Master's thesis

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

Sunniva Brevik Kværnø

Growth, survival and liver histology in lumpfish (*Cyclopterus lumpus*) larvae fed different start-feeding diets (*Artemia*, copepods, cirripeds and formulated diet)

Master's thesis in Ocean Resources
Supervisor: Elin Kjørsvik
Co-supervisor: Tu Anh Vo, Arne Malzahn & Andreas Hagemann
February 2022

NTNU
Norwegian University of
Science and Technology

Sunniva Brevik Kværnø

Growth, survival and liver histology in lumpfish (*Cyclopterus lumpus*) larvae fed different start-feeding diets (*Artemia*, copepods, cirripeds and formulated diet)

Master's thesis in Ocean Resources
Supervisor: Elin Kjørsvik
Co-supervisor: Tu Anh Vo, Arne Malzahn & Andreas Hagemann
February 2022

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

NTNU
Norwegian University of
Science and Technology

Acknowledgements

This Master's Thesis was written at the Department of Biology, at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was written under the guidance of main supervisor Elin Kjørsvik and co-supervisors Tu Anh Vo at NTNU, and Arne Malzahn and Andreas Hagemann at SINTEF Ocean AS. The experimental work was conducted in the facilities of NTNU Centre of Fisheries and Aquaculture (SeaLab) and SINTEF Ocean AS from September 9th to October 14th 2020. The experiment was part of the research project STARTRENS (901561) mainly funded by the Norwegian Seafood Research Fund (FHF), but also by the collaborative partners NTNU and SINTEF Ocean AS.

First, I would like to thank my main-supervisor Elin Kjørsvik for her guidance and valuable feedback in the writing of the thesis. I would also like to thank my co-supervisor Tu Anh Vo at NTNU for helping me with writing and statistics, as well as guidance under the experimental work. Furthermore, I would like to thank my two co-supervisors at SINTEF Ocean AS, Arne Malzahn and Andreas Hagemann for valuable feedback on the last draft of my thesis. Also, a big thanks to Frank Thomas Mlingi and Luciana Alves Musialak for your help and guidance under the experimental work. Thank you, Iurgi Imanol Salaverria-Zabalegui, Arne Kjøsnes and Dag Altin for technical help during the start-feeding experiment. I am also grateful to Tora Bardal for invaluable help during the analytical work and in the lab. Additionally, I want to thank Joachim Larsen Marthinsen for discussing the methodology with me.

Thanks to my fellow students at SeaLab for many long coffee breaks and great discussions, making these two years memorable. I would also like to thank my two fellow students in this experiment, Marte Solli Lindskog and Saba Akbar, for great teamwork and constructive discussions in retrospect of the experiment.

Lastly, I would like to thank my family and closest friends for all their love, help and encouragement, as it proved to be particularly challenging to do lab work and write a thesis during a global pandemic – I could not have done it without you.

Sunniva Brevik Kværnø

Trondheim, February 2022

Abstract

The Norwegian production of Atlantic salmon (*Salmo salar*) has since 2012 been stable, mainly due to problems with salmon lice (*Lepeophtheirus salmonis*). To combat the lice, lice-grazing cleaner fish has been deployed into the net-pens. The most used cleaner fish today is lumpfish (*Cyclopterus lumpus*), which of the majority stems from commercial farming. Nevertheless, the lumpfish production has been characterised by variable growth and survival, especially in the larval stage. This is due to the fact that there is poor knowledge about the species' biology and its nutritional needs. There are also only a few studies that have taken a closer look at the lumpfish' liver, which is necessary to interpret the fish' nutritional status, as this is an important organ in the digestive system.

The purpose of the present study was to contribute to optimizing start-feeding regimes for lumpfish larvae in commercial farming. This was achieved by comparing effects of different start-feeding regimes with respect to the growth and survival of the fish larvae. In addition, the nutritional status of the fish was examined by histological analysis of the liver. A total of five different start-feeding regimes were examined, where the larvae were introduced to the feeding regimens at 2 days post hatch (dph) and lasted until 35 dph. Larvae from one group received enriched *Artemia* before weaned to formulated diet, while another group were given cirripeds (*Semibalanus balanoides*) before the weaning. Two larval groups were fed copepods (*Acartia tonsa*) in the beginning, of which one group had an early weaning to formulated diet, whereas the other group were given cirripeds and then weaned to formulated diet. The last group were given formulated diet throughout the whole start-feeding experiment. Common for all the feeding regimes were weaning to the same formulated diet before the experiments end at 35 dph.

Lumpfish larvae fed enriched *Artemia* had the best growth and survival throughout the experiment and showed early signs that excess energy was stored in the form of vacuoles in the liver, the latter in contrast to the other larval groups. Larvae fed cirripeds grew slow and had a slight lower survival rate before the weaning to formulated diet but ended up at almost the same size as the larvae fed *Artemia* due to a higher growth rate at weaning. In contrast, the larvae fed cirripeds had few signs of high nutritional status in the liver before weaning, as they had significantly smaller hepatocytes and a lower vacuole fraction than the larvae fed *Artemia*. This suggests that the rapid growth was due to an increased ability to digest and assimilate the nutrients in the formulated diet after the period fed cirripeds. Both groups given copepods had lowered growth and survival compared to the larvae fed *Artemia* and cirripeds, especially the group that had early weaning to formulated diet. Despite the fact that the other group received cirripeds after the copepod-period, this group did not show the same tendency for rapid growth during weaning to formulated diet like the larvae fed only cirripeds prior to weaning, indicating that the copepod-period was the reason for this. Larvae fed formulated diet throughout the whole experiment also had lower growth and survival than the groups given *Artemia* and cirripeds. The results therefore indicate that a late weaning to formulated diet after a longer period of live feed could be beneficial, and that perhaps a combination of *Artemia* and cirripeds before formulated diet could be an optimal diet for lumpfish larvae. At the experiments end, when all the larval groups received the same formulated diet, the larvae showed no significant differences in neither hepatocyte size, hepatocyte nucleus size nor vacuole fraction. Thus, it is evident that changes in the liver are reversible up to a certain point and can give an indication of what the larvae eats. On the other hand, the hepatocyte size and vacuole fraction may be better suited than hepatocyte nucleus size in evaluating the lumpfish larvae's nutritional status, as the findings among the former two correlated better with the fish' growth and survival during the experimental period.

Sammendrag

Den norske produksjonen av atlantisk laks (*Salmo salar*) har siden 2012 vært stabil, hovedsakelig på grunn av problemer med lakselus (*Lepeophtheirus salmonis*). For å bekjempe lusa har det blitt satt ut lusespisende rensefisk i merdene. Rensefisken som er mest brukt i dag er rognkjeks (*Cyclopterus lumpus*), og størsteparten av denne kommer fra oppdrett. Derimot så har rognkjeksproduksjonen vært preget av variabel vekst og overlevelse, da særlig i larvestadiet. Dette begrunnes med at det er lite kunnskap om artens biologi og dens næringsbehov. Det er også kun få studier som har sett nærmere på rognkjeks larvenes lever, noe som er nødvendig for å tolke fiskens næringsstatus, da dette er et viktig organ i fordøyelsessystemet.

Formålet med denne studien var å bidra til å optimalisere startfôringsregimer til rognkjekslarver i oppdrett. Dette ble gjort ved å sammenligne effekten av ulike start-fôrings regimer med hensyn på fiskelarvenes vekst og overlevelse. I tillegg ble fiskens næringsstatus undersøkt ved histologisk analyse av leveren. Til sammen ble fem ulike startfôringsregimer undersøkt, hvor larvene ble introdusert til fôringsregimene 2 dager etter klekking og varte til 35 dager etter klekking. Larver fra en gruppe fikk anriket *Artemia* før tilvenning til tørrfôr, mens en annen gruppe ble gitt cirripedier (*Semibalanus balanoides*) før tilvenningen. To andre larvegrupper ble gitt copepoder (*Acartia tonsa*) i starten, hvorav den ene gruppen hadde en tidlig overgang til tørrfôr, mens den andre gruppen ble gitt cirripedier for deretter å bli tilvennet tørrfôr. Den siste gruppen ble gitt tørrfôr gjennom hele starfôringsperioden. Felles for fôringsregimene var at larvene ble tilvennet det samme tørrfôret innen forsøkets slutt ved 35 dager etter klekking.

Rognkjekslarver fôret anrikede *Artemia* hadde best vekst og overlevelse gjennom forsøket og viste også tidlig tegn på at det ble lagret overskuddsenergi i form av vakuoler i leveren, i motsetning til de andre larvegruppene. Larver fôret cirripedier vokste sent og hadde noe lavere overlevelse før tilvenningen til tørrfôr, men endte opp på tilnærmet lik størrelse som larvene fôret *Artemia* ved forsøkets slutt, grunnet en høyere vekstrate ved tilvenningen. Derimot hadde larvene fôret cirripedier få tegn på høy næringsstatus i leveren før tilvenningen, da de hadde signifikant mindre størrelse på hepatocyttene og lavere vakuolefraksjon enn larvene fôret *Artemia*, noe som hentyder at den raske tilveksten kom av en økt evne til å fordøye og assimilere næringsstoffene i tørrfôret etter perioden gitt cirripedier. Begge gruppene gitt copepoden *A. tonsa* hadde lav vekst og overlevelse, særlig gruppen som hadde tidlig tilvenning til tørrfôr. Til tross for at den ene gruppen ble gitt cirripedier etter copepod-perioden, viste ikke denne gruppen denne samme tendensen til rask vekst ved tilvenning til tørrfôr slik som larvene fôret kun cirripedier før tilvenningen, noe som peker på at copepod-perioden var årsaken til dette. Larver gitt tørrfôr gjennom hele forsøket hadde også lavere vekst og overlevelse enn de to gruppene gitt *Artemia* og cirripedier. Resultatene tilsier derfor at en senere tilvenning til tørrfôr etter en lengre periode med levendefôr kan være fordelaktig, samt at en kombinasjon av både Artemia og cirripedier før tørrfôr kan være en optimal diett for rognkjeks larver. Ved forsøkets slutt, da alle larvegruppene fikk det samme tørrfôret, var det ingen signifikante forskjeller i hverken hepatocytt-størrelse, hepatocytt-kjernestørrelse eller vakuolefraksjon mellom gruppene. Det er derfor tydelig at endringer i leveren er reversbare til et visst punkt og kan gi et uttrykk for hvilket fôr larvene får. Videre så kan det tyde på at hepatocytt-størrelse og vakuolefraksjon er bedre egnet en hepatocytt-kjernestørrelse i evaluering av rognkjekslarvenes næringsstatus, da funnene blant de to førstnevnte stemte bedre overens med fiskens vekst og overlevelse gjennom forsøksperioden.

Table of Contents

Abbreviations

AB-PAS	Alcian Blue-Periodic Acid Schiff. Staining method combining the properties of alcian blue (pH 2.5) and PAS to detect polysaccharides such as glycogen and other mucosubstances in tissues. Alcian-blue stains blue. Schiff's reagent stains magenta.
Art-larva	Lumpfish larva fed *Artemia* during the treatment period (2-25 dph) in the present study.
Cir-larva	Lumpfish larva fed cirripeds during the treatment period (2-25 dph) in the present study.
Cop/Cir-larva	Lumpfish larva fed copepods and weaned to cirripeds during the treatment period (2-25 dph) in the present study.
Cop/FD-larva	Lumpfish larvae fed copepods and weaned to formulated diet during the treatment period (2-25 dph) in the present study.
CPA	Cryoprotectant agent. Used in the process of cryo-freezing cirripeds.
d°	Degree-days. Water temperature (°C) multiplied by number of days. Often used to explain time of development in fish in aquaculture.
DHA	Docosahexaenoic acid (22:6*n*-3). Polyunsaturated omega-3 fatty acid essential for marine fish larvae.
Dph	Days post hatch. Number of days since a fish larva emerged from its egg. 0 dph is regarded as when about 50 % of the total eggs have hatched.
DW	Dry weight. Weight of a fish larva dried at 60°C for a minimum of 24h.
DWI	Daily weight increase. Increase in dry weight per day given as a percentage over a time interval, often dph.
EFA	Essential fatty acid. Fatty acid which must be supplied through diet as it can not be synthesized by the larvae itself.
EPA	Eicosapentaenoic acid (20:5*n*-3) Polyunsaturated omega-3 fatty acid essential for marine fish larvae.

FA	Fatty acid. Carboxylic acid consisting of a hydrocarbon chain and a terminal carboxyl group. The fatty acid can be either saturated or unsaturated (one or more double bonds in the chain).
FAA	Free amino acid. Amino acid that is present as an individual unbound unit.
FD-larva	Lumpfish larva fed formulated diet during the treatment period (2-25 dph) and therefore for the entire period in the present study.
HUFA	Highly unsaturated fatty acid. Fatty acid containing two or more double bonds and at least 20 carbon atoms in the hydrocarbon chain.
Nuclei 1	Hepatocyte nuclei with one nucleolus.
Nuclei 2+	Hepatocyte nuclei with two or more nucleoli.
PBS	Phosphate buffered saline. Commonly used in laboratories for work with cells and tissues.
PFA	Paraformaldehyde. Commonly used fixation agent for cells and tissues.
PL	Phospholipid. Lipid molecule with a hydrophilic phosphate head and two hydrophobic "tails" derived from fatty acids. Functions as key components of cell membranes.
SGR	Specific growth rate. Growth metric that measures the increase in fish dry weight over a time interval, often dph.
SL	Standard length. Length of the fish measured from the tip of the snout to the end of the notochord/caudal peduncle if notochord is not visible.
TAG	Triacylglycerol. Neutral lipid derived from glycerol and three fatty acids. Functions as energy storage.
TB	Toluidine blue. Basic dye with high affinity for acidic tissue components that stains blue. Used to increase sharpness of histological slides images in the present study.

1 Introduction

1.1 Lumpfish in salmon aquaculture

1.1.1 Lumpfish as a cleaner fish

The Atlantic salmon (*Salmo salar*) is one of the most widely farmed finfish species in the world today, with 2.4 million tonnes produced in 2018 (FAO, 2020). Norway, which is currently the world's biggest producer of Atlantic salmon, accounted for as much as 1.3 million tonnes of the total that year (Norwegian Directorate of Fisheries, 2018). The annual Norwegian production has however been quiet stable after surpassing 1.2 million tonnes already in 2012, due to sea lice being one of the main challenges (Norwegian Directorate of Fisheries, 2020; Norwegian Institute of Marine Research, 2021).

