Master's thesis

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

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Effects of phospholipids and bile salts on the growth, survival and nutritional status in the liver of Atlantic cod larvae and juveniles

Master's thesis in Ocean Resources
Supervisor: Kjell Inge Reitan
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Abstract

Atlantic cod is a species of interest to diversify Norwegian aquaculture. Despite the growth of commercial cod aquaculture, the production of juveniles remains a challenge. Early stages nutritional requirements, particularly the role of phospholipids, remain poorly understood. Phospholipids are vital in cell membrane structure and several metabolic functions, including lipid digestion. The liver is a central metabolic organ essential for a proper lipid digestion and bile production, including bile salts that aid in lipid emulsification. Although bile salts supplementation has demonstrated growth, survival and digestibility benefits, studies also show contradictory results. The impact of bile salts supplementation in diets for fish larvae was never studied.

This thesis aimed to investigate the effect of diets with two different phospholipid levels (approximately. 38 (HPL) and 32 % (LPL) of the total lipid fraction) on the growth performance, survival and nutritional status, by analysing nutritional biomarkers in the liver, in Atlantic cod larvae and juveniles. In addition, it was tested how the supplementation of those diets with bile salts (0.04 % sodium taurocholate (BS)) influences these parameters. A total of four groups were compared: HPL, HPL-BS, LPL and LPL-BS.

At the end of the trial, the larval groups did not present any significant differences in growth, survival, or liver histomorphology. However, during the trial, there were some significant differences with the HPL group demonstrating a larger dry weight than the LPL-BS and LPL groups on 38 and 45 dph, respectively. The same group kept a non-significant higher growth throughout the remaining of the experiment. Non-significant differences were found in survival, although HPL and BS both seemed to have some positive effect. The biomarkers in the liver showed a good nutritional status, especially noticeable in the HPL group. However, the LPL-BS group exhibited a poorer nutritional status at the end of the trial, with reduced, but not degraded, nucleus size. All groups had low vacuolization (glycogen or lipids) during the experiment until day 45 post hatch, but significant 35-fold increase was observed by the end of the trial.

In conclusion, this study demonstrated that a higher phospholipid amount in the diet is required for a better performance of Atlantic cod larvae and juveniles. However, to optimize the cod aquaculture industry there is a need for further research to fully understand phospholipid requirements and the possible effects of bile supplementation on early life stages.

Sammendrag

Atlantisk torsk er en art av interesse for å diversifisere norsk havbruk. Til tross for veksten i kommersielt torskeoppdrett, er produksjonen av settefisk fortsatt en utfordring. Ernæringsbehov i tidlige stadier, spesielt rollen til fosfolipider, er det fortsatt lite kunnskap om. Fosfolipider er avgjørende i cellemembranstrukturen og flere metabolske funksjoner, inkludert lipidfordøyelse. Leveren er et sentralt metabolsk organ som er essensielt for en lipidfordøyelse og galleproduksjon, inkludert gallesalter som hjelper til med lipidemulgering. Selv om tilskudd av gallesalt har vist fordeler ved vekst, overlevelse og fordøyelighet, viser studier også motstridende resultater. Effekten av tilskudd av gallesalt i dietter for fiskelarver har aldri blitt studert tidligere.

Denne oppgaven tok sikte på å undersøke effekten av dietter med to ulike fosfolipidnivåer (ca. 38 % (HPL) og 32 % (LPL) av den totale lipidfraksjonen) på vekst, overlevelse og ernæringsstatus, ved å analysere ernæringsbiomarkører i leveren, i torskelarver og -yngel. I tillegg ble det testet hvordan tilskudd av disse diettene med gallesalter (0,04 % natriumtaurokolat (BS)) påvirker disse parameterne. Totalt fire grupper ble sammenlignet: HPL, HPL-BS, LPL og LPL-BS.

Ved slutten av forsøket viste larvegruppene ingen signifikante forskjeller i vekst, overlevelse eller leverhistomorfologi. I løpet av forsøket var det imidlertid noen signifikante forskjeller i vekst, hvor HPL-gruppen hadde en større tørrvekt enn LPL-BS- og LPL-gruppene på henholdsvis dag 38 og 45 etter klekking. Den samme gruppen holdt en ikke-signifikant høyere vekst gjennom resten av forsøket. Ingen signifikante forskjeller i overlevelse ble observert, selv om både HPL og BS så ut til å ha en viss positiv effekt. Biomarkørene i leveren viste god ernæringsstatus, spesielt i HPL-gruppen. Imidlertid viste LPL-BS-gruppen en dårligere ernæringsstatus ved slutten av forsøket, med redusert, men ikke degradert, kjernestørrelse. Alle gruppene hadde lav vakuolisering (glykogen eller lipider) under forsøket til dag 45 etter klekking, men som hadde økt med en faktor på 35 ved slutten av forsøket.

Avslutningsvis viste denne studien at en høyere fosfolipidmengde i dietten er nødvendig for en bedre ytelse hos torskelarver og -yngel. For å optimalisere torskeoppdrettsnæringen er det imidlertid et behov for ytterligere forskning for å fullt ut forstå fosfolipidkrav og hvilken effekt gallesupplement kan ha på tidlige livsstadier.

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After a bachelor and two master's degree, this thesis marks the beginning of a career transition. Finally, I would like to thank for the support of my family and friends through these years. Especially my partner, João, and my cat, Knerten, for the love, support, companion and for making my time at home always special.

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Bruno Nunes

Contents

1. Introduction

Atlantic cod (*Gadus morhua*) is a fish of the *Gadidae* family which inhabits the North Atlantic Ocean. Established populations are found on both sides of the ocean in temperatures between 0 and 11°C. This species has an observed longevity of over 20 years, being able to exceed 55 kg and 1.8 meters in length (Skreslet, 2017; Marteinsdóttir and Rose, 2018). Juvenile and adult Atlantic cod are distinguished by the presence of three dorsal fins, two pelvic and anal fins. The presence of a barbell positioned at the lower jaw is also a feature characteristic of Atlantic cod and some of the species belonging to the *Gadidae* family (Figure 1).

Figure 1 – Diagram of general appearance diagram adult Atlantic cod. Cod external morphology is characterised by three dorsal fins, two anal and two pelvic fins. A barbel is present on the lower jaw.

1.1. Advances in cod farming

In the late 1800s, a significant number of Atlantic cod larvae was successfully cultivated in the Southern part of Norway in extensive systems, fed natural zooplankton (Rognerud, 1889). A century later, new trials were made using the same methods, for restocking of wild populations (Kvenseth and Øiestad, 1984; Øiestad et al., 1985). However, these extensive systems are dependent on environmental conditions. In 1984, Howell showed the intensive production of Atlantic cod juveniles was possible using cultivated live feed, i.e. rotifer and *Artemia*, and in 1993 Rosenlund further upscaled this Atlantic cod farming using the intensive method.

The extensively produced cod had far better quality than cod produced intensively. Those larvae were fed natural zooplankton, and it is known that the nutritional quality of these organisms is superior to other live feed organisms used to feed marine fish larvae (Watanabe et al., 1983; Lubzens et al., 1985; Watanabe and Ali, 1987; Evjemo and Olsen, 1997; Conceição et al., 2010; Hamre et al., 2013). Even though a great number of larvae attained metamorphosis, large mortality was observed due cannibalism associated with poor rearing conditions and suboptimal feeding protocols (Folkvord, 1991; Svåsand et al., 2004;

Puvanendran et al., 2008). Grading of juveniles can reduce cannibalism, but it can weaken the fish which could induce higher mortality (Sadoul and Vijayan, 2016). Nowadays, hatcheries are using higher flow rates and increased feeding frequencies. This will reduce interactions between large and small fish, specifically reducing cannibalism opportunities, as well as keeping the gut full (Folkvord, 1991; van der Meeren et al., 2011). Survival has also been correlated with egg quality between different batches (Petersen et al., 2016).

Atlantic cod could be a species to complement an aquaculture industry dominated by salmon farming (Rosenlund and Halldórsson, 2007), which could also be farmed in low temperature water conditions, i.e. Northernmost parts of Norway, as it produces antifreeze proteins (Nardi et al., 2021). However, in addition to the mortality, a bottleneck on intensive juvenile production made cod farming challenging, the economic crisis in the 2000 and 2010's led to lack of investment, and the increase in quotas prompted the decline in cod aquaculture (Puvanendran et al., 2022).

During larval stages, marine fish larvae, such as Atlantic cod, need to have a feeding that supports their fast growth rates and is easily digestible. So far, produced live feed is the common way to feed fish larvae at initial stages. Rotifers and *Artemia* are commonly used, but the nutritional composition of these organisms is deficient for most of marine fish larvae. These organisms especially lack essential fatty acids (EFA) which are essential for normal growth and development, and must be enriched with lipids, before feeding to the larvae (Watanabe et al., 1983; Watanabe and Ali, 1987; Hamre et al., 2013).

1.2. Atlantic cod nutrition and physiology

1.2.1. Larval nutrition

The production of fish larvae and high quality juveniles has been a bottleneck in the development of the marine aquaculture industry (Olsen et al., 2004; Kjørsvik et al., 2004). Fish larval stages have been thought to be rather primitive organisms (Webb and Rønnestad, 2011). However, larval growth and ontogeny is a rather complex process (Kjørsvik et al., 2004; Yúfera et al., 2011). The larval stage can be considered a transitional age in which substantial changes in structure, physiology, size and body shape occur (Zambonino-Infante et al., 2008). Therefore, a high energy supply and a proper nutrient composition is needed to support routine maintenance metabolism, swimming, and growth in order to enhance survival, growth performance, and transformation into a juvenile (Lazo et al., 2011).

Several studies have demonstrated that marine fish larvae fed with natural zooplankton results in better quality juveniles than larvae fed with cultivated rotifers and *Artemia*. Dietary

lipid composition has been suggested as the determining factor for this difference between natural zooplankton and cultivated live feed (Evjemo et al., 2003; Imsland et al., 2006; Olsen et al., 2014; Karlsen et al., 2015). Lipids are the most investigated nutrient in the fish nutrition field, but it is also the nutrient with less knowledge on, simply because lipid chemistry is complex and difficult to understand (Sargent et al., 2003; NRC, 2011).

Lipids can be divided by the presence or absence of FA or of the alcohol glycerol in their basic structure or according to their polarity: the so called polar and neutral, or non-polar (NL), lipids. In fish, the most important FA are the docosahexaenoic acid (DHA, 22:6*n*-3), eicosapentaenoic acid (EPA, 20:5*n*-3), arachidonic acid (ARA, 20:4*n*-6), dihomo-*γ*-linolenic acid (20:3*n*-6) and eicosatrienoic acid (20:3*n*-9) (Higgs and Dong, 2000; NRC, 2011). Polar lipids are composed mainly by phospholipids (PL) such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), and also plasmologens, sphingomyelin, cerebrosides and gangliosides, which all are essential components of membranes at a structural level (Higgs and Dong, 2000; Sargent et al., 2003; NRC, 2011). PL have hydrophobic and hydrophilic ends, which together with bile salts, will help in the emulsification of lipids in the intestine (Steinberg, 2018). Neutral lipids include triacylglycerols (TAG), wax esters, alkyl diacylglycerols and sterols esters, such as cholesterol, which are source of energy and lipid storage in the cells (Higgs and Dong, 2000; Olsen et al., 2004).

Overall, lipid requirements include a not so specific gross energy but more specific requirements for lipids classes such as cholesterol, PL, and EFA. For example, when EFA are incorporate in the PL fraction are better utilized for growth and development than when incorporated in the neutral fraction. It positively affects larval growth, survival, histological organization and maturation of the liver and intestine, digestive enzyme activities and dry feed ingestion (Izquierdo et al., 2001; Gisbert et al., 2005; Wold et al., 2007; Wold et al., 2009), and skeletal development (Kjørsvik et al., 2009). And, in fact, marine cold-water animals are known to have PL containing a high fraction of polyunsaturated FA (PUFA), which can only be supplied by the food (Olsen et al., 2004).

Usually, fish oil is incorporated into fish feed to meet the requirements for PUFA. However, fish oils are rich in TAG, which can lead to fat assimilation problems (Stoss et al., 2004). Larvae will accumulate lipids droplets in the enterocytes and liver, meaning that lipids are absorbed in the intestine while the transport into the blood is not efficient (Boulhic and Gabaudan, 1992; Bisbal and Bengtson, 1995; Sarasquete et al., 1995; Stoss et al., 2004). Fontagné et al. (1998) suggested that the addition of PL into the feed can help to reduce the

problem. This was further proved in gilthead seabream (Izquierdo et al., 2001; Gisbert et al., 2005) and in cod (Wold et al., 2009). Izquierdo et al. (2001) also found that PL rich diets promoted the consumption of feed by the larvae. Nevertheless, the way FA binds to the PL appears to have varying effects on larval performance. Marine PL sources contain higher levels of PUFA compared to vegetable PL sources, potentially leading to different performance outcomes when comparing these two sources (Kanazawa et al., 1983; Tocher et al., 2008; Wold et al., 2009; Betancor et al., 2012; Hamre et al., 2013).

Commercial dry feed for fish larvae has a highly variable lipid fraction, which may occur because determining the optimal level for a given species at early developmental stages is extremely difficult (Pinto et al., 2022). Regarding PL requirements in marine fish larvae, it seems to vary between 5 and 12% (Tocher et al., 2008). In Atlantic cod larvae, Wold et al. (2009) found a better performance with diets containg 12.5 % of PL than 107%. But Hansen et al. (2018) found that increasing PL levels from 5 to 8 % did not improved the overall performance. The latter also hypothesised that either an earlier introduction of the dry feed would have been beneficial or that the lower PL level composition was already enough to fulfil the nutritional requirements of the larvae. However, PL quantitative requirements during early life stages of Atlantic cod is still to be found.

In general, PL requirements are expected to be larger in fish larvae than in juveniles due to their fast growth rates (Reitan et al., 1993; Otterlei et al., 1999; Wold et al., 2007; Øie et al., 2017) and incapability to synthetise PL *de novo* (Coutteau et al., 1997). However, Li et al. (2015) found that the gene expression for PL synthesis in cod larvae remained relatively consistent during development, but with a limitation on PC synthesis. PC is the most common PL class in fish lipoproteins and in the bile (Moschetta et al., 2005; Tocher et al., 2008). Biliary PLs are re-esterified in the intestine and transported in the blood as lipoproteins, contributing to lipoprotein synthesis and lipid transport (Tocher et al., 2008). Nonetheless, metabolic constraints in early life stages should be considered, as the immature liver in cod larvae may potentially limit the supply of biliary PC to the intestine (Li et al., 2015). Therefore, when studying PL requirements in developing cod, a nutritional evaluation of the liver should be taken into account as this organ is sensitive to nutritional imbalances (Hoehne-Reitan and Kjørsvik, 2004).

1.2.2. Liver development

Contrary to other marine fish larvae at hatching, such as gilthead seabream, in which the liver is a small undifferentiated rounded cell mass (Yúfera et al., 2011), Atlantic cod have a differentiated liver, located behind the heart, in the abdominal cavity under the foregut

(Pedersen and Falk-Petersen, 1992; Morrison, 1993; Kamisaka and Rønnestad, 2011). As the larva grows, the volume of the liver increases (Wold et al., 2009) (Figure 2). In adult Atlantic cod, the liver can make up to 25% of the weight of the fish (Rust, 2003). The liver is a central metabolic organ, with important functions in digestion and physiology, similarly to mammals, such as absorption of nutrients, production of bile, detoxification, and maintenance of the body metabolic homeostasis. The liver also participates in the production of blood plasma proteins, such as albumin, fibrinogen, and complement factors (Hoehne-Reitan and Kjørsvik, 2004; Genten et al., 2009; Kjørsvik et al., 2011).

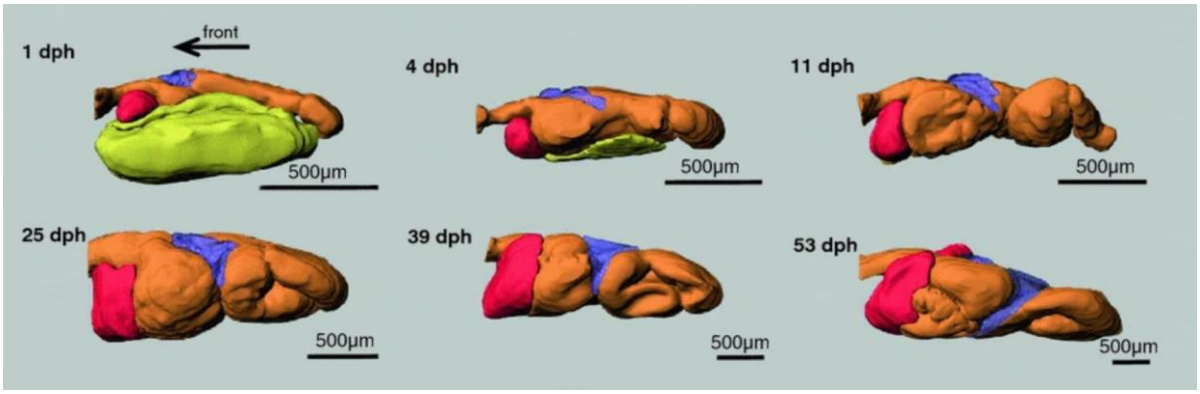

Figure 2 - Ontogeny of the digestive organs in Atlantic cod larvae in 3D models. The arrow shows the direction of the front (mouth). Orange outer layer of digestive tract, red shows the liver, green the gallbladder, purple the pancreas, pink the islet of Langerhans, and yellow the yolk-sac. Modified from Kamisaka and Rønnestad (2011).

Generally, the liver parenchyma is covered by a thin capsule of fibroconnective tissue and a serosa (Genten et al., 2009; Wilczyńska and Wołczuk, 2019). The hepatocytes are arranged cylindrically around a bile duct, with their bases turned to the sinusoids (Figure 3) (Wilczyńska and Wołczuk, 2019). After the start of exogenous feeding, and as the larva grows, it is observed an increase of blood vessels, tubules, sinusoids and bile canaliculi, and vacuolization is observed in the liver tissue (Kryvi and Poppe, 2016). Sinusoids, supported by the arterial and venous system, transport nutrients and oxygen rich blood into the hepatocyte cells (Young et al., 2006).

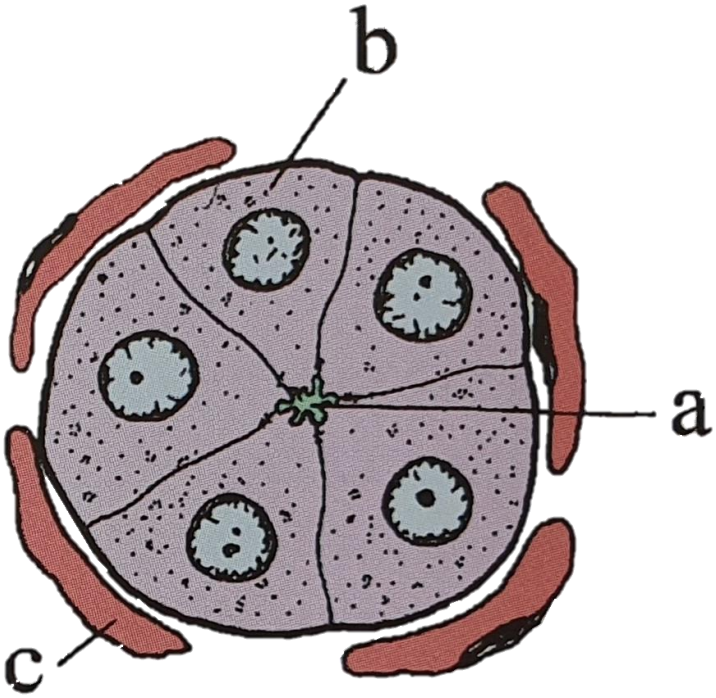

Figure 3 – Illustration of the live hepatocyte organization. A: bile duct; b: hepatocyte; c: sinusoid. Modified from Kryvi and Poppe (2016)

Bile is synthesised in the liver parenchymal tissue and consists of a mixture of bile salts (BS), PL, cholesterol and bile pigments and stored in the gallbladder (Moschetta et al., 2005). BS are the end-product derived from cholesterol, which is a simple lipid that do not contain FA. BS facilitate intestinal absorption of lipids, enhance proteolytic cleavage of dietary proteins and have an antimicrobial activity in the intestine (Hofmann and Hagey, 2008; Hagey et al., 2010) .When lipids are detected in the intestine, cholecystokinin (CCK) is secreted, which signals the gallbladder to release bile to the intestine (Aldman and Holmgren, 1987; Le et al., 2019).

In the intestine, bile acts as an emulsifier for lipids and lipid-soluble vitamins to be absorbed and transported, by reabsorption of the BS through the enterohepatic circulation (EHC) to the liver (Figure 4). Bile results in a pool of BS that circulates between the intestine and the liver, with a minimal amount being produced in the liver. As BS are not completely recycled, close to 5% is lost in the faeces (Hofmann et al., 2010; Romano et al., 2020; Wang et al., 2023).

