the alimentary canal was open at both ends and distinctly divided into the esophagus, stomach anlage, midgut and hindgut. This indicated a high developmental status at first feeding compared to altricial species like Atlantic cod, Atlantic halibut, white seabream and yellowtail kingfish (*Seriola lalandi*) larvae (Luizi *et al.*, 1999; Ortiz-Delgado *et al.*, 2003; Chen *et al*., 2006; Wold *et al*., 2008). But unlike precocial species such as Atlantic salmon and Atlantic wolffish (Gorodilov, 1996; Falk-Petersen & Hansen, 2001), the lumpfish stomach was still under development at onset of exogenous feeding and did not seem to fully differentiate until 34 dph. The lumpfish digestive system thus exhibited an intermediate developmental status at hatching compared to characteristic altricial and precocial species. This correlates well with the fact that lumpfish have considerably longer time to develop between fertilization and hatching compared to the altricial Atlantic cod (300 versus 90 d°) but shorter time than the precocial Atlantic salmon (300 versus 500 d°) (see review by Kjørsvik *et al*., 2004). Although first feeding commenced shortly after the lumpfish larvae hatched, this species also had a longer lecithotrophic phase (> 21 dph) compared to several altricial fish larvae (Kjørsvik *et al*., 1991; Ortiz-Delgado *et al.*, 2003; Chen *et al*., 2006). A prolonged period with mixed endogenous and exogenous feeding combined with the well-developed digestive apparatus at first feeding can possibly explain why larval survival rates in lumpfish larviculture are much higher compared to what is observed in rearing of for instance in Atlantic cod and ballan wrasse (Øie *et al*., 2015).

Stomach ontogeny is of high relevance when establishing commercial feeding regimes in lumpfish as it influences when the larvae are physiologically able to digest formulated diets (Govoni *et al.*, 1986; Kolkovski, 2001). Some authors have proposed that weaning should be synchronized with gastric gland formation (Zaiss *et al*., 2006), as these glands secrete pepsin and HCl to optimize proteolytic capacity (Tanaka, 1971). The first gastric glands in lumpfish were observed by 10 dph (S_2: 6.6 – 6.8 mm SL) and coincided with notochord flexion, which can serve as a potential weaning indicator since it is easily examined and does not require any histological investigation. However, as shown for red porgy (*Pagrus pagrus*), gastric glands are not necessarily functional at the time they develop. In this species, gastric glands are differentiated by 25 dph, while pepsinogen expression and acidic stomach pH is not evident until 30-35 dph (Darias *et al.*, 2005). In contrast, larval winter flounder (*Pseudopleuronectes americanus*) develop gastric glands 20 dph, by which

time expression of both pepsinogen and proton pump subunits responsible for HCl secretion can be detected (Douglas *et al.*, 1999). It is thus not possible from the present study alone to conclude at which time the lumpfish gastric glands became functional. The fundic region of the stomach formed by 34 dph (S_3: 8.0 – 9.1 mm SL) and was accompanied by an expanded stomach lumen volume. This allows ingested feed to be stored and exposed to the acidic and proteolytic gastric environment for an extended time period, thereby increasing larval protein digestion capacity. Accordingly, it can be claimed that lumpfish larvae should be weaned after the fundic stomach is developed to ensure optimized growth and utilization of feed resources. This practice is also recommended when rearing yellowtail kingfish larvae (Chen *et al*., 2006).

Epithelial cells lining the lumpfish stomach mucosa were initially cubical but gradually developed a columnar shape, as also seen in white seabream larvae (Ortiz-Delgado *et al.*, 2003). In lumpfish, this elongation was observed after larvae reached the final ontogenetic stage (S_3) around 34 dph, as reflected in the increased stomach epithelium height from 34-50 dph. Columnar epithelium generally performs absorptive or secretory functions (Young *et al*., 2006), and various studies indicate that stomach epithelial cells of teleosts are involved in both of these processes. Stomach epithelial cells of several species contain neutral glycoconjugates that are secreted to form a mucinous layer on their apical surface, thereby protecting the stomach from autodigestion by HCl and pepsin (Ferraris *et al.*, 1987; Ortiz-Delgado *et al.*, 2003). Potentially increased secretion of gastric juices with formation of additional gastric glands must therefore be accompanied by increased capacity to synthesize and secrete neutral glycoconjugates. This can possibly explain why stomach epithelium height was positively correlated to gastric gland abundance in the current study, since elongation of cubical into more voluminous columnar cells imply more space to both synthesize and store neutralizing substances. It has also been proposed that neutral glycoconjugates mediate uptake of some easily digestible nutrients such as disaccharides and short-chained fatty acids by stomach epithelial cells (Grau *et al.*, 1992). Despite which functions are performed by the cells lining the stomach mucosa in lumpfish, it can be hypothesized that the change from a cubical to columnar shape is part of the functional maturation of these cells.

Absorptive capacity in lumpfish most certainly increased gradually during ontogeny of the gut as microvillus and villus height correlated well with larval size, both parameters of which contribute to increased absorptive surface area (Caspary, 1992; Pirarat *et al*., 2011). Microvilli elongation might also have increased digestive capacity by embedding additional brush border enzymes such as alkaline phosphatase (Zambonino-Infante & Cahu, 2001), whose activity increase accordingly with microvilli surface area in larval Senegal sole (*Solea senegalensis*) (Ribeiro *et al.*, 1999). Since lumpfish microvilli were considerably longer in the midgut compared to the hindgut, as also found in channel catfish (*Ictalurus punctatus*) (Krementz & Chapman, 1975), it is possible that brush

border digestion along the lumpfish intestine is primarily occurring in the anterior half. Seeing as the enteroendocrine-like cells were restricted to the midgut and pyloric caeca, it is likely that also luminal digestion is predominant in the anterior parts of the lumpfish gut. Identical findings have also been made for other species with a coiled gut (Kamisaka *et al*., 2001; Kamisaka *et al*., 2002). As stated by these studies' authors, it is logical that these cells are located where the chyme is retained to allow control of the secretory activities of the exocrine pancreas and gallbladder. The hindgut did, however, exhibit pinocytotic activity that gave rise to acidophilic supranuclear vesicles. Pinocytosis of dietary proteins diminishes after the gastric function is attained in several species (Iwai, 1969; Noaillac-Depeyre & Gas, 1976; Ortiz-Delgado *et al.*, 2003; Chen *et al*., 2006), whereas the present study found no indication of reduced pinocytotic activity in lumpfish during or after stomach development. These findings suggest that the intracellular mode of protein digestion remains significant even in metamorphosed lumpfish individuals, which has also been found for African catfish (*Clarias lazera*) (Stroband & Kroon, 1981).

Goblet cells occurred in the intestine and esophagus from hatching and proliferated as larvae aged, spreading to the pyloric caeca with its formation by 34 dph. These cells contained neutral mucins in the posterior esophagus and intestine, which could be attributed to a neutralizing function to prevent proteolysis by gastric juices from the stomach (Smith, 1989). As the most anterior part of the gastrointestinal tract, the esophagus is also lubricated by secretory products from goblet cells to prevent physical damage by ingested feed particles (Kapoor *et al*., 1975). The seemingly large abundance of esophageal goblet cells at hatching thus indicate that the alimentary canal already by this time was ready for passage of exogenous food, relating well with the fact that lumpfish larvae start feeding shortly after hatching. The acidic goblets cells observed in the anterior half of the esophagus has previously also been found in larval gilthead seabream and Siberian sturgeon (*Acipenser baeri*) (Domeneghini *et al.*, 1998; Gisbert *et al.*, 1999). Secretion of acidic residues might be a response to shifting abiotic factors or maintaining osmotic balance (Domeneghini *et al*., 1998), but their function in lumpfish is not possible to clarify from this study alone.

The lumpfish liver was fully differentiated at hatching, as also shown in Atlantic salmon, Atlantic wolffish and white seabream (Morrison, 1993; Ortiz-Delgado *et al.*, 2003; Hoehne-Reitan & Kjørsvik, 2004). Additionally, numerous vacuoles were observed in the liver from hatching and throughout the experimental period in seemingly well-fed larvae. These findings suggest that lumpfish are ontogenetically programmed to synthesize and store nutrients in the liver during the entire larval stage, which is also true for rainbow trout (Vernier & Sire, 1977). Selective accretion of hepatic nutrients indicates that fed lumpfish larvae are able to maintain nutritional homeostasis and sustain short episodes of food scarcity by mobilizing these nutrients, a potential factor contributing to the overall high survival rates in lumpfish larviculture.

Teleostean metamorphosis is the size dependent transformation of larvae into juveniles that has important implications for all organ systems (Youson, 1988; Kjørsvik *et al*., 2004). Juveniles have optimized digestive capacity (Govoni *et al*., 1986), hence the question of when metamorphosis occurs in lumpfish is of high relevance regarding digestive system development in this species. Metamorphosis is initiated with formation of stomach gastric glands (Tanaka, 1971), which in lumpfish occurred simultaneously with notochord flexion around 10 dph at 6.6 – 6.8 mm SL. This was followed by fin fold resorption and development of a diffuse pancreas by 21 dph, the latter of which has been used to characterize metamorphosis in Japanese flounder (*Paralichthys olivaceus*) (Kurokawa & Suzuki, 1996). Pyloric caeca formation is the final morphological change in the digestive system of fish larvae (Bisbal & Bengtson, 1995), and coincided with expansion of the stomach volume and differentiation of the fundic stomach region around 34 dph at a size of 8.0 – 9.1 mm SL. This overlaps well the 7.7 – 9.7 mm SL size range where external morphometric characters in lumpfish from the present study changed from an allometric to isometric growth pattern (van Mil, 2018). As isometric growth is characteristic for metamorphosed fish (Osse & van den Boogaart, 1995), it can be hypothesized that the lumpfish digestive system is fully developed and functional before reaching 10 mm SL.