The salmon louse *Lepeophtheirus salmonis* has been the primary issue since it targets salmonid species, but also the less host-specific louse *Caligus elongatus* has been problematic, especially in northern parts of Norway (Torrissen *et al.*, 2013; Braden, Monaghan & Fast, 2020; Hemmingsen *et al.*, 2020). High-density farming of Atlantic salmon in open net-pens has created an ideal environment for such ectoparasitic sea lice, which infects and feeds on the fish' skin, mucus and blood (Braden, Monaghan & Fast, 2020). These feeding activities can result in skin and fin erosion, making the fish more prone to secondary infections as well as osmoregulatory stress, and can in severe cases be lethal (Finstad *et al.*, 2000; Johnson *et al.*, 2004; Costello, 2006; Braden, Monaghan & Fast, 2020). The infestations are not only detrimental to the farmed fish, but also poses a threat to wild salmonids, attributed to the free movement of pathogens, including the sea lice itself, between wild and farmed fish (Thorstad & Finstad, 2018).

Treatment of infected salmon has had large economic cost for the salmon industry (Torrissen *et al.*, 2013). Treatment to remove lice has commonly been done by using chemotherapeutants, mainly in form of bath treatments, but also as in-feed additives (Salte *et al.*, 1987; Burridge *et al.*, 2010; Overton *et al.*, 2019). However, as the lice has become increasingly resistant to these chemical treatments and there is now more knowledge on the chemicals potentially harmful effects on non-target organisms, there has been a shift towards more preventative and environmentally friendly treatments, such as biological control using cleaner fish (Burridge *et al.*, 2010; Aaen *et al.*, 2015; Barrett *et al.*, 2020; Strachan & Kennedy, 2021).

Biological control in the aquatic environment can be defined as the control of pests using other living organisms (Treasurer, 2002). In the Norwegian salmon aquaculture mainly the lumpfish (*Cyclopterus lumpus*) and four different wrasse species; goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*), ballan wrasse (*Labrus bergylta*) and rock cook wrasse (*Centrolabrus exoletus*) have been deployed into the net-pens for this purpose (Treasurer, 2002; Powell *et al.*, 2018). Cleaner fish is a functional term used to describe the fish performing this task. Using cleaner fish is argued to be a less stressful way to delice since it does not require any handling of the salmon, as opposed to other alternatives to chemical treatments, e.g. mechanical-, thermal- and freshwater treatments (Overton *et al.*, 2019). These treatments typically require crowding and pumping of the fish, which stresses the fish and can cause physical damage like scale loss

and gill-bleeding (Overton *et al.*, 2019). Cleaner fish is also more cost-effective in use and has less of an ecological impact than the use of chemical treatments (Liu & Bjelland, 2014).

Cleaner fish were first used in Norwegian aquaculture in 1988, when different wrasse species were deployed to combat the sea lice (Bjordal, 1991; Treasurer, 2018). However, there has been several challenges regarding the use of wrasses. For instance, all the wrasse species used as cleaner fish currently are sensitive to temperature fluctuations. The wrasses are active at temperatures above 10 °C, but when the temperature drops below 6-8°C they enter a hypometabolic state and lice-grazing efficiency drops significantly (Sayer & Reader, 1996; Blanco Gonzalez & de Boer, 2017). The lumpfish on the other hand remains active and feed at temperatures close to 4°C, suggesting that the lumpfish is a more suited cleaner fish in colder waters (Nytrø *et al.*, 2014). This is also supported by the fact that the lumpfish generally has a much broader geographical distribution in the wild compared to the wrasses, especially towards northern latitudes (Blacker, 1983; Skiftesvik *et al.*, 2013; Rueness *et al.*, 2019). Additionally, the vast majority of the lumpfish used today originate from commercial farming, whereas the wrasse supply still mainly depends on wild catch, with only the ballan wrasse being cultivated today (Blanco Gonzalez & de Boer, 2017; Norwegian Directorate of Fisheries, 2021) (Figure 1.1). This is because lumpfish has proven easier to cultivate, with a generally much higher survival rate than ballan wrasse in several studies (Romundstad, 2015; Dahle *et al.*, 2017; Øie *et al.*, 2017; Marthinsen, 2018; Rian, 2019). The lumpfish can also be ready for deployment as fast as 4 months, whereas the ballan wrasse usually require 18 months (Skiftesvik *et al.*, 2013; Powell *et al.*, 2018). However, although more successful in terms of numbers, there are still challenges in the commercial production of lumpfish in need of attention.

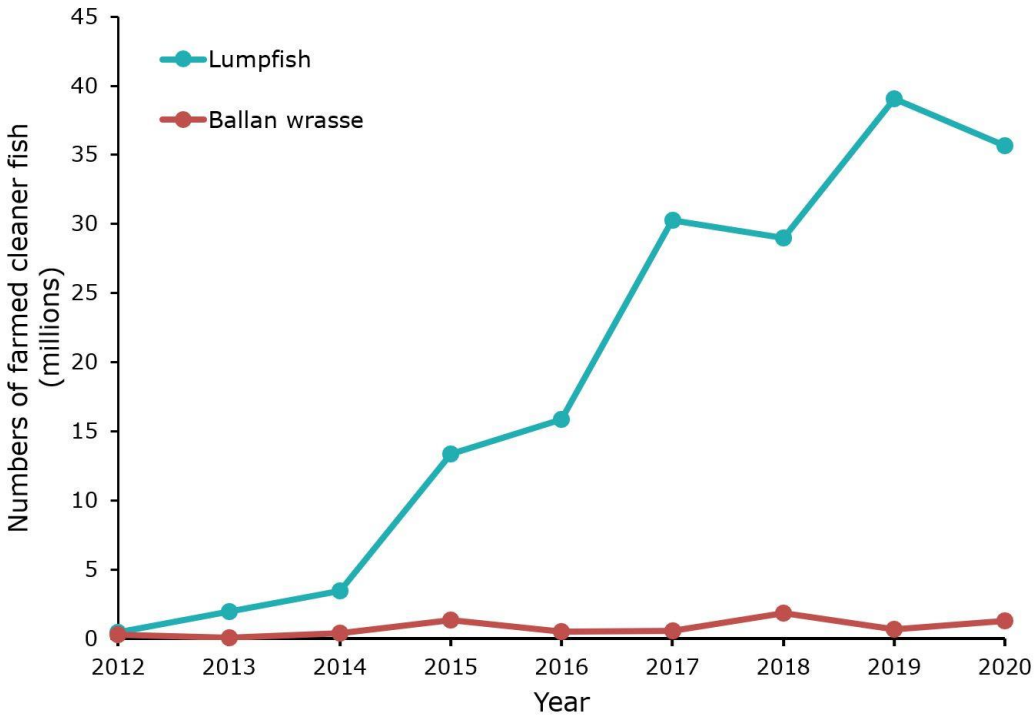


Figure 1.1. Farmed cleaner fish in Norwegian aquaculture 2012-2020. Farmed lumpfish and ballan wrasse in millions sold to Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) producers in Norway to combat sea lice between the years of 2012-2020. Source: Norwegian Directorate of Fisheries (2021).

1.1.2 Challenges in commercial production of lumpfish

One of the main areas in the lumpfish production cycle where research is needed is in the larviculture, described as the rearing of fish larvae from hatching to ongrowing (Marthinsen, 2018). The larval stage is characterised by rapid growth and development and is therefore a crucial life stage if the aim is to mass rear individuals of high quality (Kjørsvik, Pittman & Pavlov, 2004; Powell *et al.*, 2018). After all, the transition from an endogenous energy supply provided by the yolk sac to exogenous supply from the diet, marks one of the most critical phases in a fish' life (De Silva & Anderson, 1995). Today, lumpfish larval rearing can be described as varying in terms of survival, quality, and size of the larvae (Dahle *et al.*, 2017; Powell *et al.*, 2018; FHF, 2019; Garcia de Leaniz *et al.*, 2021). High post-weaning mortality has also been reported, which is assumed to be caused by the shift from a live to a formulated diet (Powell *et al.*, 2018). Since the lumpfish is a relatively new species in aquaculture, the varying rearing success has often been linked to the lack of knowledge on appropriate feeding regimes and nutritional requirements for the species (Imsland *et al.*, 2018a; Imsland *et al.*, 2018b; Powell *et al.*, 2018; FHF, 2019). As a result, start-feeding regimes so far have been based upon what has been used for other marine fish species in aquaculture (Benfey & Methven, 1986; Brown, 1986; Powell *et al.*, 2018). To overcome the inconsistent rearing success, it is therefore vital to find feeding regimes suited to the lumpfish specifically.

In nature, the lumpfish hatch from demersal eggs at an approximate length of 5-6 mm (Benfey & Methven, 1986; Dahle *et al.*, 2017; Marthinsen, 2018; Rian, 2019). The larvae are found in shallow rockpools either around or attached to floating seaweed, feeding mainly on small crustaceans, such as harpacticoid and calanoid copepods (Ingólfsson & Kristjánsson, 2002). Other potential prey items that were abundant, but small, sessile or slow-moving were however largely ignored, such as ostracods, rotifers and polychaetes (Ingólfsson & Kristjánsson, 2002). Lumpfish also feed differently than most other cultured fish, as it uses the advantage of its suction disc to stay in one spot and feed passively when prey is abundant (Brown, 1986; Killen, Brown & Gamperl, 2007). This is expected to lower the overall cost of foraging and allows for more energy to be allocated towards growth (Brown, 1986; Killen, Brown & Gamperl, 2007). However, a switch from passive clinging to actively swimming and hunting for prey do appear when prey is scarce (Brown, 1986; Killen, Brown & Gamperl, 2007).

Today, most producers use formulated diets directly, given at 2-5 dph (Dahle *et al.*, 2017; Jonassen, Lein & Nytrø, 2018). Different live feeds prior to weaning to formulated diets, such as *Artemia* nauplii, copepods and cirripeds has also been tried (Jonassen, Lein & Nytrø, 2018; Planktonic AS, 2022). However, there is still little known about how these different feed types and feeding-regimes used currently affect the fish' development and survival further, as well as their ultimate objective of lice-grazing (FHF, 2019). To design an optimal feeding regime, it is therefore essential to have knowledge on the nutritional requirements in fish larvae in general, and how the current industry standard (formulated diet) and different live prey meet these.

1.2 Commercial diets used in lumpfish larviculture

1.2.1 Nutritional requirements in fish larvae

Proteins are important for the development of fish in general, as they make up the major organic material in fish tissue, accounting for 65-75 % of the fish' dry weight (Wilson, 2002). It is therefore crucial for fish to get a consistent protein intake from the diet once its yolk sac reserves has been depleted. This is especially the case in larvae, since the larval stage is characterised by rapid growth the requirements for proteins are often higher in larvae than in adult fish (De Silva & Anderson, 1995). The building blocks of the proteins, amino acids, are continuously used to either build new protein (growth) or to replace existing proteins (maintenance) (Wilson, 2002). Amino acids have also been shown to be a significant energy source in the early life stages of marine fish (Rønnestad *et al.*, 2003). The amino acids are provided by the diet is either incorporated in proteins or as free amino acids (FAA), with the FAAs being more easily absorbed by the gut of marine fish larvae, as opposed to larger proteins (Rønnestad *et al.*, 2003). This is because FAAs and small peptides are regarded as water-soluble protein and can thus be absorbed directly from the digestive tract without any prior digestion (Tonheim *et al.*, 2007).

Lipids are also essential for larval development as they perform a wide range of biological functions (Olsen, van der Meeren & Reitan, 2004). For instance, neutral lipids like triacylglycerols (TAG) function as storage lipids, whereas polar lipids like phospholipids (PL) are fundamental components of cell membranes (Sargent *et al.*, 1999a; Sargent *et al.*, 1999b; Olsen, van der Meeren & Reitan, 2004; Feller, 2008; Tocher, 2010). Lipids also provide fatty acids (FA), which are necessary for normal growth and development of fish larvae as they are important sources of cellular energy (Tocher, 2003; 2010). Marine fish larvae especially have high requirements for the *n*-3 highly unsaturated fatty acids (HUFA), mainly docosahexaenoic acid (DHA, 22:6*n*-3) and eicosapentaenoic acid (EPA, 20:5*n*-3) (Sargent, McEvoy & Bell, 1997). These two HUFAs are essential fatty acids (EFAs), meaning they can't be synthesized by the larvae themselves and must therefore be supplied through the diet (Watanabe, 1982; Sargent, McEvoy & Bell, 1997). It also matters whether these fatty acids are supplied in neutral or polar lipids, as HUFAs stored in polar lipids are more bioavailable and hence more effectively utilized by the larvae. This has been demonstrated for various marine fish larvae, as both European seabass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) showed significantly greater development and survival with diets providing HUFAs in polar lipids than neutral lipids (Gisbert *et al.*, 2005; Wold *et al.*, 2009).

1.2.2 Formulated diet

Formulated diets have frequently been favoured by the industry, as they are less expensive and labour-demanding compared to using live prey (Hamre *et al.*, 2013). Formulated diets also come in a variety of shapes, sizes, and nutrient profiles, making them suitable for numerous fish species (Hamre *et al.*, 2013; Rian, 2019). One of the challenges in larval formulated diets is however the retention of nutrients (Kvåle *et al.*, 2006; Hamre *et al.*, 2013). When rehydrated, hydrosoluble nutrients such as FAAs are often lost due to leaching (Kvåle *et al.*, 2006; Hamre *et al.*, 2013). This happens especially in microdiets, which are often used for the larval stage, as the surface/volume ratio is high and the diffusion distance from the core of the feed particle to the surface is very short (Hamre *et al.*, 2013). In the worst case, as much as 95 % of FAAs can be lost from certain diets during the first few minutes of rehydration (Kvåle *et al.*, 2006; Nordgreen, Tonheim & Hamre, 2009; Hamre *et al.*, 2013). Additionally, the uneaten feed particles and leaching can result in

poor tank hygiene and reduced water quality with respect to a high load and unfavourable composition of bacteria (Dahle *et al.*, 2017). Biofilm will quickly form on the tank walls under such conditions, and for the lumpfish which mainly likes to sit attached and feed passively (Killen, Brown & Gamperl, 2007), this could increase the risk of disease (Dahle *et al.*, 2017). It has therefore been suggested that the period with the smallest formulated feed which lasts about 20 days, may be desirable to avoid (Dahle *et al.*, 2017).

Formulated diet particles must also be identifiable by the larvae as a food item to be ingested (Hamre *et al.*, 2013). Most fish larva use their vision when hunting for prey (Hunter, 1981), which is likely also the case of the lumpfish, as they have functional eyes from the moment they hatch (Brown, 1986). However, the movement of the feed particles are limited to those generated by the water currents in the tanks and can if the currents are low end up sinking and settling at the tank bottom (D'Abramo, 2002). Live prey on the other hand naturally have more movement, and the hunting instinct of the fish larvae is perhaps more triggered by live prey as opposed to a 'sessile' feed particle (D'Abramo, 2002). Once ingested, the feed must also be digested. Most fish larvae lack a functional stomach and their ability to digest and assimilate the nutrients from formulated diet is thought to be less effective than in adult fish (Govoni, Boehlert & Watanabe, 1986). Although the lumpfish is relatively large and have been able to feed on formulated diets from start (Brown, 1986; Kjørsvik, Pittman & Pavlov, 2004), there are still factors advocating for having a live feed period first. It has been shown that the lumpfish larvae's stomach is not fully functional before 21-34 days post hatch (Marthinsen, 2018), and that feeding formulated diet can have negative effects on the gut epithelium and energy storage in the liver of lumpfish larvae (Dahle *et al.*, 2017).