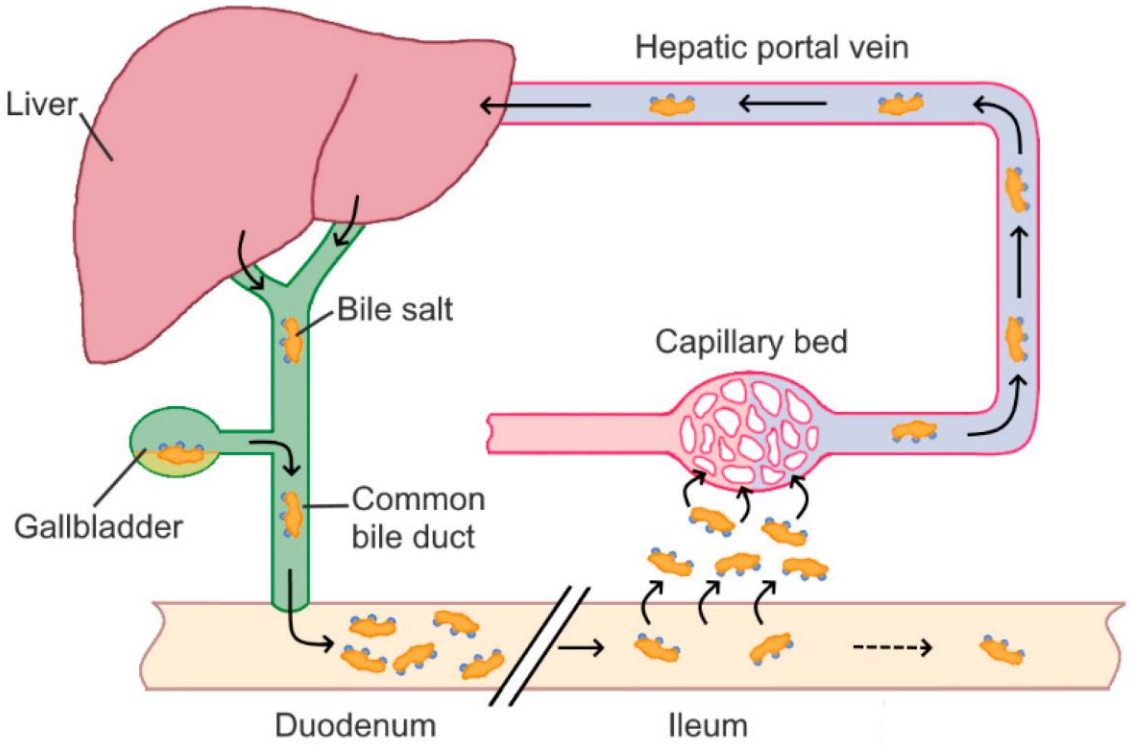

Figure 4 - Simplified lipid digestion, representing the enterohepatic circulation. In this process, the bile salts released into the intestine are reabsorb by intestinal lumen and transported back to the liver via the hepatic portal vein. Source: Naumann et al. (2020)

There is evidence that an interaction between BS and PL in the liver is required so that PL are released into the bile fluid, namely PC (Coleman and Rahman, 1992; Elferink et al., 1997; Wüstner et al., 2000). In theory, this means that the presence of PL and supplementation with BS in a diet could allow EHC of these dietary ingredients directly to the liver and subsequently to be secreted into the bile, sparing energy in metabolizing bile acids as well. Zhou et al. (2018) found that bile acid (BA) supplementation in grass carp juveniles (*Ctenopharyngodon idella*) reduced *de novo* synthesis of cholesterol, allowing cholesterol to be spared for lipid transport. Gu et al. (2017) confirmed this by supplementing juvenile turbot diets with sodium taurocholate. Ruiz et al. (2023) found enhanced growth performance and reduced the levels of perivisceral, hepatic and intestinal fat in juvenile gilthead seabream when supplementing 0.06% BS in a diet. The latter also found that 0.12% BS supplementation enhanced lipid digestibility and increased activity of BS-activated lipase in the anterior intestine. In addition, BS supplementation may aid digestion of feeds containing high starch and lipid levels, and ingredients of vegetable origin (Yamamoto et al., 2007b; Guo et al., 2020; Xu et al., 2022).

The addition of BS in feeds has been tested in some species to evaluate mainly fish growth performance and survival. However, these parameters may also depend on the feed

formulation, species and the type and amount of BS added (Yamamoto et al., 2007a; Yamamoto et al., 2007b; Wang et al., 2023). For example, Ding et al. (2022) found that the use of 0.03% sodium taurocholate, a BS, per kg feed in Nile Tilapia juveniles (*Oreochromis niloticus*) did not influence the growth performance while 0.06% mg sodium taurocholate per kg feed translated into liver damage and cholesterol accumulation. In turbot juveniles, the use of 0.09% (dry matter basis) of a BS improved growth performance, survival and decreased lipid deposition, but increasing to 0.12% BS supplementation did not bring any further improvement (Huang et al., 2015). And in juvenile leopard coral grouper (*Plectropomus leopardus*), 0.3% of BS supplementation in the feed gave the best growth performance. Concentrations above gave a poorer growth (Gao et al., 2023). To our best knowledge, supplementation of dry feed with BS in fish larvae was never done. However, the youngest stage where BS supplementation was tested was in 0.5 g European seabass (*Dicentrarchus labrax*) juveniles. Sallam et al. (2017) found that 0.04 % of the BS sodium taurocholate supplementation in a diet, improved the overall performance.

1.2.3. Nutritional evaluation of the liver

The most accurate indicator of nutritional status in early life stages such as in fish larvae is a histological assessment (Ferron and Leggett, 1994; Gisbert et al., 2008). The small size of the larvae makes it difficult to chemically analyse the nutrients content in an individual larva and organs. Although histological observations can be made in several tissues, the most commonly used for larval physiology, nutritional condition and early development are the liver, exocrine pancreas, intestine, muscular and cartilaginous tissue (Kjørsvik et al., 2011; Gisbert et al., 2008). This thesis will focus on the histological changes in the liver of Atlantic cod during development from larval to juvenile stage.

The liver is a sensitive organ that respond quickly to nutritional disorders (Gisbert et al., 2008), and nutritionally unbalanced feeding or feed deprivation will lead to histomorphological changes in the organization of the liver. The morphological development together with the onset of bile synthesis and secretion, are key factors to determine functionality in the larval liver (Hoehne-Reitan and Kjørsvik, 2004; Gisbert et al., 2008).

The nutrient storage function in the liver, i.e. glycogen deposition, is not observed in Atlantic cod during the lecithotrophic stage but increases after yolk-sac absorption. After metamorphosis, both glycogen and lipids are stored in the hepatocytes. (Hoehne-Reitan and Kjørsvik, 2004). Fish larvae with low energy storage present large nuclei centrally located in the hepatocytes, while larvae with high energy storage present hepatocytes with peripherally

located nuclei (Gisbert et al., 2008). These energy stores can quickly respond to nutritional changes.

If food intake is reduced or during long starvation periods, lipids and glycogen in the liver hepatocytes are the first to be mobilized. In this case, histological section of the liver shows hepatocytes with more densely compacted cellular materials (Green and McCornick, 1999). Other cellular components also respond quickly to dietary change, such as: decrease of nuclear volume, swollen mitochondria, dilated sinusoids, large intercellular spaces, vascularization, increase in lysosomes, cytoplasmatic necrosis and hypertrophy of the bile canaliculi and the gall bladder (Gisbert et al., 2008).

The liver histopathological changes can be assessed through quantitative and semiquantitative methods. Quantitative methods, or morphometric parameters, are the most used when assessing changes in the liver tissue, i.e. hepatocytes number, hepatocytes surface area, hepatocytes nuclear area, and glycogen and lipids storage in the hepatocytes. For example, changes in the size of the hepatocyte nuclear area are translated into changes in liver metabolism. Large nucleus have an increased surface area of contact, allowing increased exchanges between nucleus and cytoplasm indicating a high metabolic activity (Ghadially, 1997). Stereological methods, also a quantitative method, measure the volume of liver constituents, such as hepatocyte nuclear volume and hepatocyte cell volume (Wold et al., 2009; Raskovic et al., 2011). Semiquantitative methods classify the organs in a degree of alteration such as pyknotic nuclei, and small and indistinct cell (See Gisbert et al. (2008) for more detailed information)

1.3. Aims, hypothesis and research question

The aims of this study were to test the effects of dietary phospholipid levels (38 and 32 % of the total lipid fraction) and bile salt supplementation (0.04% sodium taurocholate) on (1) the growth and survival of Atlantic cod larvae and juveniles, and on (2) the histomorphological development and nutritional status of the liver. Specifically, dry weight (DW) and standard length (SL) will be used to analyse growth performance, while hepatocyte cell area size, hepatocyte nucleus area size, and vacuoles fraction will be used to analyse the development and nutritional status in the liver.

To achieve the aims, Atlantic cod larvae were fed four different microencapsulated diets from 17 dph to 61 dph. The phospholipid levels were chosen in consultation with the feed manufacturer, assuming isolipidic diets. Sodium taurocholate is the most common bile salt

found in gadiformes (Goto et al., 1996; Hagey et al., 2010), and the supplementation level was chosen based on the results obtained by Sallam et al. (2017).

The following hypothesis was formulated:

Hypothesis: Atlantic cod larvae and juveniles are expected to have a better growth performance and higher survival when fed a diet containing a higher phospholipid level. The fish fed diets containing higher phospholipids are expected to present a bigger nucleus size and smaller accumulation of vacuoles in the liver hepatocytes.

As for the bile salt supplementation, the results are conflicting. Therefore the following research question was formulated:

Research question: How is the growth, survival and nutritional status in the liver of Atlantic cod larvae affected when the same diets are supplemented with sodium taurocholate?

This project is part of the PhD project "Phospholipid requirements in relation to functional liver development and dietary factors in Atlantic cod (*Gadus morhua* L.) larvae and juveniles" at the Department of Biology at NTNU in which the overall aim is to obtain a better understanding dietary phospholipids requirements during early life stages of Atlantic cod. Specifically, the project intends to (1) quantify dietary phospholipid requirements in cod juveniles and (2) investigate how the production and secretion of bile components is related to functional development of the liver and dietary phospholipid content and bile salts in cod larvae.

2. Materials and methods

2.1. Eggs and larval rearing

Atlantic cod eggs (65 d°) were shipped from Havbruksstasjonen i Tromsø AS (Tromsø, Norway) to NTNU Centre of Fisheries and Aquaculture – Sealab (Trondheim, Norway). At arrival, the eggs were disinfected with glutaraldehyde (Carl Roth GmbH + Co. KG, Germany) in seawater (34 % salinity) [400 mg L^{-1}] for 6 minutes at 8°C, following Salvesen et al. (1997). After rinsed in seawater, the eggs were equally distributed between 12 cylindrical flat-bottomed tanks holding 200 L of seawater with a density of approximately 145 eggs L^{-1}.

The eggs were kept at ca. 6.5 °C with a flow of 1.10 L min^{-1} and strong aeration to keep the eggs under constant suspension in complete darkness. A small number of eggs were kept in beakers for assessment of hatching success and embryological development. As the hatching rate approached 50 %, at 104 d°, the aeration and water flow were reduced to avoid mechanical damage on the newly hatched larvae. The temperature was increased by 0.5 °C every day until a maximum of 12.0 °C was reached at 12 dph. The flow was adjusted from 0.14 L min^{-1} to a maximum of 1.81 L min^{-1}. For a detailed description of temperature and flow adjustment see appendix 2.

The tanks were equipped with a central outlet pipe at the bottom. The outlet pipe was covered with a sieve, going through the total water height in the tank, to allow the live prey to be flushed out. The sieve mesh size was changed during the rearing period and ranged from 300 µm at the beginning to 1000 µm at the end of the experiment. The mesh size was changed accordingly to the size of the larvae, water quality and prey size. The sieves were removed and washed once a week, increasing to twice a week in the last two weeks of the experiment.

The seawater used in the tanks was pumped from 70 meters depth in Trondheimsfjorden, filtered and microbially matured, following Skjermo and Vadstein (1999). The water was continuously degassed to prevent supersaturation of dissolved gases before entering the fish tanks.

From the onset of exogenous feeding cod larvae were fed with microalgae and rotifers. The microalgae *Tisochrysis lutea* was continuously added to the tanks with a peristaltic pump (Kronos 50, SEKO S.p.A., Italy) keeping a concentration of 1 mg C L^{-1} (Reitan et al., 2021). Overhead LED light tubes (FlexTube SC 4000 K of 75 W, Vanpee AS, Norway) were turned on and set to a photoperiod of 24h/0h (light/darkness) with a measured surface energy of 5.4 µE m^{-2} s^{-1}. At the same time, rotifers *Brachionus spp*. were added to the tanks with a peristaltic

pump (505U/520Sa; 313X 3 Roller Extension Pumphead, Watson-Marlow Fluid Technology Solutions, United Kingdom). Rotifers feeding was done four times per day, with 6 hours between each feeding. Rotifers and microalgae were added until the larvae reached 18 dph. *Artemia* AF strain (Inve Aquaculture, Belgium) was added in the tanks from 15 to 25 dph, together with the rotifers. At 21 dph, *Artemia* EG strain (Inve Aquaculture, Belgium) was also added to the tanks until 34 dph. *Artemia* strains were fed four times per day, using the same pump as the one that fed rotifers. Each tank was equipped with 2 feeding reservoirs were the live feed and microalgae were stored prior to feeding (Figure 5). The live feed protocol is shown in Table 1.

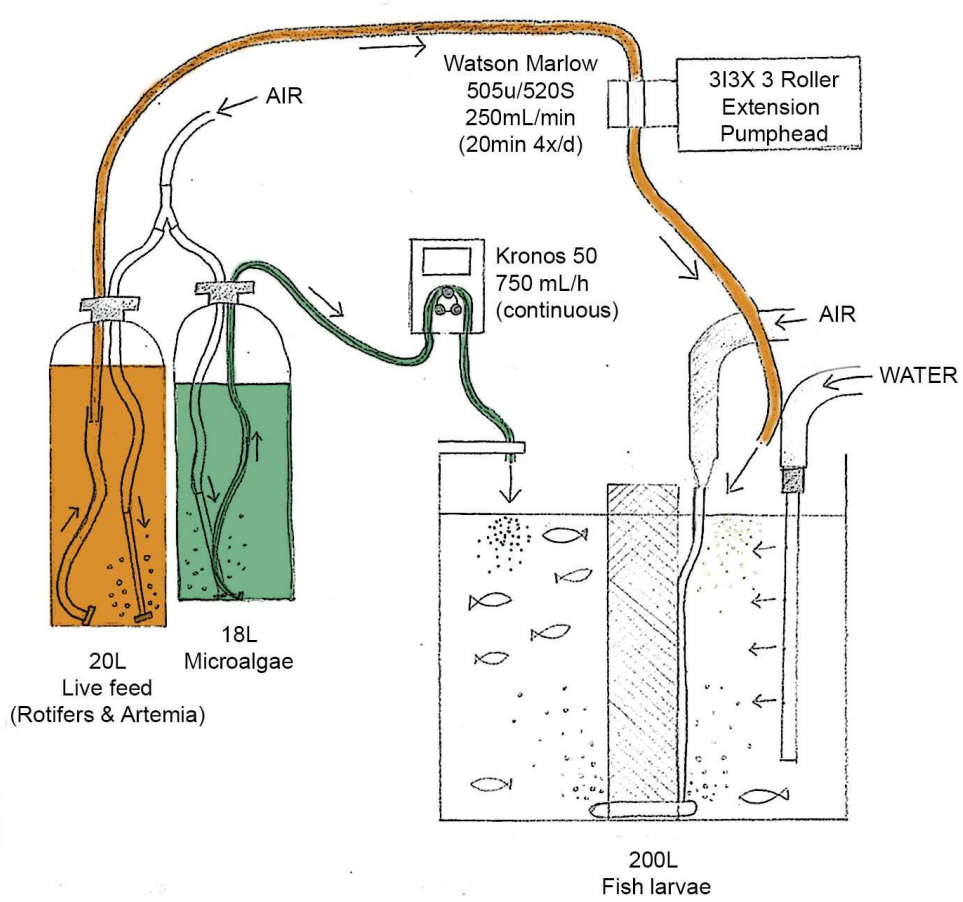

Figure 5 - Illustration of the fish tanks and feeding reservoirs where the live feed and microalgae were stored prior to feeding. Drawing made by Joachim Larsen Marthinsen.

Weaning from live feed started at 17 dph, and larvae were handfed daily until 20 dph. At 21 dph, calibrated automatic feeders (Sterner 905, Fish Tech AS, Norway) were programmed to feed at a frequency of 4 feeding cycles per day, feeding 3 times in each cycle up to 34 dph. At 35 dph, feeders were set to feed ca. 34 times per day with 42 minutes between each feeding. The amount of feed fed per day is shown in Table 1.

Table 1 – Feeding protocol used in the rearing of Atlantic cod larvae. Rotifers (Rot), *Artemia* AF (Art AF) and *Artemia* EG (Art EG) are expressed as number of individuals per litre per feeding. The amount of dry feed is expressed as milligrams per day.

Feeding periods	dph	Rot (# L^{-1})	Art AF (# L^{-1})	Art EG (# L^{-1})	Dry feed (mg day^{-1})	Dry feed size (µm)
Live feed	3 - 14	8000				
	15 - 16	8000	3000			
Co-feeding	17 - 19	8000	3000		1	100
	20 - 24		2000	1000	4	100/200
	25			1000	6	100/200
	26			1000	8	100/200
	27 - 30			1000	12	100/200
	31 - 34			500	16	200/300
Dry feed	35 - 44				20	200/300
	45 - 55				24	300/400
	56 - 60				32	300/400

Daily operations and registrations were implemented such as measurements of oxygen saturation and temperature, and cleaning of the tanks to remove dead eggs and eggshells, dead larvae, and debris such as faeces and uneaten feed.

2.2. Experimental setup

A feeding experiment with Atlantic cod larvae fed dry feed was run from day 17 post hatch until larvae reached 61 dph, from May to July 2022. Four isoproteic and isolipidic diets were produced by Biomar SAS (Nersac, France), according to a 2 x 2 factorial design. The first factor was the phospholipid (PL) level, formulated at 42% (HPL) and 32 % (LPL) of the total lipid fraction and the source differed from fish meal, marine and vegetable PL to fish meal and vegetable PL, respectively. The second factor was BS (sodium taurocholate ≥97.0%, Sigma-Aldrich, USA) supplementation level (0% vs 0.04% of the diet dry matter). The diets were fractioned in 100, 200, 300 and 400 µm fractions. The nutrient composition of the diets was analysed by the Institute of Aquaculture at the University of Stirling (Scotland) and shown in Table 2. The four experimental treatments were randomized between 12 tanks with three replicates each.

Table 2 – Ingredients and nutritional composition of the formulated diets used in the experiment. HPL: High Phospholipid: LPL: Low Phospholipid: HPL-BS: High Phospholipid-Bile salt; LPL-BS: Low Phospholipid-Bile salt.

Ingredients	HPL	HPL-BS	LPL	LPL-BS
Meal mix (%)	89	88.96	89.5	89.46
Fish oil (%)	3	3	6	6
Marine phospholipid (%)	2	2		
Vegetable phospholipid (%)	6	6	4.5	4.5
Sodium taurocholate 95%		0.04		0.04
Nutrient composition				
Energy (J kg^{-1})[1]	1839	1844	1842	1864
Protein (% dry matter)	60.99	60.80	61.33	61.04
Lipids (% dry matter)	21.77	21.97	21.70	22.42
Polar lipids (PL) (% dry matter)	8.05	8.52	7.16	7.20
Polar lipids (PL) (% in lipid fraction)	37.0	38.8	33.0	32.1
- Unknown glycolipid	5.0	4.7	3.5	3.6
- Phosphatidylethanolamine	7.0	6.4	5.3	5.2
- Phosphatic acid/Phosphatidylglycerol/Cardiolipin	2.7	2.4	1.8	2.1
- Phosphatidylinositol	4.0	5.2	3.7	3.3
- Phosphatidylserine	0.9	1.3	1.0	0.7
- Phosphatidylcholine	12.2	13.0	12.2	11.6
- Sphingomyelin	0.8	1.0	0.7	0.7
- Lysophosphatidylcholine	1.8	2.0	1.8	1.7
- Pigmented material	2.6	2.8	3.0	3.2
Neutral lipids (NL) (% dry matter)	13.72	13.45	14.54	15.22
Neutral lipids (NL) (% in lipid fraction)	63.0	61.2	67.0	67.9
- Wax/ Sterol esters	2.1	2.3	2.5	2.1
- Triacylglycerols	37.4	37.4	42.1	43.7
- Free fatty acids	11.7	10.4	11.0	11.3
- Cholesterol/ Sterols	8.9	8.7	8.7	8.3
- Diacylglycerol	2.9	2.4	2.7	2.5
EPA/DHA ratio	0.86	0.83	0.83	0.94
Ash	11.69	11.75	11.23	11.48

[1]Energy calculated as: fat x 37.7 J kg^{-1} + protein x 16.7 J kg^{-1}

2.3. Live feed production

2.3.1. Microalgae

For the green water conditions (Reitan et al., 1993; Reitan et al., 2021), the microalgae *T. lutea* was cultivated in a semi-continuous production system. A start culture was obtained from the microalgae cultures kept at Sealab and inoculated in disinfected seawater, in two cylindrical polycarbonate bioreactors with a volume of 200 L and 160 L each (Figure 6). After inoculation, Conway medium was added to the culture at a concentration of 1 mL per L of seawater added. The culture was continuously supplied with CO_2 and light. The cultivation cylinders were illuminated with 3 fluorescent light tubes at three different points at an irradiance of ca. 443 µE m^{-2} s^{-1} (Universal Light Meter ULM-500 connected to a Submersible Spherical Micro Quantum Sensor US-SQS/L (Heinz Walz GmbH, Germany)).

Figure 6 – Bioreactors used to produce microalgae *Tisochrysis. lutea*. The culture was continuously supplied with carbon dioxide.