4.2 Larval performance in relation to start feeding diets

4.2.1 Growth, development and survival

The present study found that use of enriched *Artemia* during the start feeding period (2-22 dph) improved larval somatic growth and survival compared to the use of *A. tonsa* and formulated diet. The differences between larvae fed *Artemia* and inert diet are in accordance with the hypothesis and results from previous studies (Brown, 1986; Belova, 2015). On the other hand, a prolonged feeding with *A. tonsa* only had a minor benefit on growth observed for the period *A. tonsa* was supplied exclusively to Copepod larvae (9-22 dph). The slightly increased growth of Copepod larvae compared to FD larvae might have been due to differences in feed intake. FD larvae had been weaned 6-8 dph, hence it is possible that ingestion rates in this group were lower following weaning as predation is often hampered after fish are introduced to new food sources (Dutton, 1992). Additionally, ingestion might be reduced when feeding fish larvae inert diets over live prey due to limited movement to trigger predatory behaviour (D'Abramo, 2002). Still, no difference in larval size or survival was observed between Copepod and FD larvae after weaning to Gemma micro 300 (20-22 dph). The current findings are thus not enough to confirm the hypothesis that *A. tonsa* is advantageous for larval growth and survival of lumpfish compared to formulated diet. Similarly, Dahle *et al*. (2017) only found slightly improved growth and survival of lumpfish larvae fed *A. tonsa* rather than inert diet and were unable to detect consistent significant differences.

The better growth obtained by Artemia larvae compared to Copepod and FD larvae was due to large differences in daily weight increase (DWI) for the period the groups received different larval diets (2-22 dph). *Artemia* instar I and *A. tonsa* copepodites CI-CIII are about similar in linear dimensions (Miller *et al.*, 1977; Dhont & Van Stappen, 2003). However, the *Artemia* strain used in this experiment achieve an individual size of 2.2-2.5 µg/1.2-1.3 mm when enriched over 24 hours (Jan Ove Evjemo, SINTEF Ocean AS, pers. comm., 2018). This is considerably larger than the individual size of the used *A. tonsa* stages, ranging from 0.56-1.40 µg/410-580 µm ('On growing of copepods', user's manual from C-Feed AS). Differences in larval growth might thus have been due to a larger biomass of *Artemia* being available to Artemia larvae. The larger prey size administrated to Artemia larvae were also more energetically favorable due to a higher nutrient content (Pastorok, 1981; Olsen, 2004), indicating that less energy had to be invested in foraging to reach food satiation. A behavioural study reported that lumpfish larvae attach to surfaces and adopt a passive foraging mode when prey is abundant, while more active foraging is observed when feed is scarce (Killen *et al*., 2007a). As Copepod and FD larvae were comparably more active during the treatment period (2-22 dph), it can be suggested that Artemia larvae grew faster because they had more energy available for growth (Killen *et al.*, 2007a). Moreover, the active foraging mode observed in the Copepod and FD groups indicate that these larvae were not fed to satiation. Several factors could have contributed to this, such as differences in biomass, particle sizes, capture success or acceptance.

Stomach ontogeny was strongly correlated to larval size, hence the larger DWI of Artemia larvae induced more rapid stomach morphogenesis (transition from S_1 to S_2 and S_2 to S_3) and growth of the stomach epithelium after complete stomach differentiation (S_3) compared to Copepod and FD larvae. This is important to consider when establishing feeding protocols in lumpfish, as a diet that can promote rapid growth and stomach development will potentially allow for faster weaning to formulated diet. Studies have shown that proper development of several species require that DHA and EPA are located in phospholipids (PL) rather than neutral lipids (NL) (Evjemo *et al*., 2003; Gisbert *et al*., 2005; Kjørsvik *et al*., 2009; Wold *et al.*, 2009). As DHA and EPA are mainly incorporated into the NL fraction during *Artemia* enrichment (Conceição *et al.*, 2010), the rapid growth and stomach development of Artemia larvae suggest that lipid class is not limiting the availability of DHA and EPA that are efficiently incorporated into lumpfish larval tissues. This is also supported by the rapid hepatocyte hypertrophy of Artemia larvae, as increased cell sizes imply a proportional increase in cell membrane surface area and integration of DHA and EPA required for proper membrane functioning (Sargent *et al.*, 1999a). These findings correlate well with the fact that lumpfish had a well-developed digestive system at hatching, which separates them from some altricial species that require DHA and EPA in the PL fraction for proper growth and development (Hamre *et al.*, 2002; Evjemo *et al*., 2003; Kjørsvik *et al*., 2009; Wold *et al*., 2009).

The overall exponential increase in DW and linear increase in SL resulted in an exponential DW/SL relationship, which can probably be attributed to the allometric growth of many organs during the larval stage (Osse & van den Boogaart, 1995; Gagnat *et al.*, 2016). The DW/SL slope coefficient differed significantly between all groups and was considerably lower in Artemia larvae, indicating reduced organ growth in *Artemia*-fed larvae compared to similar sized larvae from the other groups. However, as shown for instance by Gagnat *et al*. (2016), increase in organ tissue volumes typically changes from an allometric to isometric growth pattern when fish larvae metamorphose into juveniles. This implies that growth of the whole fish changes from allometric to isometric as well, thereby changing the DW/SL ratio from exponential to linear. In a parallel study it was found that the inflection point where growth in DW in relation to SL changed from allometric to isometric occurred around 9.5 mm SL (van Mil, 2018). A probable explanation for the lower DW/SL in the exponential model for Artemia larvae is thus that a comparably larger fraction of all sampled larvae from this group had a SL > 9.5 mm and were thus growing isometrically (linearly).

The apparent drop in survival of Copepod and FD larvae around 30 dph is consistent with common experience in commercial lumpfish production (Powell *et al*., 2017; MoreFish AS, Tjeldbergodden, Norway, pers. comm., 2018). Powell *et al*. (2017) claimed that this mortality is caused by weaning from live to formulated diet, which is also a problem in other species (Bengtson *et al.*, 1999; Hamlin & Kling, 2001). As Copepod and FD larvae were not weaned to inert diet at the same time (20-22 versus 6-8 dph) but the survival drop occurred around 30 dph in both groups, mortality induced by weaning itself seems unlikely. Progressively starved Atlantic cod and European flounder (*Platichthys flesus*) larvae grow slowly and encounter a point of no return shortly after yolk resorption where they are too weak to continue feeding and die (Yin & Blaxter, 1986). Likewise, Copepod and FD larvae exhibited limited growth and lower nutritional status prior to yolk resorption between 21 and 34 dph, which overlaps well with the observed mortality after 30 dph in these groups. Lower nutritional status was reflected in both liver and gut histology of these larvae, such as smaller hepatonuclear sizes and epithelium height in the midgut (Ehrlich *et al.*, 1976; Segner *et al.*, 1988). On the other hand, *Artemia*-fed larvae showed much larger DWI prior to yolk resorption, high nutritional status in the liver and gut, and hardly any mortality during the experiment. These results indicate that the observed mortality in the Copepod and FD group was due to malnourishment. Consequently, it can also be hypothesized that the survival bottleneck in lumpfish larviculture around 30 dph is caused by inadequate ingestion of exogenous food prior to yolk resorption. Still, it cannot be disregarded that mortality was also caused by the bacteria that proliferated from 26 dph and entangled larvae. As Artemia larvae were much larger and probably also stronger than Copepod and FD larvae by the time bacteria flourished, it is possible that it was easier for these larvae to avoid being entangled. This is supported by the fact that Artemia larvae were rarely found within bacterial filaments.

4.2.2 Nutritional status – liver and gut histology

The lumpfish liver and gut exhibited different histological characteristics in response to the start feeding regimes. Copepod and FD larvae showed significantly smaller hepatocytes and hepatocyte nuclei compared to Artemia for the period they were fed or shortly after fed *A. tonsa* (Copepod larvae: 10 and 21 dph, FD larvae: 10 dph), at which time the livers of these larvae also were deprived of hepatic vacuoles. Differences in larval gut histology between dietary treatments were most pronounced in terms of epithelium height, which was significantly lower in both the midgut and hindgut of Copepod and FD larvae compared to Artemia larvae during the start feeding period (2-22 dph). These effects on the liver and gut tissue coincided with the low larval growth rates observed in the Copepod and FD groups. The present findings thus suggest that histological organization of the liver and gut in lumpfish are sensitive towards dietary factors and indicative of larval nutritional status. This is in accordance with studies on Atlantic cod, Atlantic herring, European flounder, European plaice and pejerrey (*Odontesthes bonariensis*) (Ehrlich *et al*., 1976; Segner & Möller, 1984; Strüssmann & Takashima, 1990; Kjørsvik *et al*., 1991).