1.2.3 Copepods

Copepods are small crustaceans and functions as the natural prey organism of marine fish larvae (Støttrup, 2003), which has also been observed in lumpfish larvae (Ingólfsson & Kristjánsson, 2002). Copepods are believed to be an appealing live prey, as they stimulate the larvae's hunting and visual instincts by exhibiting typical zigzag motions (FAO, 1996). Copepods are also very nutrient-dense, and it is believed that fish larvae have adapted to hunt for and consume these prey organisms through evolution, and thus could have high requirements for the nutrients found in copepods (Hamre *et al.*, 2013). Although copepods appear to have a suitable nutrient profile, there are some disadvantages to using them, such as higher manufacturing costs, as they can be more labour-demanding and difficult to produce when compared to *Artemia* and formulated diets (FAO, 1996).

Copepods have a naturally high content of EFAs needed for proper growth in fish, and these are primarily incorporated in the PLs (Albers, Kattner & Hagen, 1996; van der Meeren *et al.*, 2008). Lumpfish fed copepods have also resulted in higher wet weight, length and myotome height, as well as a cleaner tank environment compared to formulated diet (Dahle *et al.*, 2017). However, in other studies, copepods resulted in lower growth and survival in lumpfish when compared to both *Artemia* and formulated diet (Marthinsen, 2018; Rian, 2019). These contrasting findings highlight the importance of learning more about the nutritional requirements and appropriate feeding regimes for lumpfish larvae. Copepods are also rich in protein (Hamre *et al.*, 2013), and of this protein a considerable fraction is FAAs (van der Meeren *et al.*, 2008).

1.2.4 Artemia

Artemia or "brine shrimp" are small shrimp-like crustaceans and are the most widely used live prey in marine aquaculture because of its commercial availability and practicality (Van Stappen, 1996). *Artemia* reproduce using dormant embryos, so-called cysts, which is arguably the main trait as to why it is deemed such a practical live feed to use. After a 24-hour incubation period in seawater to rehydrate the cysts, free-swimming nauplii hatch from the cysts. Already at this first larval stage, named nauplii instar I (400-500 µm long), it can be used as live prey immediately after hatching (Merchie, 1996; Van Stappen, 1996). Although *Artemia* is easily cultured and readily used as a live feed, it has been debatable whether its sufficient in meeting the nutritional needs of marine fish larvae.

The biggest concern regarding *Artemia's* nutritional value is its low lipid and fatty acid content (Sorgeloos, Dhert & Candreva, 2001). After hatching, it has a FA profile of practically no DHA and only low amounts of EPA (Sorgeloos, Dhert & Candreva, 2001; Hamre *et al.*, 2013). Of their total lipids, as much as 70 % are neutral lipids (Hamre *et al.*, 2013). To deal with these insufficient HUFA-levels, it is necessary to boost the *Artemia's* nutritional content (Sorgeloos, Dhert & Candreva, 2001; Hamre *et al.*, 2013). However, even after enrichment, the EFAs are still mainly found in the neutral lipid fraction (Conceição *et al.*, 2010). Maintaining high levels of DHA and a good ratio of DHA/EPA after enrichment is also troublesome, as the *Artemia* selectively metabolizes DHA and converts it to EPA.

Artemia is thought to contain sufficient levels of protein required for normal growth of larvae (Hamre *et al.*, 2013), and both nauplii and adult stages contain sufficient levels of the 10 essential amino acids for fish (Dhont & Van Stappen, 2003). Approximately 50 % of all the protein in *Artemia* is water soluble, which is thought to be the same ratio as in copepods (Hamre *et al.*, 2013). Additionally, FAAs constitute about 9-10 % of the total amino acids in *Artemia* (Hamre *et al.*, 2013). However, the FAA levels are somewhat lower than that of copepods (12-13 %) and could therefore be a limiting factor for growth (Rønnestad *et al.*, 2003; van der Meeren *et al.*, 2008; Hamre *et al.*, 2013).

1.2.5 Cirripeds

The subclass Cirripedia, or more commonly known as barnacles, are sessile crustaceans that spends the majority of their lives permanently attached to substrates such as rock and coral (López, Pham & Isidro, 2012). Most often the cirripeds are seen forming communities and are densely packed on these substrates (López, Pham & Isidro, 2012). The cirripeds incubate eggs within their body cavity until the eggs becomes a nauplius larva (stage I) (López, Pham & Isidro, 2012). The nauplius larva is then released into the water column, beginning a planktonic, free-swimming life stage that can last 2-4 weeks (López, Pham & Isidro, 2012).

The company Planktonic AS has utilized this nauplii stage and promoted it as a new and viable alternative to traditional live feeds (Planktonic AS, 2022). The nauplii are harvested and then cryopreserved in containers with liquid nitrogen (Planktonic AS, 2022). The fish farmer simply has to choose the amount of cirripeds needed, thaw them in seawater to remove the cryoprotectant agent (CPA), and once cleaned put them in another seawater tank to revitalize (Planktonic AS, 2022). According to the producer, the cirripeds are still alive and able to swim again after the revitalization period, as the cryopreservation process has only stopped their metabolism (Planktonic AS, 2022). The cirripeds can then be fed directly to the fish, as they are said to have an optimal nutrient profile for marine fish larvae (Planktonic AS, 2022). This is due to high proportions of the fatty acids DHA and EPA in the phospholipids of the cirripeds (Planktonic AS, 2022).

1.3 Nutritional status and histological biomarkers in the liver

Conducting research to monitor the nutritional status of the fish is critical for learning more about their nutritional requirements and feeding habits (Imsland *et al.*, 2018b). Various digestive organs in fish have been found to use different cellular mechanisms in response to diet quantity and quality (Lazo, Darias & Gisbert, 2011). Therefore, the use of the gut and accessory digestive organs, such as the liver, as target organs for assessing the nutritional status of fish is well established (Lazo, Darias & Gisbert, 2011). The liver performs various tasks in the body, as it is functions as both an endocrine and exocrine gland (Ostaszewska & Sysa, 2004). For instance, the liver is crucial for maintaining nutritional homeostasis since it is responsible for nutrient processing and storage (Hoehne-Reitan & Kjørsvik, 2004). The liver also aids in digestion by producing bile that is secreted to the gut and emulsifies lipids, and also plays an important role in detoxification processes (Akiyoshi & Inoue, 2004).

The histomorphological organization of the liver is regarded to accurately reflect feeding conditions, regardless of whether that is due to an unbalanced diet or deprivation of feed altogether (Gisbert, Ortiz-Delgado & Sarasquete, 2008). As previously mentioned, one of the primary tasks of the liver is energy metabolism and storage, which is one of the main reasons as to why it is a regularly targeted organ for assessing nutritional status. Dietary effects can often be seen on the liver in form of intracellular changes in the hepatocytes, which are the parenchymal cells of the liver (Gisbert, Ortiz-Delgado & Sarasquete, 2008). If the larvae have been starved, there will likely be an absence of intracellular vacuoles containing glycogen and lipids in the hepatocytes, as these energy sources will be rapidly mobilized in order to maintain nutritional homeostasis (Hoehne-Reitan & Kjørsvik, 2004; Gisbert, Ortiz-Delgado & Sarasquete, 2008). Correspondingly, if the larvae have been given an adequate diet according to its nutritional needs, there will likely be vacuoles present in the hepatocytes (Hoehne-Reitan & Kjørsvik, 2004; Gisbert, Ortiz-Delgado & Sarasquete, 2008). Hepatocyte size is also affected by the level of vacuolization, as more vacuoles yield larger hepatocytes (Gisbert, Ortiz-Delgado & Sarasquete, 2008). However, storage and mobilization of vacuoles might be different between species, as there are indicators that hepatic glycogen storage could be related to the specific larval energy metabolism of different species (Hoehne-Reitan & Kjørsvik, 2004). There is however an overall trend, that vacuole storage increases after the onset of exogenous feeding if given adequate diet (Gisbert, Ortiz-Delgado & Sarasquete, 2008). Additionally, the positioning of the hepatocyte nucleus is affected by the accumulation of vacuoles in the cytoplasm. If there is high intracellular vacuolization, the nucleus will be displaced peripherally, whereas when vacuoles are absent the nucleus takes a central position in the hepatocyte (Gisbert *et al.*, 2005; Zambonino Infante *et al.*, 2008).

Nutritional status also influences the size of the hepatocyte nucleus. Strüssmann & Takashima (1990) found that the larval pejerrey's (*Odontesthes bonariensis*) hepatocyte nuclei were affected by the diet, as the nuclei size remained constant when given an adequate diet. In contrast, it decreased rapidly during starvation, indicating that changes in nuclei size were linked to nutritional status and growth (Strüssmann & Takashima, 1990). This relationship could be explained by the rate of metabolic activity, as a larger nucleus will have an increased surface area. This allows for higher exchange rates between the nucleus and cytoplasm and is therefore indicative of higher metabolic activity (Ghadially, 1997; Wold *et al.*, 2009). As a result, it's plausible that the liver's metabolic activity is higher during times of rapid energy transfer and growth (Wold *et al.*, 2009). Aside from the hepatocytes, there are many other biomarkers for determining nutritional status in the liver. Dilatation of the sinusoidal capillaries, larger intercellular spaces and hypertrophy of the bile canaliculi are some histopathological changes that can be seen in the event of starvation (Gisbert, Ortiz-Delgado & Sarasquete, 2008).

1.4 Aims and hypotheses

The present study was motivated by the fact that an upscaling in commercial production of lumpfish needs to be accompanied by more comprehensive knowledge on appropriate start-feeding regimes for lumpfish larviculture to be sustainable. Therefore, to contribute to optimalization of feeding regimes in lumpfish aquaculture the study aims were:

Aim 1: Evaluate dietary effects of different start-feeding diets on lumpfish larvae's growth and survival.

Aim 2: Evaluate dietary effects of different start-feeding diets on histological biomarkers of the liver and their potential use for assessing nutritional status in lumpfish larvae.

The aims were approached by conducting a start feeding experiment with lumpfish larvae from hatching until 35 days post hatch (dph). In the experiment today's industry standard, which is formulated diet exclusively, were used as a control against four other feeding regimes composed of different live feed organisms: *Artemia*, copepods and cirripeds.

Past experiments have shown that using enriched *Artemia* has resulted in both higher survival and better growth in lumpfish larvae compared to the copepod *A. tonsa* and formulated diet (Marthinsen, 2018; Rian, 2019). Marthinsen (2018) also found that lumpfish larvae fed *Artemia* had the largest hepatocyte nucleus size, hepatocyte size and generally a larger vacuole fraction than those fed copepods and formulated diet. Furthermore, cirripeds are said to have an optimal nutrient profile for fish larvae, as it is similar to that of copepods (Planktonic AS, 2022). Based on these claims, the following was hypothesised:

Hypothesis 1: Lumpfish fed *Artemia* will have better growth and survival than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof.

Hypothesis 2: Lumpfish fed cirripeds will have better growth and survival than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof.

Hypothesis 3: Lumpfish fed *Artemia* will have larger hepatocytes, hepatocyte nuclei and vacuole fractions than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof.

Hypothesis 4: Lumpfish fed cirripeds will have larger hepatocytes, hepatocyte nuclei and vacuole fractions than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof.

The experiment was part of project "STARTRENS", which main goal was to improve cleaner fish welfare and performance through optimizing start-feeding of cleaner fish larvae and assessment of egg quality (FHF, 2019). STARTENS was mainly funded by the Norwegian Seafood Research Fund (FHF), but also by the collaborative partners NTNU and SINTEF Ocean AS. Live feed organisms were purchased of C-FEED AS and Planktonic AS, who also contributed to the design of the feeding regimes. Two other master students were partaking in the experiment, namely Marte Solli Lindskog who investigated skeletal muscle development and Saba Akbar who performed lipid and fatty acid analysis.

2 Materials and methods

2.1 Egg incubation, tank setup and larval rearing

The start-feeding experiment of lumpfish and analyses were conducted in the facilities of NTNU Centre of Fisheries and Aquaculture (SeaLab) and SINTEF Ocean AS from September 9th to October 14th 2020. Eggs from eight lumpfish females and milt from one male were supplied by Akvaplan-niva AS from Tromsø, Norway. The eggs of the different females were pooled and then divided into 15 groups of 300 mL, before fertilized with milt from the one male. The egg groups were then put into separate, circular cake tins and flattened to ensure equal oxygen distribution. The eggs were left to harden for approximately 30 minutes in running sea water. Next, each of the hardened egg groups were placed into 15 separate incubators (FT family hatcher, Fish Tech AS, Norway) with continuous water flow (34 ppt salinity), a gradual increase in temperature from 5 to 10°C, and complete darkness. Sea water used in the experiment had been pumped from the Trondheimsfjord, filtered through a sand filter and 1µm filter. The incubators were designed with an upwelling current and an outlet at top (Figure 2.1A), allowing newly hatched larvae at approximately 300 d° to go directly into the rearing tanks (Figure 2.1B), aiming for a density of a 100 larvae L^{-1}. The average density was 69 larvae L^{-1}, after calculating based on the survival.

The rearing tanks were cylindrical and flat-bottomed with a mesh-covered cylindrical outlet placed at the centre (Figure 2.1C). Each tank contained 100 L of seawater and was continuously aerated with tubes placed around the outlet. The water inlet was positioned at an angle aiding in circulation of the tank water. Water exchange rates and outlet mesh sizes (Appendix 1; Table A1) were adjusted throughout the experiment to accommodate for the different feed types, ranging from 300-2400 % and 100-750 µm, respectively. For environmental enrichment and additional surface area, 1-4 small silicon mats were added in the tanks at 2 dph (Figure 2.1C). These were removed from all tanks at 22-24 dph, as they had become a place for floc to collect. Continuous lighting was provided by fluorescent tubes above the tanks for the whole experimental period.

To ensure a stable and healthy tank environment, temperature and oxygen saturation was measured daily by placing a probe in the outlet (ProODO Optical Dissolved Oxygen Instrument, YSI, USA). Temperature and oxygen saturation was on average 10.3°C and 7.8 mg L^{-1}, respectively. Additionally, dead fish, excess feed and floc were removed once or twice daily by using siphons to hover the tank bottoms and walls. Live fish caught in the siphons were put back into their respective tanks, whereas dead fish were counted. A cleaning-arm was started at 8 dph, aiding in cleaning the tank by rotating and collecting dirt along its wipers (Figure 2.1D).