Microalgae density was measured with a UviLine 900 spectrophotometer (Schott Instruments GmbH, Germany). Cell density was calculated using the obtained calibration curve, as follows:

Equation 1:

$$y = 5.0e^{-8}x + 0.2656$$

Where *y* is the absorbance measured at 750 nm and *x* is the cell concentration in cells mL^{-1}.

Cell density was maintained at 10 million cells mL^{-1} and 25% of each culture was removed daily and replaced with new seawater and Conway medium. The removed volume was distributed between 12 x 20L reservoirs. Seawater used for *T. lutea* cultivation was sourced at 70 meters depth in Trondheimsfjorden and disinfected by chlorination for approximately 24 h, after which was neutralized with sodium thiosulfate ($Na_2S_2O_3$).

2.3.2. Rotifers

A start culture of 500 million rotifers (*Brachionus sp.*) was obtained from Ode AS (Stadsbygd, Norway). The culture was divided into 4 conical 250 L tanks with aerated seawater. The culture tanks were equipped with a 100 µm mesh size sieve with constant water renewal at a rate of one tank volume per day. Temperature and oxygen saturation were measured daily, ranging between 19 to 22 °C and 70 to 85%, respectively. Each tank was flushed once a day to remove debris, dead rotifers and organic matter sedimented at the bottom. The cultures were filtered and washed weekly.

Rotifers and eggs were counted daily to calculate density and egg ratio. The egg ratio is an indicator of the culture health status and predicts the state of the culture for the next 24 hours (Lubzens and Zmora, 2003). Rotifers were continuously fed with fresh baker's yeast (*Saccharomyces cerevisiae*) and Rotifer Diet (Reed Mariculture, United States of America) diluted in fresh water, according to SINTEF (Trondheim, Norway) cultivation routines.

Circa 100 million rotifers were harvested daily and transferred to a conical 150 L tank to be enriched with Larviva Multigain (Biomar AS, Norway) immediately after transfer and 12 h later, according to the manufacturer instructions. 24 h after the first enrichment, rotifers were washed and chilled with cold seawater. After determining density, rotifers were distributed between 12 x 20L feeding reservoirs. The temperature in the enrichment tanks ranged from 20 to 22 °C and the oxygen saturation from 30 to 80%.

2.3.3. Artemia

Iron-coated *Artemia* AF nauplii cysts were weighed and distributed in 60L conical tanks containing seawater at approximately 26 °C and supplied with bottom aeration. Shells were separated from newly hatched *Artemia* AF using a magnetic separator (Inve Aquaculture, Belgium), 24 hours after incubation. *Artemia* AF was washed and chilled with cold seawater. *Artemia* AF was then counted and distributed into the same feeding reservoirs used for rotifers.

Artemia EG strain cysts were treated in the same way as the AF strain, with an additional enrichment step. After shell separation, the newly hatched *Artemia* EG were transferred to

fresh seawater at 26 °C, and was enriched with Larviva Multigain (Biomar AS, Norway) immediately after shell separation and 12 h later. 24 h after the first enrichment dose, *Artemia* EG was washed and chilled with cold seawater. *Artemia* EG was then counted and distributed into the same feeding reservoirs used for *Artemia* AF. Both *Artemia* strains were cultivated in accordance with the supplier instructions.

2.4. Growth, survival, and liver histology analysis

2.4.1. Larval growth

The sampled larvae from each tank were euthanized with an overdose of tricaine mesylate (Finquel vet. MSD, Ireland) in seawater by adding small amounts of a stem solution [1 g L^{-1}]. Larvae were sampled from each tank at 2 (n=6), 8 (n=6), 15 (n=6), 23 (n=8), 30 (n=8), 38 (n=16), 45 (n=16), 52 (n=16), 60 dph (n=32) and 61 dph (n=20) (Table 3). All sampled larvae were rinsed in distilled water and photographed using a stereo microscope Leica MZ7.5 (Leica Camera AG, Germany) coupled with a camera (Axiocam ERc 5s, Carl Zeiss AG, Germany) and using the image software Zen Core software (Carl Zeiss AG, Germany). Standard length (SL) of the photographed larvae was measured using the software Image J (Schneider et al., 2012), measuring the larvae from the tip of the snout to the end of the notochord. Larvae were then placed individually in pre-weighted tin or aluminium capsules and dried at 60°C for at least 48 hours. The capsules containing the dried larvae were weighed on an Ultra-microbalance UMX2 (Mettler Toledo, USA) to determine dry weight. Due to biased sampled towards smaller fish, DW and SL from larvae at 60 dph were disregarded from the growth results but used for DW/SL correlation. At 61 dph, larvae were measured and fixated in 4 % formaldehyde phosphate buffered (VWR International LLC; USA) at 4°C until further analysis.

Table 3 - Number of Atlantic cod larvae sampled for growth and histology per tank.

dph	2	8	15	23	30	38	45	52	60	61
DW	6	6	6	8	8	16	16	16	32	-
SL	6	6	6	8	8	10	10	10	20	80
Histology	2	2	2	2	2	2	2	2	2	2
Total	8	8	8	10	10	18	18	18	34	82

The average DW was used to calculate the specific growth rate (SGR), expressed in mg day^{-1}, according to Ricker (1979):

Equation 1:

$$SGR\ (g\ day^{-1}) = \frac{\ln W2 - \ln W1}{t2 - t1}$$

Where *W1* and *W2* are the initial and final dry weights, respectively. *t1* and *t2* are the corresponding time in days between each weighing.

With the values obtained from the SGR calculation, the daily weight increase (DWI), expressed as percentage, was calculated as follows:

Equation 2:

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

2.4.2. Survival

Dead larvae were removed from the tanks during the cleaning routines from mouth opening. However, as larvae were too small to identify it was difficult to get an accurate mortality estimate. To calculate survival, dead larvae were removed and counted from 20 dph onwards. In this way, mortality was followed since the addition of dry feed into the tanks. At the end of the experiment, the remaining larvae in the tanks were euthanized with tricaine mesylate and counted. Sampled larvae were removed from the total number of larvae. Survival was then calculated as follows:

Equation 3:

$$Survival\ (\%) = \frac{number\ of\ larvae\ at\ the\ end}{number\ of\ larvae\ at\ 20\ dph} \cdot 100\ \%$$

2.4.3. Liver histology analysis

Two larvae per tank were sampled and euthanized together with the larvae used for growth analysis (see Table 3). These larvae were fixated in 4 % formaldehyde phosphate buffered at 4°C until further analysis. Liver histology was done in larvae aged 15, 30, 45 and 61 dph in Technovit 7100 (Heraeus Kulzer GmbH, Germany), a plastic material for histological studies. The preparation of the samples was done according to the protocol at Sealab (Table 4), and it followed five main steps: washing, dehydration, pre-infiltration, infiltration and embedding. All steps were performed at room temperature. After the washing step, SL was measured so all further results could be related to larval size. The head and/or tail were excised on larvae sampled at 30, 45 and 61 dph, so that the ethanol and infiltration solution could easily penetrate the tissue. The samples were then embedded and left to harden overnight.

Table 4 – Dehydration and embedding protocol used in the fish samples for liver histology. Samples from fish at 15 and 30 dph were processed whole. Samples from fish at 45 and 61 dph had the head and tail excised.

Step	Reagent	Time
Washing	10% PBS	3*5’
	50% Ethanol:Water	10’
Dehydration	70% Ethanol:Water	10’
	96% Ethanol:Water	2*10’
Pre-infiltration	2:1 Technovit 7100 + Hardener 1:96% Ethanol	2 h
Infiltration	Technovit 7100 + Hardener 1	overnight
Embedding	Tehcnovit 7100 + Hardener 1+ Hardener 2	Until set

The embedded larvae were cut longitudinally on samples from 15, 30 and 45 dph, and transverse on samples from 61 dph due to the larger size of the larvae (Figure 7). Using a microtome Leica HistoCore AUTOCUT (Leica Biosystems Nussloch GmbH, Germany), 2 µm sections were removed from the block. When liver tissue was observed, three sections were collected with 10 µm spacing between each other and placed on a glass slide (Brand, GmbH, Germany). The sections were stained with toluidine blue (TB) 0.05% for 15 seconds. Each slide was mounted with a glass cover (VWR, USA) and Neo-Mount™ (Merck KGaA, Germany) before scanned with a digital slide scanner (Nano-Zoomer, Hamamatsu Photonics K.K., Japan). Sections were scanned with 40 times magnification in three layers of 0.5 µm. The section was then checked with the NDP2 viewer software (Hamamatsu Photonics K.K., Japan). Further analysis was performed using QuPath (Bankhead et al., 2017) and ImageJ (Schneider et al., 2012).

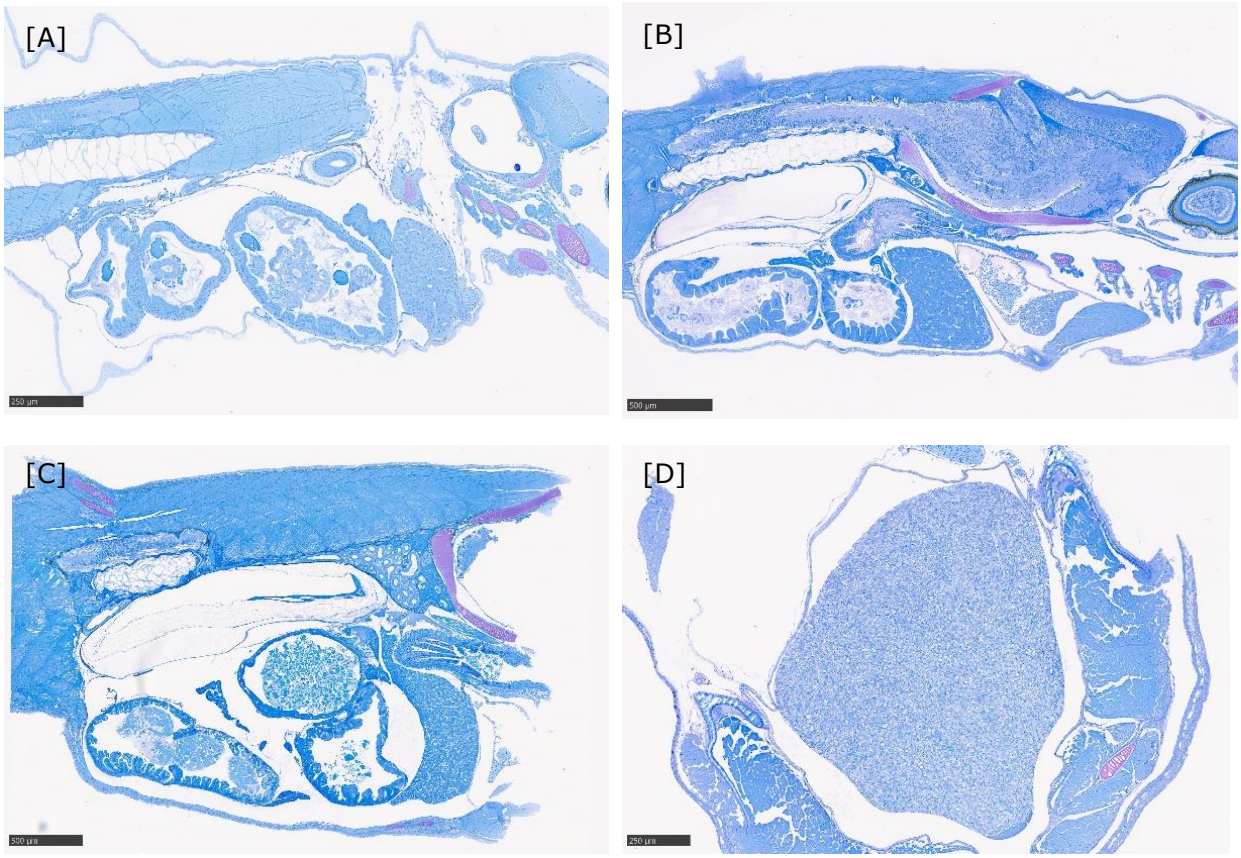

Figure 7 – Example of sections removed from samples for histology. A: 15 dph, longitudinal cut; B: 30dph, longitudinal cut; C: 45 dph, longitudinal cut; D: 61 dph, transversal cut. Each sample was stained with 0.05% toluidine blue for 15 seconds after being attached to the glass slide.

2.4.4. Liver analysis

The scanned sections were analysed for hepatocyte cell area size, nucleus area size, nucleus area fraction and hepatocyte vacuole fraction using QuPath, in which was divided into areas of 250 µm x 250 µm. Enough area was analysed to include at least 200 nuclei (after Wold et al. (2009)). The nucleus area (μm^2) was obtained by measuring the area of each nucleus with a visible nucleolus, using the polygon tool (Figure 8). The same tool was used to measure the area of blood vessels, bile ducts and sinusoids, called "other" from this point onwards. The analysed liver tissue was then imported to Image J to measure the vacuoles area fraction. A point grid tool was applied ensuring a good coverage of the tissue (Wold et al., 2009), which corresponded to 256 points, represented by a cross. Using the multi-point tool, each vacuole touching the right upper part of the cross was marked. The vacuoles are defined as containing glycogen or lipids, without distinction.

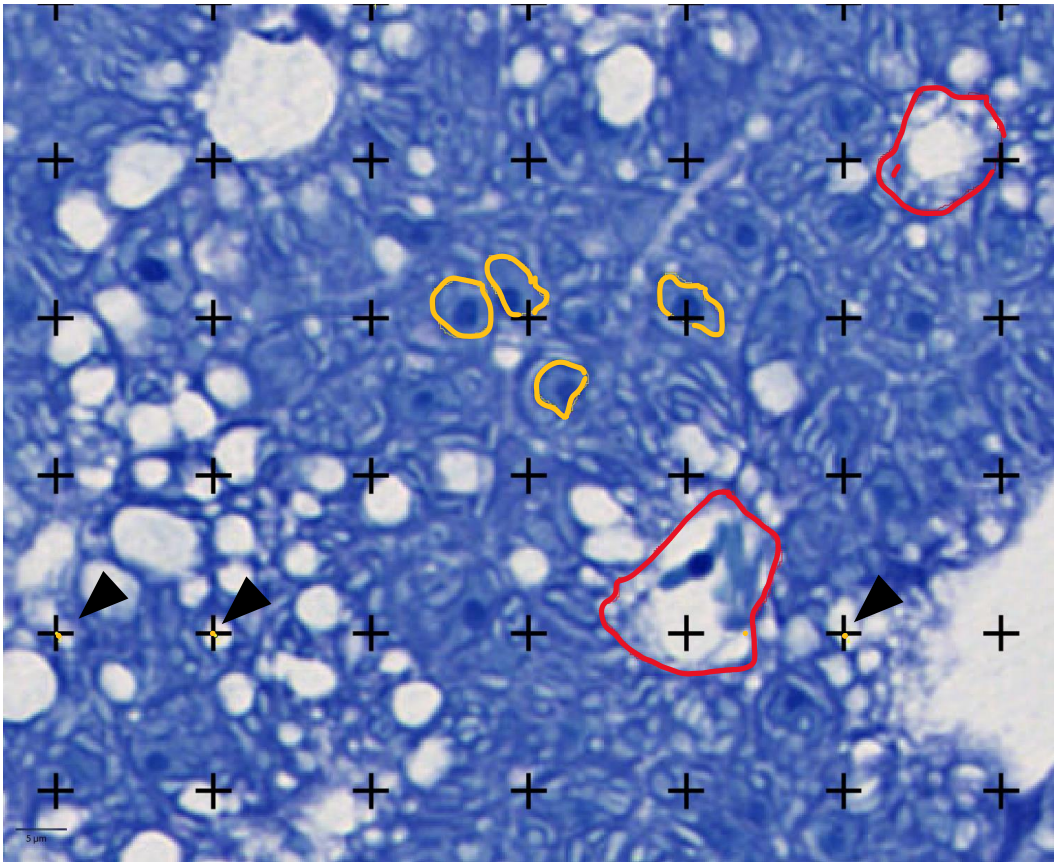

Figure 8 - Example of how the measurements were made in the liver sections. The nucleus with visible nucleolus are circled in yellow; Black arrowhead with a yellow dot in the cross represent a vacuole touching the upper right part of the cross; The "other" area is surrounded in red..

The "other" area was removed from the analysed area to get a more accurate estimation of the hepatocyte cell area size. To estimate the hepatocyte cell area size, the defined area was divided by the total number of observed nuclei (A_{liver}/N_{nuclei}). The nucleus area size was estimated as the average of all nuclei measurements per fish. To estimate the nucleus area fraction, the sum of nuclei areas was divided by the liver tissue area ($\Sigma\ A_{nuclei}/A_{liver}$). To estimate the vacuoles area fraction, the points hitting vacuoles P_{struc} were divided by the points hitting the liver tissue P_{sect} ($\Sigma\ P_{struc}/\Sigma\ P_{sect}$) (Gundersen et al., 1988). All area measurements and area fractions are expressed as μm^2 and percentage (%), respectively.

2.5. Data and statistical analysis

All statistical analysis and data visualization were performed with R (R Development Core Team, 2022) equipped with RStudio (version 2022.07.2+576 "Spotted Wakerobin" Release (RStudio Team, 2022)). The list of packages used can be consulted in Appendix 1. The calculation for the DWI, survival, and area fractions were made with Microsoft® Excel® for

Microsoft 365 MSO (Version 2301 Build 16.0.16026.20196) 64-bit. All parameter estimates are expressed with mean and standard error of mean (± SEM).

DW, SL, hepatocyte cell area and nucleus area were analysed on natural log transformed data. Linear mixed effects models (lmer) were applied with tank as random factor. The distribution of the mean residuals was tested for normality before statistical inference with ANOVA followed by post hoc Tukey test. The distribution of the residuals was assessed with a fitness plot of residuals, histogram and Shapiro-Wilk normality test. The ANOVA tested the main effects, PL levels and BS supplementation, and the interaction between these two (PL x BS) All hypotheses were tested with a confidence level of $1 - \alpha = 0.95$. DW/SL correlations were tested with a linear model (lm) and normality of the residuals was tested with the Kolmogorov-Smirnov test (Mishra et al., 2019). Due to the observed overestimation in predicted DW (data not shown), DW/SL regression was analysed with segmented regression to check if there was any change in the slope (Muggeo, 2003; Muggeo, 2016; Muggeo, 2017). Davies' test tested for differences in the slopes.

DWI, survival, nucleus and vacuoles area fractions data were analysed with a generalized linear model (glm) due to a non-normal distribution of the residuals. The models were first fitted with a binomial distribution family, but due to overdispersion of the residuals the quasibinomial family was chosen. Estimated marginal means function (emmeans) and post hoc Tukey test checked for differences and interactions between groups.

In case of no statistical differences found between treatments, a linear model (lm) and ANOVA test was used to test differences within treatments in the different days.

3. Results

3.1. Morphological development

Developmental characteristics from 2 to 60 dph are described in Table 5. The characteristics described on Table 5 are to be seen in Figure 9 and consulted with the growth results on section 3.2. The description provided are on larval size across all treatment.

Table 5 - Morphological development description of Atlantic cod (*G. morhua*) larvae from 2 to 60 dph. Descriptions are common to the four feeding treatments used in the experiment and no distinction is done.

Age (dph)	Morphological development description
2	The body of the larva was surrounded by a large fin-fold. The fin-fold was covered by round protuberances. Some pigmentation was already present in the head, gut and caudal regions. The mouth had started to develop being especially prominent the lower jaw. An empty straight digestive tube was observed laying on a large yolk-sac. The liver, ball shaped, was observed on the front part of the digestive apparatus. Live observations denoted a beating heart and lymphatic blood running along the notochord to the end of the tail. Larvae in the tanks were active presenting an anguilliform swimming pattern. Pectoral fins were developed.
8	Mouth was open at this stage. The yolk-sac was still visible in some sampled larvae and the gut was full of live feed. Some more pigmentation had developed around the larva's body, including on the top the head. The liver seemed to have grown and the gut was still a straight tube but enlarged. It was possible to distinguish the midgut from the hindgut.
15	The gut had coiled, and the liver was bigger than previously observed. More pigmentation had developed. Besides the black pigmentation spots, yellow pigmentation appeared on the top of the larva's body. The head had grown and elongated. The fin-fold was reduced on the head and gut region. Trunk musculature seemed to have increased in size. The swim bladder had started to inflate.
23	The larva's body was more pigmented, including on the gut region. The swim bladder was more inflated than previously. Tail fin rays had started to develop, as well as a slight anal and dorsal fin growth. Fin-fold also decreased, especially on the head region. Gills were developed.

30	Darker pigmentation was pronounced on the head as well as on the frontal part of the gut region. A more elongated swim bladder was observed. Anal and dorsal fin rays were completely noticeable. Most larvae observed in the tanks at this stage had less anguilliform swimming pattern, switching to carangiform swimming mode.
38	Most larvae had the fin fold almost completely absorbed, with clearly visible anal, dorsal and tail fins. A pair of small pectoral fins were also observed. Swim bladder seemed more inflated than previously. Dissected fish presented a fully differentiated gut with oesophagus, stomach, and pyloric caeca. Notochord end seemed the have shortened and was barely visible. Live observations under the microscope also revealed that some teeth were already present on the upper and lower jaw Most fish in the tanks were swimming counter current, appearing to be schooling.
45	The fin fold was completely absorbed, and the fish were clearly more pigmented. The gut region was practically covered by dark pigmentation and a silvery blue coloration was developing under the epidermis. All fins seemed to be developing pigmentation as well. Fish resembled a small adult Atlantic cod.
52-60	The silvery blue coloration observed previously was also observed on the head. Live observations denoted the presence of the barbel on the lower jaw.