The teleostean liver grows allometrically during the larval stage (Gagnat *et al*., 2016), and a study with Atlantic cod found that larger sized larvae displayed the largest livers and hepatocytes (Wold *et al*., 2009). Present findings indicate that a similar relation might be true for lumpfish larvae, as limited hepatocyte hypertrophy was observed in Copepod and FD larvae during the period larval growth rates were low in these two groups. At this time, these larvae also had seemingly smaller livers than Artemia larvae. It has been shown that hepatocyte size in larval turbot is determined by the amount of nutrients stored within hepatocytes (Segner *et al*., 1994). Similarly, Fontagné *et al*. (1998) attributed larger hepatocytes in common carp (*Cyprinus carpio*) larvae to an increased flux of lipoproteins from the intestine to the liver. The observed hepatocyte hypertrophy in larvae from the Copepod and FD treatments might thus have been due to the significant accretion of vacuolated nutrients after weaning from *A. tonsa* to inert diet. This can explain why hepatocytes of FD larvae but not Copepod larvae had increased in size by 21 dph, at which time FD larvae had been fed formulated diet for roughly two weeks while Copepod larvae were still receiving *A. tonsa*. Interestingly, larvae from all groups also showed significant hypertrophy towards the end of the experiment. In contrast to vacuoles observed 0-21 dph, the vacuoles residing in hepatocytes by 34 dph seemed larger and were clearly of lipid origin (Caballero *et al*., 2004; Gisbert *et al*., 2005). It is thus possible that the continued hypertrophy after 34 dph can be attributed to a change in the type of nutrients stored in the liver. This could either be due to weaning, an ontogenetic change in liver metabolism, or a combination of dietary and ontogenetic factors (Segner & Witt, 1990). Larger hepatocytes are also related to essential FA deficiency (Watanabe *et al*., 1989). But as hepatocyte size related positively with growth rates and hepatic vacuole content, it seems more likely that hypertrophy of these cells is associated with a high nutritional status in lumpfish.

Hepatocyte area sizes were positively correlated with hepatonuclear sizes. This was reflected in the significantly smaller nuclei of Copepod and FD larvae compared to Artemia larvae at the same time as hepatocyte sizes differed between these groups during the treatment period (2-22 dph). A start feeding experiment with Atlantic cod by Wold *et al*. (2009) found smaller hepatocyte nuclei in larvae from the treatment group that showed lowest growth. This is consistent with present findings, as small hepatonuclear area sizes coincided with low growth rates in Copepod and FD larvae. In contrast, large nuclei are associated with high metabolic activity as the increased membrane surface area allows for more rapid transfer of molecules between the nucleus and cytoplasm (Ghadially, 1997). The positive linear correlation between hepatonuclear area size and hepatic vacuole content in the present study thus indicate that larvae with large nuclei and a high nutrient content in their liver were more metabolically active. No such relationship was found in the Artemia group, probably only because these larvae exhibited high nutritional status in terms of large nuclei and a high vacuole content at all times during the experiment. Hepatonuclear sizes correlated negatively with lipid content in the livers of Atlantic cod and common carp larvae (Fontagné *et al*., 1998; Wold *et al*., 2009). On the other hand, area sizes of hepatocyte nuclei in the present study were largest in larvae from all groups during the period the vacuoles contained lipids. It can thus be hypothesized that a high lipid content in the liver of lumpfish is a sign of high nutritional status.

Previous studies with fish have shown that hepatic glycogen and lipids are depleted during fasting (Weis, 1972; Leatherland, 1984; Segner & Möller, 1984; Watanabe, 1985), an indication of average or degraded hepatocytes in fish larvae (Grade 1/2, Table 1.1). The evident reduction of hepatic vacuoles in Copepod and FD larvae during the treatment period (2-22 dph) thus suggest that these larvae were malnourished. This can explain why some larvae from these groups showed signs of histopathological effects associated with starvation, such as sinusoidal dilatation, less prominent hepatocyte boundaries and central hepatocyte nuclei (Gisbert *et al*., 2008). Strüssmann and Takashima (1990) found that the hepatocyte nuclei of starved pejerrey larvae gradually shrank and reached a minimum size just before death. Although the small hepatonuclear sizes displayed by Copepod and FD larvae during the treatment period (2-22 dph) were not significantly reduced from hatching, it is likely that malnourishing conditions can explain why the nuclei of these larvae were significantly smaller than that of Artemia larvae. Differences in supplied live prey biomass, prey sizes or larval capture success of the prey can possibly explain why larvae fed *A. tonsa* showed signs of starvation. Absence of histopathological liver effects and replenishment of hepatic vacuoles after the Copepod and FD larvae had been weaned to inert diet suggest reversal of starving conditions (Segner & Möller, 1984). Moreover, it indicates that dietary effects on the liver accurately reflected larval nutritional status at the time these larvae were sampled.

Lipid absorption in the midgut was most noticeable in Artemia larvae 10 and 21 dph, matching the period when these larvae were fed enriched *Artemia*. Most lipid inclusions constituted large droplets with a diameter > 5 µm, which could be attributed to a high dietary NL content in enriched *Artemia* (Deplano *et al*., 1989; Gisbert *et al*., 2005). Some researchers relate intestinal steatosis with reduced absorption capacity due to cellular abrasion, necrosis or inflammatory reactions (Deplano *et al*., 1991; Segner *et al*., 1993). As no such effects were observed along the intestinal mucosa in any larvae, it seems unlikely that the extensive lipid assimilation in the gut of Artemia larvae was a pathological condition. The intestinal lipid droplets could have formed because the absorption rate exceeded the rate of export from midgut enterocytes (Gisbert *et al*., 2005; Wold *et al*., 2008). Still, the rapid growth rates and high nutritional liver status of *Artemia*-fed larvae suggest that the absorbed lipids only constituted a temporary storage in midgut enterocytes and were successfully exported to peripheral tissues. Comparably fewer lipid inclusions in Copepod larvae 10-21 dph and FD larvae 10 dph coincided with a seemingly low content of exogenous food sources in the midgut lumen of these larvae. This provides a probable explanation for the low larval growth rates in these groups. As FD larvae still had seemingly low gut contents and nutritional liver status by 10 dph but not 21 dph, it is likely that larval ingestion rates were still low in this group after weaning 6-8 dph. As previously discussed, this could be due to introduction of a new type feed type (Dutton, 1992).

Of the measured parameters in the gut, epithelium height in the midgut exhibited largest variation in response to supplied larval diet. Midgut enterocytes grew rapidly in Artemia larvae and reached a maximum height already 10 dph, thus establishing fully hypertrophied enterocytes at an early ontogenetic stage as described for Atlantic cod (Wold *et al.*, 2008). On the other hand, two larvae from the FD group exhibited 22 and 43 % reduction in midgut epithelium height from 10-21 dph, during which time mean midgut epithelium height also reduced significantly in Copepod larvae. Several authors have found reduced intestinal mucosa in fish larvae during starvation (Ehrlich *et al*., 1976; Oozeki *et* al., 1989; Kjørsvik *et al*., 1991; Segner *et al*., 1993), which seems like a probable explanation for the reduced epithelium height of these larvae. This also correlates well with the low larval growth rates and nutritional status of Copepod and FD larvae during the start feeding period (2-22 dph). The reduced epithelium height observed 21 dph did however not correspond to degraded enterocytes but rather an average condition (Grade 2, Table 1.1). As previously stated, histopathological effects on the liver tissue were pronounced already by 10 dph. Present findings thus indicate that the liver reflected larval nutritional status more accurately than the gut, which is in accordance with previous studies with larvae of Atlantic cod and European sea bass (Catalán & Olivar, 2002; Wold *et al*., 2009; Norheim, 2011). This might be attributed to lower energy investment in renewal of enterocytes during starvation followed by a gradual rather than immediate degeneration of intestinal tissue (Rios *et al*., 2004).

The greatly enlarged midgut epithelium height in Copepod and FD larvae from 21-34 dph indicate that these larvae had not been yet been starved to such an extent that regrowth of the intestinal mucosa was irreversible (Watanabe, 1985; Kjørsvik *et al*., 1991). This is supported by the fact that no distortion of microvilli or villi was observed in seemingly starved Copepod and FD larvae, which are common histopathological features of malnourished fish (Gas & Noailliac-Depeyre, 1976; Kjørsvik *et al*., 1991; McFadzen *et al*., 1994; Rios *et al*., 2004; Krogdahl & Bakke-McKellep, 2005). It is however probable that the observed increase in midgut epithelium height 21-34 dph was due to the significant mortality in some Copepod and FD tanks around 30 dph, in which case starved (and dead) larvae were omitted from the sampling 34 dph. Studies with Atlantic cod and gilthead seabream larvae have shown that larvae reach a point of irreversible starvation where the intestine is degenerated to such an extent that death is inevitable (Kjørsvik *et al*., 1991; Yúfera *et al*., 1993). The same can be expected to be true for lumpfish. Hypothetically, the observed reduction in midgut epithelium height continued from 21 dph and terminated with functional shutdown of the intestine prior to the apparent drop in survival around 30 dph. Nevertheless, the present findings suggest that midgut epithelium height might be a sensitive parameter indicative of long term starvation in lumpfish.