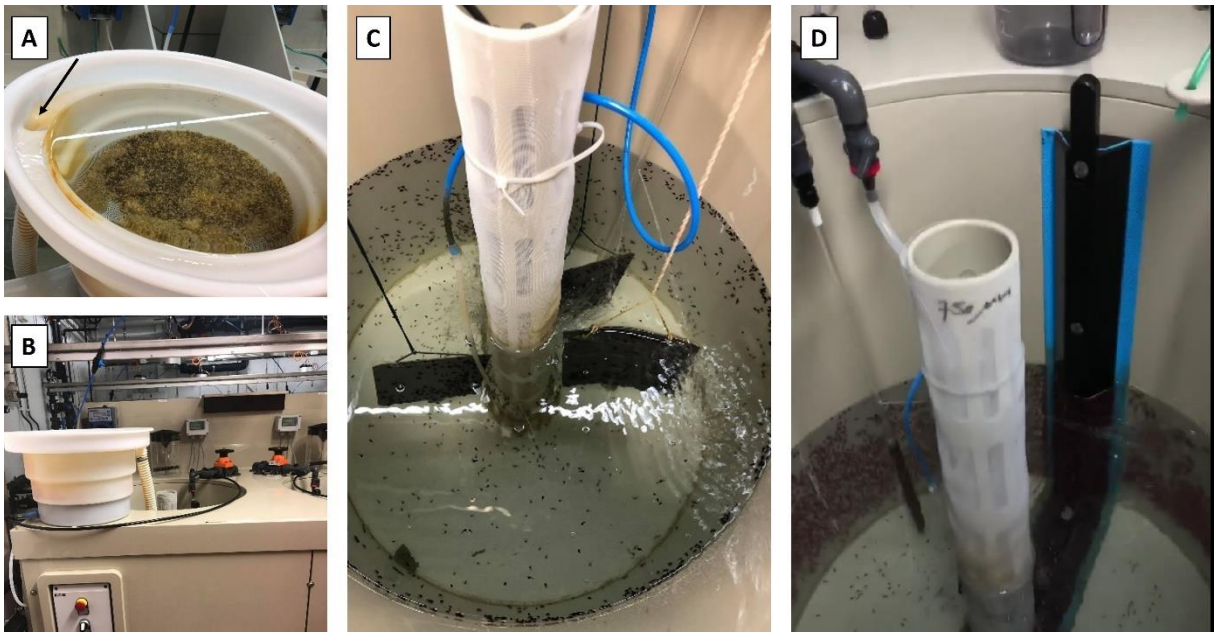


Figure 2.1. Egg incubator and tank set up in the start feeding experiment of lumpfish (0-35 dph). A) Egg incubator seen from above containing a flattened mass of yellow and brown lumpfish eggs. Outlet at top left (black arrow). B) Egg incubator positioned besides the rearing tank with a tube from its outlet, allowing newly hatched lumpfish larvae to enter the tank. C) The cylindrical fish tank seen from above with a mesh-covered central outlet going through the flat bottom and silicon mats added to the tank. D) Cleaning arm with blue wipers running along the tank wall and bottom.

2.2 Start-feeding regimes

There were five different feeding regimes replicated in three fish tanks each. This gave 15 tanks in total, which were randomized (Appendix 2; Figure A1) to avoid bias. Introduction to feed started at 2 dph and lasted until 35 dph. The feeding regimes schedule and the feed types/size used is shown in Table 2.1 and Table 2.2, respectively. The feeding amounts of live feed and formulated diet for each of the five feeding regimes are given in Appendix 3, Table A2. The different feeding regimes were:

1. ***Artemia* (Art)**
   Lumpfish larvae were fed enriched *Artemia* for 18 days (2-20 dph) before weaned to formulated feed (GEMMA micro mix) for 5 days (21-25 dph). GEMMA micro mix means a 1:1 mix of GEMMA micro 150 and 300. The larvae were then fed formulated feed (GEMMA micro 300) until the experiments end (26-35 dph).

2. **Cirripedia (Cir)**
   Lumpfish larvae were fed cirripeds for 18 days (2-20 dph) before weaned to formulated feed (GEMMA micro mix) for 5 days (21-25 dph). The larvae were then fed formulated feed (GEMMA micro 300) until the experiments end (26-35 dph).

3. **Formulated diet (FD)**
   Lumpfish larvae were fed formulated diet exclusively the entire experiment, starting with GEMMA micro 150 (2-9 dph) before weaned to the GEMMA micro mix for 7 days (10-16 dph). The larvae were then fed GEMMA micro 300 (17-35 dph) until the experiments end.

4. **Copepod and formulated diet (Cop/FD)**
   Lumpfish larvae were fed copepods for 7 days (2-9 dph) before weaned early to formulated feed (GEMMA micro mix) for 7 days (10-16 dph). The larvae were then fed formulated feed (GEMMA micro 300) until the experiments end (17-35 dph).

5. **Copepod and Cirripedia (Cop/Cir)**
   Lumpfish larvae were fed copepods for 7 days (2-9 dph) before weaned to cirripeds for 6 days (10-16 dph). The larvae were fed cirripeds (17-20 dph) before weaned to formulated feed (GEMMA micro mix) for 5 days (21-25 dph). The larvae were then fed formulated feed (GEMMA micro 300) until the experiments end (26-35 dph).

Table 2.1. Feeding regimes and sampling times in the start-feeding experiment of lumpfish 0-35 dph. Five different feeding regimes were used, replicated in three tanks each. Feeding started at 2 dph and lasted until 35 dph. Days with overlapping feed types indicates a weaning period. Sampling days are given for standard length (SL), dry weight (DW), histology of the liver and survival. The sampling days were in general 2, 9, 15, 21, 29, 34 and 35 dph.

Dph	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Art			Enriched *Artemia* nauplii																																	
																						GEMMA micro mix					GEMMA micro 300								
Cir			Cirripedia nauplii																																	
																						GEMMA micro mix					GEMMA micro 300								
FD			GEMMA micro 150																																	
											GEMMA micro mix							GEMMA micro 300																			
Cop/FD			Copepod nauplii																																	
											GEMMA micro mix							GEMMA micro 300																			
Cop/Cir			Copepod nauplii																																	
											Cirripedia nauplii																										
																						GEMMA micro mix					GEMMA micro 300								
Sampling																																				
SL			X							X						X						X								X					X	X
DW			X							X						X						X								X					X	
Histology			X							X												X														X
Survival			Every day from 2 dph																																	

Table 2.2. Feed types and their size used in the start-feeding experiment of lumpfish 0-35 dph. Feed types used and respective sources for size: *Artemia* (FAO, 1996), Cirripedia (CryoPlankton data sheet, user's manual from Planktonic AS), copepod (C-FEED AS, 2014) and formulated diet (Skretting, 2021a; 2021b). The copepod size is given as an interval as the copepods grew in their delivered tank and started over again when given a new shipment (tank) of young copepods.

Feed type		Size
Live feed	Enriched *Artemia franciscana* instar III meta nauplii	800 µm long
	Cirriped *Semibalanus balanoides* nauplii	350 µm long, 150 µm wide
	Copepod *Acartia tonsa* nauplii (N3-N6)	185-394 µm long
Formulated feed	GEMMA micro 150 pellet	100-200 µm
	GEMMA micro 300 pellet	200-500 µm

2.3 Live feed production

2.3.1 Artemia

A work protocol for *Artemia*-production was made by following the recommendations of the cyst- and enrichment manufacturers (INVE Aquaculture & BioMar AS; Appendix 4). *Artemia* cysts (EG ® INVE Aquaculture, INVE Aquaculture, Thailand) were incubated for 24-hours until hatching in cylindroconical tanks with 60 L heavily aerated seawater at 25-29 °C under constant lighting. The hatched nauplii were separated from unhatched cysts and debris using a magnetic separator (SEP-Art Magnetic Artemia SEPARATOR, INVE Aquaculture, Australia) and a plankton net (60 µm). The harvested nauplii were then transferred to a new tank for enrichment. The Artemia was enriched twice over a 24-hour period with 10 g LARVIVA Multigain (BioMar AS, France) per 60L water each time before added to the feed reservoirs. The *Artemia* strain used in this experiment (*Artemia franciscana*) was approximately 800 µm long when fed to the fish, as it had been enriched and become an instar III meta-nauplii (FAO, 1996).

2.3.2 Cirripedia

Frozen cubes containing Cirripedia nauplii (*Semibalanus balanoides*) and cryoprotectant agent (CPA) stored in a Dewar flask with liquid nitrogen (-196 °C) were provided by Planktonic AS twice. Cirripedia-production was done by following a protocol given by the provider (Appendix 5). The frozen cubes were thawed in seawater, and then transferred to a plankton net (100 µm) to rinse off the CPA. The cirripeds were then transferred to a cylindroconical revitalization tank with 50-55 L heavily aerated seawater in a temperature regulated room of 5 °C. The cirripeds were then left in the revitalization tank for a minimum of 6 hours before transferred to the feed reservoirs. Cirripedia nauplii fed to the larvae was approximately 350 µm long and 150 µm wide (CryoPlankton data sheet, user's manual from Planktonic AS).

2.3.3 Copepods

Live copepods of the species *Acartia tonsa* were delivered in a $1m^3$ plastic tank. To feed the copepods, the microalgae *Rhodomonas baltica* were delivered in several 20 L plastic cannisters in the same shipment. The copepods and algae were provided by C-FEED AS and kept in temperature regulated room at 5 °C. How to harvest the copepods was explained by the provider and a work protocol was made (Appendix 6). The copepods were harvested using a plankton net (53 µm) and placed into buckets and added aerated seawater until desired copepod-density was met. The copepods were then fed *R. baltica* before added to the feed reservoirs. The plastic tank containing the copepods was also replenished with seawater and the copepods in the tank were fed *R. baltica* after each harvest to maintain the culture. As the copepods grew bigger in the tank over time, four shipments of new *A. tonsa* and their feed was required to ensure their size was appropriate for the fish larvae. Thus, the *A. tonsa* fed to the fish was between stage N3-N6 and approximately 185-394 µm long (C-FEED AS, 2014).

2.4 Distribution of live feed and formulated diet

Live feeds were transferred to 20 L plastic beer kegs (KeyKeg 20L, OneCircle, The Netherlands) used as feed reservoirs (Figure 2.2A) and filled up with seawater until the 20 L mark. The reservoirs were continuously aerated from the bottom through silicone hoses. The live feeds were transported from the reservoirs into the rearing tanks through transparent silicone hoses using peristaltic pumps (Kronos 50, Seko, Italy). The formulated feed was distributed by feed automats (Sterner 905, Fish Tech AS, Norway; Figure 2.2B), dropping the feed directly into the rearing tanks 4-24 times per day.

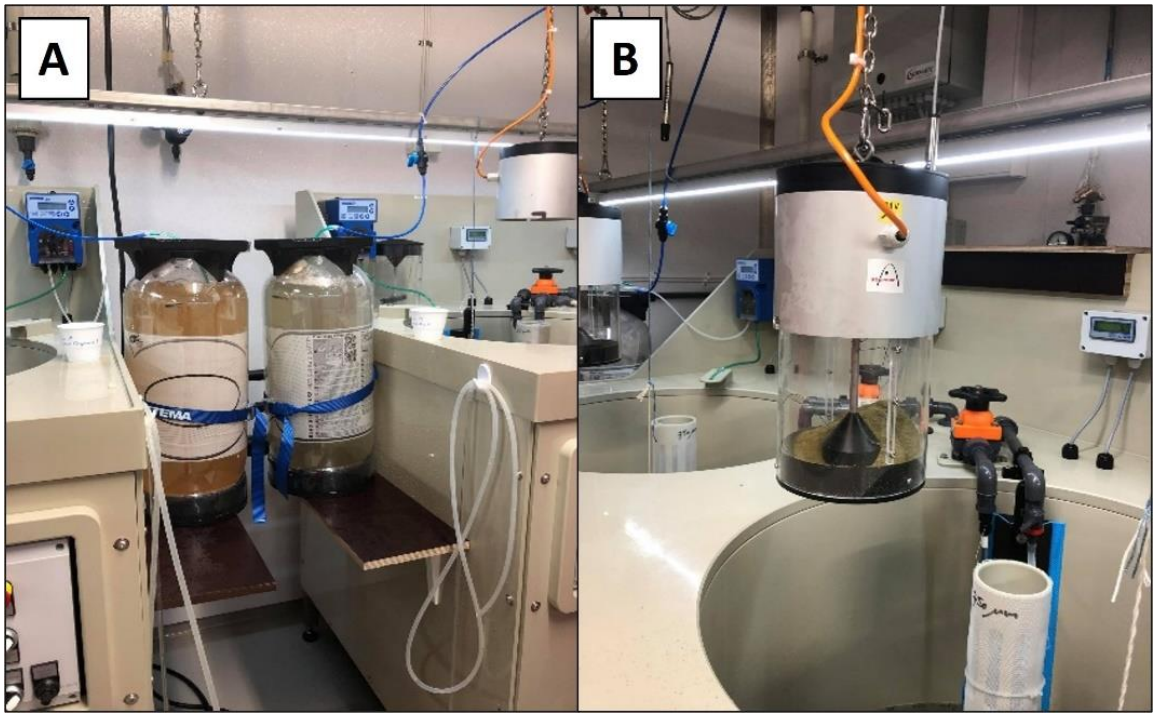


Figure 2.2. Feed reservoirs and feed automat used in the start-feeding experiment of lumpfish 0-35 dph. A) Two live feed reservoirs filled to the brim. The left reservoir is filled with *Artemia*, as seen by the typical orange colour, and cirripeds to the right. B) Feed automat containing formulated diet.

2.5 Larval sampling and analyses

2.5.1 Larval sampling

All larvae were randomly sampled and euthanized by an overdose of tricaine methanesulfonate (MS-222 Finquel ®, Argent Chemical Laboratories Inc., USA) mixed in with seawater from the rearing tanks. Larvae sampled for growth, meaning dry weight (DW) and standard length (SL), were rinsed in distilled water prior to analysis. Larvae sampled for histology of the liver were pooled, rinsed in distilled water, and fixated in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) with a pH of 7.4 (Apotekproduksjon AS, Norway), and measured SL of later. Fixated larvae were stored in vials at 4 °C until further analysis. The sampling days were chosen according to the feeding regimes, and therefore represent days before or after weaning to different feed types (Table 2.1). At the last day of the experiment (35 dph) larvae from each tank were pooled in separate buckets and then euthanized by an overdose of tricaine methanesulfonate (MS-222 Finquel ®, Argent Chemical Laboratories Inc., USA). From the euthanized larvae, 250 fish per bucket (tank) were sampled to measure SL only, to check for sampling bias. Number of larvae sampled for growth and histology is summarized in Table 2.3.

Table 2.3. Number of lumpfish larvae sampled for growth and histology. Larvae on 2 dph were sampled from random tanks before feeding started and is the total sample size (black). From 9-35 dph, the sample sizes represent the number of fish sampled per tank for growth (light grey) and per treatment for histology (dark grey). Note that on 35 dph the sample for growth was only used for SL and therefore marked with an asterisk (*).