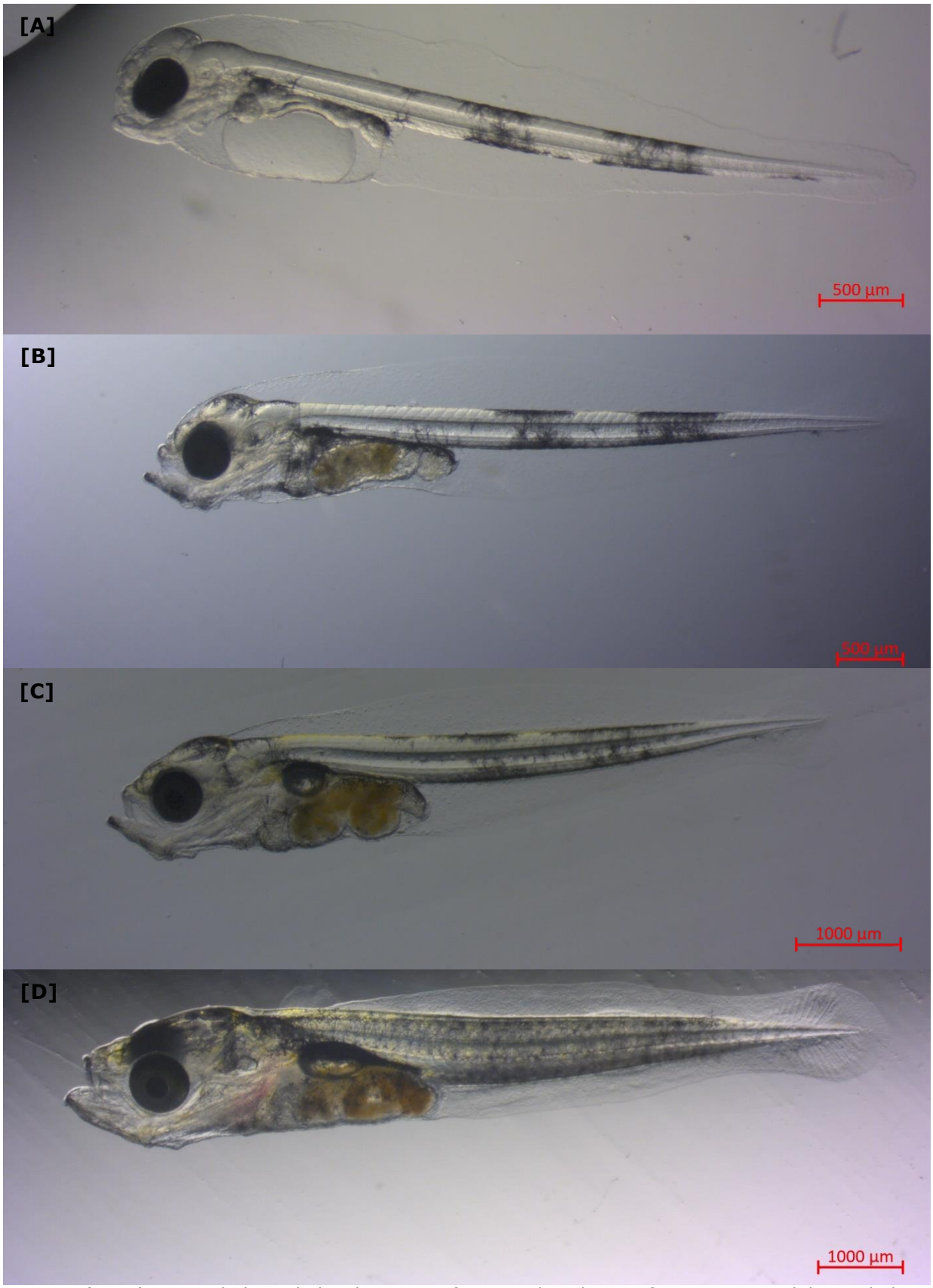

Figure 9 (cont.) - Morphological development of *G. morhua* larvae from 2 to 60 dph. Each larva corresponds to a different developmental age: A: 2 dph; B: 8 dph; C: 15 dph; D: 23 dph, E: 30 dph; F: 38 dph; G: 45 dph; H: 52 dph; I: 60 dph. Photographs by Richard Hasan Bjørklund and Bruno Nunes (NTNU). Scale bar on each picture.

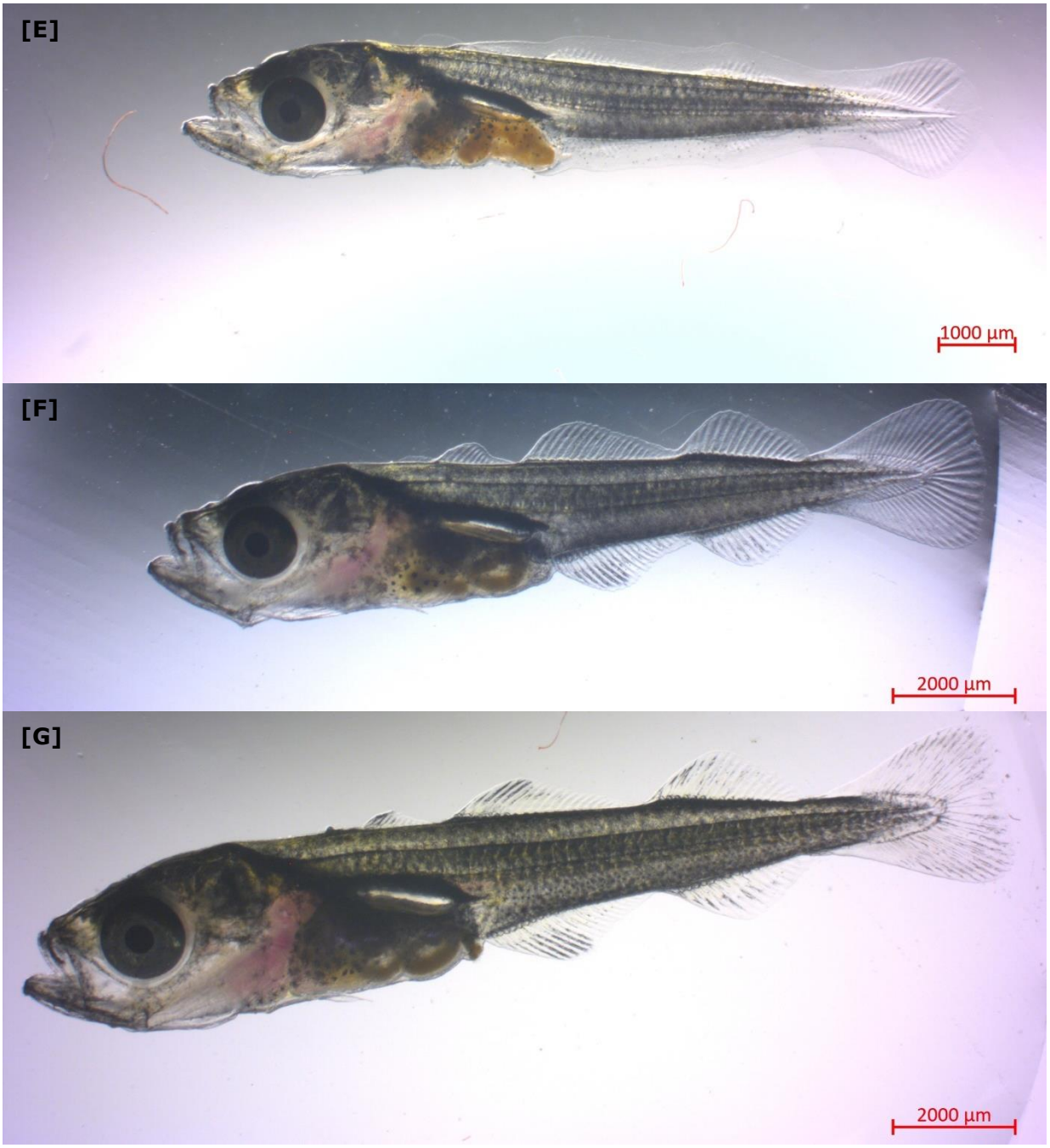

Figure 9 (cont.) - Morphological development of *G. morhua* larvae from 2 to 60 dph. Each larva corresponds to a different developmental age: A: 2 dph; B: 8 dph; C: 15 dph; D: 23 dph, E: 30 dph; F: 38 dph; G: 45 dph; H: 52 dph; I: 60 dph. Photographs by Richard Hasan Bjørklund and Bruno Nunes (NTNU). Scale bar on each picture.

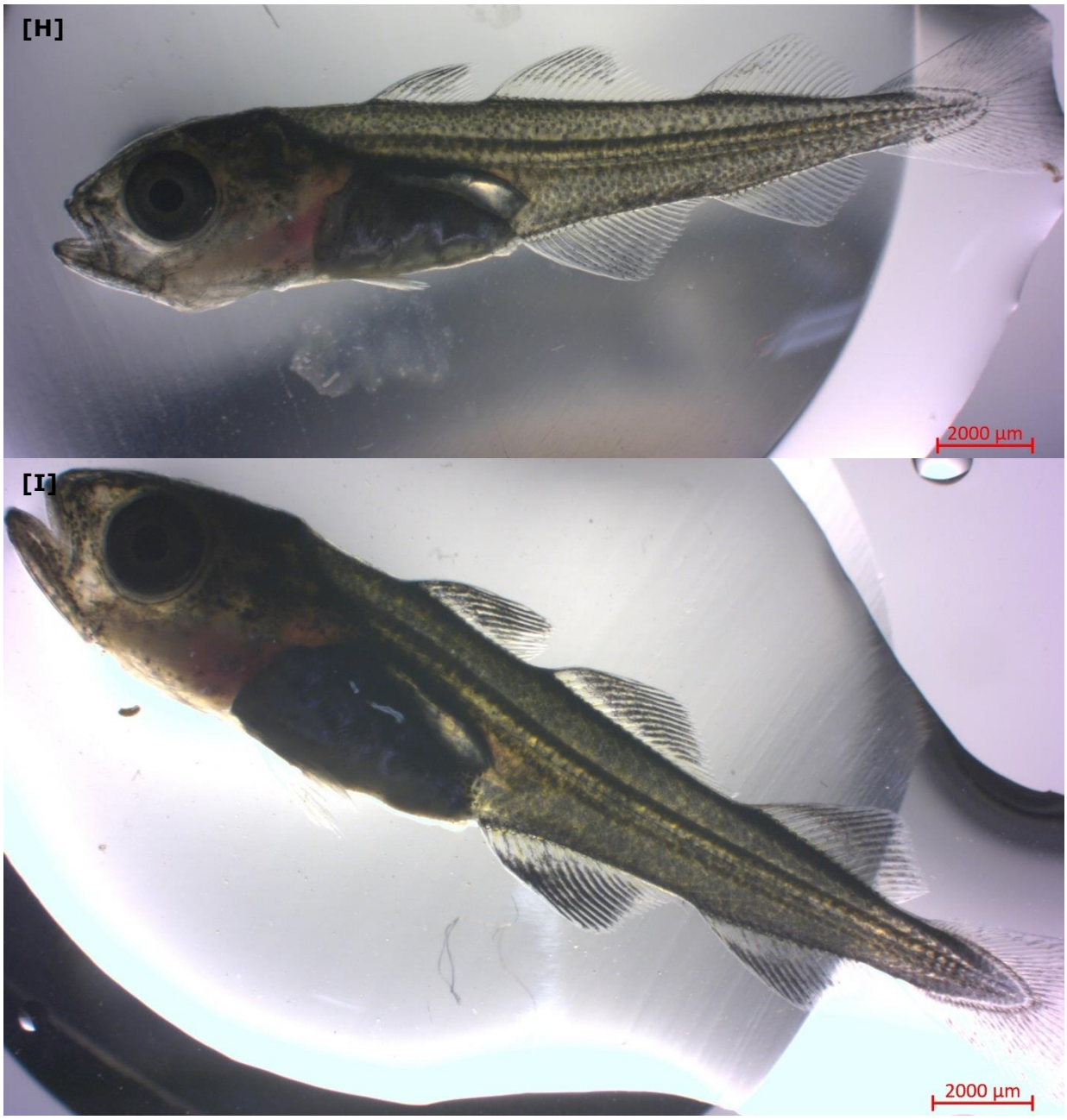

Figure 9 - Morphological development of *G. morhua* larvae from 2 to 60 dph. Each larva corresponds to a different developmental age: A: 2 dph; B: 8 dph; C: 15 dph; D: 23 dph, E: 30 dph; F: 38 dph; G: 45 dph; H: 52 dph; I: 60 dph. Photographs by Richard Hasan Bjørklund and Bruno Nunes (NTNU). Scale bar on each picture.

3.2. Larval growth

While larvae were feeding on live feed, the mean DW was 52±0.001, 69±0.002 and 192±0.01 µg at 2, 8 and 15 dph (Figure 10). During the co-feeding period, no significant differences in mean DW were observed between the larval groups. After weaning, larvae from the HPL group exhibited significantly greater DW compared to larvae from the LPL-BS group (p=0.0311) on

day 38 post hatch and the LPL group (p=0.0241) on day 45 post hatch. The greater DW in the HPL group resulted in more exponential growth compared to the other groups. At 52 dph, no significant DW differences were found between the larval groups, with DW ranging from 6.30±0.48 to 7.89±0.65 mg.

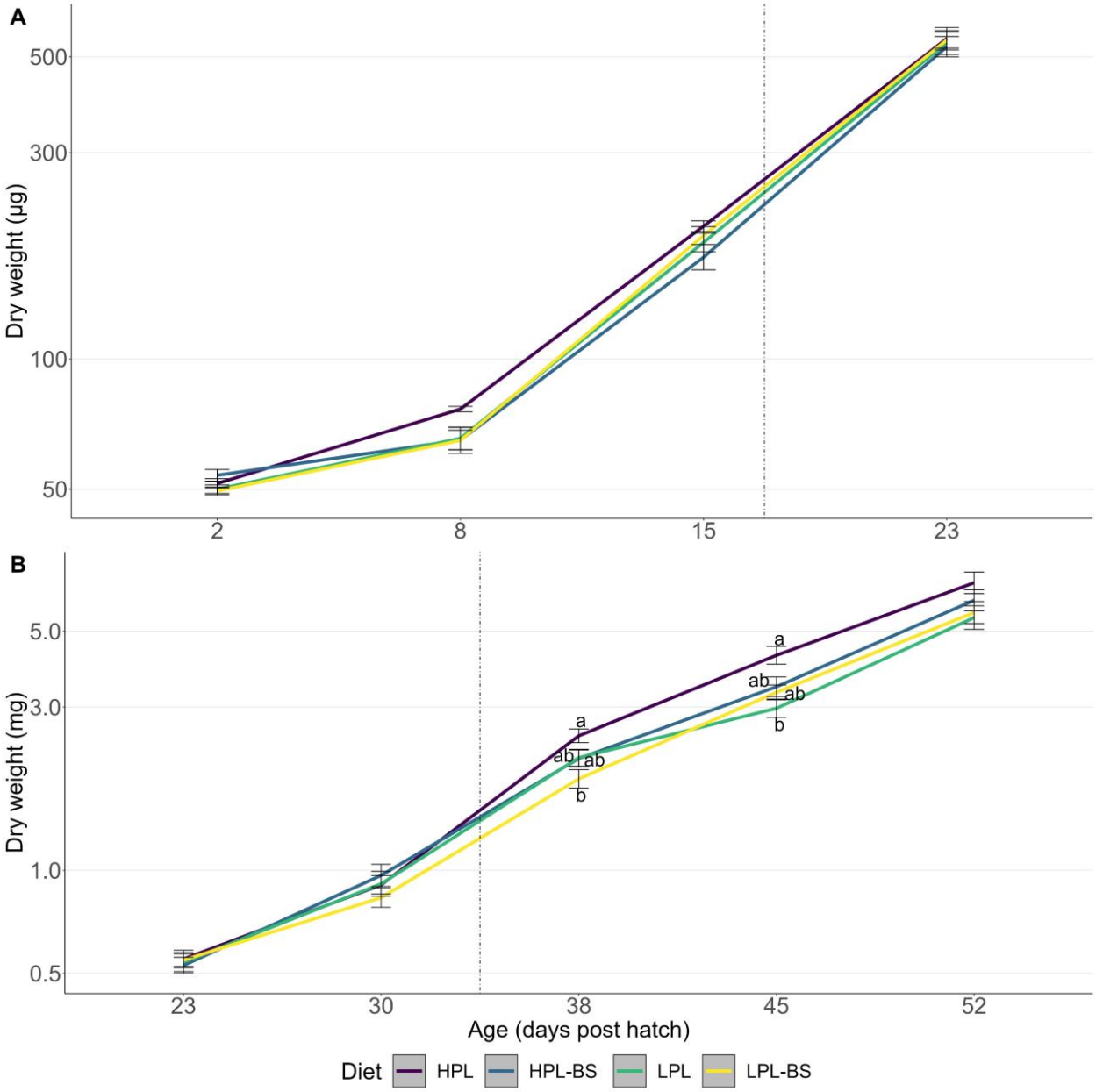

Figure 10 – Larval mean dry weight (DW) expressed as µg and mg ± SEM during (A) the live feed period (left of vertical line), , and (B) the co-feeding (left of vertical line) and dry feed periods (right of the vertical line). Different letters indicate a statistical difference between larval groups (p < 0.05) at the respective age. Different colours indicate different larval groups fed the respective dry feed.

During the live feed period (2-15 dph), larvae exhibited a growth rate of approximately 10.4% per day, with a lower DWI initially (2-8 dph) (Figure 11). During the co-feeding period (15-38 dph), the DWI decreased across all larval groups during the period in which the live feed

was reduced (23-30 dph), in which the HPL groups presented a more variable DWI than the LPL groups. However, these did not affect the overall DWI during that period, in which the larval groups grew approximately 11.4 % per day. During the last co-feeding period (30-38 dph) the HPL group had a higher, but not significant, DWI which was not reflected in the whole co-feeding period but reflect in the DW results. After weaning (38-52 dph), the LPL group presented a significantly lower DWI than the LPL-BS ($p=0.0095$) and HPL ($p=0.0174$) groups (38-45 dph). This difference was not observed in the next sampling period, as all groups exhibited similar DWI trends. Throughout the whole dry feed period, the larval groups grew slower compared to the live feed and co-feeding periods, with higher variation in both LPL groups. Considering the whole experimental period (15-52 dph), the DWI was similar across all groups, ranging from 9.82±0.42 % to 10.40±0.20 %. While not significantly different, the HPL groups had a slightly higher and less variable DWI compared to the LPL groups.

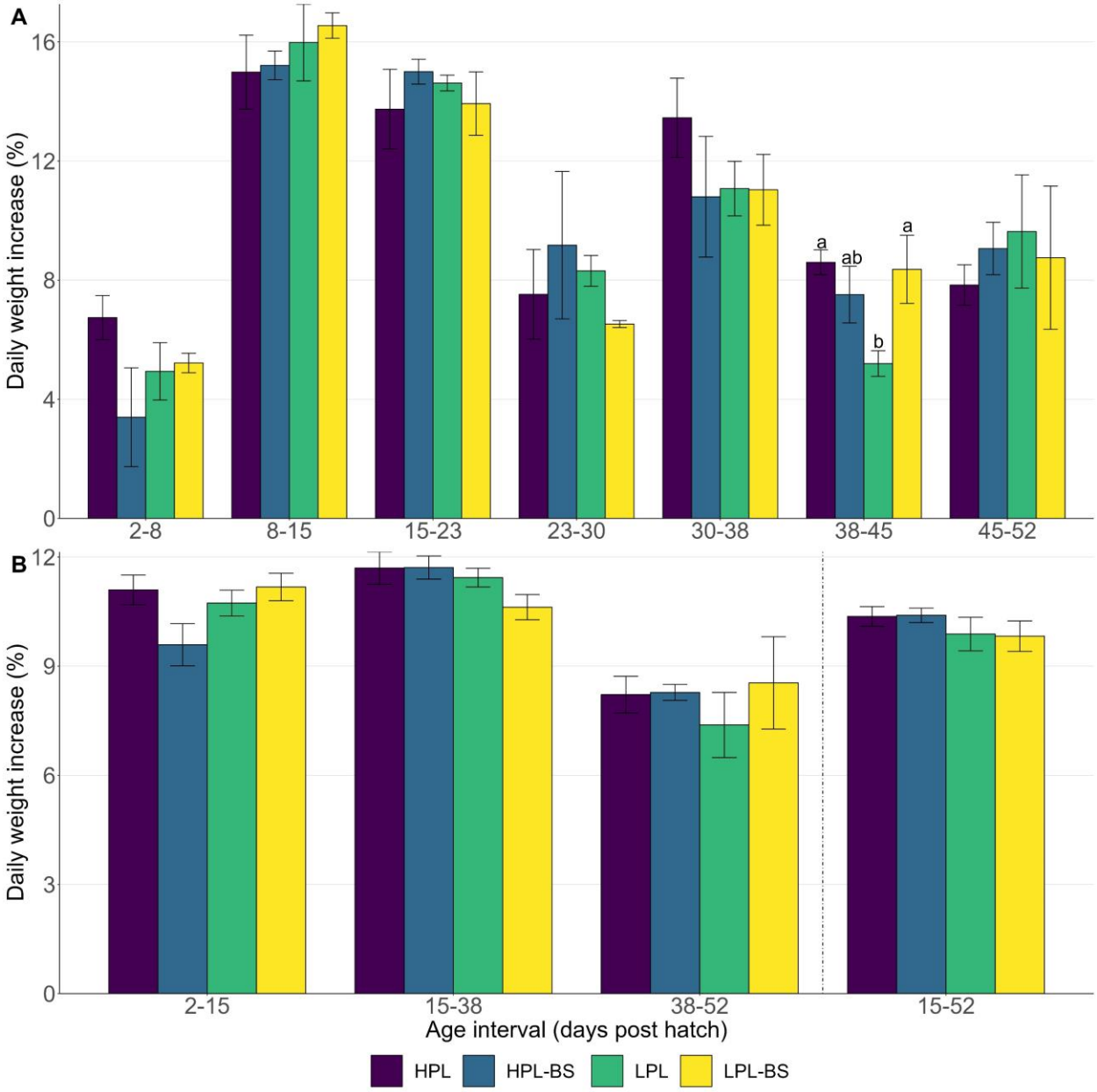

Figure 11 – Mean daily weight increase (DWI) expressed in percentage (%) ± SEM, during (A) the total duration of the fish rearing period and during (B) three distinct feeding phases and experimental time. Different letters indicate a statistical difference between larval groups on the respective time interval ($p < 0.05$). The vertical line distinguishes the DWI of the three feeding phases from the experimental time. Different colours indicate different larval groups fed the respective dry feed.