Hindgut enterocyte height showed overall slower growth than midgut enterocytes, and even more so in Copepod and FD larvae although no sign for degeneration was observed. Accordingly, the hindgut was not as useful to distinguish treatments as the midgut and liver. Similarly, hepatocyte diameter and muscle fiber separation were more sensitive to dietary treatments than hindgut epithelium height in European sea bass larvae (Catalán & Olivar, 2002). It has previously been found that lumpfish larvae fed formulated diet had seemingly smaller and fewer intestinal villi than larvae fed *A. tonsa* (Dahle *et al.*, 2017). In the present study, one FD larva aged 21 dph had very few villi in the midgut. This larva also had thin midgut epithelium (42 % reduction from average value 10 dph) and a much smaller size than other larvae from the same treatment, suggesting that villi abundance is related to nutritional status in lumpfish. Few consistent differences were found in villus height, but the slope coefficient between midgut villus height and SL was significantly larger Copepod larvae than Artemia larvae. It is likely that this difference was due to the fast growth rates of Artemia larvae and that relative villus growth was slower in larger larvae. Likewise, a study with Atlantic cod larvae demonstrated that intestinal length and volume increased rapidly during early larval stages but then deaccelerated with increasing larval size (Wold *et al*., 2009).

4.3 Methodological limitations

Due to unequal *A. tonsa* and *Artemia* biomass it is difficult to conclude if differences in larval growth, survival, as well as developmental and conditional status of the digestive organs were due to qualitative or quantitative aspects of the larval diets. It was also difficult to obtain equal larval densities between individual rearing tanks, which could have affected larval growth and survival besides the dietary treatments (Holm *et al*., 1990). Each treatment consisted of a triplicate number of tanks (n = 3). This limited the number of independent values for DWI and survival estimates as these variables are estimated on basis of per tank and not larva. Relatively small sample sizes were also used for histological analyses (n = 5) due to the time-consuming procedure of tissue processing, sectioning and analysis. Small sample sizes have lower statistical power as it increases the probability of incorrectly accepting the null hypothesis (H_0: no significant difference) in favor of the alternative hypothesis (H_1: significant difference) (Keppel & Wickens, 2004). Moreover, small sample sizes provide less reliable and precise results (Sandelowski, 1995; Hackshaw, 2008). The present findings should thus be interpreted with caution but can prove useful if designing larger and more comprehensive studies.

Measurements of the morphometric parameters may have been over- or underestimated as the angle of the section is not always perfectly transverse. Another problem with histological and stereological methods is the low objectivity and reliance on the experience of the histologist (Catalán, 2003). As an example, complete morphogenesis of the lumpfish stomach with formation of the fundic region (S_3) was not observed until 34 dph. This is much later than 16 dph in cobia (*Rachycentron canadum*) and 18 dph in yellowtail kingfish (Chen *et al*., 2006; Faulk *at al*., 2007), both species of which develop gastric glands around the same time as lumpfish. Accordingly, it is possible that stomach development in lumpfish completed somewhat earlier than identified in this study. Investigation of some additional age groups < 34 dph would thus be advantageous to get more accurate information regarding stomach development and potentially other digestive organs as well.

5 Conclusions

The present study demonstrated that the digestive system of lumpfish follows an ontogenetic pattern similar to that of other teleosts. The gastrointestinal tract of newly hatched larvae was divided into the esophagus, stomach anlage, midgut and hindgut, at which time the liver exhibited high nutritional status with abundant nutrient inclusions. Both the mouth and anus were open at hatching, indicating that the alimentary canal was ready to process exogenous food long before yolk resorption 21-34 dph. These findings indicated a well-developed digestive system at hatching compared to many altricial species but lower developmental status than precocial species. Gastric glands had formed in the stomach by 10 dph (6.6 – 6.8 mm standard length). Digestive system morphogenesis was completed by 34 dph when the stomach volume had expanded (8.0 – 9.1 mm standard length) and the fundic stomach and pyloric caeca were differentiated.

Using enriched *Artemia* as live prey for lumpfish enhanced growth rates during the start feeding period (2-22 dph) compared to using *Acartia tonsa*, regardless of whether *A. tonsa* was fed for the full start feeding period or with an early weaning to formulated diet. This resulted in larger larval sizes throughout the experiment and was proposed to be decisive for the high larval survival rates in this group. Stomach development correlated strongly to larval size, and the improved growth rates associated with the use of *Artemia* as a start feed induced more rapid stomach differentiation and stomach epithelium growth. Histological organization of the liver and gut exhibited different characteristics in response to supplied diet and indicated lower nutritional status in larvae fed *A. tonsa*. The liver accurately reflected larval nutritional status at the time of sampling in terms of hepatocyte area sizes, hepatonuclear area sizes and vacuole content. Of the measured parameters in the gut, midgut epithelium height exhibited largest variation in response to the diet. Feeding with *A. tonsa* was associated with a gradual rather than immediate reduction in midgut epithelium height, which was suggested to be a useful biomarker for long term starvation in lumpfish.

Present findings provide useful information applicable when optimizing rearing protocols for lumpfish, as a successful production requires that feeding regimes are adapted to developmental status of the fish and not *vice versa*. Inert diets should ideally be fed to lumpfish after complete stomach development when protein digestion capacity is maximized and not immediately after hatching, a common practice in commercial lumpfish production today. It is however important to stress that a morphologically differentiated organ is not necessarily functional in a fish that is still under development. Findings from the present study should thus be combined with other methodological approaches to get a holistic view of digestive system development in lumpfish. Further research should also aim to find the start feed most suited for lumpfish, which require that start feeding experiments with equal biomasses of supplied live feed are conducted.

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7 Appendices

Appendix 1. Dehydration and embedding (Technovit® 7100)

Component 1:	Basic resin (monomer), 500 mL	2-hydroxyethylmethacrylate (HEMA)
Component 2:	Activator (powder), 5 pkg. a 1 g	dibenzoyl peroxide
Component 3:	Hardener II (herder), 40 mL	dimethyl sulfoxide

Dehydration fluids:

A: 15 mL distilled water + 5 mL Basic Resin (3 mL/mL)

B: 10 mL distilled water + 10 mL Basic Resin (1 mL/mL)

Samples were immersed in the first dehydration fluid (A) at 4 °C for 3 hours, before transferred to the second dehydration fluid (B) at room temperature for 2 hours.

Infiltration fluid:

50 ml Basic Resin + 0,5 g Activator (10 mg/mL)

A magnetic stirrer was used until the powder was completely dissolved. The dehydrated samples were immersed in the infiltration fluid and placed on a digital shaker (ROCKER 3D digital, IKA®, USA) overnight (12 hours or more). The infiltration fluid was stored at 4 °C for a maximum of four weeks.

Polymerization fluid:

30 mL infiltration fluid + 2 mL Hardener II (15 mL/mL)

The two components were mixed for 3-5 minutes and used immediately, as the polymerization process was very rapid. The samples were placed and oriented in individual embedment molds, which were partly filled with the polymerization fluid. An adapter (holding the block steady when attached to the microtome) was then placed over the mold containing the sample, and the mold was filled with polymer through a hole in the adapter. The embedment was completed after 40-120 minutes at room temperature.

Appendix 2. Staining protocols and tests

Four different staining methods (A-D) were tested on the larvae embedded in Technovit® 7100 (Kulzer, Germany), with some modifications:

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.

Note: A concentration of 0.05 % TB was compared to 0.1 % TB to examine if it affected resolution of the tissue. The desired concentration was obtained by diluting the stain with saturated borate buffer.

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.

Note: Immersion of the sections in periodic acid for a 15-minute duration was compared to a 30-minute duration. This was done to examine if the duration affected the extent of glucose oxidation.

C. Alcian Blue-Periodic acid Schiff (AB-PAS)

1. Stain with Alcian blue for 5 minutes.
2. Rinse with running tap water.
3. Follow steps 1-7 for PAS staining (B).

D. Hematoxylin and Eosin (H&E)

1. Stain with Hematoxylin for 5 minutes.
2. Rinse with running tap water for 3 minutes to allow stain to develop.
3. Quick immersion in acid alcohol (1% HCl in 70 % ethanol). Repeat 5 times.
4. Rinse with running tap water for 3 minutes.
5. Stain with Eosin (0.5 % aqueous) for 2 minutes.
6. Quick immersion in 70 % ethanol.
7. Quick immersion in distilled water.
8. Dry on hot plate (75 °C) for 20 minutes or more.