Dph	2	9	15	21	29	34	35
Growth							
Sampled	15	5	5	10	15	15	250*
Analysed	15	5	5	10	15	15	250*
Histology							
Sampled	15	15	15	15	15	0	15
Analysed	5	5	0	5	0	0	5

2.5.2 Standard length

Standard length (SL) was measured from the tip of the lumpfish' snout to the end of the notochord (Figure 2.3A) until the notochord was no longer clearly visible, then the caudal peduncle was used as the measuring point for the posterior end (Figure 2.3B). SL measurements was done using the image processing program ImageJ (Schneider, Rasband & Eliceiri, 2012) on pictures taken using a mounted camera on a stereo microscope (Zeiss Axiocam ERc 5s, Zeiss Inc., Germany; Leica MZ75, Leica Microsystems, Germany). Images taken for SL were also used for assessing external morphology of larvae throughout the experimental period.

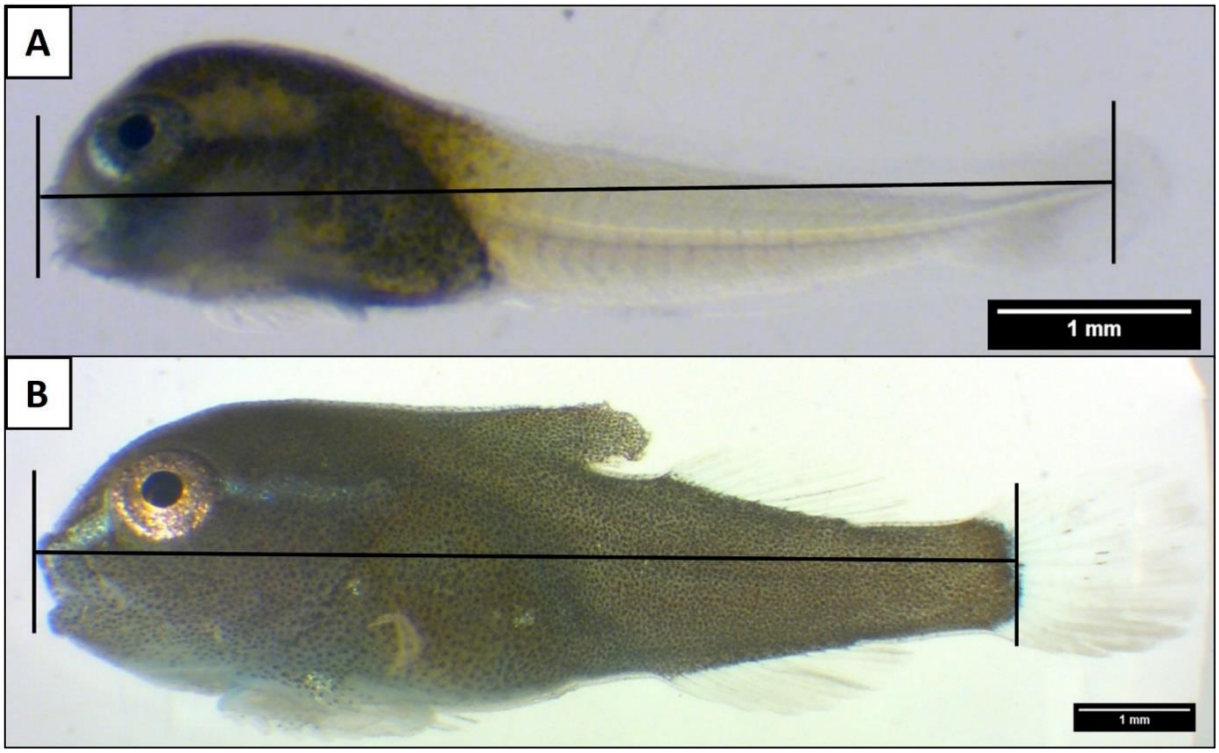


Figure 2.3. Standard length measurements of lumpfish larvae A) Standard length (SL) measured from the tip of the lumpfish' snout to the end of the notochord on a 2 dph larvae. B) SL measured from the tip of the snout to the caudal peduncle on a 34 dph FD-larva. Scale bar: 1 mm.

2.5.3 Dry weight and DWI

Dry weight (DW) measurements were done on the same larvae sampled for SL, except for the last larger sample at 35 dph which was only used for SL. After pictures for SL were taken, the larvae were placed individually in pre-weighed tin capsules. The larvae were then dried at 60 °C for at least 24 hours before weighed on a micro-balance (UMX2 Ultra-microbalance, Mettler-Toledo, USA) to measure DW. Daily weight increase (DWI) in percentage was calculated by first determining the specific growth rates (SGR) between specific sampling intervals by using the following equations (Houde & Schekter, 1981), where W_2 and W_1 is an individual larva's dry weight at time t_2 and t_1, respectively:

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

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

2.5.4 Survival

As previously stated, all remaining larvae were taken out from the tanks at the end of the experiment (35 dph) and concentrated into buckets according to tank numbers and euthanized with an overdose of tricaine methanesulfonate (MS-222 Finquel ®, Argent Chemical Laboratories Inc., USA). 250 fish per bucket (tank) were taken out for measuring SL. The remaining fish larvae were then counted. The number of larvae at the experimental end together with registered samples and mortality data were used to estimate survival throughout the experiment. Survival (S_t) throughout the experiment was calculated for each tank using the following equation:

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

where N_t is the number of larvae alive at time t (dph), whereas N_0 is the total number of larvae alive at the start of the experiment, calculated using the mortality and sampling data. In the calculation, it was assumed that all sampled larvae would have been alive if not for sampling.

2.5.5 Histological analysis of the liver

The larvae were retrieved from fixation, rinsed in PBS and first taken pictures of to measure SL using ImageJ (Schneider, Rasband & Eliceiri, 2012) the same way as described in chapter 2.5.2. Next, the larvae were embedded in resin (Technovit ® 7100, Kulzer, Germany; Appendix 7). Embedded larvae were sectioned longitudinally in the sagittal plane in 2 µm thin sections using a microtome (Jung Autocut 2055, Leica Biosystems, Germany) with carbide metal blades (Leica TC65 Microtome Blade, Leica Biosystems, Germany). Sections were taken out for every 10 µm as a precaution to avoid measuring the same nuclei/hepatocyte twice. The sections were placed on a glass slide (SuperFrost ®, Menzel-Gläser, Germany) and dried on a heating table (Stretching table OTS 40, Medite, Germany) for a minimum of 10 minutes at 75 °C. The sections were then stained with 0.05 % toluidine blue (TB) (Honeywell Riedel-de-Haën™, Germany). Additionally, one fish larva from each treatment from 21 and 35 dph was also stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) to examine for glycogen deposits. Staining protocols for TB and AB-PAS is given in Appendix 8. After staining, the slides were mounted (Neo-Mount®, Merck, Germany) with coverslides (Microscope Cover Glasses, VWR, Germany). Mounted slides were scanned in three 0.5 µm layers at 40x magnification using a slide scanner (NanoZoomer SQ, Hamamatsu Photonics, Japan). Thereafter the image viewer software NDP.view2 v.2.9.22

(Hamamatsu Photonics, Japan) was used to examine the quality of the scanned sections before picking the sections with the most liver tissue visible to analyse further.

The quantitative analysis of the liver was done by using the image analysis programs QuPath v.0.2.3 (University of Edinburgh, Scotland) and ImageJ v.1.53k (Schneider, Rasband & Eliceiri, 2012). In QuPath, grids for analysis were placed randomly either on the same section of liver (Figure 2.4A) or onto a series of sections of the fish's liver (Figure 2.4B), depending on the quantity of liver tissue in each section. For 2 dph a total of 0.18375 mm^2 (6 grids at 175µmx175µm) were analysed per fish, whilst for 9, 21 and 35 dph an area of 0.18750 mm^2 (3 grids at 250µmx250µm) were analysed per fish. The liver area analysed was based upon Marthinsen (2018), which measured an area of 0.1752663 mm^2 on average per larva.

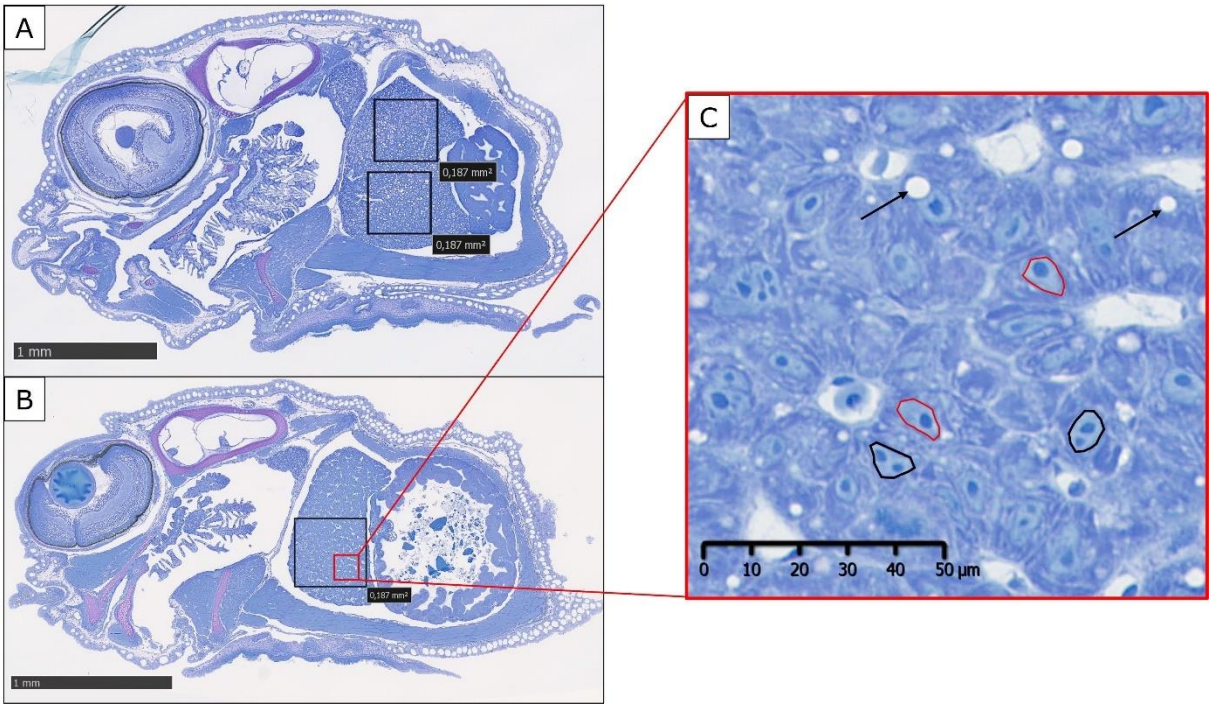


Figure 2.4. Examples of grid placement and nuclei types in the histological analysis of the liver in lumpfish 2-35 dph. A) An Art-larva from 35 dph clearly fits two grids at 250x250 µm, thus only needing one more section to place the last grid. Scale bar: 1 mm. B) The liver of a Cop/FD-larva from 21 dph barely fits one grid at 250x250 µm and thus several sections were used to place all three grids. Scale bar: 1 mm. C) Close-up of the liver of the Cop/FD-larva from 21 dph. In the analysis it was distinguished between nuclei with one prominent nucleolus (circled in red) and nuclei with two or more nucleoli (circled in black). The black arrows indicate vacuoles in the hepatocytes. Scale bar: 50 µm.

The polygon tool in QuPath was used to trace the borders of the hepatocyte nuclei, separating between nuclei with one nucleolus and nuclei with two or more nucleoli (Figure 2.4C). In average 264 nuclei were measured per fish. Nuclei touching the edges of the grid, or missing one or more distinct nucleoli, were not measured.

This data was further used to estimate hepatocyte size, assuming that the hepatocytes were mononuclear, calculated as analysed liver area of the grids in total divided by the total number of nuclei measured in the grids (A_{liver}/N_{nuclei}). The total nuclear area fraction was also derived from this data, calculated as the total nuclear area in the grids divided by the analysed liver area in the grids (A_{nuclei}/A_{liver}). Each grid was also exported to ImageJ, and a point-grid of crosses was applied to estimate the vacuole fraction in the hepatocytes

$(A_{vacuoles}/A_{liver})$ using the multi-point tool for counting. The point grid had a density ensuring 225 crosses to hit the liver tissue (Hamilton, 2004; Wold *et al.*, 2009). A hit on a vacuole was counted when the vacuole was within the top right-hand corner of the cross. Lastly, the area fraction of 'other' liver components such as sinusoids, central veins, organelles, etc. was calculated by subtracting the sum of nuclear and vesicular fractions: $'Other' = 100\% - nuclear\ area\ fraction - vesicular\ area\ fraction)$.

Note that for 21 and 35 dph the sampled larvae were shared with Marte Solli Lindskog which cut the larvae through the transversal plane behind the anus (Appendix 7, Figure A2) to investigate the skeletal muscle development. The remaining front part of the fish was thus used for histological analysis of the liver and explains why in some images the fish is missing its tail.

2.6 Statistical analyses

All statistical analyses were done using SigmaPlot v.14 (Systat Software Inc., USA), a graphing program with in-built tools for data analysis. A significance level of α=0.05 was used for all statistical tests. Arcsine transformation was applied to percentage data before statistical testing. Graphs were primarily made using SigmaPlot, but also Microsoft Excel v.2109 (Microsoft, USA). Tables were made exclusively in Microsoft Word v.2109 (Microsoft, USA).

For detecting differences either between treatment groups at the same day or within the same treatment over the experimental period, a One-Way Analysis of Variance (ANOVA) was done. When doing the ANOVA, the program also tested for normal-distribution of the data using the Shapiro-Wilk test as well as homogeneity of variance with the Brown-Forsythe test. If the test passed for both normality and equal variance, Tukey was used for post-hoc test. If the data was non-normal and of unequal variance, the non-parametric equivalent for ANOVA, Kruskal-Wallis, was used. Subsequently, the Dunn's post-hoc test was used for the non-normal data.

Linear regressions were made of data from the liver analysis and the corresponding SL. A One-Way Analysis of Covariance (ANCOVA) was used to investigate if the linear equations differed significantly between the treatments, and whether the data were significantly affected by the treatment, SL or the combination thereof. Further, the Holm-Sidak method was used for pairwise multiple comparisons to investigate whether the linear equations of the different treatments were significantly different.

When managing the data, it was decided to pool the results for nuclei with one nucleolus and nuclei with two or more nucleoli, as there was much fewer of the latter. Therefore, results presented are to be assumed to come from the pooled dataset, except for chapter 3.4.5 where the different nuclei types are compared.