During the live feed period, larvae were of 4.44±0.03, 4.99±0.03 and 6.39±0.05 mm at 2, 8 and 15 dph, respectively (Figure 12). During the co-feeding period, the larval groups had a similar SL to each other at the different time points. After weaning, at 38 dph the LPL group had a significant bigger SL than LPL-BS (p=0.0275) group. During the remaining of the experiment, no more significant differences were found, and at the end of the experiment the SL ranged from 22.79±0.21 to 23.80±0.25 mm.

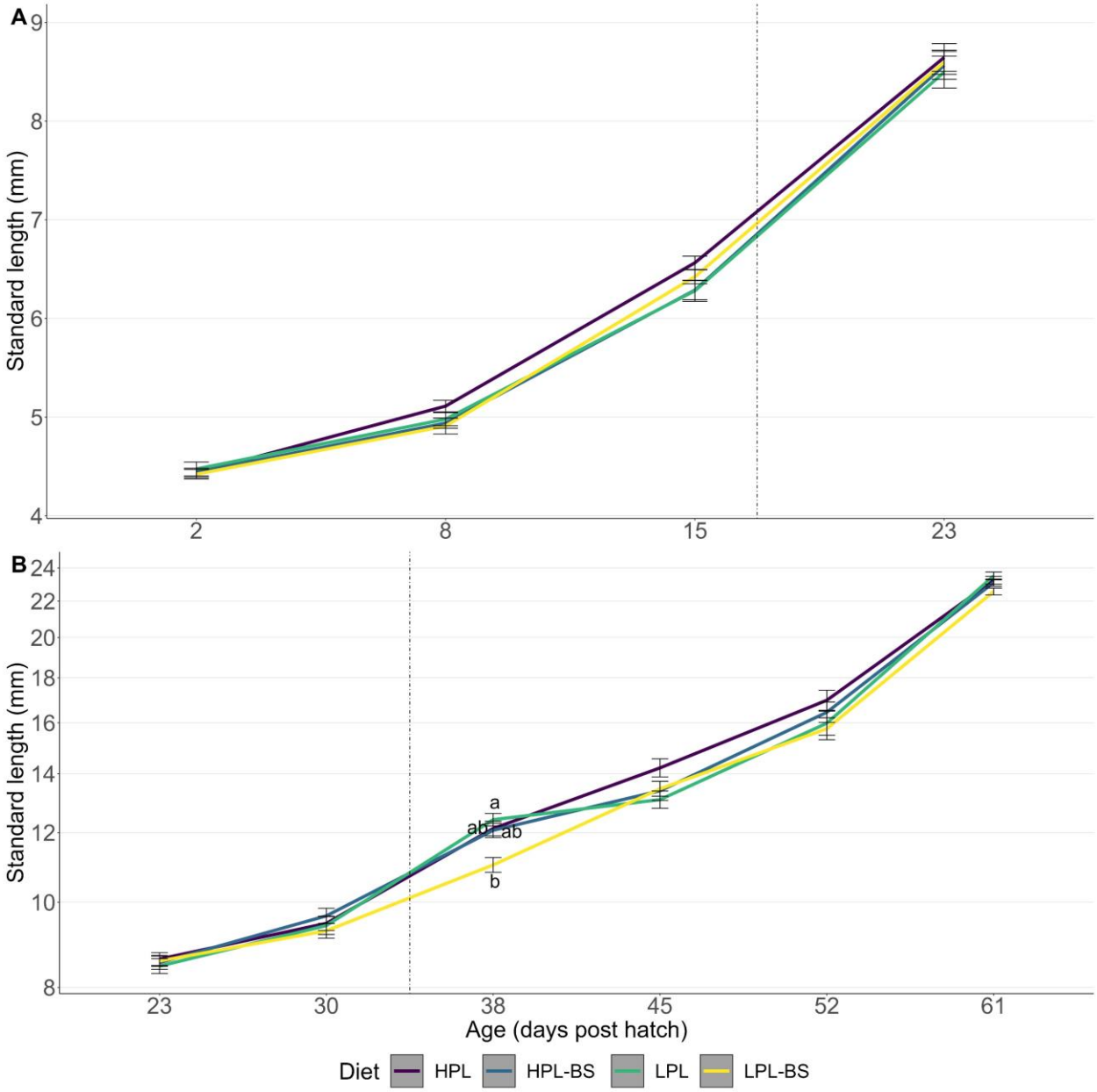

Figure 12 - Larval mean standard length (SL) expressed as mm ± SEM during (A) the live feed period (left of vertical line) and (B) the co-feeding (left of vertical line) and dry feed periods (right of the vertical line). Different letters indicate a statistical difference between larval groups ($p < 0.05$), at the respective age. Different colours indicate different larval groups fed the respective dry feed.

The relationship between SL and DW was highly correlated (R^2 =0.996) and presented a positive linear relationship (Figure 13). The equations in Figure 15 shows a change in the growth pattern when larvae reached SL 13.59±1.02 mm, differing significantly (p<2.2e-16). All the larval groups reached this size around day 45 post hatch. Up to the inflection point, larval DW increased by 3.87% for each 1% increase in SL, while after the inflection point, for each 1% increase in SL larval DW increased by 3.26%.

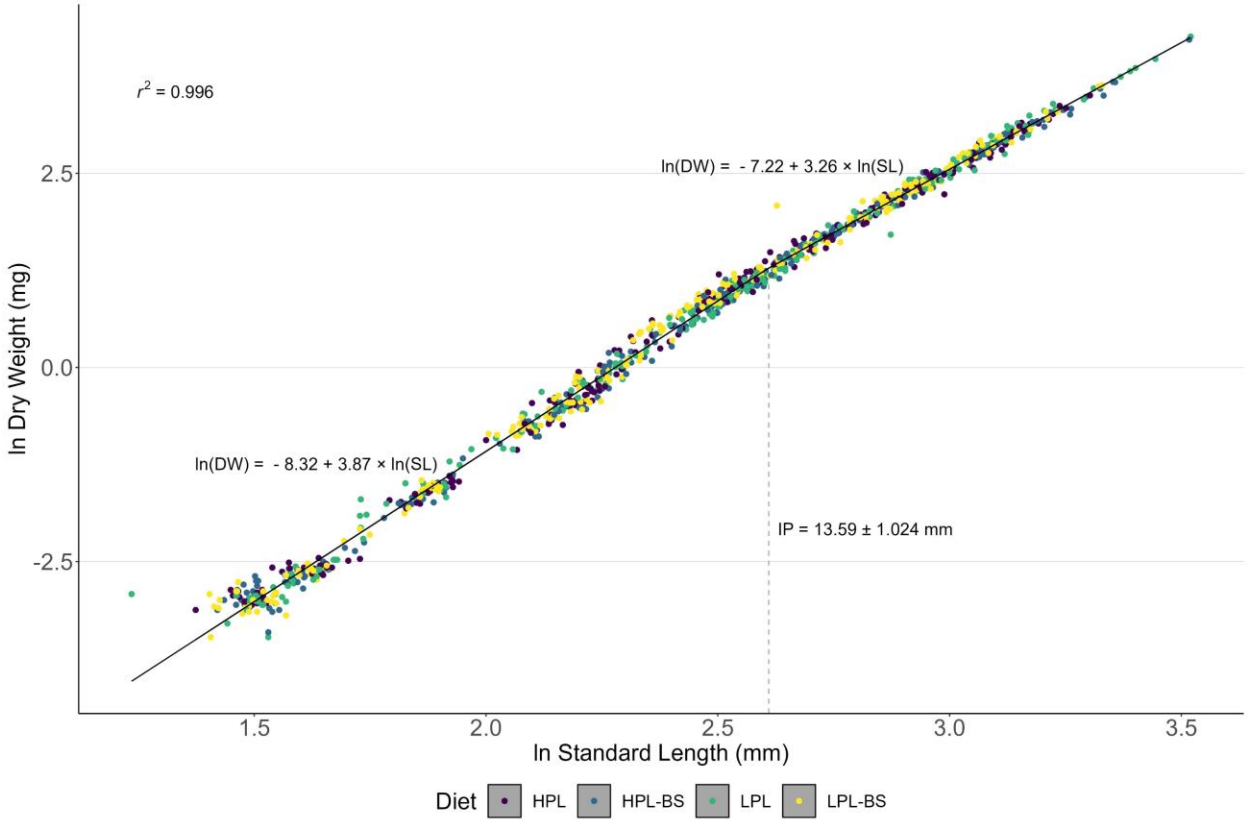

Figure 13 – Linear regression between standard length (SL), expressed as natural log mm, and dry weight (DW), expressed as natural log mg. Data includes values from fish sampled at 2, 8, 15, 23, 30, 38, 45, 52 and 60 dph (n = 1008). The linear relationship has an inflection point at 13.59±1.02 mm, where the slopes differ (p<2.2e-16), and the growth can be explained by both equations (R^2 = 0.996). Different colours indicate different larval groups fed the respective dry feed.

3.3. Survival

The survival difference between larval groups was not significant, varying from 42±11 to 59±2 % (Figure 14). The bigger variation in the LPL group resulted from a big mortality event in one tank. There was a small trend in which the HPL groups exhibited a higher survival than the LPL groups, while the groups supplemented with BS were inclined to have a higher survival than the groups without BS.

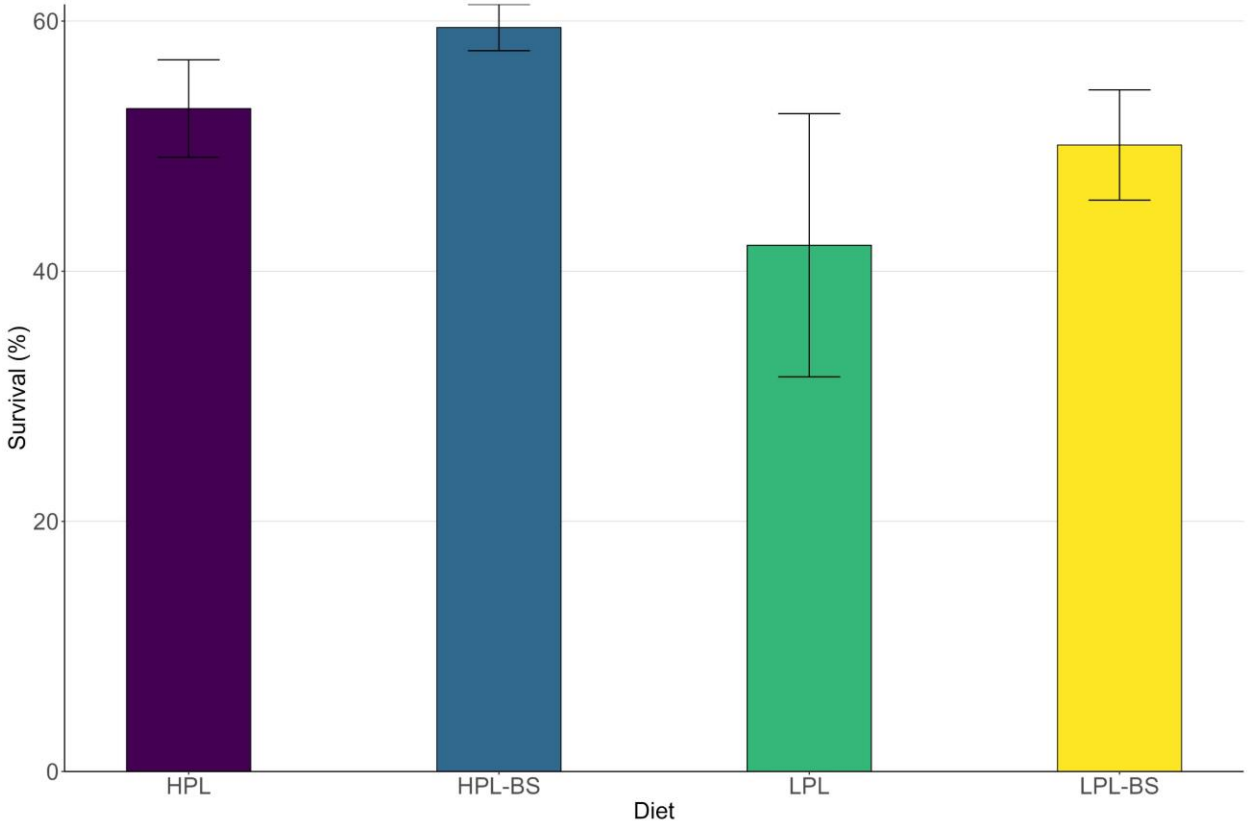

Figure 14 – Atlantic cod juvenile mean survival, expressed as percentage ± SEM. Each column represents a larval group fed the respective dry feed. Significance between groups was tested with a confidence interval of 95%

3.4. Liver histology

3.4.1. Liver morphology

At 15 dph the liver occupied the front part of the abdominal cavity, centrally located beneath the oesophagus, and the pancreas located behind the liver (Figure 15). Polygonal hepatocytes had a more or less distinct border with central polygonal nuclei. Red blood cells, veins and bile ducts were identified in the liver tissue. The hepatocytes seemed to be arranged cylindrically with a bile duct running through the centre. However, at 30 and 45 dph, hepatocyte borders were difficult to identify due to the cellular material being so densely stained. At 61 dph, the hepatocyte borders were visible in most of the analysed samples.

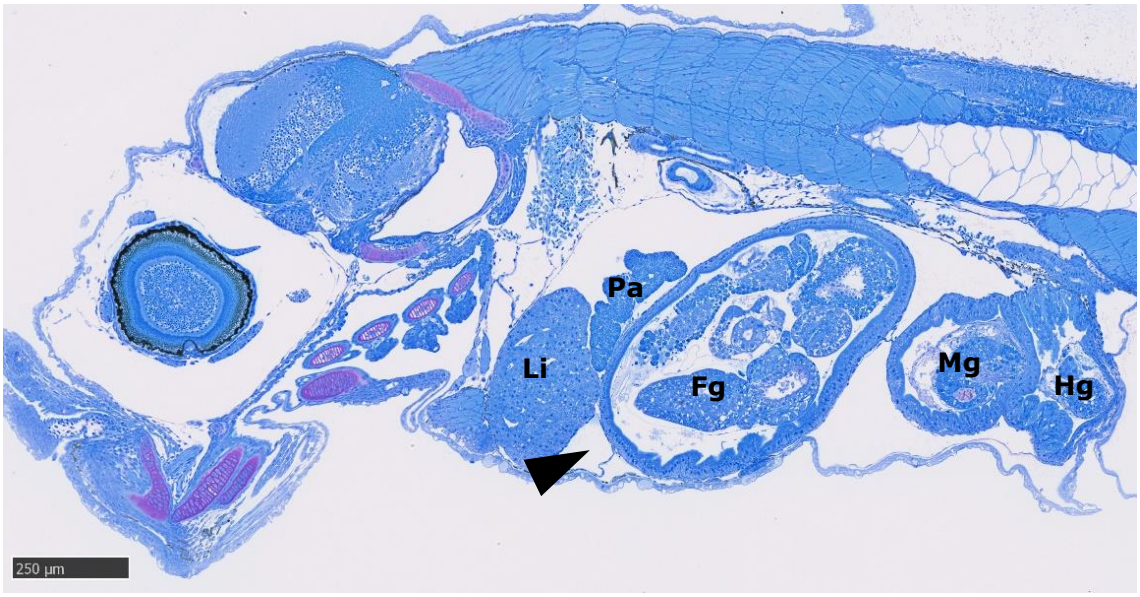

Figure 15 – Longitudinal section of Atlantic cod larvae used for histological analysis at 15 dph. The liver (Li) occupied the foremost part of the abdominal cavity in the larvae. The pancreas (Pa) laid behind the liver. The gut (Fg, Mg and Hg) occupied the majority of the abdominal cavity. The arrowhead indicates the remaining yolk-sac. Scale bar = 250 µm.

As the fish grew, the nuclei density decreased size, and the nuclei moved more peripherally in the hepatocytes. The nuclei had a visible nucleolus with a clear border, varying in shape. i.e. round, oval, without a clear shape and with irregular border. Double nucleolus was observed in some nuclei. The degree of vacuolization was low at 15, 30 and 45 dph, even though in these last two sampling points some vacuolization was observed in all fish. At 30 dph the vacuoles were not equally distributed through the liver, as the majority was surrounding the sinusoids and veins, and even observed inside these (not considered). At 45 dph, the existing vacuoles were more scattered in the liver. At 61 dph, the liver tissue in all larval treatments had a high degree of spherical shaped vacuoles in the liver. Figure 16 shows examples of the structures analysed.

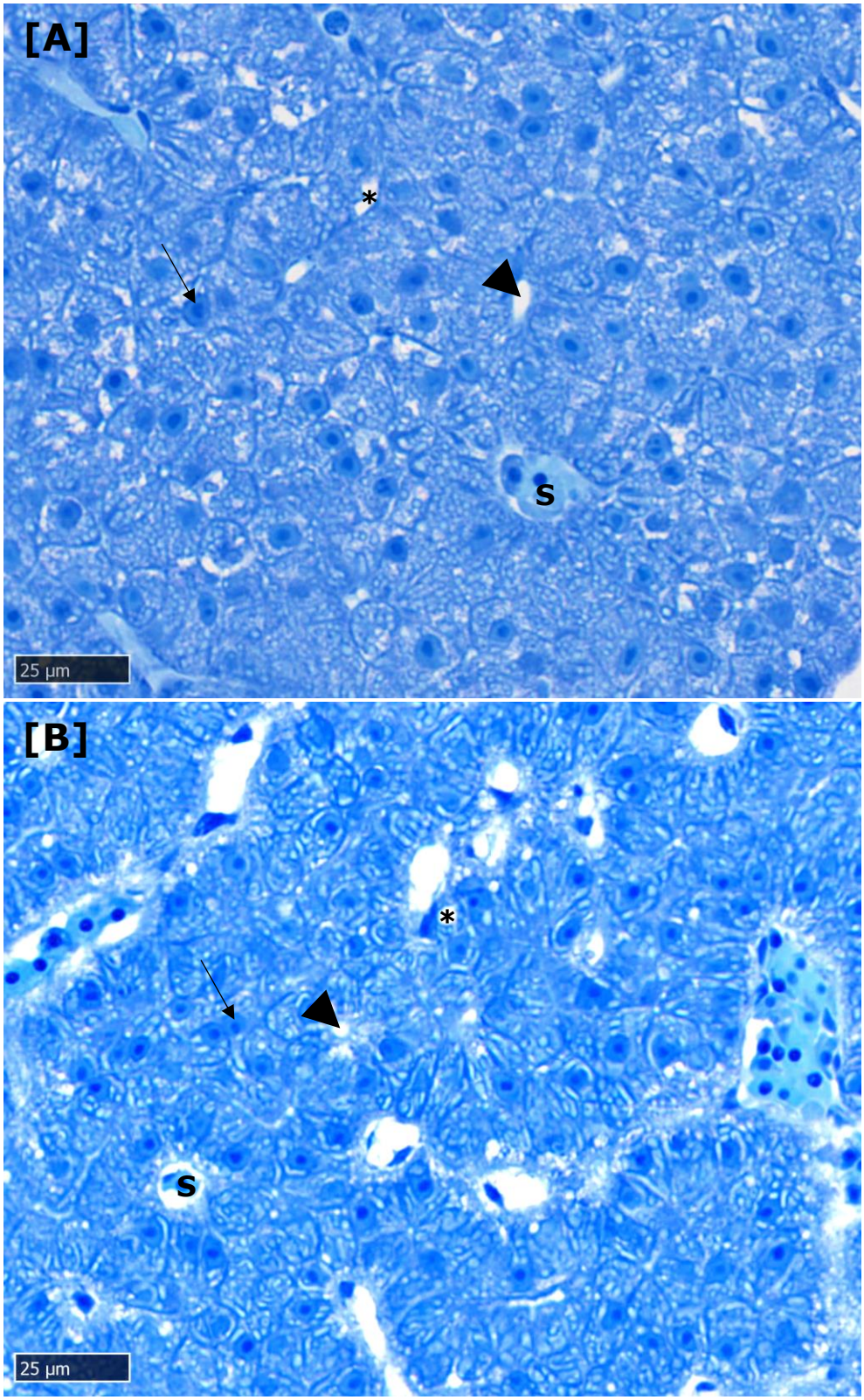

Figure 16 (cont.) - Histological sections containing liver tissue at 15 (A), 30 (B), 45 (C) and 61 (D) dph. The sections were randomly chosen from all the samples. Larvae belong to different treatments: A – HPL-BS; B – HPL; C – LPL; D– HPL. Scale bar = 25 µm. The black arrow indicates nuclei. The black arrowhead indicates the bile canaliculi. The asterisk indicates vacuole. The letter S indicates a sinusoid.