Appendix 3. Results from the staining methods

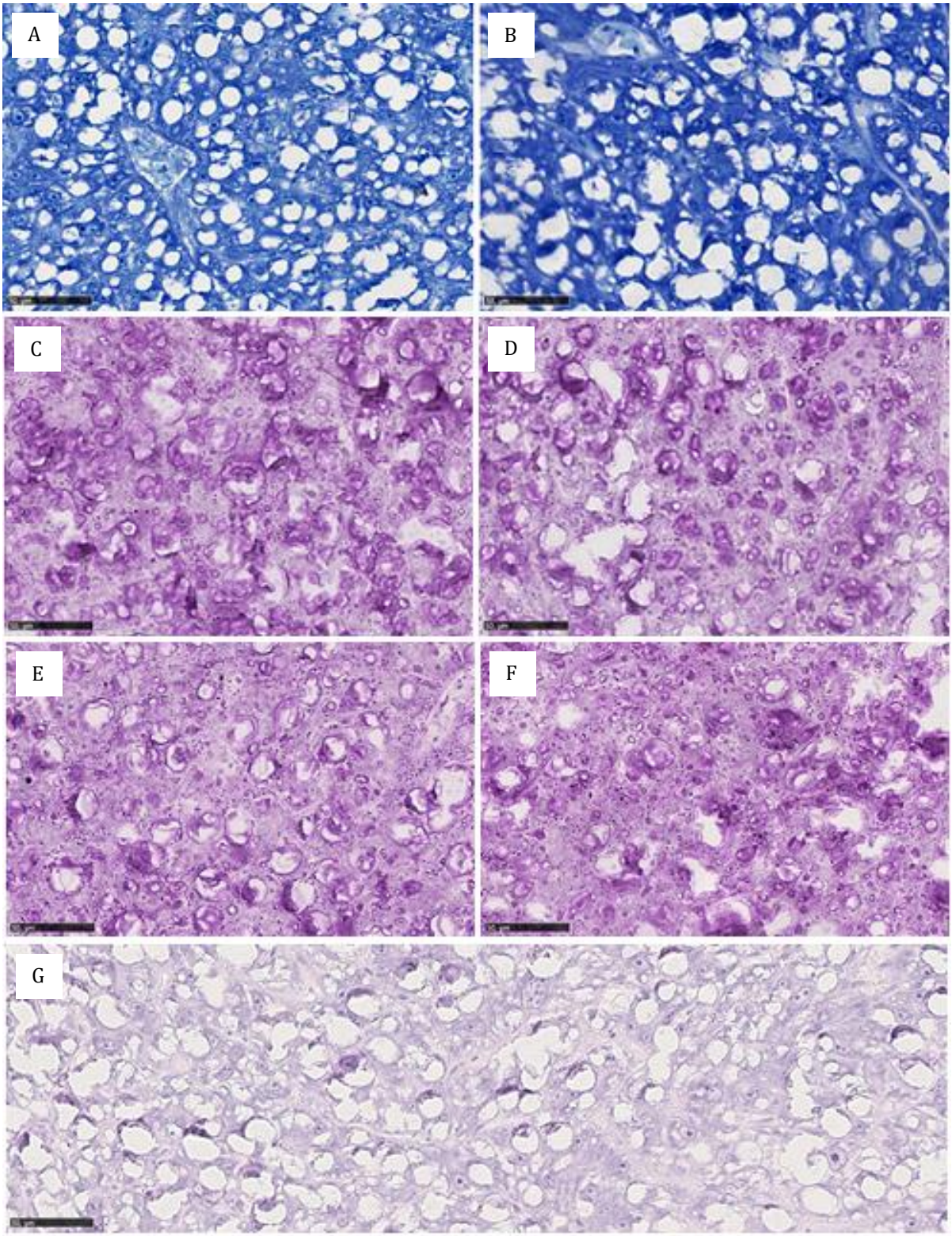

Figure A1. Cross-section of *C. lumpus* liver 50 dph. The sections are stained according to the protocols in Appendix 2: A) TB 0.05 %, B) TB 0.1 %, C) PAS 15 min, D) PAS 30 min, E) AB-PAS 15 min, F) AB-PAS 30 min, G) H&E. All sections are embedded in Technovit® 7100 and scanned with 40x magnification. Scale bars: 50 µm.

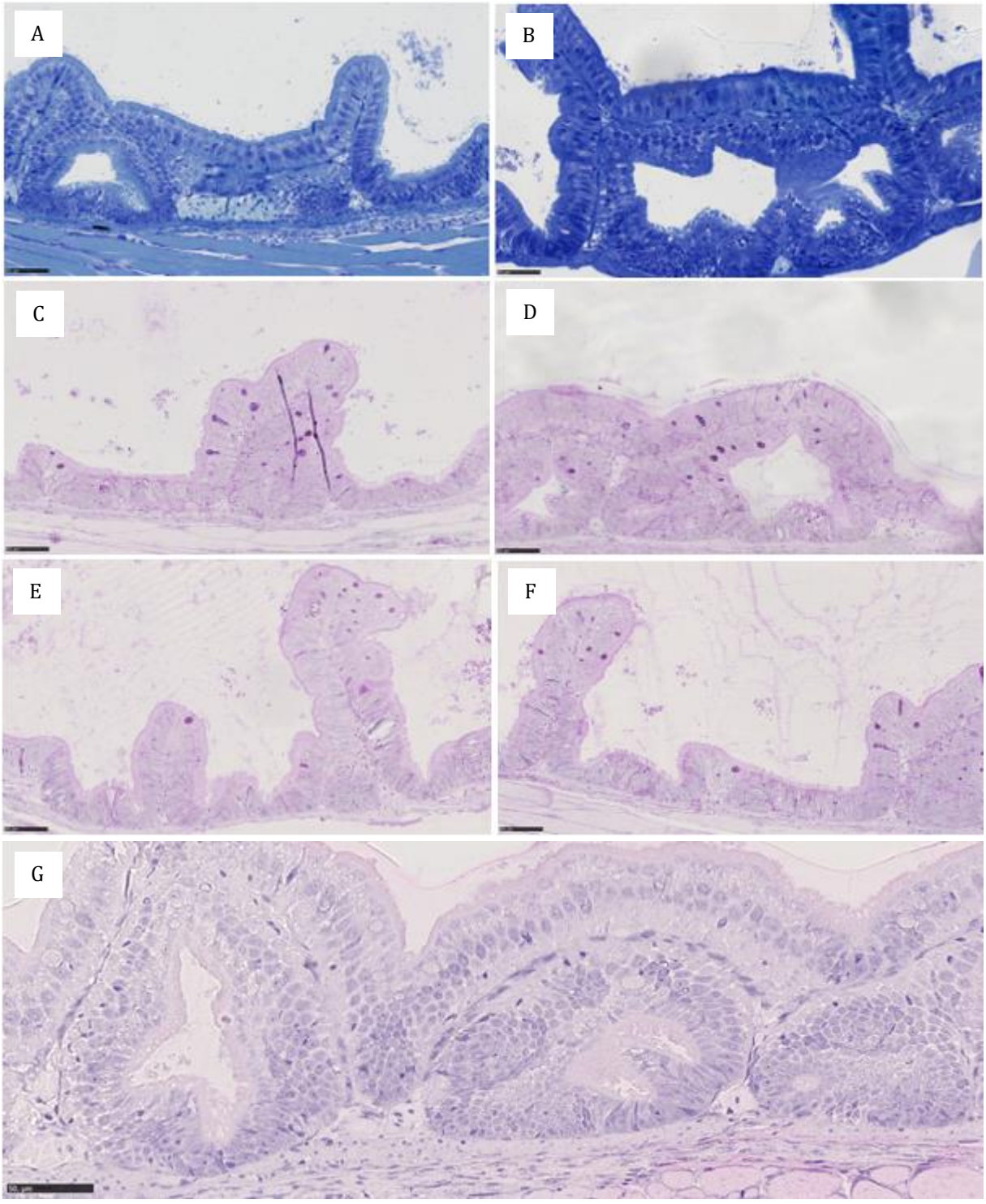

Figure A2. Cross-section of *C. lumpus* gut epithelium 50 dph. The sections are stained according to the protocols in Appendix 2: A) TB 0.05 %, B) TB 0.1 %, C) PAS 15 min, D) PAS 30 min, E) AB-PAS 15 min, F) AB-PAS 30 min, G) H&E. All samples are embedded in Technovit® 7100 and scanned with 40x magnification. Scale bars: 50 µm.

Appendix 4. Functions in RStudio used for statistical analyses

Table A1. R functions used for statistical analyses. Functions used when comparing treatments and different age groups within each treatment are listed, including the R package where the functions can be found.

Statistical test	R function	R package	Comments
Shapiro-Wilk	`shapiro.test()`	Default	
Q-Q plot	`qqnorm()` `qqline()`	Default	The function `qqnorm()` was used to plot theoretical versus sample quantiles, while `qqline()` was used to plot the line used to evaluate normality.
Levene	`leveneTest()`	car[1]	The argument `center` was set equal to `mean` to compare the means for the different treatments: `center=mean`
One-way ANOVA	`lm()` `anova()`	Default	The function `lm()` was used to fit a linear model with the dependent and independent variable as arguments, while `anova()` was used to conduct the actual test.
Welch's ANOVA	`oneway.test()`	Default	The argument `var.equal` was set equal to `FALSE` to assume heteroscedasticity: `var.equal=FALSE`
Kruskal-Wallis	`kruskal.test()`	Default	
Student-Newman-Keuls' post hoc	`snk()`	mutoss[2]	The argument `alpha` was set equal to `0.05` to control the significance level: `alpha=0.05`
Dunnett's T3 post hoc	`DunnettT3Test()`	PMCMRplus[3]	
Dunn's post hoc	`dunnTest()`	FSA[4]	The argument `method` was set equal to `"holm"` to use Holm's correction for adjustment of *p*-values: `method="holm"`
Paired sample t-test	`t.test()`	Default	The argument `paired` was set equal to `TRUE`: `paired=TRUE`
Wilcoxon's signed-rank tests	`wilcox.test()`	Default	The argument `paired` was set equal to `TRUE`: `paired=TRUE`
Pearson correlation	`cor.test()`	Default	The argument `method` was set equal to `"pearson"` to acquire Pearson's correlation coefficient (*r*): `method="pearson"`
ANCOVA	`lm()` `summary()`	Default	The function `lm()` was used to fit the linear function with the dependent and independent variable as arguments, including the interaction effect of the different treatments: `lm(y~x*group)`. The function `summary()` was used to conduct the actual test.