As mentioned earlier, a larger sample of SL was performed at 35 dph (n= 750 larvae per treatment group) to see if there was a sampling bias. This was investigated by making a prediction of how long the larvae sampled at 34 dph (n=45 larvae per treatment group) would have been at 35 dph, using the SGR between 29-34 dph. Welch's t-test was applied to see if there was any significant difference in the predicted length at 35 dph versus the actual length measured in the large sample within treatment groups.

3 Results

3.1 Morphological development and general observations

The lumpfish larvae's external morphology changed considerably throughout the start-feeding experiment (Figure 3.1). Visual assessment of developing features like the larvae's overall body shape, skin colour and fin development revealed that overall external morphological development was however quite similar between treatments. Therefore, a general description was made based upon Art-larvae (Table 3.1), as these larvae presented the best growth throughout the experiment. Although the description is based upon Art-larvae and the colour of the larvae is therefore described accordingly, it should be noted that the lumpfish larvae had a wide range of colours; all from pale yellow, brown, and green shades to dark red colour. However, the dark red colour depicted was of the most abundant colours of the larvae, especially among the Art-larvae.

Table 3.1. External morphology description of lumpfish 2-34 dph. General morphology description of lumpfish larvae at 2, 9, 21 and 34 dph from the present start-feeding experiment. The description is based on pictures of Art-larvae depicted in Figure 3.1.

Dph	Description external morphology
2	The larvae had a large, rounded head with big eyes situated on each side. The trunk was short and lacked distinction from the head, whereas the tail was long (about 2/3 of the total body length) and laterally compressed. The rounded head and trunk together with the tail made the body shape of the larvae similar to a tadpole. Yellow colour pigments speckled with brown and black dominated the larvae's head and trunk. The larvae had a distinct band of lighter pigments on each side of the eyes, starting from the mouth and ending above the operculum. Although the larvae were pigmented, the yolk sac was visible through its skin. Faint yellow pigmentation covered the tail, except for the continuous median finfold that were still transparent. The notochord was visible amid the tail. Fin rays were appearing along the dorsal side of the finfold, in the pectoral fins, and in the functional suction disc located ventrally to the centre of the trunk region.
9	The tail was less laterally compressed and more pigmented, however the notochord was still easily visible. The band of pigments on each side of the eyes were now reflective, making it stand out against the fish' overall dull yellow and brown colour. The finfold had begun reabsorbing and had formed two dorsal fins and one ventral fin. Fin rays had formed in all these. The anterior dorsal fin had become slightly overgrown by epidermal tissue. The suction disk had become pigmented and the fin rays in it were more prominent.
21	Opaque red and brown pigmentation covered the larval body. As the head/trunk region were now more oval and the tail had grown thicker, the transition between the regions were more seamless, giving the larvae a more streamlined shape. The finfold were completely reabsorbed and fin rays were prominent in all fins. The caudal fin had become a rounded shape. Epidermal tissue had now overgrown the first dorsal fin completely.
34	The larvae's body shape was not tadpole-like anymore and had become streamlined. The larvae's overall colour was red with brown speckles. Pigmentation had also spread to the suction disk. The reflective pigment-band beside the eyes were still prominent. Faint yellow pigmentation was spotted on the posterior ventral fin, dorsal fin and caudal fin.

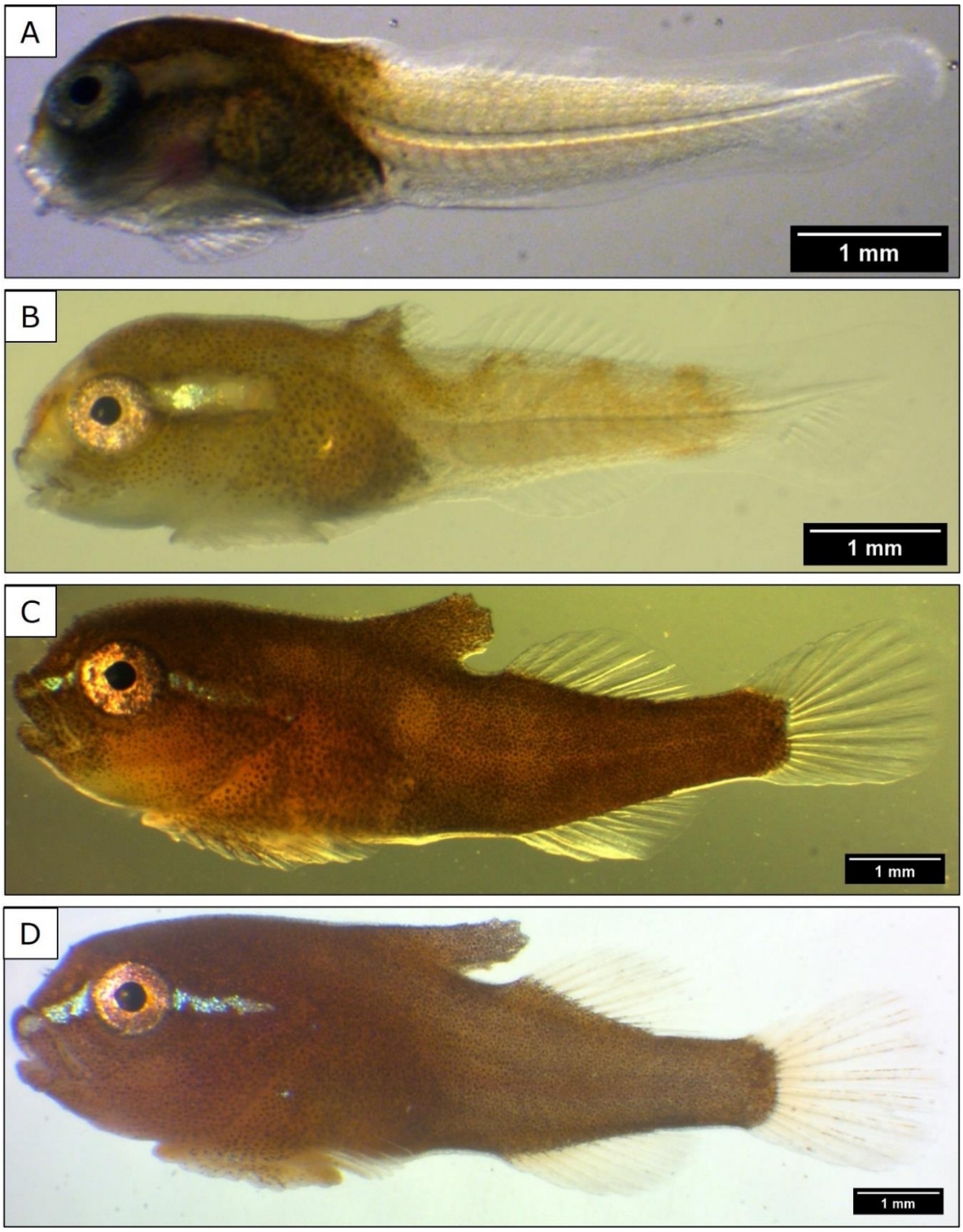


Figure 3.1. External morphology of lumpfish 2-34 dph. Morphology of lumpfish larvae in the present study at: A) 2 dph, B) 9 dph, C) 21 and D) 34 dph. Each picture is of an Art-larva. Scale bar: 1 mm.

Behavioural changes observed were mainly in form of the lumpfish either attaching to surfaces using its suction disc or swimming around the tank. Between hatching and first feeding (0-2 dph) all larvae were quite active and swam around the tanks. After introducing feed, larvae seemed to settle and attach to surfaces in the tank or the silicon mats. As the larvae grew bigger, they seemed to switch more easily between swimming and attaching compared to when they were younger and were mostly attached. Art-larvae sat attached the most, with little swimming observed compared to the other treatment groups.

3.2 Growth and survival

3.2.1 Dry weight

At 2 dph the larvae's mean dry weight was 0.91±0.05 mg (Figure 3.2). By 9 dph the Art-larvae were significantly bigger than Cir-larvae, but not the other larval groups. From then, the Art-larvae grew steady and had a signifcantly higher DW than all the other larvae at 15 and 21 dph. The Cir-, Cop/Cir- and FD-larvae also experienced growth between 15 to 21 dph, but not to the same extent as the Art-larvae. In contrast, the Cop/FD-larvae's DW had only kept stable and was at 21 dph significantly lower than all the other larval groups. By the last two sampling days, 29 and 34 dph, the Cir-larvae's DW had increased to the same range as the Art-larvae's and were no longer significantly different. Art- and Cir-larvae were both significantly heavier than larvae from the three other larval groups at 29 and 34 dph. The three other larval groups, Cir-, Cop/Cir- and FD-larvae also experienced an increase in DW at 30 and 34 dph, but not enough to reach the same range as the Art- and Cir-larvae's dry weights. The Cir-, Cop/Cir- and FD-larvae's dry weights were not significantly different from each other on neither of the last two sampling days. Mean DW per tank for each of the treatment groups are given in Appendix 9, Table A3.

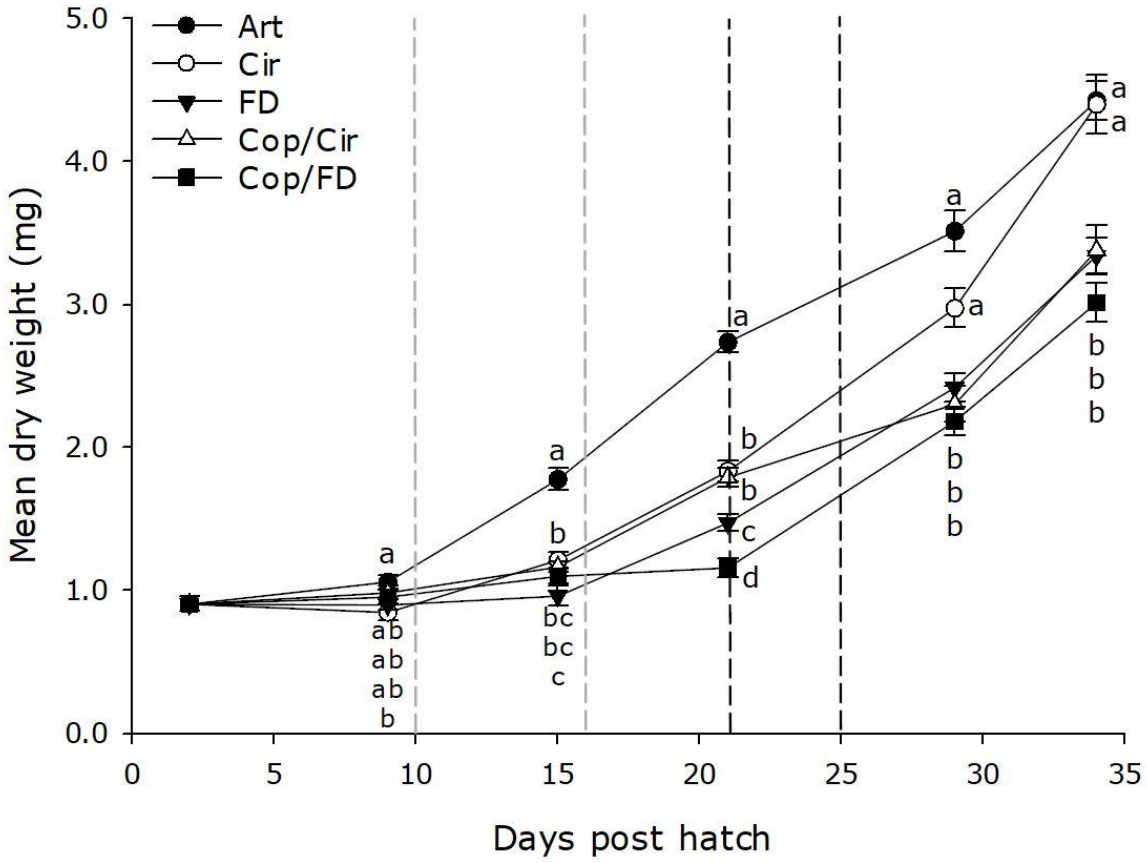


Figure 3.2. Mean dry weight in lumpfish 2-34 dph. Means (mg) are based upon increasing sample sizes: n=15 (2 dph), n= 15 per treatment (9-15 dph), n= 30 per treatment (21 dph) and n= 45 per treatment (29-34 dph). Grey dashed lines indicate weaning from copepods to formulated diet for the Cop/FD-larvae, and to cirripeds for the Cop/Cir-larvae. Black dashed lines indicate weaning from respective feeds to formulated diet for the remaining larvae groups. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters. Error bars indicate ± standard error (SE).

3.2.2 Daily weight increase

At the introduction to the feeding regimes between 2-9 dph, only the Cir- and FD-larvae had a negative daily weight increase (DWI) (Figure 3.3). However, no larval groups were significantly different at this interval. DWI were positive in all larvae for later time intervals. At the interval of 9-21 dph, the Art-larvae had the highest DWI and the Cop/FD-larvae the lowest. The Cir-larvae had the second highest DWI at this time interval, and both Art- and Cir-larvae were significantly different from the Cop/FD-larvae. In the last time interval 21-34 dph, when formulated diet was introduced for the first time to the Art-, Cir- and Cop/Cir-larvae, the Art-larvae had a large decrease in DWI as opposed to the two larval groups weaned from cirripeds, which exhibited a similar DWI as the previous time interval. The FD- and Cop/FD-larvae, which already were on formulated diet at this time, experienced an increased DWI compared to the former time interval. Most notably the Cop/FD-larvae increased from having the lowest DWI at 9-21 dph, to the highest at 21-34 dph. When averaging the whole experimental period into one interval (2-34 dph), both the Art- and Cir-larvae had a significantly higher DWI than all the other treatment groups but were not significantly different from each other. The larvae with the lower DWIs, the FD-, Cop/Cir- and Cop/FD-larvae were also not significantly different from each other for the 2-34 dph interval. Mean DWI per tank for each of the treatment groups are given in Appendix 10, Table A4.