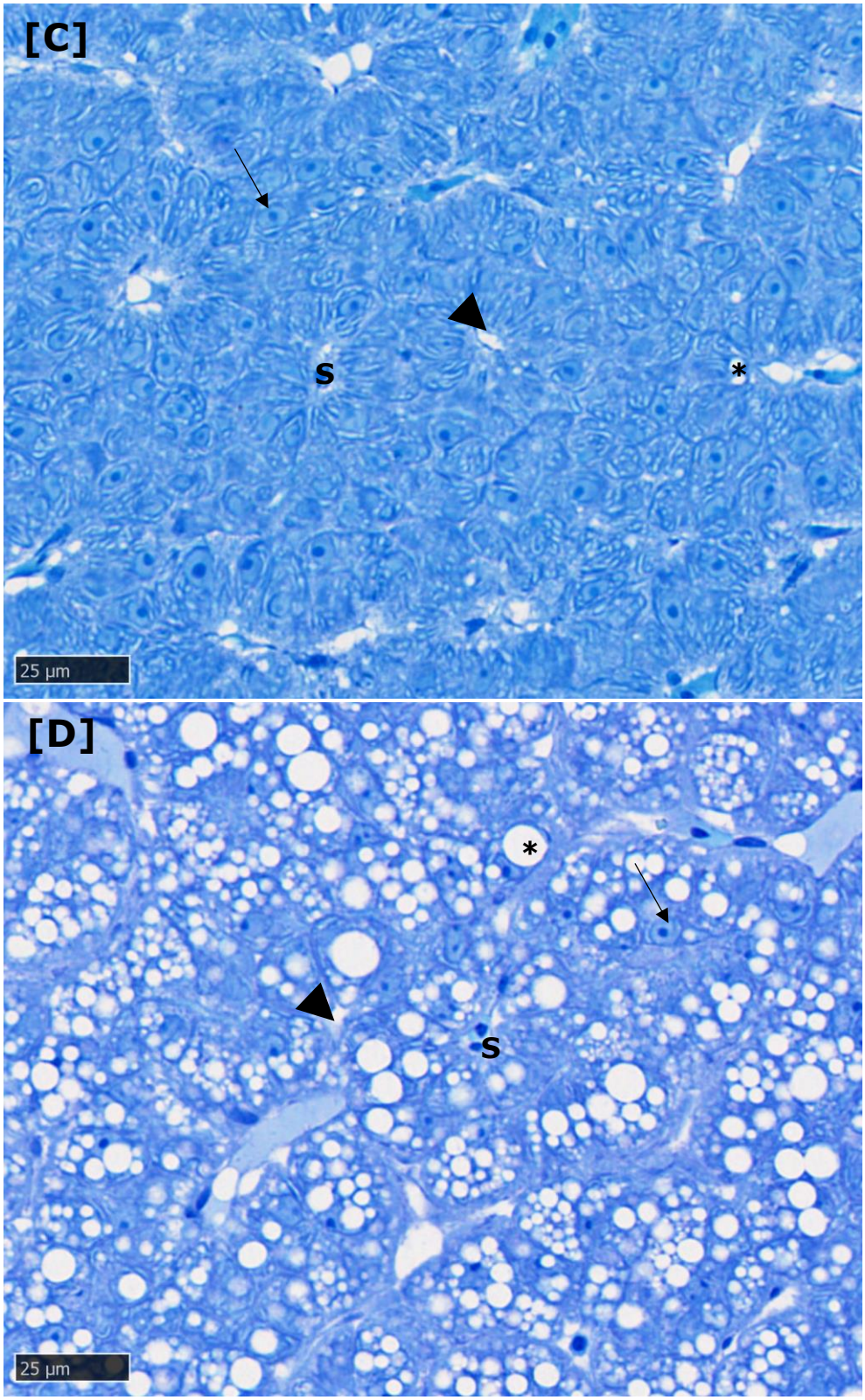

Figure 16 - Histological sections containing liver tissue at 15 (A), 30 (B), 45 (C) and 61 (D) dph. The sections were randomly chosen from all the samples. Larvae belong to different treatments: A – HPL-BS; B – HPL; C – LPL; D– HPL. Scale bar = 25 µm. The black arrow indicates nuclei. The black arrowhead indicates the bile canaliculi. The asterisk indicates vacuole. The letter S indicates a sinusoid.

3.4.2. Hepatocyte nucleus size

Mean hepatocyte nucleus size was 20.45 μm^2 for all groups at 15 dph (Figure 17). At 30 dph, a significant decrease in nucleus size was observed in all groups, except for the LPL group (Table 6). This group presented a similar nucleus size to what was observed at 15 dph. At 45 dph, the HPL and LPL groups had a nucleus size comparable to what was observed earlier. However, both groups supplemented with BS did not show any alteration in nucleus size compared to was observed at 30 dph and was still significantly smaller than the size observed at 15 dph. At 61 dph, all larval groups had a similar nucleus area size compared to what was observed initially, expect the LPL-BS group which did not show any significant improvement from day 30 post hatch. On the other hand, no significant differences were found between the groups at any time during the experiment, except on day 15 post hatch where the LPL presented a significantly smaller nucleus size than the HPL-BS ($p = 0.0121$) and LPL-BS ($p = 0.0399$) groups. This difference was due to tank differences ($p = 0.00313$).

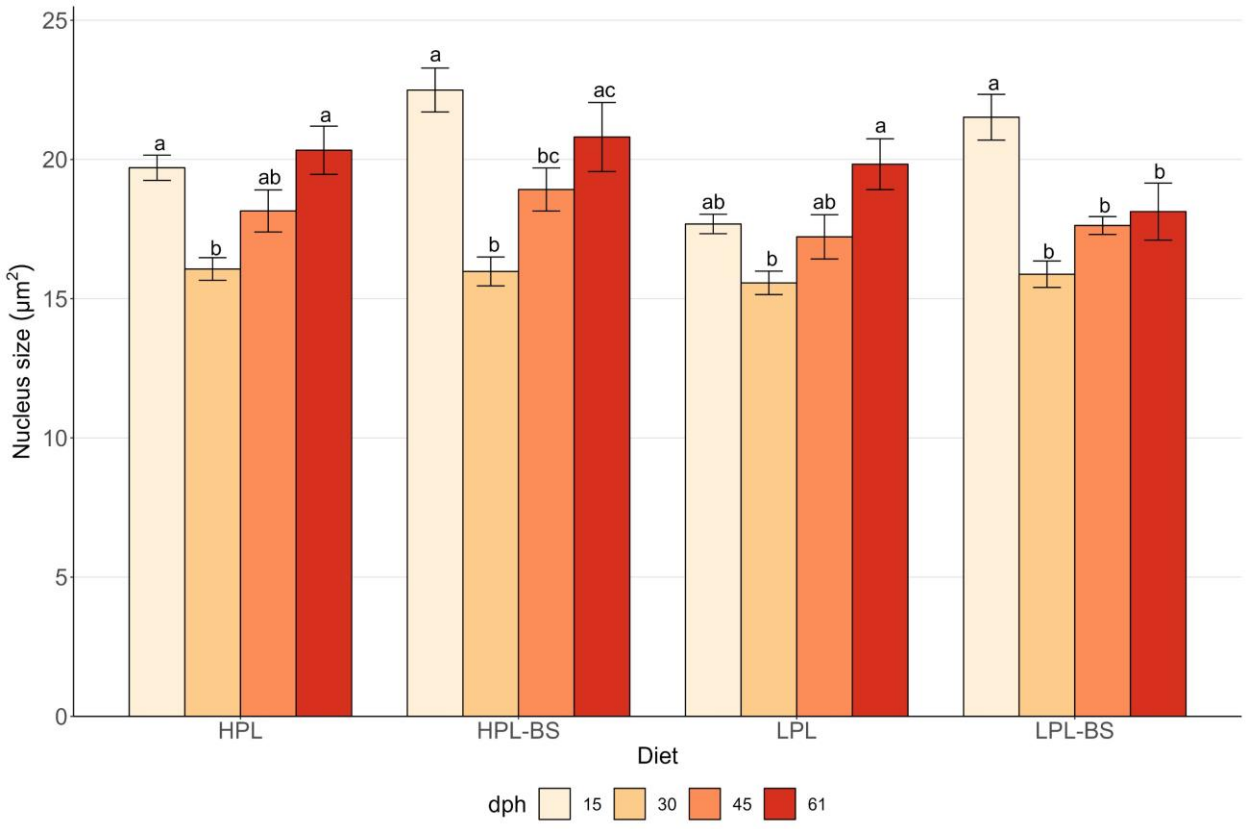

Figure 17 - Mean nucleus area size of the liver expressed as μm^2 ± SEM at 15 (n=23), 30 (n=24), 45 (n=24) and 61 (n=24) dph and diet. Different letters indicate statistical difference ($p<0.05$) between dph within the same diet larval groups. Each column represents a dph. The x-axis represents each larval group fed the respective diet.

Table 6 - Pairwise comparisons of sampling points within each larval group for hepatocyte nucleus size using ANOVA and Tukey Test (95% Confidence Interval).

	p-value			
dph	HPL	HPL-BS	LPL	LPL-BS
15-30	0.0040	0.0002	0.1827	0.0001
15-45	0.3561	0.0402	0.9662	0.0052
15-61	0.9020	0.5306	0.1728	0.0157
30-45	0.1391	0.1107	0.3328	0.3363
30-61	0.0008	0.0043	0.0013	0.1517
45-61	0.1155	0.4375	0.0574	0.9596

3.4.3. Hepatocyte size

Mean hepatocyte size was ca. 359 μm^2 for all groups at 15 dph (Figure 18). The HPL and LPL groups had similar hepatocyte size at 15 and 30 dph, but a significant increase was observed on day 45 post hatch (Table 7). The HPL-BS and LPL-BS groups had an estimated hepatocyte size similar at 15, 30 and 45 dph. The hepatocyte size significantly increased in all groups at 61 dph, doubling the size compared to the other sampling days. A bigger size variation was also observed on the groups supplemented with BS at 61 dph. At any time during the experiment, the hepatocyte size did not differ between the groups.

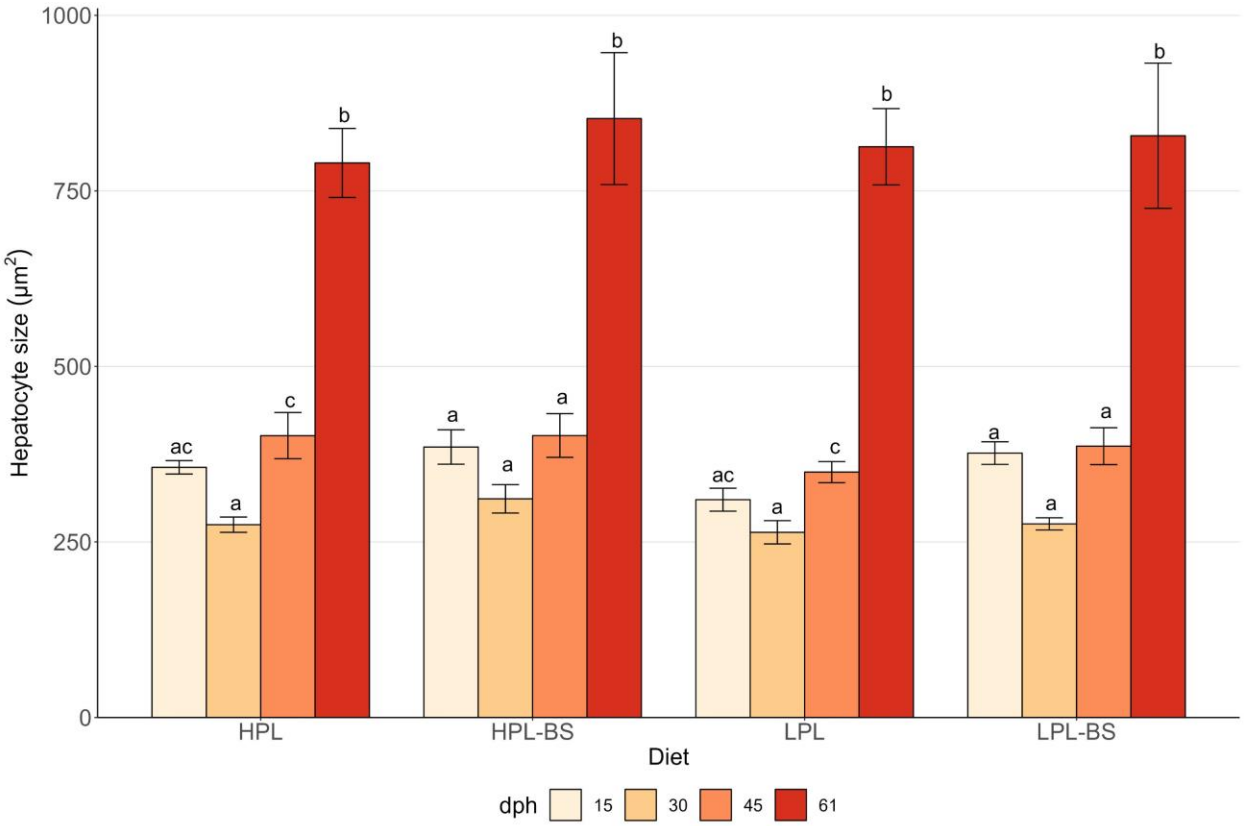

Figure 18 - Mean hepatocyte cell area size of the liver expressed as μm^2 ± SEM at 15 (n=5), 30 (n=6), 45 (n=6) and 61 (n=6) dph and diet. Different letters indicate statistical difference ($p<0.05$) between dph within the same diet larval groups. Each column represents a dph. The x-axis represents each larval group fed the respective diets.

Table 7 - Pairwise comparisons of sampling points within each larval group for hepatocyte size using ANOVA and Tukey Test (95% Confidence Interval).

	p-value			
dph	HPL	HPL-BS	LPL	LPL-BS
15-30	0.2604	0.7486	0.2342	0.5622
15-45	0.7234	0.9960	0.4935	0.9992
15-61	<0.0001	<0.0001	<0.0001	<0.0001
30-45	0.0368	0.6175	0.0101	0.4865
30-61	<0.0001	<0.0001	<0.0001	<0.0001
45-61	<0.0001	<0.0001	<0.0001	0.0001

3.4.4. Area fractions of liver components

The mean degree of vacuolization consistently low in all groups until day 45 post hatch, even though a significant increase was observed in the LPL and LPL-BS groups (Figure 19, Table 8). The HPL-BS group showed a large mean vacuole content variation compared to the other groups, primarily due to high individual variation. From 15 to 61 dph, there was a noticeable

and substantial average 35-fold increase in vacuole content observed in the liver of larvae across all experimental groups. Throughout the course of the experiment, no significant differences in vacuole content were observed at any of the time points.

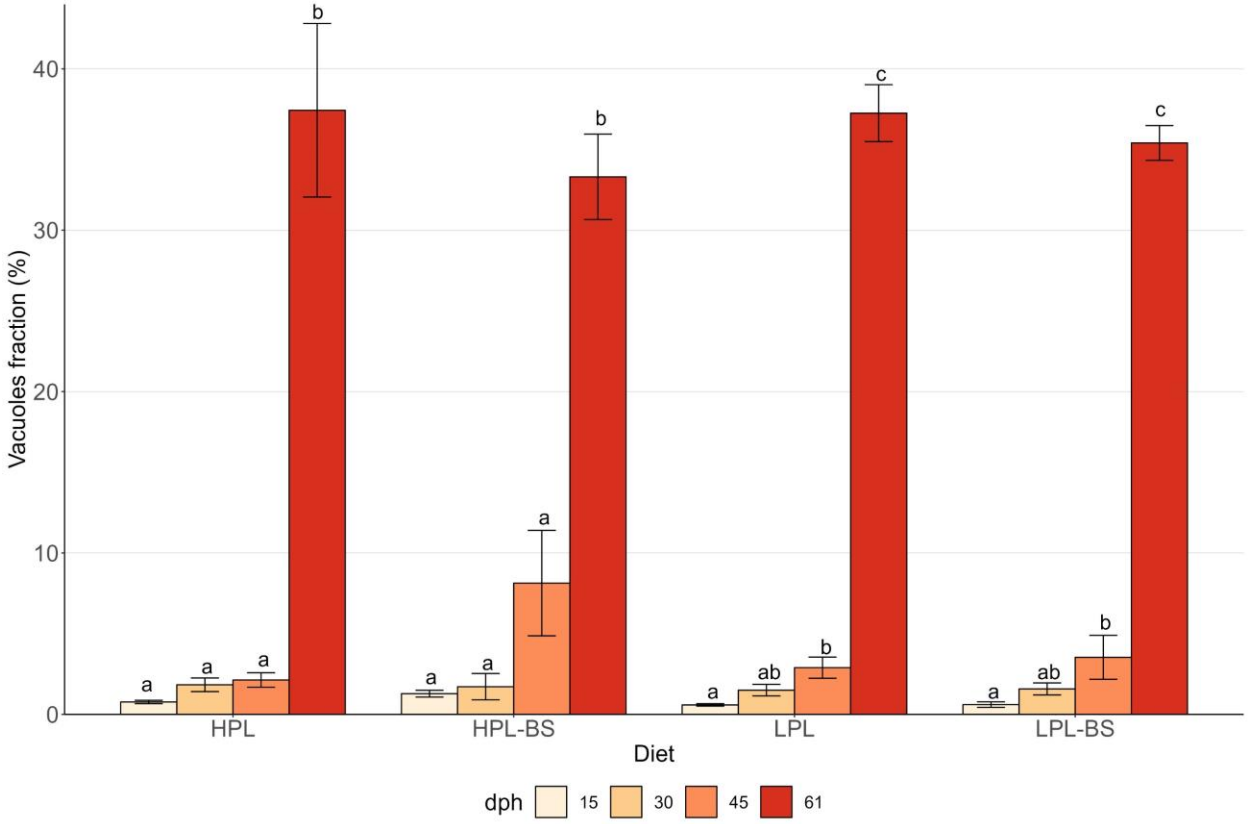

Figure 19 - Mean vacuoles fraction of the liver hepatocytes expressed as percentage ± SEM at 15 (n=5), 30 (n=6), 45 (n=6) and 61 (n=6) dph and diet. Different letters indicate statistical difference ($p<0.05$) between dph within the same diet larval groups. Each column represents a dph. The x-axis represents each larval group fed the respective diets.

Table 8 - Pairwise comparisons of sampling points within each larval group for vacuole fraction using ANOVA and Tukey Test (95% Confidence Interval).

	p-value			
dph	HPL	HPL-BS	LPL	LPL-BS
15-30	0.7192	0.9996	0.2863	0.3545
15-45	0.5739	0.0647	0.0063	0.0112
15-61	<0.0001	<0.0001	<0.0001	<0.0001
30-45	0.9948	0.0790	0.2078	0.2988
30-61	<0.0001	<0.0001	<0.0001	<0.0001
45-61	<0.0001	<0.0001	<0.0001	<0.0001

The nuclear fraction in the parenchymal tissue was constant up to day 30 post hatch (Figure 20, Table 9). At 45 dph all the groups had a significant nuclear density decline, with exception

of the HPL-BS. This group presented a similar nuclear fraction to what was observed earlier in the study. At 61 dph, a 50% significant reduction in the nuclear fraction was observed across all the groups when compared to earlier stages. At any time of the study were detected significant differences between the experimental groups.

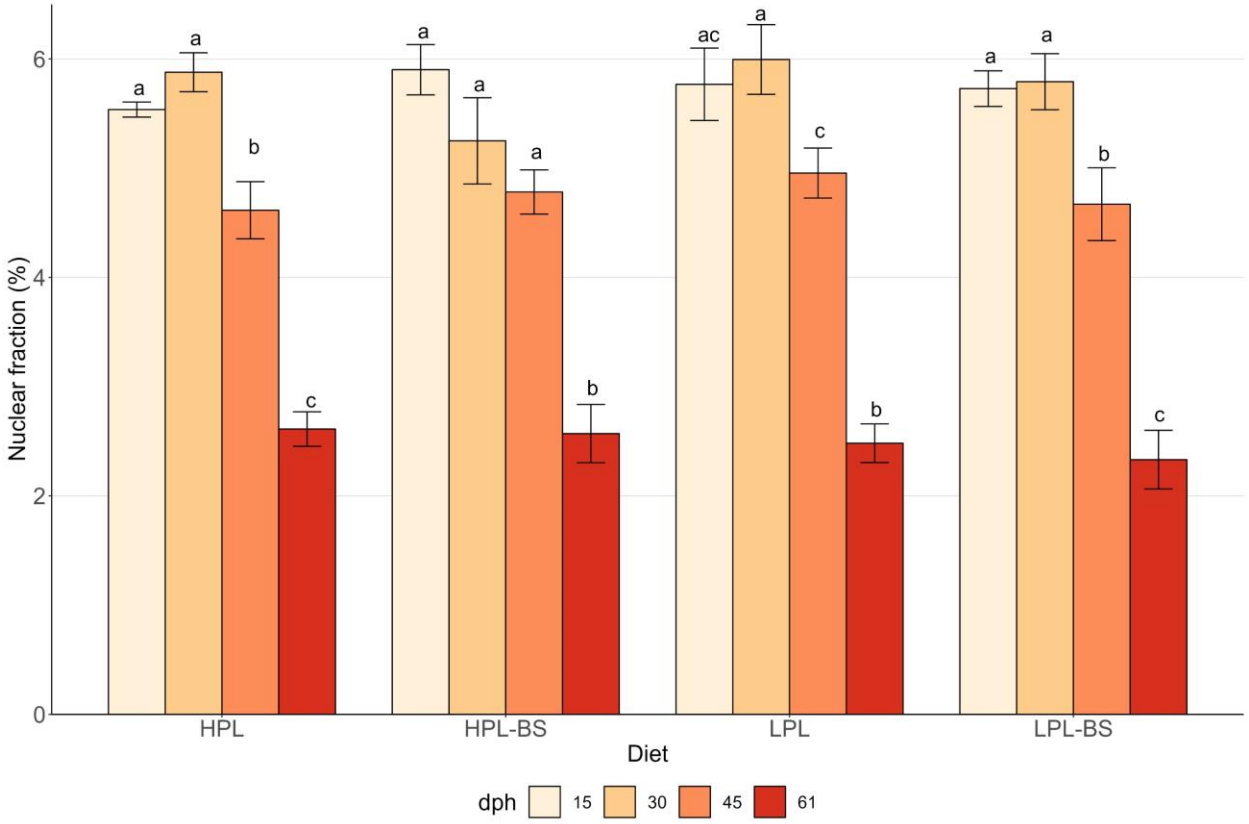

Figure 20 - Mean nuclear fraction of the liver expressed as percentage ± SEM at 15 (n=5), 30 (n=6), 45 (n=6) and 61 (n=6) dph and diet. Different letters indicate statistical difference ($p<0.05$) between dph within the same diet larval groups. Each column represents a dph. The x-axis represents each larval group fed the respective diets.