1. Fox J, Weisberg S, Adler D, Bates D, Baud-Bovy G, Ellison S, *et al.* (2017). car: Companion to applied regression. R package version 2.1-6. Available from: https://CRAN.R-project.org/package=car
2. Team MC, Blanchard G, Dickhaus T, Hack N, Konietschke F, Rohmeyer K, *et al.* (2017). mutoss: Unified multiple testing procedures. R package version 0.1-12. Available from: https://github.com/kornl/mutoss/
3. Pohlert T (2018). PMCMRplus: Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended. R package version 1.0-0. Available from: https://CRAN.R-project.org/package=PMCMRplus
4. Ogle DH (2017). FSA: Fisheries Stock Analysis. R package version 0.8-17. Available from: https://github.com/droglenc/FSA

Appendix 5. Mean dry weight per tank

Table A2. Mean dry weight in *C. lumpus* 0-51 dph. The averages (mg larva^{-1}) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean DW ± SE (mg larva^{-1})	Total *N*	Dph	Group	Tank	Mean DW ± SE (mg larva^{-1})	Total *N*
0	Yolk sac	Hatching tank	1.10 ± 0.01	16					
2	Yolk sac	Hatching tank	1.07 ± 0.01	16					
6	Artemia	1 2 9	1.17 ± 0.02 1.23 ± 0.04 1.23 ± 0.03	5 5 5	21	Artemia	1 2 9	2.80 ± 0.13 3.05 ±0.14 3.08 ± 0.06	5 5 5
	Copepod	3 6 10	1.06 ± 0.01 1.04 ± 0.03 1.02 ± 0.04	5 5 5		Copepod	3 6 10	1.20 ± 0.07 1.23 ± 0.04 1.59 ± 0.15	5 5 5
	FD	4 5 7	1.07 ± 0.01 1.03 ± 0.03 1.04 ± 0.01	5 5 5		FD	4 5 7	1.30 ± 0.13 0.90 ± 0.10 1.44 ± 0.14	5 5 5
10	Artemia	1 2 9	1.43 ± 0.03 1.37 ± 0.05 1.40 ± 0.05	5 5 5	24	Artemia	1 2 9	3.34 ± 0.39 2.88 ± 0.15 3.60 ± 0.29	5 5 5
	Copepod	3 6 10	1.07 ± 0.05 1.12 ± 0.03 1.08 ± 0.04	5 5 5		Copepod	3 6 10	1.43 ± 0.19 1.13 ± 0.05 1.42 ± 0.06	5 5 5
	FD	4 5 7	1.06 ± 0.05 1.08 ± 0.04 1.07 ± 0.04	5 5 5		FD	4 5 7	1.44 ± 0.14 0.88 ± 0.13 1.36 ± 0.10	5 5 5
13	Artemia	1 2 9	1.57 ± 0.07 1.71 ± 0.09 1.91 ± 0.07	5 5 5	29	Artemia	1 2 9	4.37 ± 0.39 4.35 ± 0.64 3.88 ± 0.32	5 5 5
	Copepod	3 6 10	1.18 ± 0.03 1.16 ± 0.04 1.16 ± 0.04	5 5 5		Copepod	3 6 10	1.40 ± 0.38 1.01 ± 0.03 1.84 ± 0.31	5 5 5
	FD	4 5 7	1.06 ± 0.03 0.80 ± 0.10 1.08 ± 0.04	5 5 5		FD	4 5 7	2.35 ± 0.13 2.08 ± 0.29 2.35 ± 0.19	5 5 5
17	Artemia	1 2 9	2.12 ± 0.14 2.25 ± 0.13 2.33 ± 0.12	5 5 5	34	Artemia	1 2 9	5.96 ± 0.57 4.87 ± 0.34 5.79 ± 0.19	5 5 5
	Copepod	3 6 10	1.27 ± 0.11 1.30 ± 0.04 1.53 ± 0.03	5 5 5		Copepod	3 6 10	1.97 ± 0.15 2.40 ± 0.32 4.03 ± 0.14	5 5 5
	FD	4 5 7	1.08 ± 0.07 1.15 ± 0.07 1.09 ± 0.08	5 5 5		FD	4 5 7	3.03 ± 0.18 3.17 ± 0.27 3.13 ± 0.17	5 5 5

Table A2. Mean dry weight in *C. lumpus* 0-51 dph (continued). The averages (mg larva^{-1}) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean DW ± SE (mg larva^{-1})	Total *N*	Dph	Group	Tank	Mean DW ± SE (mg larva^{-1})	Total *N*
38	Artemia	1	7.24 ± 0.43	5	50	Artemia	1	13.16 ± 0.90	10
		2	5.61 ± 0.81	5			2	14.88 ± 0.56	10
		9	6.18 ± 0.69	5			9	18.49 ± 1.42	10
	Copepod	3	3.50 ± 0.40	5		Copepod	3	7.29 ± 0.55	10
		6	3.75 ± 0.12	5			6	8.45 ± 0.80	10
		10	4.62 ± 0.75	5			10	12.83 ± 1.09	10
	FD	4	3.74 ± 0.37	5		FD	4	10.30 ± 0.70	10
		5	4.46 ± 0.63	5			5	10.05 ± 0.62	10
		7	4.23 ± 0.18	5			7	8.51 ± 0.73	10
42	Artemia	1	10.52 ± 0.99	10	51	Artemia	1	18.70 ± 0.92	14
		2	11.06 ± 0.80	10			2	19.49 ± 1.92	10
		9	12.14 ± 0.93	10			9	28.67 ± 1.14	15
	Copepod	3	4.98 ± 0.55	10		Copepod	3	9.79 ± 0.77	14
		6	4.93 ± 0.51	10			6	9.99 ± 0.80	16
		10	5.54 ± 0.50	10			10	13.78 ± 0.65	16
	FD	4	5.29 ± 0.53	10		FD	4	11.01 ± 0.86	16
		5	6.69 ± 0.49	10			5	13.15 ± 0.78	16
		7	6.04 ± 0.43	10			7	11.60 ± 0.99	16
45	Artemia	1	12.42 ± 0.76	10					
		2	11.40 ± 0.77	10					
		9	13.73 ± 0.86	10					
	Copepod	3	5.62 ± 0.57	10					
		6	6.98 ± 0.66	10					
		10	8.35 ± 0.83	10					
	FD	4	6.57 ± 0.20	10					
		5	6.64 ± 0.61	10					
		7	5.76 ± 0.61	10					

Appendix 6. Mean wet weight per tank

Table A3. Mean wet weight in *C. lumpus* 0-51 dph. The averages (mg larva^{-1}) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean WW ± SE (mg larva^{-1})	Total *N*	Dph	Group	Tank	Mean WW ± SE (mg larva^{-1})	Total *N*
2	Yolk sac	Hatching tank	3.24 ± 0.16	16					
6	Artemia	1 2 9	5.55 ± 0.54 5.82 ± 0.28 6.30 ± 0.33	5 5 5	21	Artemia	1 2 9	18.78 ± 0.93 20.04 ± 0.85 19.63 ± 0.31	5 5 5
	Copepod	3 6 10	5.35 ± 0.26 5.33 ± 0.17 5.38 ± 0.22	5 5 5		Copepod	3 6 10	9.65 ± 0.66 9.35 ± 0.23 12.11 ± 1.23	5 5 5
	FD	4 5 7	5.87 ± 0.08 5.75 ± 0.18 5.45 ± 0.18	5 5 5		FD	4 5 7	8.84 ± 0.62 7.17 ± 0.43 9.60 ± 0.70	5 5 5
10	Artemia	1 2 9	8.65 ± 0.17 8.04 ± 0.44 8.37 ± 0.32	5 5 5	24	Artemia	1 2 9	21.86 ± 2.79 19.73 ± 1.27 24.88 ± 1.98	5 5 5
	Copepod	3 6 10	7.21 ± 0.36 6.92 ± 0.21 6.35 ± 0.41	5 5 5		Copepod	3 6 10	10.94 ± 1.36 9.17 ± 0.46 11.04 ± 0.51	5 5 5
	FD	4 5 7	6.70 ± 0.33 6.89 ± 0.43 6.69 ± 0.37	5 5 5		FD	4 5 7	9.72 ± 0.92 6.77 ± 0.76 9.47 ± 0.64	5 5 5
13	Artemia	1 2 9	9.65 ± 0.46 10.45 ± 0.55 11.61 ± 0.47	5 5 5	29	Artemia	1 2 9	29.26 ± 2.59 30.25 ± 3.97 29.07 ± 2.34	5 5 5
	Copepod	3 6 10	7.78 ± 0.18 7.39 ± 0.41 7.30 ± 0.28	5 5 5		Copepod	3 6 10	10.46 ± 2.30 8.54 ± 0.32 13.22 ± 1.84	5 5 5
	FD	4 5 7	7.25 ± 0.32 5.93 ± 0.12 7.27 ± 0.35	5 5 5		FD	4 5 7	15.98 ± 0.95 14.42 ± 2.16 15.49 ±1.17	5 5 5
17	Artemia	1 2 9	13.33 ± 0.78 14.64 ± 0.83 14.98 ± 0.90	5 5 5	34	Artemia	1 2 9	44.00 ± 3.86 35.52 ± 2.72 41.47 ±2.01	5 5 5
	Copepod	3 6 10	8.87 ± 0.97 9.11 ± 0.31 10.91 ± 0.25	5 5 5		Copepod	3 6 10	14.47 ± 1.39 16.74 ± 2.12 26.91 ± 1.10	5 5 5
	FD	4 5 7	7.30 ± 0.42 7.96 ± 0.45 7.39 ± 0.53	5 5 5		FD	4 5 7	21.35 ± 1.51 21.44 ± 1.72 22.00 ± 1.35	5 5 5