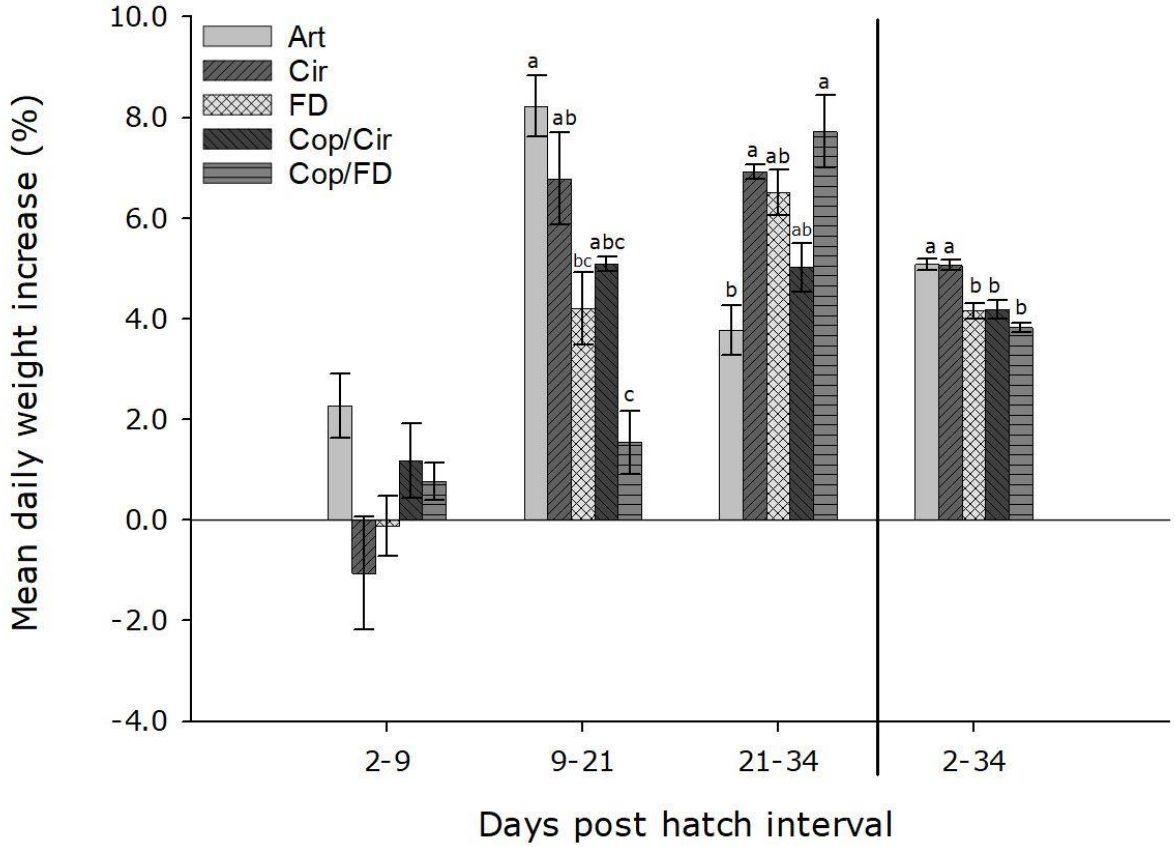


Figure 3.3 Mean daily weight increase in lumpfish 2-34 dph. Mean daily weight increase (%) as a function of specific time intervals (dph). The means are based upon increasing sample sizes: n=15 (2 dph), n= 15 per treatment (9-15 dph), n= 30 per treatment (21 dph) and n= 45 per treatment (29-34 dph). The solid vertical line separate consecutive intervals in the treatment period and the whole experimental period (2-34 dph). Signficant differences (p<0.05) between treatment groups at a given dph interval are indicated by different letters. Error bars indicate ± standard error (SE). Note that vertical axis starts at -4.0 %.

3.2.3 Standard length

At 2 dph larvae was 5.94±0.10 mm long (Figure 3.4). Already by 15 dph, Art-larvae were significantly longer than Cir-, Cop/FD- and FD-larvae. The Art-larvae were also significantly longer than all other larvae at 21 dph. However, after weaning to formulated diet for the Art-, Cir- and Cop/Cir- larvae between 21-25 dph, the Cir-larvae's length increased to the same range as the Art-larvae's at 29 and 34 dph. In contrast, Cop/Cir-larvae's SL did not increase immediately after weaning. After the weaning, both Art- and Cir-larvae were significantly longer than all the other larval groups at 29 and 34 dph. The last larger sample for standard length at the experimental end (35 dph) of 250 fish per tank, i.e. 750 fish per treatment, presented a different result than 34 dph. Art-larvae were significantly longer than all other larvae, including the Cir-larvae at 35 dph. Additionally, FD- and Cop/Cir-larvae were significantly longer than Cop/FD-larvae at 35 dph but were not significantly different from each other. However, there was found no significant difference within treatments between the predicted length at 35 dph (based on 34 dph larvae and SGR) and the large sample of SL at the experiments end. Mean SL per tank for each of the treatment groups are given in Appendix 11, Table A5.

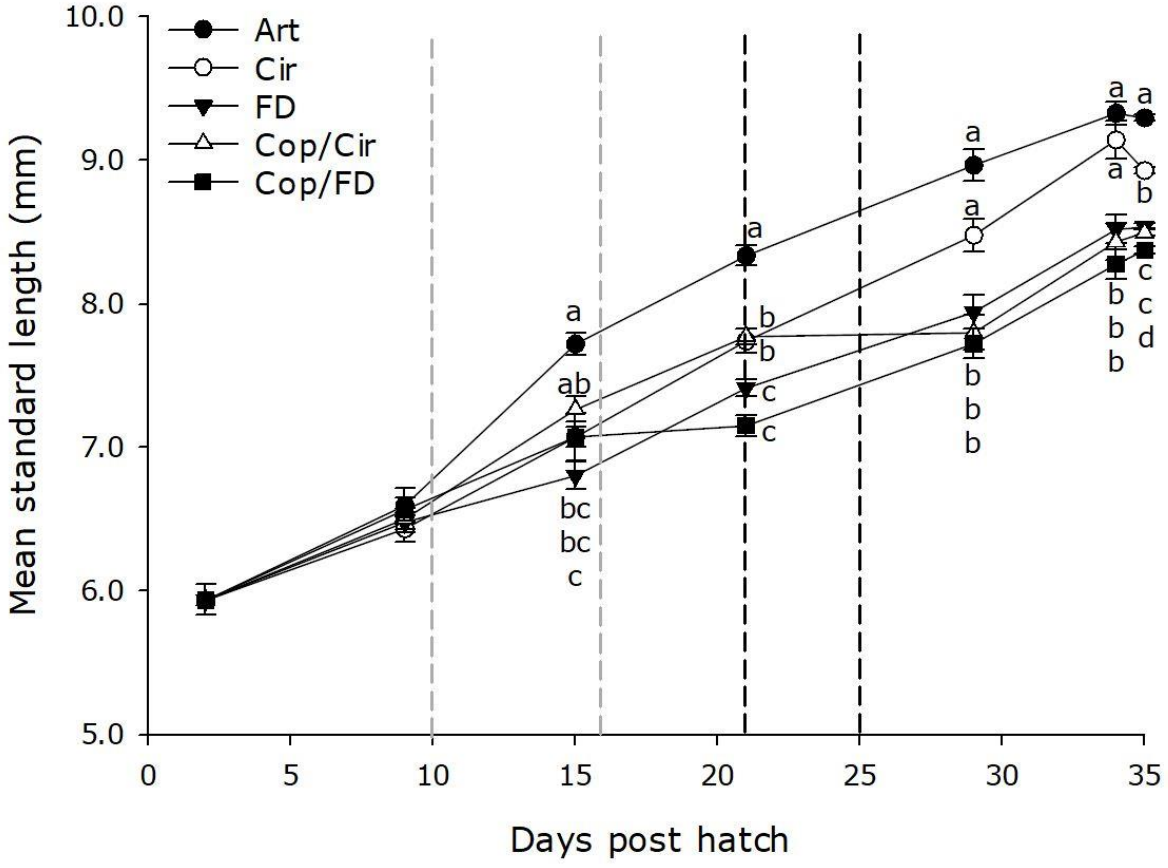


Figure 3.4. Mean standard length in lumpfish 2-35 dph. Means (mm) are based upon increasing sample sizes: n=15 (2 dph), n= 15 per treatment (9-15 dph), n= 30 per treatment (21 dph), n= 45 per treatment (29-34 dph) and n=750 per treatment (35 dph). Grey dashed lines indicate weaning from copepods to formulated diet for the Cop/FD-larvae, and to cirripeds for the Cop/Cir-larvae. Black dashed lines indicate weaning from respective feeds to formulated diet for the remaining larvae groups. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters. Error bars indicate ± standard error (SE). Note that the vertical axis starts at 5.0 mm.

3.2.4 Correlation between dry weight and standard length

The DW and SL had a positive polynomial correlation for the pooled data of the five treatments groups (Figure 3.5), meaning that the larvae's DW increased exponentially as SL increased. Pearson-correlation value were r=0.970 for the pooled data, proving a strong correlation between DW and SL for all larvae, regardless of treatment.

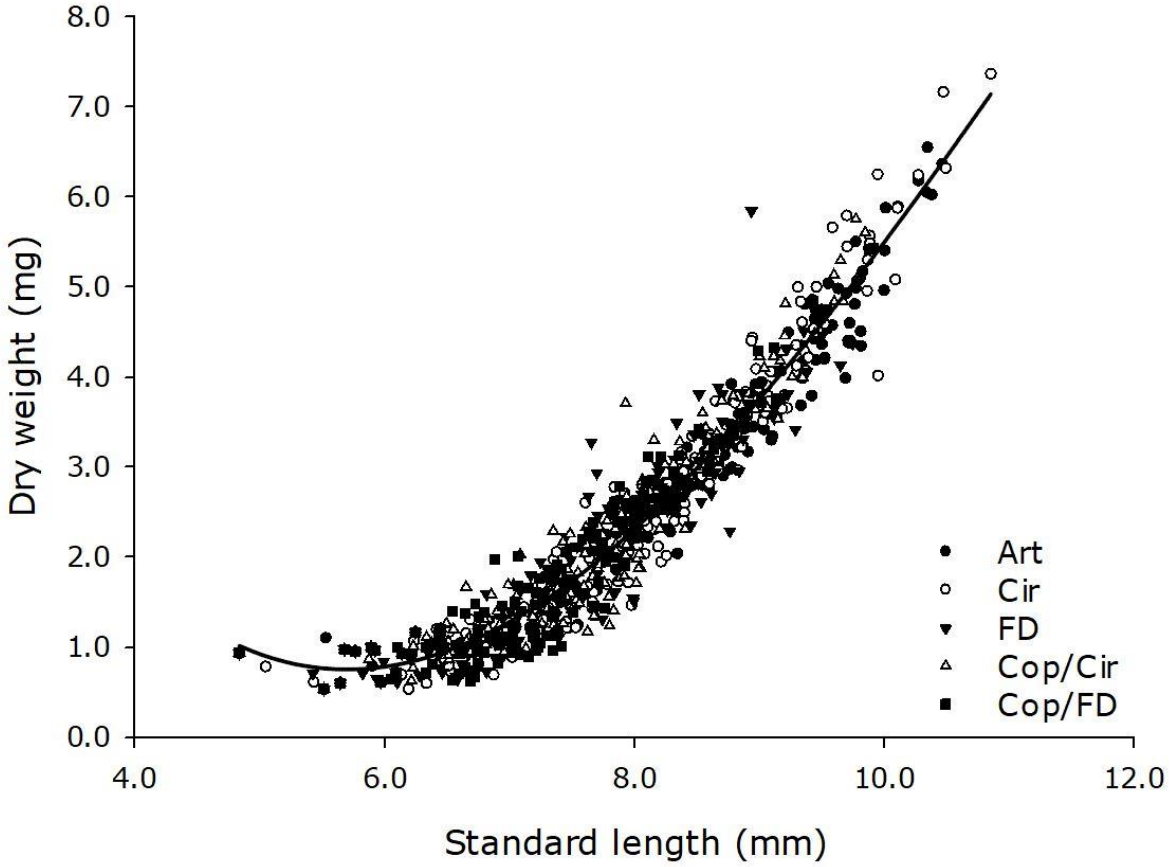


Figure 3.5. Correlation between standard length and dry weight of lumpfish 2-34 dph. Larval dry weight (mg) as a function of standard length (mm) made from pooled data of all treatment groups. Each point represents the SL and corresponding DW of an individual larva, with a total of n=166 larvae per treatment for Art- and Cir-larvae, and n=165 per treatment for FD-, Cop/Cir- and Cop/FD-larvae. Pearson correlation and r^2 for the pooled data: r= 0.97, r^2=0.94.

3.2.5 Survival

After introduction of feed at 2 dph and until the start of the first weaning period at 10 dph, both Cir- and Cop/FD-larvae had a slightly lower survival rate than the other larval groups, although not significantly different (Figure 3.6). However, the Cir-larvae's survival stabilized at about 10 dph, whereas the Cop/FD-larvae's, which were weaned to formulated diet early (10-16 dph) experienced a drop in survival after weaning which lasted until the end of the experiment. At the start of the last weaning period at 21 dph, Art-larvae had a significantly higher survival rate than the Cop/FD-larvae. This pattern went on until the last sampling day at 35 dph, with the Art-larvae still having the highest survival rate at 95±0 % survival, but not significantly different from Cir-, Cop/Cir- and FD-larvae, as there was large variation within these treatments replicate tanks. Lowest survival at 35 dph was in the Cop/FD-larvae at 86±1 % survival, nearly 10 % less survival than the best survival rate found among Art-larvae. Number of larvae alive tank for each of the treatment groups from 2-35 dph are given in Appendix 12, Table A6.

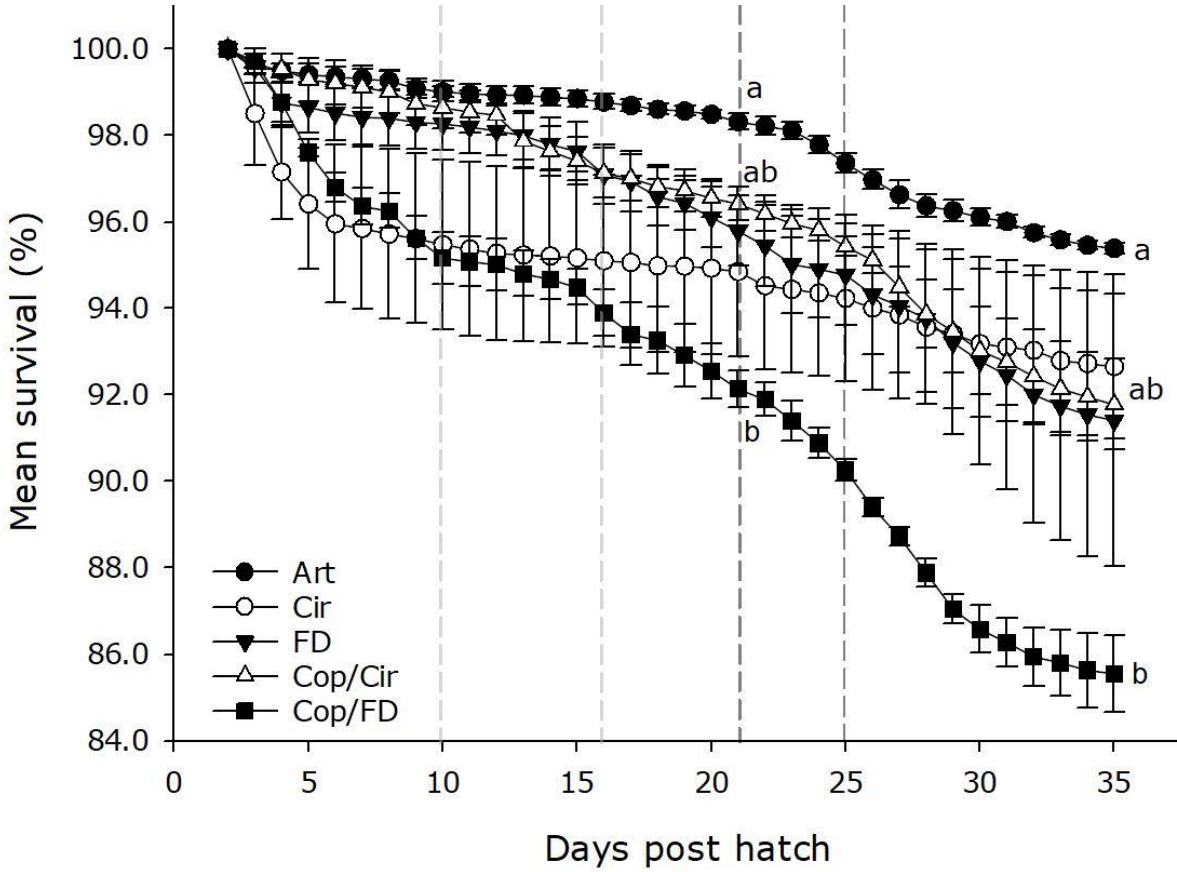


Figure 3.6. Mean survival in lumpfish 2-35 dph. Mean survival (%) are based upon the average of the three replicate tanks of each treatment group. Grey dashed lines indicate weaning from copepods to formulated diet for the Cop/FD-larvae, and to cirripeds for the Cop/Cir-larvae. Black dashed lines indicate weaning from respective feeds to formulated diet for the remaining larvae groups. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters and was tested at 9, 15, 21, 29 and 35 dph. Error bars indicate ± standard error (SE). Note that the vertical axis starts at 84.0 %.