Table 9 – Pairwise comparisons of sampling points within each larval group for nuclear fraction using ANOVA and Tukey Test (95% Confidence Interval).

	p-value			
dph	HPL	HPL-BS	LPL	LPL-BS
15-30	0.5488	0.3871	0.9349	0.9982
15-45	0.0086	0.0504	0.1886	0.0447
15-61	<0.0001	<0.0001	<0.0001	<0.0001
30-45	0.0004	0.6527	0.0493	0.0313
30-61	<0.0001	<0.0001	<0.0001	<0.0001
45-61	<0.0001	<0.0001	<0.0001	<0.0001

4. Discussion

This study investigated the effect two dietary phospholipid levels supplemented or not with sodium taurocholate, a bile salt, on the growth, survival and the histomorphological development and nutritional status of the liver in Atlantic cod larvae and juveniles. In general, the four diets supported a positive growth but did not influence differently the performance between the larval groups. At the end of the trial no significant differences were found between treatments, neither in DW at 52 dph or SL at 61 dph. Nevertheless, the growth of larvae from the HPL group seemed more stable and exponential than in the other groups from days 30-38 post hatch, even though not significant at the end. After ending the co-feeding period, the weight increase in this larval group tended to be higher than in the other larval groups. The opposite was observed for the LPL group, that presented a worse performance during the dry feed period, although not significant. It is also important to highlight that both LPL groups presented a larger DWI variation during the dry feed period compared to the HPL groups.

The performance observed in the HPL group shows the same tendency as in Wold et al. (2009) study, where a better growth was found when feeding Atlantic cod larvae with higher PL diets (12.5 %) than with lower PL diets (10.7 %). These results demonstrates that diets containing higher PL levels are essential for a better growth performance. However, these findings are related not only to the amount of PL but also to the origin. Kanazawa et al. (1983) found that ayu (*Plecoglossus altivelis*) larvae tended to have a better growth performance when fed bonito egg lecithin than soybean lecithin. Similar findings were found for cod and rainbow trout (*Oncorhynchus mykiss*), where not only growth but also digestive enzymes production was affected by the origin and mount of dietary PL sources (Wold et al., 2007; Azarm et al., 2013).

The developmental stage should also be taken into account when considering PL requirements (Tocher et al., 2008). In Macqueen Leifson et al. (2003), the use of higher PL of marine origin yielded a better growth at initial stages. In fact, when changing the ratio of marine and vegetable PL, Wold et al. (2009) obtained a lower DWI initially, but after weaning, the DWI was higher than when fed a higher marine PL amount, which might be explained by an increased digestive capacity (Wold et al., 2007). Hansen et al. (2018) found that increasing the PL fraction did not improve growth in Atlantic cod larvae but hypothesised that if higher PL diets were introduced earlier, it could have positively impacted the growth. In our results, one can observe a substantial increase in the LPL group from the period 38-45 to 45-52 dph, after being significantly lower than the other groups. The significant difference between the

LPL and LPL-BS groups between the DWI at 38-45 dph might be related to the BS inclusion. Some bile acids, such as cholic acid (CA), were found to serve as attractants in species such as Large Yellow Croaker (*Larimichthys crocea*) (Zhu et al., 2022) However this should be interpreted carefully since there is no data regarding ingestion rates

There were found some interactions between the PL level and the supplementation of BS in our study. At 38 dph the SL was negatively affected by the BS at low PL, while on day 45 post hatch, the DW was negatively affected by the BS at high PL levels. However, these findings were not translated into differences in the growth (DWI) when comparing with the corresponding diets without bile salts. The level of BS used in this study, in a diet for Atlantic cod larvae and juveniles did not bring any improvement into the performance and can be considered negligible.

Larval DW was strongly correlated with larval SL, which resulted in an exponential DW/SL relationship due to the allometric growth pattern of the many organs and musculature (Osse and Boogaart, 2004; Gagnat et al., 2016). The overall growth was allometric throughout the experiment, and not different between the larval groups. However, the growth pattern changed when larvae reached approximately 13.59 mm. From this point on, the slope changed and even though the growth was still allometric, became more isometric. Meaning that the DW increase slowed down as the fish increased in SL. At the SL at which the slope changed, larvae were feeding solely on dry feed and is associated with the start of metamorphosis (Folkvord et al., 1994; Pedersen and Falk-Petersen, 1992; Brown et al., 2003). At this point, a differentiated gut with the presence of stomach tissue was already observed , even though one cannot be sure if gastric digestive functions were already active. Pedersen and Falk-Petersen (1992) found the presence of gastric glands in 18-20 mm larvae, whereas Kamisaka and Rønnestad (2011) observed these glands in larvae with approximately 10 mm. These differences can either be due to protocol differences or sampling time. Anyway, in the latter study, the age at which they found the gastric gland, the fish were 39 dph. In our study, at the same age, the fish were approximately 12 mm, which is close to the length associated with the start of metamorphosis.

Although survival was not significantly different, it appears that the groups fed with the HPL diets had a tendency to have higher survival than the LPL groups. It is worth denoting that the larger variation in the LPL group was related to lower oxygen levels in a tank with higher fish biomass. Cahu et al. (2009) reviews the advantages of using higher PL amounts, which in some cases improves both growth and survival, although this can vary depending on factors such as PL quantity, composition and origin (Kanazawa et al., 1983; Kanazawa, 1993;

Geurden et al., 1998; Cahu et al., 2003; Azarm et al., 2013; Pinto et al., 2022). Copeman and Laurel (2010) also proved that changes in the FA composition affects survival and growth in Pacific cod (*Gadus macrocephalus*). Regarding the BS supplementation, there seems to be a slight trend indicating that supplementing the diets with BS may improve survival independently of the PL level. Li et al. (2021) and Zhang et al. (2022) found the same trend in tongue sole (*Cynoglossus semiliaevis*) and Chinese perch (*Siniperca chuatsi*) juveniles. The later hypothesised that BS enhances the immunity state and modules the gut microbiota, improving survival in Chinese perch. In mice, BS supplementation improved survival on individuals with intestinal disease (Perrone et al., 2010). However, it's important to note that numerous complex factors are at play, and it remains uncertain if the fish survival in our study was influenced by the BS in the diets.

The variation in hepatocyte nucleus size in the larvae were associated with changes in the feeding protocol. The decrease observed in all groups from 15 to 30 dph was related to the decrease in live feed, as also seen in the DWI from 23 to 30 dph, and all groups recovered to the nucleus size observed initially, except the LPL-BS group. In this group, the nucleus size at the end was not significantly different between the larval groups, but the nucleus size development in the LPL-BS group might be a sign of a poorer nutritional status of this larval group. While no signs of degradation of the nuclei was observed, i.e. pyknotic nuclei, smaller nuclei are sign of decreased metabolic activity. Being a central metabolic organ, it necessitates elevated metabolic activity to facilitate rapid energy transfer and growth (Ghadially, 1997; Wold et al., 2009). Wold et al. (2009) found a similar trend in which Atlantic cod larvae fed vegetable PL had smaller nuclei. However, those larvae also presented a smaller hepatocyte size, which was not observed in our study.

The hepatocyte size did not suffer a significant decrease in size from 15 to 30 dph in all groups. However, the HPL-BS and LPL-BS groups at 45 dph still had a similar hepatocyte size to what was observed initially, while the HPL and LPL groups had a small but significant increase. But as the hepatocyte size doubled at 61dph, this cannot be considered detrimental, but related to the increase of glycogen and lipids in the cells (Hoehne-Reitan and Kjørsvik, 2004; Gisbert et al., 2008). Glycogen and lipids vacuoles accumulation (not differentiated) in the liver was low in all larval groups until day 45 post hatch, and not enough to influence hepatocyte size. However, the significant increase observed in the LPL and LPL-BS groups can be a sign of disturbances in lipid transport (Segner and Witt, 1990). Similar findings have been found in gilthead seabream and European seabass larvae fed with reduced levels marine PL, showing lipids accumulation in the hepatocytes (Salhi et al., 1999; Gisbert et al., 2005).

These findings together with the nucleus size in the LPL-BS group might be a sign of a negative effect of the bile salt supplementation in the diet.

Hamre et al. (2011) concluded that 40 dph Atlantic cod larvae has a high capacity to absorb and metabolize lipids up to 100% of the total lipid fraction in the diets. However, that experiment did not investigate the absorption and metabolism of lipids in the liver. In our study, at 61 dph, the vacuolization degree in the liver had increased greatly, and the observed vacuoles were large and equally dispersed in the liver parenchymal cells. These vacuoles were spherical, which is sign of lipids accumulated in the liver cells (Rodrigues et al., 2017). The observed lipid accumulation cannot be considered steatosis at this point, especially because cod stores fat in the liver (dos Santos et al., 1993; Albrektsen et al., 2006; Jobling et al., 2008; Ingebrigtsen et al., 2014). Instead it indicates a good nutritional status and larvae are accumulating lipids for metabolism (Hoehne-Reitan and Kjørsvik, 2004), though one cannot be sure if the observed vacuoles are lipids without a confirmation with a proper staining method. Toluidine blue stains acidic tissue components, having affinity for nucleic acids, binding to nuclear material of tissues with a high DNA and RNA content (Sridharan and Shankar, 2012), and does not affinity for glycogen detection. The reduction of the nucleus fraction is an expected observation since cell space is being occupied by the vacuoles and hepatocytes expand.

To our knowledge, this is the first study where any fish larvae were fed dry feeds supplemented with bile salts. In juvenile and adult marine and freshwater fish the supplementation with bile salts has improved lipid digestibility, especially when high levels of vegetable ingredients are incorporated in the diets (Gu et al., 2017; Yamamoto et al., 2007b). In mammals, bile salts supplementation helps to regulate the lipids metabolism (Clifford et al., 2021). However, sodium taurocholate at a concentration of 0.6 % induced liver damage by cholesterol accumulation in Nile Tilapia, probably due to the cholesterol not being utilized to produce bile salts, while 0.3 % did not show any significant improvements in growth (Ding et al., 2022). The level of BS supplementation in our study might have been insufficient to demonstrate a noticeable impact of its utilization.

But might also be possible that Atlantic cod larvae does not utilize sodium taurocholate at early stages. For example, Hagey et al. (2010) found that immature Senegalese sole (*Solea senegalensis*) and European eel (*Anguilla anguilla*) had a different bile acid profile than the adults. Immature Senegalese sole contained C_{27} bile alcohols and mainly unconjugated cholic acid (CA), while immature European eel contained C_{27} and C_{24} bile acids, and C_{27} and C_{26} bile alcohols. CA belongs to the C_{24} and C_{27} bile acids groups which conjugates with taurine. C_{27}

bile alcohols conjugate with sulphates instead of taurine. Both adult species had mainly taurocholic acid. However, sodium taurocholate is highly digestible at intestinal pH 6-7 (Hofmann and Hagey, 2008), which is in the range observed in Atlantic cod larvae (Tibbetts et al., 2011), gilthead seabream larvae (Yúfera et al., 2004), and of several fresh water species, such as carp, perch (*Perca fluviatilis*) and pikeperch (*Sander lucioperca*) (Solovyev et al., 2015). In turbot larvae, the intestinal pH is slightly higher than 7 (Hoehne-Reitan et al., 2001). Another hypothesis might be related to a shorter gut transit at early stages, which can reduce digestion and absorption of the nutrients and diet components i.e. bile salts (Tonheim et al., 2005; Rønnestad et al., 2009).

The liver histological parameters analysed together with the growth parameters give a good impression of the nutritional status and PL requirements. Our results suggest that a higher PL level, preferably of marine origin, are better utilised for growth. This is also suggested by Wold et al. (2009), that also denotes that the PL requirements are still to be found for Atlantic cod. Further research is needed, especially comparing with intestinal histological parameters as well, to check whether there is a degradation and/or inflammation of the intestinal cells. Gene expression could also be a useful tool to find if the genes involved in bile salts production and absorption are present (Marthinsen et al., in preparation).

5. Conclusion

This study demonstrated that Atlantic cod had a growth performance and high survival independently of the phospholipid level, comparable to other studies. However, there is a pattern in which the higher level of phospholipid gave a better performance throughout the experiment. The same pattern was seen in the survival, where the groups fed higher phospholipid diets might be positively affected than the groups fed lower phospholipid diets. These findings are aligned with previous research where was demonstrated the essentiality of the presence of marine phospholipids for growth. The supplementation of sodium taurocholate in the diet does not seem to have affected the growth of the Atlantic cod larvae and juveniles. Nonetheless, it demonstrated a very slight yet not significant positive effect on survival which was independent of the level of phospholipid in the diet.

The changes in the nutritional biomarkers in the liver, especially the nucleus size, are associate with changes in the feeding protocol, suggesting variations in the nutritional status. After an initial decrease, towards the end of the experiments all groups had recovered to the initial nucleus size observed, except the LPL-BS group. Although the nucleus size in this group did not differ from the other larval groups, at the end of experiment these larvae had a poorer nutritional status. This might be a sign that the sodium taurocholate present in the diet might have negatively influenced the lipid metabolism. Both groups fed low phospholipid diets presented a small significant increase in vacuole content at 45 dph, but only the one fed bile salts supplemented diets showed a reduced nucleus size. The liver vacuolization increased significantly by the end of the experiment in all groups, which can be considered a sign of healthy lipid metabolism and energy storage. These results also demonstrated how sensitive the liver is to nutritional imbalances, especially the nucleus size, which is associate to metabolic activity in the cell.

In summary, Atlantic cod larvae and juveniles fed higher phospholipids levels grow better, might have a positively affected survival rate, and present a good nutritional status in the liver. Even though, no significant effects of the bile salt supplementation in the diets were observed in the fish, our results are inconclusive regarding bile salts in a diet for Atlantic cod at these stages.

In the future, should be possible to do a dose-response experiment with different levels of phospholipids, with different origins, i.e. vegetable and marine, and with different bile salts supplementation amounts.

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7. Appendices

Appendix 1: R packages used for data and statistical analysis.

Package name	Version	Description
car	3.1-0	Companion to Applied Regression
cowplot	1.1.1	Streamlined Plot Theme and Plot Annotations for 'ggplot2'
dplyr	1.1.0	A Grammar of Data Manipulation
emmeans	1.8.2	Estimated Marginal Means, aka Least-Squares Means
ggpattern	1.0.1	'ggplot2' Pattern Geoms
ggplot2	3.4.1	Create Elegant Data Visualisations Using the Grammar of Graphics
ggpubr	0.4.0	"ggplot2" Based Publication Ready Plots
ggtext	0.1.2	Improved Text Rendering Support for 'ggplot2'
glmm	1.4.4	Generalized Linear Mixed Models via Monte Carlo Likelihood Approximation
glmmTMB	1.1.7	Generalized Linear Mixed Models using Template Model Builder
grid	4.2.1	The Grid Graphics Package
lme4	1.1-30	Linear Mixed-Effects Models using "Eigen" and S4
lmerTest	3.1-3	Tests in Linear Mixed Effects Models
magick	2.7.3	Advanced Graphics and Image-Processing in R
MASS	7.3-58.1	Support Functions and Datasets for Venables and Ripley's MASS
MuMIn	1.47.1	Multi-Model Inference
olsrr	0.5.3	Tools for Building OLS Regression Models
plotrix	3.8-2	Various Plotting Functions
plyr	1.8.7	Tools for Splitting, Applying and Combining Data
reshape	0.8.9	Flexibly Reshape Data
Rmisc	1.5.1	Ryan Miscellaneous
scales	1.2.1	Scale Functions for Visualization
segmented	1.6-2	Regression Models with Break-Points / Change-Points (with Possibly Random Effects) Estimation
sjPlot	2.8.14	Data Visualization for Statistics in Social Science
survival	3.4-0	Survival Analysis
survminer	0.4.9	Drawing Survival Curves using 'ggplot2'

tidyr	1.3.0	Tidy Messy Data
tidyverse	2.0.0	Easy Instal and Load of the "Tidyverse" The tidyverse encompasses the repeated tasks at the heart of every data science project: data import, tidying, manipulation, visualisation, and programming.
TMB	1.9.1	Template Model Builder: A General Random Effect Tool Inspired by 'ADMB'
viridis	0.6.2	Colorblind-Friendly Color Maps for R

Appendix 2: Temperature and water flow adjustments during the experiment.

dph	0	1	2	3	4	5	6	7	8	9	10	11	12-14
Temperature (°C)	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12
Water flow (Tank volume per day)	8	1	2										
Water flow (L min^{-1})	1.11	0.14	0.28										

dph	15-24	25-30	31-50	51-61
Temperature (°C)	12			
Water flow (Tank volume per day)	4	6	8	13
Water flow (L min^{-1})	0.56	0.83	1.11	1.81

Appendix 2: Growth results for the whole duration of the experiment.

Table A3.1: Mean dry weight, expressed in mg, and SEM per tank from 2 to 23 dph.

dph	Diet	Tank	DW±SEM ($mg.larva^{-1}$)	n	dph	Diet	Tank	DW±SEM ($mg.larva^{-1}$)	n
2	Live feed	1	0.049±0.003	6	15	Live feed	1	0.182±0.021	6
		2	0.052±0.002	6			2	0.201±0.013	6
		3	0.051±0.002	6			3	0.182±0.005	6
		4	0.058±0.002	6			4	0.170±0.016	6
		5	0.055±0.003	6			5	0.182±0.019	6
		6	0.047±0.001	6			6	0.203±0.011	6
		7	0.055±0.003	6			7	0.224±0.005	6
		8	0.050±0.003	6			8	0.198±0.008	6
		10	0.050±0.002	6			10	0.207±0.011	6
		11	0.049±0.001	6			11	0.170±0.020	6
		12	0.052±0.002	6			12	0.192±0.014	6
		14	0.052±0.002	6			14	0.195±0.019	6
8	Live feed	1	0.072±0.003	6	23	HPL-BS	1	0.536±0.036	8
		2	0.062±0.009	6			4	0.551±0.049	8
		3	0.079±0.003	6			5	0.545±0.054	8
		4	0.061±0.004	6		HPL	3	0.567±0.039	8
		5	0.066±0.007	6			7	0.519±0.066	8
		6	0.066±0.004	6			10	0.629±0.046	8
		7	0.075±0.002	6		LPL	2	0.589±0.048	8
		8	0.070±0.005	6			8	0.577±0.073	8
		10	0.076±0.001	6			11	0.526±0.057	8
		11	0.069±0.004	6		LPL-BS	6	0.500±0.028	8
		12	0.067±0.007	6			12	0.611±0.059	8
		14	0.068±0.010	6			14	0.564±0.034	8

Table A3.2: Mean dry weight, expressed in mg, and SEM per tank from 30 to 52 dph.

dph	Diet	Tank	DW±SEM (mg.larva^{-1})	n	dph	Diet	Tank	DW±SEM (mg.larva^{-1})	n
30	HPL-BS	1	0.867±0.083	8	45	HPL-BS	1	3.155±0.445	16
		4	1.388±0.139	8			4	4.487±0.558	16
		5	0.836±0.088	8			5	3.781±0.256	16
	HPL	3	0.838±0.039	8		HPL	3	4.395±0.485	16
		7	1.044±0.113	8			7	4.399±0.544	16
		10	0.964±0.138	8			10	5.084±0.450	16
	LPL	2	1.059±0.193	8		LPL	2	3.225±0.330	16
		8	1.048±0.132	8			8	3.530±0.430	16
		11	0.860±0.104	8			11	3.020±0.357	16
	LPL-BS	6	0.768±0.100	8		LPL-BS	6	3.733±0.342	16
		12	0.956±0.100	8			12	3.377±0.254	16
		14	0.887±0.056	8			14	3.423±0.338	16
38	HPL-BS	1	2.052±0.167	16	52	HPL-BS	1	6.415±0.929	16
		4	2.392±0.212	16			4	7.510±0.909	16
		5	2.385±0.213	16			5	6.859±0.781	16
	HPL	3	2.601±0.159	16		HPL	3	6.938±1.193	16
		7	2.380±0.185	16			7	7.391±0.968	16
		10	2.808±0.245	16			10	9.340±1.172	16
	LPL	2	2.200±0.150	16		LPL	2	4.822±0.555	16
		8	2.403±0.195	16			8	7.888±0.780	16
		11	2.243±0.227	16			11	6.196±0.972	16
	LPL-BS	6	2.048±0.254	16		LPL-BS	6	4.907±0.807	16
		12	2.223±0.136	16			12	6.830±0.628	16
		14	1.759±0.187	16			14	7.419±0.721	16