Table A3. Mean wet weight in *C. lumpus* 0-51 dph (continued). The averages (mg larva^{-1}) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean WW ± SE (mg larva^{-1})	Total *N*	Dph	Group	Tank	Mean WW ± SE (mg larva^{-1})	Total *N*
38	Artemia	1	50.46 ± 2.94	5	45	Artemia	1	84.50 ± 5.17	10
		2	41.07 ± 4.65	5			2	79.51 ± 5.55	10
		9	43.21 ± 4.54	5			9	91.08 ± 5.39	10
	Copepod	3	25.12 ± 2.85	5		Copepod	3	38.20 ± 3.99	10
		6	26.70 ± 0.47	5			6	46.87 ± 5.06	10
		10	31.20 ± 4.59	5			10	54.83 ± 5.51	10
	FD	4	26.83 ± 2.36	5		FD	4	43.53 ± 1.51	10
		5	31.74 ± 3.98	5			5	42.83 ± 3.80	10
		7	29.47 ± 1.08	5			7	38.75 ± 4.38	10
42	Artemia	1	69.22 ± 6.22	10	50	Artemia	1	90.84 ± 5.85	10
		2	74.04 ± 5.15	10			2	102.70 ± 3.90	10
		9	78.97 ± 5.87	10			9	130.62 ± 10.60	10
	Copepod	3	33.58 ± 3.68	10		Copepod	3	50.97 ± 4.05	10
		6	31.22 ± 3.34	10			6	56.83 ± 5.60	10
		10	38.27 ± 3.55	10			10	82.45 ± 6.77	10
	FD	4	35.22 ± 3.39	10		FD	4	68.38 ± 4.88	10
		5	43.84 ± 3.09	10			5	66.05 ± 4.16	10
		7	38.68 ± 2.80	10			7	58.28 ± 5.14	10

Appendix 7. Daily weight increase per tank

Table A4. Daily weight increase in *C. lumpus* 0-51 dph. The values (% day^{-1}) represent daily percentage increase in dry weight (mg larva^{-1}) for individual tanks during specific time intervals.

Dph interval	Group	Tank	DWI (% day^{-1})	Dph interval	Group	Tank	DWI (% day^{-1})
0-2	Yolk sac	Hatching tank	-1.44				
2-10	Artemia	1 2 9	3.64 3.13 3.43	42-51	Artemia	1 2 9	6.60 6.49 10.02
	Copepod	3 6 10	0.03 0.51 0.14		Copepod	3 6 10	7.81 8.17 10.67
	FD	4 5 7	-0.07 0.15 0.00		FD	4 5 7	8.48 7.80 7.51
10-21	Artemia	1 2 9	6.34 7.55 7.41	0-21	Artemia	1 2 9	4.55 4.97 5.01
	Copepod	3 6 10	1.04 0.89 3.56		Copepod	3 6 10	0.42 0.52 1.76
	FD	4 5 7	1.85 -1.67 2.76		FD	4 5 7	0.80 -0.95 1.30
21-34	Artemia	1 2 9	5.97 3.65 4.98	0-51	Artemia	1 2 9	5.71 5.79 6.60
	Copepod	3 6 10	3.86 5.27 7.43		Copepod	3 6 10	4.38 4.42 5.08
	FD	4 5 7	6.72 10.17 6.14		FD	4 5 7	4.62 4.98 4.72
34-42	Artemia	1 2 9	7.36 10.81 9.69				
	Copepod	3 6 10	12.30 9.41 4.04				
	FD	4 5 7	7.20 9.76 8.56				

Appendix 8. Mean standard length per tank

Table A5. Mean standard length in *C. lumpus* 0-51 dph. The averages (mm) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean SL ± SE (mm)	Total *N*	Dph	Group	Tank	Mean SL ± SE (mm)	Total *N*
2	Yolk sac	Hatching tank	6.4 ± 0.1	16					
6	Artemia	1 2 9	7.0 ± 0.1 7.0 ± 0.1 6.9 ± 0.1	5 5 5	21	Artemia	1 2 9	8.3 ± 0.2 8.5 ± 0.1 8.4 ± 0.0	5 5 5
	Copepod	3 6 10	6.8 ± 0.1 6.6 ± 0.1 3.4 ± 0.0	5 5 4		Copepod	3 6 10	7.5 ± 0.1 7.5 ± 0.1 7.9 ± 0.2	5 5 5
	FD	4 5 7	6.7 ± 0.1 6.7 ± 0.1 6.7 ± 0.1	5 5 5		FD	4 5 7	7.3 ± 0.1 7.0 ± 0.1 7.3 ± 0.1	5 5 5
10	Artemia	1 2 9	7.3 ± 0.0 7.2 ± 0.1 7.2 ± 0.1	5 5 5	24	Artemia	1 2 9	8.8 ± 0.3 8.6 ± 0.2 9.1 ± 0.2	5 5 5
	Copepod	3 6 10	7.2 ± 0.1 7.0 ± 0.1 7.0 ± 0.1	5 5 5		Copepod	3 6 10	7.6 ± 0.3 7.2 ± 0.1 7.7 ± 0.1	5 5 5
	FD	4 5 7	6.9 ± 0.1 7.0 ± 0.1 6.8 ± 0.1	5 5 5		FD	4 5 7	7.4 ± 0.1 6.7 ± 0.1 7.4 ± 0.1	5 5 5
13	Artemia	1 2 9	7.5 ± 0.1 7.6 ± 0.1 7.8 ± 0.1	5 5 5	29	Artemia	1 2 9	9.5 ± 0.3 9.6 ± 0.4 9.4 ± 0.2	5 5 5
	Copepod	3 6 10	7.3 ± 0.1 7.3 ± 0.1 7.3 ± 0.1	5 5 5		Copepod	3 6 10	7.2 ± 0.4 6.9 ± 0.2 7.6 ± 0.2	5 5 5
	FD	4 5 7	7.0 ± 0.1 6.7 ± 0.0 7.2 ± 0.1	5 5 5		FD	4 5 7	7.8 ± 0.2 7.6 ± 0.4 7.8 ± 0.2	5 5 5
17	Artemia	1 2 9	8.1 ± 0.1 8.2 ± 0.1 8.3 ± 0.1	5 5 5	34	Artemia	1 2 9	10.6 ± 0.3 9.8 ± 0.3 10.4 ± 0.1	5 5 5
	Copepod	3 6 10	7.5 ± 0.2 7.4 ± 0.1 7.6 ± 0.1	5 5 5		Copepod	3 6 10	7.6 ± 0.2 7.9 ± 0.3 9.2 ± 0.1	5 5 5
	FD	4 5 7	7.0 ± 0.2 7.3 ± 0.1 7.0 ± 0.1	5 5 5		FD	4 5 7	8.6 ± 0.2 8.5 ± 0.2 8.7 ± 0.2	5 5 5

Table A5. Mean standard length in *C. lumpus* 0-51 dph (continued). The averages (mm) are shown for individual tanks on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Group	Tank	Mean SL ± SE (mm)	Total *N*	Dph	Group	Tank	Mean SL ± SE (mm)	Total *N*
38	Artemia	1	11.0 ± 0.2	5	45	Artemia	1	12.7 ± 0.3	10
		2	10.2 ± 0.5	5			2	12.4 ± 0.3	10
		9	10.5 ± 0.3	5			9	13.0 ± 0.3	10
	Copepod	3	9.0 ± 0.4	5		Copepod	3	10.1 ± 0.3	10
		6	9.3 ± 0.1	5			6	10.6 ± 0.3	10
		10	9.6 ± 0.4	5			10	11.0 ± 0.3	10
	FD	4	9.1 ± 0.3	5		FD	4	10.7 ± 0.1	10
		5	9.7 ± 0.4	5			5	10.6 ± 0.3	10
		7	9.6 ± 0.1	5			7	10.1 ± 0.4	10
42	Artemia	1	12.0 ± 0.3	10	50	Artemia	1	13.0 ± 0.3	10
		2	12.2 ± 0.2	10			2	13.5 ± 0.2	10
		9	12.4 ± 0.3	10			9	14.5 ± 0.3	10
	Copepod	3	9.7 ± 0.4	10		Copepod	3	10.9 ± 0.3	10
		6	9.4 ± 0.3	10			6	11.3 ± 0.4	10
		10	10.2 ± 0.3	10			10	11.3 ± 0.3	10
	FD	4	9.9 ± 0.4	10		FD	4	11.9 ± 0.2	10
		5	10.7 ± 0.2	10			5	12.1 ± 0.2	10
		7	10.3 ± 0.3	10			7	11.4 ± 0.3	10

Appendix 9. Number of larvae per tank

Table A6. Number of *C. lumpus* larvae 0-51 dph. Estimated number of larvae alive per tank on each day of the start feeding experiment. The estimates are based on number of sampled larvae, registered mortality and remaining larvae 51 dph.