Table A3.3: Mean dry weight, expressed in mg, and SEM per treatment from 2 to 52 dph.

dph	Diet	DW±SEM (mg.larva^{-1})	n	dph	Diet	DW±SEM (mg.larva^{-1})	n
				38	HPL	2.596±0.116	48
2	Live feed	0.052±0.001	72		HPL-BS	2.276±0.115	48
8	Live feed	0.069±0.002	72		LPL	2.282±0.110	48
15	Live feed	0.192±0.01	72		LPL-BS	2.010±0.116	48
23	HPL	0.571±0.030	24	45	HPL	4.626±0.283	48
	HPL-BS	0.544±0.026	24		HPL-BS	3.808±0.260	48
	LPL	0.564±0.034	24		LPL	3.258±0.214	48
	LPL-BS	0.559±0.025	24		LPL-BS	3.511±0.179	48
30	HPL	0.949±0.061	24	52	HPL	7.889±0.648	48
	HPL-BS	1.031±0.079	24		HPL-BS	6.928±0.499	48
	LPL	0.989±0.084	24		LPL	6.302±0.481	48
	LPL-BS	0.870±0.051	24		LPL-BS	6.386±0.437	48

Table A3.5: Mean daily weight increase, expressed in %, and SEM per treatment from 2 to 52 dph.

dph	Diet	DWI±SEM (%)	dph	Diet	DWI±SEM (%)
			45-52	HPL	7.84±0.68
				HPL-BS	9.06±0.88
2-8	Live feed	5.07±0.57		LPL	9.63±1.90
8-15	Live feed	15.68±0.45		LPL-BS	8.75±2.41
15-23	HPL	13.74±1.34	2-15	HPL	11.10±0.41
	HPL-BS	15.00±0.42		HPL-BS	9.59±0.58
	LPL	14.62±0.27		LPL	10.74±0.35
	LPL-BS	13.93±1.06		LPL-BS	11.18±0.38
23-30	HPL	7.52±1.51	15-38	HPL	11.70±0.44
	HPL-BS	9.17±2.48		HPL-BS	11.71±0.32
	LPL	8.31±0.52		LPL	11.43±0.26
	LPL-BS	6.53±0.12		LPL-BS	10.62±0.35
30-38	HPL	13.45±1.33	38-52	HPL	8.22±0.50
	HPL-BS	10.80±2.03		HPL-BS	8.28±0.22
	LPL	11.08±0.92		LPL	7.38±0.90
	LPL-BS	11.03±1.19		LPL-BS	8.54±1.27
38-45	HPL	8.60±0.42	15-52	HPL	10.37±0.27
	HPL-BS	7.52±0.95		HPL-BS	10.40±0.20
	LPL	5.20±0.43		LPL	9.88±0.46
	LPL-BS	8.36±1.14		LPL-BS	9.82±0.42

Table A3.5: Mean standard length, expressed in mm, and SEM per tank from 2 to 23 dph.

dph	Diet	Tank	SL±SEM (mm.larva^{-1})	n	dph	Diet	Tank	SL±SEM (mm.larva^{-1})	n
2	Live feed	1	4.48±0.11	6	15	Live feed	1	6.36±0.20	6
		2	4.57±0.05	6			2	6.41±0.17	6
		3	4.30±0.07	6			3	6.32±0.08	6
		4	4.40±0.04	6			4	6.21±0.17	6
		5	4.55±0.03	6			5	6.30±0.15	6
		6	4.35±0.08	6			6	6.48±0.06	6
		7	4.51±0.10	6			7	6.78±0.06	6
		8	4.23±0.16	6			8	6.29±0.16	6
		10	4.49±0.04	6			10	6.60±0.11	6
		11	4.63±0.05	6			11	6.15±0.23	6
		12	4.53±0.06	6			12	6.29±0.15	6
		14	4.39±0.10	6			14	6.40±0.15	6
8	Live feed	1	5.02±0.08	6	23	HPL-BS	1	8.50±0.21	8
		2	4.95±0.15	6			4	8.58±0.26	8
		3	5.13±0.15	6			5	8.62±0.29	8
		4	4.81±0.08	6		HPL	3	8.68±0.16	8
		5	4.99±0.10	6			7	8.33±0.33	8
		6	4.92±0.14	6			10	8.93±0.18	8
		7	5.07±0.06	6		LPL	2	8.70±0.21	8
		8	4.99±0.12	6			8	8.56±0.33	8
		10	5.13±0.10	6			11	8.23±0.29	8
		11	5.01±0.09	6		LPL-BS	6	8.40±0.15	8
		12	4.84±0.16	6			12	8.76±0.23	8
		14	4.97±0.15	6			14	8.63±0.25	8

Table A3.6: Mean standard length, expressed in mm, and SEM per tank from 30 to 61 dph.

dph	Diet	Tank	SL±SEM (mm.larva^{-1})	n	dph	Diet	Tank	SL±SEM (mm.larva^{-1})	n
30	HPL-BS	1	9.39±0.26	8	45	HPL-BS	1	12.13±0.47	16
		4	10.47±0.35	8			4	14.63±0.62	16
		5	9.23±0.26	8			5	13.78±0.38	16
	HPL	3	9.22±0.19	8		HPL	3	14.22±0.61	16
		7	9.68±0.34	8			7	14.06±0.59	16
		10	9.61±0.39	8			10	14.72±0.63	16
	LPL	2	9.47±0.42	8		LPL	2	13.29±0.52	16
		8	9.64±0.37	8			8	13.13±0.48	16
		11	9.28±0.38	8			11	13.12±0.58	16
	LPL-BS	6	8.91±0.36	8		LPL-BS	6	13.55±0.53	16
		12	9.70±0.31	8			12	13.63±0.35	16
		14	9.34±0.21	8			14	13.41±0.51	16
38	HPL-BS	1	11.97±0.26	16	52	HPL-BS	1	16.04±0.87	16
		4	12.69±0.37	16			4	17.54±0.79	16
		5	11.74±0.47	16			5	16.64±0.64	16
	HPL	3	12.61±0.33	16		HPL	3	17.16±0.85	16
		7	11.92±0.44	16			7	16.72±0.77	16
		10	12.05±0.41	16			10	15.83±0.95	16
	LPL	2	12.26±0.26	16		LPL	2	15.25±0.60	16
		8	12.85±0.23	16			8	17.54±0.69	16
		11	12.27±0.45	16			11	15.83±1.08	16
	LPL-BS	6	11.33±0.26	16		LPL-BS	6	13.71±0.69	16
		12	11.46±0.27	16			12	17.27±0.53	16
		14	10.46±0.44	16			14	16.83±0.51	16
61	HPL-BS	1	21.64±0.35	20	61	LPL	2	24.76±0.51	20
		4	24.27±0.36	20			8	24.20±0.42	20
		5	24.09±0.29	20			11	22.45±0.30	20
	HPL	3	21.88±0.41	20		LPL-BS	6	22.15±0.34	20
		7	23.23±0.31	20			12	22.04±0.33	20
		10	25.45±0.36	20			14	24.18±0.38	20

Table A3.7: Mean standard length, expressed in mm, and SEM per treatment from 2 to 61 dph.

dph	Diet	SL±SEM (mm.larva^{-1})	n	dph	Diet	SL±SEM (mm.larva^{-1})	n
				38	HPL	12.194±0.229	48
2	Live feed	4.443±0.026	72		HPL-BS	12.135±0.222	48
8	Live feed	4.985±0.033	72		LPL	12.460±0.191	48
15	Live feed	6.389±0.045	72		LPL-BS	11.086±0.204	48
23	HPL	8.646±0.140	24	45	HPL	14.334±0.343	48
	HPL-BS	8.566±0.142	24		HPL-BS	13.512±0.389	48
	LPL	8.497±0.161	24		LPL	13.179±0.296	48
	LPL-BS	8.597±0.122	24		LPL-BS	13.532±0.262	48
30	HPL	9.502±0.180	24	52	HPL	17.151±0.482	48
	HPL-BS	9.695±0.198	24		HPL-BS	16.622±0.440	48
	LPL	9.467±0.217	24		LPL	16.208±0.489	48
	LPL-BS	9.319±0.177	24		LPL-BS	15.938±0.437	48
				61	HPL	23.52±0.23	60
					HPL-BS	23.33±0.21	60
					LPL	23.80±0.25	60
					LPL-BS	22.79±0.21	60

Appendix 4: Survival results at the end of the experiment

Table A4.1: Mean survival, expressed as percentage (%) per tank at 61 dph.

dph	Diet	Tank	SL±SEM (mm.larva^{-1})
61	HPL-BS	1	56.18
		4	59.68
		5	62.59
	HPL	3	46.04
		7	53.45
		10	59.52
	LPL	2	60.93
		8	40.74
		11	24.55
	LPL-BS	6	57.38
		12	50.74
		14	42.15

Table A4.2: Mean survival, expressed as percentage (%) and SEM per treatment at 61 dph.

dph	Diet	SL±SEM (mm.larva^{-1})
61	HPL-BS	59.48±1.85
	HPL	53.00±3.90
	LPL	42.07±10.52
	LPL-BS	50.09±4.41

Appendix 5: Histomorphological results for the whole duration of the experiment.

Table A5.1: Mean standard length, nucleus size, hepatocyte size, vacuoles fraction and nucleus fraction, and SEM per tank at 15 and 30 dph.

dph	Diet	Tank	SL±SEM (mm.larva^{-1})	Nucleus size (µm^2.larva^{-1})	Hepatocyte size (µm^2.larva^{-1})	Vacuoles fraction (%)	Nucleus fraction (%)	n
15	HPL-BS	1	6.34±0.27	24.17±0.88	439.54±49.25	1.29±0.03	5.55±0.42	2
		4	5.54±0.23	22.15±1.82	388.41±17.47	1.55±0.23	5.70±0.21	2
		5	6.09±0.58	21.16±0.72	327.64±4.47	0.99±0.68	6.46±0.31	2
	HPL	3	5.72±0.24	20.17±0.55	369.47±24.96	0.82±0.02	5.47±0.23	2
		7	5.96±0.19	18.42±0.17	334.00±0.03	0.81±0.01	5.52±0.05	2
		10	6.21±0.15	20.52±0.47	365.30±2.83	0.66±0.38	5.62±0.08	2
	LPL	2	5.72±0.25[1]	18.17	316.57	0.63	5.74	2
		8	5.62±0.58	17.00±0.23	289.48±43.48	0.64±0.14	6.02±0.98	2
		11	5.66±0.27	18.12±0.61	327.50±4.94	0.51±0.17	5.54±0.10	2
	LPL-BS	6	5.81±0.21	22.80±1.38	382.47±52.62	1.10±0.18	6.03±0.47	2
		12	6.04±0.32	22.22±1.23	389.99±11.70	0.22±0.00	5.69±0.14	2
		14	6.16±0.15	19.52±0.80	357.32±19.69	0.48±0.05	5.47±0.08	2
30	HPL-BS	1	9.59±0.07	15.25±0.77	367.22±26.22	3.88±1.68	4.19±0.51	2
		4	10.27±0.43	15.48±0.79	289.02±20.22	0.78±0.23	5.36±0.10	2
		5	9.42±0.02	17.22±0.71	277.96±18.16	0.48±0.24	6.20±0.15	2
	HPL	3	9.25±0.28	16.09±0.22	290.44±7.20	1.12±0.56	5.54±0.21	2
		7	9.55±0.24	15.78±1.14	253.62±4.65	2.53±1.13	6.22±0.33	2
		10	9.00±0.38	16.33±0.98	279.54±31.22	1.84±0.30	5.88±0.30	2
	LPL	2	9.98±0.22	15.81±0.43	308.33±0.03	1.10±0.13	5.13±0.14	2
		8	9.10±0.06	14.46±0.24	223.91±16.65	2.47±0.68	6.50±0.59	2
		11	9.50±0.46	16.43±0.62	258.86±16.14	0.92±0.12	6.36±0.15	2
	LPL-BS	6	8.57±0.01	15.15±1.18	276.94±4.40	1.29±0.33	5.48±0.51	2
		12	9.16±0.04	16.94±0.15	286.52±11.22	2.14±1.08	5.86±0.18	2
		14	8.89±0.19	15.55±0.46	260.50±23.81	1.28±0.54	6.04±0.73	2

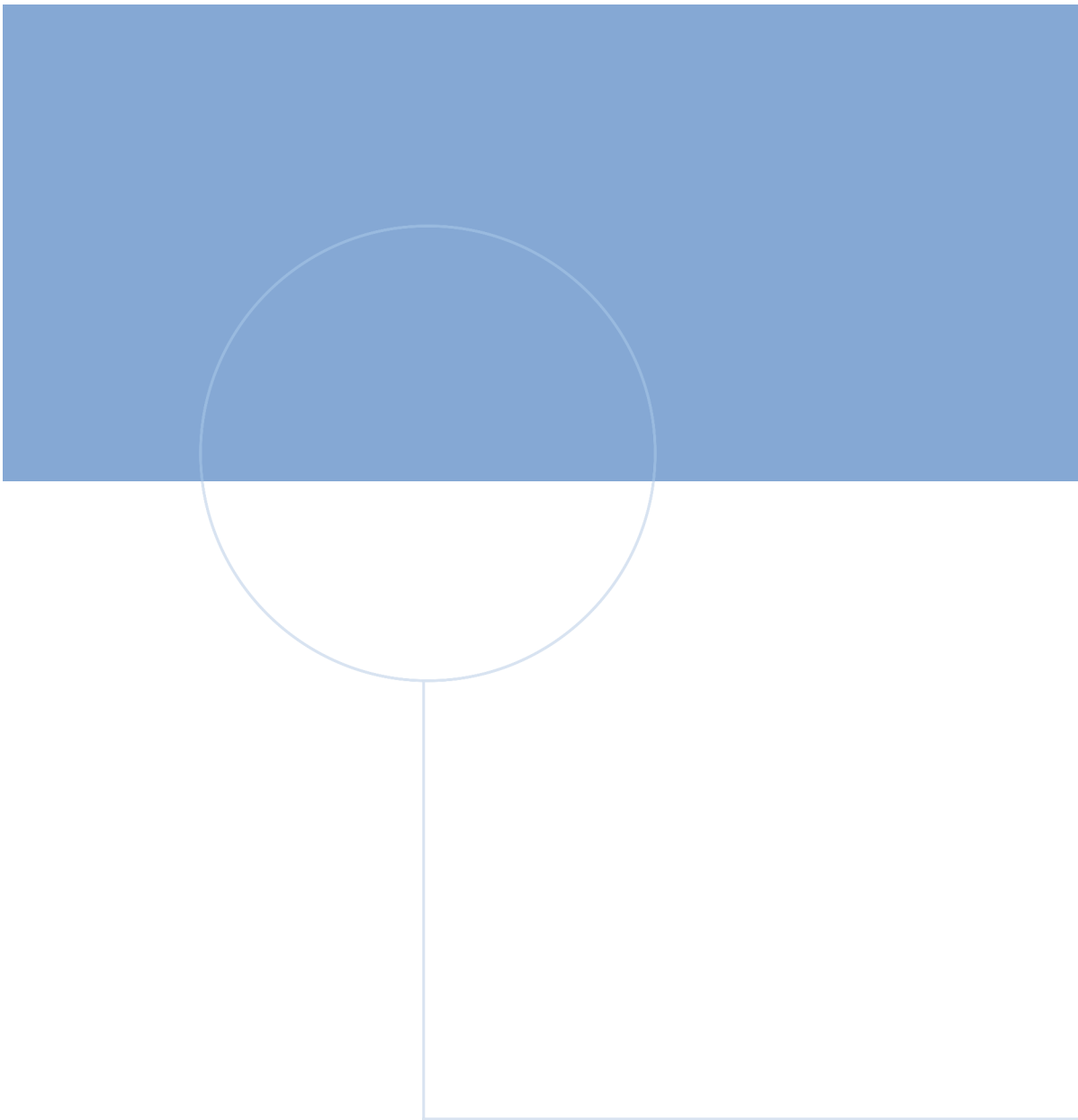
[1]Standard length was measured in two larvae. However, all the histomorphological parameters were only measured in one larva due to loss of the second during histological works.

Table A5.2: Mean standard length, nucleus size, hepatocyte size, vacuoles fraction and nucleus fraction, and SEM per treatment at 45 and 61 dph.

dph	Diet	Tank	SL±SEM (mm.larva^{-1})	Nucleus size (µm^2.larva^{-1})	Hepatocyte size (µm^2.larva^{-1})	Vacuoles fraction (%)	Nucleus fraction (%)	n
45	HPL-BS	1	12.22±0.37	17.88±0.26	346.54±11.70	5.36±1.11	5.17±0.25	2
		4	13.19±0.35	19.44±2.34	414.08±48.38	8.90±8.51	4.70±0.02	2
		5	12.72±1.00	19.44±1.33	444.06±84.02	10.14±8.61	4.48±0.55	2
	HPL	3	12.98±1.01	18.36±0.71	420.86±21.81	3.24±0.40	4.38±0.40	2
		7	13.49±0.58	16.47±1.73	332.15±28.62	2.09±0.47	4.96±0.09	2
		10	14.11±1.18	19.62±0.11	451.23±85.26	1.04±0.45	4.50±0.82	2
	LPL	2	14.81±0.77	18.23±2.75	343.90±30.85	3.59±1.83	5.27±0.33	2
		8	12.67±0.32	16.65±0.09	333.74±13.84	2.02±1.15	5.00±0.18	2
		11	12.47±0.02	16.78±0.59	370.66±39.01	3.06±0.73	4.60±0.64	2
	LPL-BS	6	12.82±0.47	17.58±0.22	364.22±57.89	1.01±0.18	4.94±0.73	2
		12	12.63±1.21	17.32±1.13	351.25±27.65	4.02±0.17	4.98±0.72	2
		14	13.32±0.10	17.98±0.00	443.66±34.94	5.56±4.12	4.08±0.32	2
61	HPL-BS	1	24.64±1.60	22.66±2.59	914.78±152.20	28.73±3.04	2.50±0.13	2
		4	25.93±0.21	19.65±1.43	1035.98±79.92	31.59±4.46	1.92±0.29	2
		5	26.18±1.31	20.11±3.00	608.08±73.59	39.62±3.48	3.30±0.09	2
	HPL	3	24.58±0.92	22.42±1.44	891.66±30.45	34.39±8.71	2.51±0.07	2
		7	26.63±2.12	19.66±0.17	712.98±126.37	44.20±16.98	2.85±0.53	2
		10	28.34±1.47	18.91±1.51	764.76±49.46	33.72±0.54	2.47±0.04	2
	LPL	2	25.91±0.39	21.80±1.21	925.07±117.91	34.62±6.01	2.38±0.17	2
		8	25.03±2.36	18.32±1.05	768.70±31.25	38.86±0.32	2.38±0.04	2
		11	25.81±1.54	19.37±1.88	744.71±100.93	38.28±0.00	2.68±0.62	2
	LPL-BS	6	25.26±1.49	16.84±0.36	677.31±188.56	34.16±1.00	2.71±0.81	2
		12	25.14±1.37	18.84±0.08	947.52±197.50	35.80±1.95	2.08±0.42	2
		14	25.47±1.84	18.70±3.61	860.91±218.52	36.26±3.19	2.21±0.14	2

Table A5.3: Mean standard length, nucleus size, hepatocyte size, vacuoles fraction and nucleus fraction, and SEM per treatment at 15, 30, 45 and 61 dph.

dph	Diet	SL±SEM (mm.larva^{-1})	Nucleus size (µm^2.larva^{-1})	Hepatocyte size (µm^2.larva^{-1})	Vacuoles fraction (%)	Nucleus fraction (%)	n
15	HPL	5.96±0.13	19.70±0.45	356.26±9.60	0.76±0.10	5.54±0.07	6
	HPL-BS	5.99±0.23	22.49±0.79	310.11±24.53	1.28±0.21	5.90±0.23	6
	LPL	5.67±0.18	17.68±0.35	385.20±16.32	0.58±0.08	5.77±0.33	5
	LPL-BS	6.00±0.12	21.52±0.82	376.59±16.08	0.60±0.17	5.73±0.16	6
30	HPL	9.27±0.17	16.07±0.41	274.54±10.84	1.83±0.42	5.88±0.18	6
	HPL-BS	9.76±0.20	15.98±0.52	311.40±20.27	1.71±0.82	5.25±0.39	6
	LPL	9.52±0.21	15.57±0.42	263.70±16.61	1.50±0.36	6.00±0.32	6
	LPL-BS	8.87±0.12	15.88±0.48	275.65±8.70	1.57±0.37	5.79±0.26	6
45	HPL	13.52±0.47	18.15±0.76	401.41±32.89	2.13±0.45	4.62±0.26	6
	HPL-BS	12.71±0.34	18.92±0.77	401.56±31.12	8.13±3.27	4.78±0.20	6
	LPL	13.31±0.52	17.22±0.79	349.43±15.04	2.89±0.66	4.96±0.23	6
	LPL-BS	12.92±0.36	17.63±0.32	386.38±26.26	3.53±1.36	4.67±.033	6
61	HPL	26.52±0.99	20.33±0.87	789.80±49.16	37.44±5.38	2.61±0.16	6
	HPL-BS	25.58±0.61	20.81±1.24	852.94±93.90	33.31±2.65	2.57±0.27	6
	LPL	25.58±0.76	19.83±0.91	812.83±54.32	37.26±1.77	2.48±0.18	6
	LPL-BS	25.29±0.71	18.13±1.02	828.58±103.40	35.41±1.08	2.33±0.27	6



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