Dph	Artemia			Copepod			FD		
	Tank 1	Tank 2	Tank 9	Tank 3	Tank 6	Tank 10	Tank 4	Tank 5	Tank 7
0	7531	6491	5625	6956	7725	5431	6459	6602	6439
1	7531	6491	5625	6956	7725	5431	6459	6602	6439
2	7531	6491	5625	6956	7725	5431	6459	6602	6439
3	7531	6491	5625	6956	7725	5431	6459	6602	6439
4	7508	6480	5621	6944	7722	5430	6450	6580	6431
5	7506	6480	5609	6943	7717	5430	6450	6579	6407
6	7504	6477	5605	6937	7717	5426	6438	6576	6404
7	7502	6476	5604	6937	7717	5425	6435	6574	6402
8	7502	6476	5604	6937	7717	5425	6435	6574	6402
9	7502	6476	5604	6937	7717	5425	6435	6574	6400
10	7502	6476	5603	6937	7717	5425	6435	6574	6400
11	7502	6476	5603	6936	7717	5425	6435	6574	6400
12	7502	6476	5603	6936	7717	5425	6435	6574	6400
13	7502	6476	5603	6936	7717	5425	6435	6574	6400
14	7502	6476	5602	6936	7717	5423	6435	6574	6400
15	7502	6475	5602	6936	7716	5422	6435	6573	6400
16	7501	6474	5602	6936	7716	5422	6435	6573	6399
17	7500	6473	5602	6936	7716	5422	6435	6573	6399
18	7499	6471	5600	6936	7715	5421	6435	6566	6396
19	7499	6471	5600	6936	7715	5421	6431	6562	6393
20	7498	6471	5600	6936	7715	5421	6428	6558	6392
21	7497	6470	5597	6934	7715	5421	6424	6555	6390
22	7495	6467	5597	6932	7713	5419	6423	6539	6375
23	7495	6465	5597	6932	7713	5382	6422	6533	6375
24	7495	6463	5597	6931	7709	5367	6417	6516	6371
25	7495	6462	5594	6928	7709	5349	6412	6512	6371
26	7491	6460	5594	6926	7707	5317	6399	6479	6363
27	7490	6459	5592	6922	7696	5298	6334	6382	6337
28	7486	6455	5590	6919	7683	5256	6257	6150	6307
29	7482	6452	5589	6910	7664	5186	6190	6027	6225
30	7479	6450	5588	6885	7634	5139	6117	5953	6179
31	7474	6447	5567	6816	7532	4923	5917	5874	6065
32	7469	6446	5565	6737	7474	4581	5884	5802	6026
33	7466	6442	5556	6468	7059	3742	5848	5707	5920
34	7464	6441	5554	6433	7034	3627	5821	5648	5909
35	7462	6438	5552	6317	6970	3553	5795	5604	5886
36	7461	6437	5547	6223	6948	3526	5786	5583	5878
37	7460	6435	5545	6186	6931	3509	5776	5567	5876
38	7457	6434	5537	6170	6905	3496	5763	5542	5871
39	7451	6431	5528	6149	6891	3488	5749	5523	5867
40	7448	6428	5523	6134	6871	3481	5745	5513	5862
41	7445	6422	5519	6114	6866	3478	5725	5498	5859
42	7433	6421	5509	6090	6853	3477	5709	5484	5852
43	7421	6407	5491	6064	6847	3474	5699	5467	5844
44	7417	6404	5484	6052	6841	3470	5691	5460	5835
45	7417	6394	5483	6033	6831	3467	5678	5452	5834
46	7415	6384	5472	6018	6824	3463	5673	5445	5828
47	7415	6384	5472	5991	6822	3460	5552	5442	5827
48	7414	6383	5470	5963	6813	3458	5004	5433	5818
49	7376	6279	5449	5869	6796	3453	4924	5421	5813
50	7373	6279	5449	5865	6784	3453	4907	5407	5808
51	7373	6279	5449	5865	6784	3453	4907	5407	5808

Appendix 10. Histological analyses: Liver, stomach and gut

Table A7. Mean hepatocyte nucleus and cell area size in *C. lumpus* 0-50 dph. Average hepatocyte nucleus area size (µm²) and estimated hepatocyte cell area size (µm²) is shown per treatment on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Treatment	Mean area size ± SE (µm²)		Total *N*
		Nucleus	Cell	
0	Yolk sac	35.1 ± 1.5	558.0 ± 8.0	5
10	Artemia	39.5 ± 0.7	759.3 ± 22.0	5
	Copepod	34.1 ± 1.1	569.4 ± 16.7	5
	FD	30.8 ± 0.8	473.0 ± 8.5	5
21	Artemia	39.9 ± 1.2	868.8 ± 23.8	5
	Copepod	31.9 ± 0.9	527.2 ± 6.3	5
	FD	37.0 ± 2.0	837.5 ± 49.5	5
34	Artemia	46.7 ± 1.0	799.7 ± 13.4	5
	Copepod	41.0 ± 1.2	693.1 ± 12.4	5
	FD	43.4 ± 1.4	765.2 ± 20.0	5
42	Artemia	46.6 ± 2.7	1167.3 ± 233.5	5
	Copepod	45.3 ± 2.5	951.5 ± 32.7	5
	FD	40.8 ± 1.2	822.2 ± 24.8	5
50	Artemia	41.6 ± 1.0	1090.4 ± 31.3	5
	Copepod	40.2 ± 1.0	846.5 ± 26.2	5
	FD	41.5 ± 0.7	999.9 ± 8.3	5

Table A8. Mean stomach epithelium height in *C. lumpus* 0-50 dph. Average stomach epithelium height (µm) is shown per treatment on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*). Abbreviations: SEH = stomach epithelium height.

Dph	Treatment	Mean SEH ± SE (µm)	Total *N*	Dph	Treatment	Mean SEH ± SE (µm)	Total *N*
0	Yolk sac	-	5	34	Artemia	21.3 ± 1.0	5
					Copepod	19.0 ± 0.6	5
					FD	19.2 ± 0.2	5
10	Artemia	19.1 ± 0.2	5	42	Artemia	28.5 ± 0.8	5
	Copepod	18.8 ± 0.2	5		Copepod	25.8 ± 0.5	5
	FD	19.5 ± 0.3	5		FD	22.3 ± 1.0	5
21	Artemia	20.5 ± 0.7	5	50	Artemia	32.1 ± 0.4	5
	Copepod	17.2 ± 0.8	5		Copepod	25.2 ± 1.5	5
	FD	19.0 ± 1.2	5		FD	27.9 ± 0.6	5

Table A9. Mean gut epithelium, microvillus and villus height in *C. lumpus* 0-50 dph. Average gut epithelium height (µm), microvillus height (µm) and villus height (µm) is shown for the midgut (*S/Mg*) and hindgut (*Hg*) sections per treatment on each sampling day, together with respective standard errors (SE) and total number of sampled larvae (Total *N*). Abbreviations: GEH = gut epithelium height, MVH = microvillus height, VH = villus height.

Dph	Treatment	Mean GEH ± SE (µm)		Mean MVH ± SE (µm)		Mean VH ± SE (µm)		Total *N*
		S/Mg	*Hg*	*S/Mg*	*Hg*	*S/Mg*	*Hg*	
0	Yolk sac	29.9 ± 1.3	25.2 ± 0.7	1.5 ± 0.1	1.8 ± 0.1	81 ± 5	71 ± 1	5
10	Artemia	40.8 ± 0.6	35.3 ± 1.1	2.5 ± 0.1	2.5 ± 0.0	104 ± 2	93 ± 3	5
	Copepod	37.3 ± 0.7	33.8 ± 1.5	2.5 ± 0.1	2.4 ± 0.0	94 ± 2	100 ± 1	5
	FD	32.7 ± 0.4	31.4 ± 0.8	2.1 ± 0.1	2.4 ± 0.1	94 ± 5	87 ± 1	5
21	Artemia	43.7 ± 0.8	41.0 ± 0.8	3.2 ± 0.2	3.1 ± 0.1	128 ± 4	119 ± 3	5
	Copepod	32.8 ± 0.8	32.2 ± 1.3	3.2 ± 0.1	2.8 ± 0.1	99 ± 8	83 ± 10	5
	FD	29.3 ± 3.1	35.0 ± 0.9	3.2 ± 0.1	2.7 ± 0.1	82 ± 11	113 ± 6	5
34	Artemia	42.2 ± 0.6	42.2 ± 1.2	4.3 ± 0.1	3.7 ± 0.2	14 ± 0 8	146 ± 11	5
	Copepod	39.3 ± 0.9	41.0 ± 2.2	4.1 ± 0.1	3.4 ± 0.3	114 ± 6	124 ± 9	5
	FD	42.2 ± 0.7	42.3 ± 0.6	3.9 ± 0.2	3.3 ± 0.3	130 ± 4	144 ± 5	5
42	Artemia	44.4 ± 0.6	42.8 ± 1.4	4.9 ± 0.2	3.8 ± 0.1	171 ± 11	153 ± 7	5
	Copepod	44.3 ± 0.7	39.5 ± 1.7	5.2 ± 0.3	3.5 ± 0.1	164 ± 3	154 ± 2	5
	FD	42.4 ± 0.4	37.3 ± 1.3	4.4 ± 0.2	3.7 ± 0.2	163 ± 11	146 ± 8	5
50	Artemia	46.0 ± 1.6	43.6 ± 0.4	5.3 ± 0.1	3.8 ± 0.2	211 ± 16	176 ± 5	5
	Copepod	42.0 ± 0.8	39.5 ± 1.0	4.9 ± 0.1	3.3 ±0.1	192 ± 18	149 ± 17	5
	FD	45.8 ± 0.5	41.3 ± 0.9	4.7 ± 0.2	3.5 ± 0.1	197 ± 7	149 ± 3	5