3.3 Liver histology

3.3.1 Liver morphology

Prior to first feeding at 2 dph the liver was located in the left half of the anterior abdominal cavity, above the yolk sac (Figure 3.7A and B) and ended approximately at the centre of the sagittal plane in the fish (Figure 3.7C). Although the size of the liver was not measured, it was generally observed that Art-larvae had the largest liver throughout the experiment as it was required fewer sections of these larvae to place the grids used for analysis. In contrast, it was necessary to have more sections of smaller fish, such as the Cop/FD-larvae, as their livers were not as big.

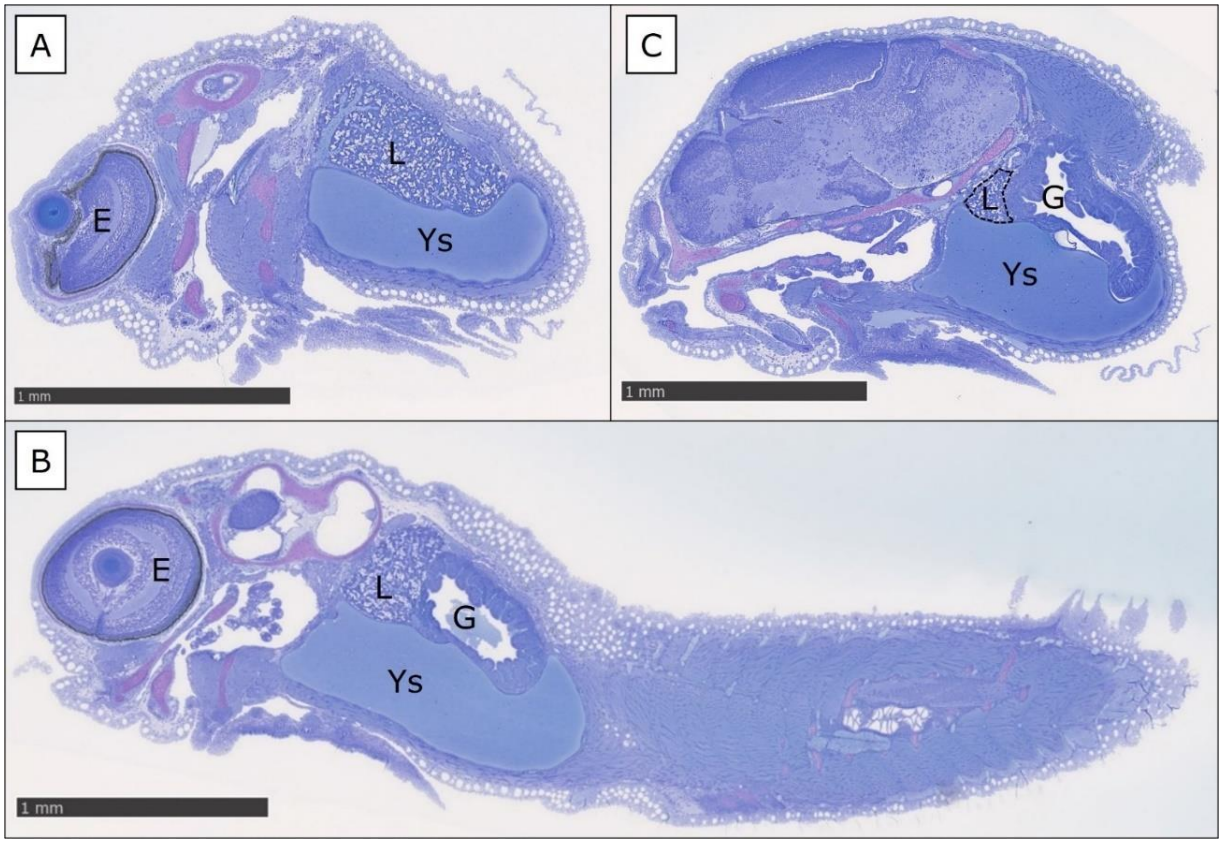


Figure 3.7 Longitudinal sections of lumpfish 2 dph. A) The liver was situated dorsally to the yolk sac in the left half of the anterior abdominal cavity at 2 dph. B) A 150 µm deeper section of the larvae in Figure 3.8 A, showing the liver becoming smaller closer to the centre of the sagittal plane. C) Sectioned past the eye and approximately in the centre of the sagittal plane of the fish. The liver (outlined in black) was now barely visible. Scale bars: 1 mm. The sections were stained with TB 0.05%. Abbreviations: E=eye, L=liver, Ys=yolk sac, G=gut.

The liver parenchyma was fully differentiated at 2 dph and was composed of polyhedral hepatocytes arranged around central veins with sinusoids and bile canaliculi scattered in between. Nuclei in the hepatocytes were nearly spherical and were distinguished from the sinusoidal erythrocytes as the hepatocyte nuclei stained darker and had more prominent borders. Within each nucleus there was mainly one prominent, darkly stained nucleolus. However, some nuclei with more nucleoli were observed, most often two. The sinusoids appeared tubular and irregularly shaped in between the hepatocytes. Endothelial cells lining the sinusoids were visible in some areas as they appeared darkly stained. In most

larvae, the cytoplasm was filled with large irregularly shaped vacuoles, which displaced the nucleus to the periphery of the cell (Figure 3.8A). At times, glycogen-like deposits could be observed in the vacuoles surface as faint orange to completely clear blobs depending on the section (Figure 3.8A). In some of the larvae, such large vacuoles were absent at this time, and the nuclei were situated more to the centre of the hepatocytes (Figure 3.8B).

Presence of vacuoles and their content differed greatly between larvae during the experimental period, not only in between treatment groups but also somewhat within treatment groups. However, a general pattern was observed, with more glycogen-like vacuoles among all treatment groups observed in the first part of the experiment (2 and 9 dph) in the larvae where vacuoles were present (Art-larvae), whereas more lipid-like vacuoles (droplets) were observed towards the end (21 and 35 dph). By 9 dph, only the Art-larvae had stable and high vacuolization of the hepatocytes since 2 dph (Figure 3.9A). The other larvae were all quite uniform with low degree of vacuolization, and a central positioning of the nuclei, however the FD larvae stood out with some signs of sinusoidal dilatation (3.9B).

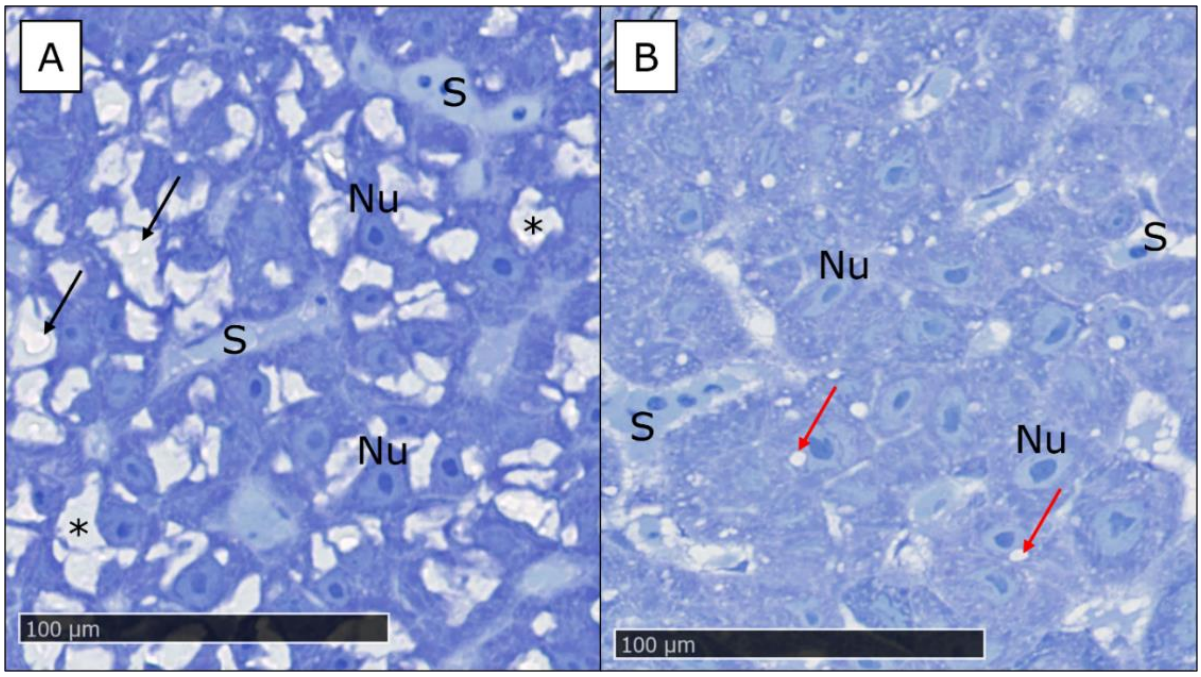


Figure 3.8. Liver structure of lumpfish 2 dph. A) In most larvae, the liver parenchyma appeared fully differentiated with hepatocytes containing irregularly shaped vacuoles (*). Glycogen-like deposits were seen as clear blobs (black arrow) in some vacuoles. B) In some larvae, there were only small vacuoles (red arrow) present. Scale bars: 100 µm. The sections were stained with TB 0.05%. Abbreviations: Nu=nucleus, S=sinusoid.

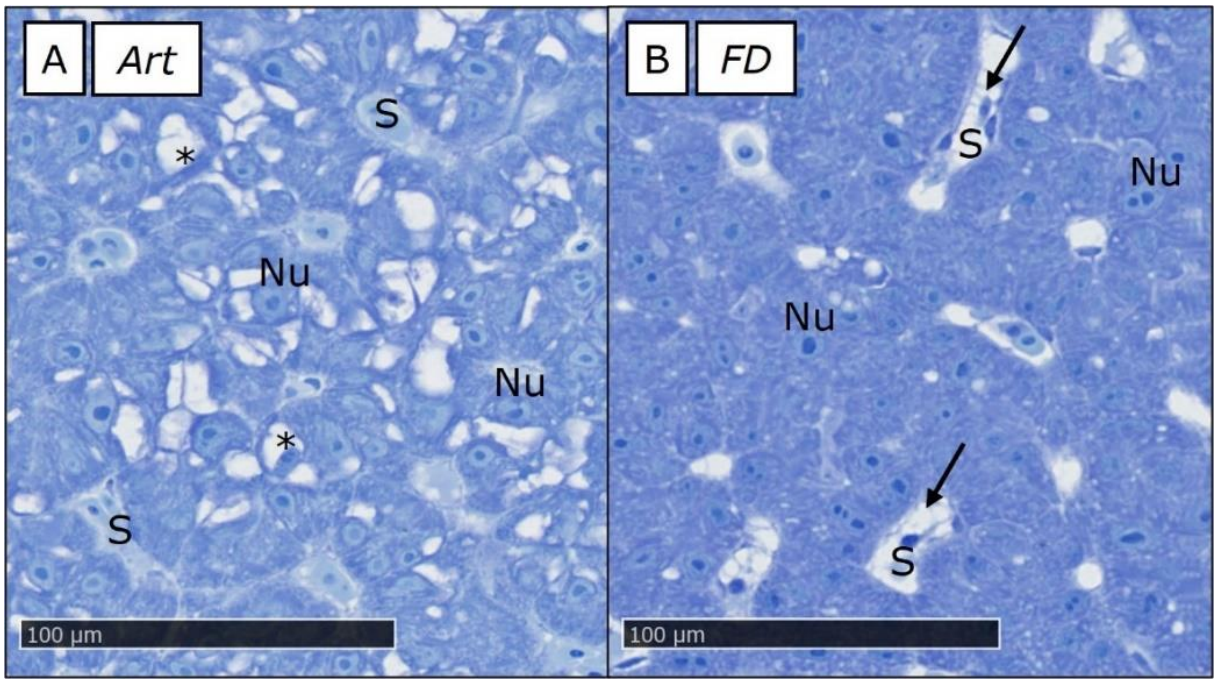


Figure 3.9. Liver structure of lumpfish 9 dph. A) An Art-larva used to represent the respective larval group, showing a high degree of vacuolization (*), similar to that in larvae at 2 dph. B) A FD-larva used to represent the trend among the four other larval groups, showing an absence of vacuoles. Unique for the FD-larvae were that they showed signs of sinusoidal dilatation (black arrow). Scale bars: 100 µm. The sections were stained with TB 0.05%. Abbreviations: Nu=nucleus, S=sinusoid.

At 21 dph Art-larvae clearly had the highest amount of vacuoles, seemingly still of glycogen origin (Figure 3.10A). This was confirmed by positive AB-PAS staining of the contents of the vacuoles in the Art larvae (Figure 3.10B). In contrast, vacuoles were nearly absent in the Cir-larvae (Figure 3.10C). Cir-larvae's nuclei also had a seemingly higher occurrence of two or more nucleoli. The FD-larvae also had quite high vacuolization (Figure 3.10D), but not as big and abundant as in the Art-larvae. Notably, many large nuclei could also be spotted in FD-larvae. Cop/Cir-larvae, which were fed cirripeds at the time, had an absence of vacuoles (Figure 3.10E), similar to Cir-larvae that were also fed cirripeds at this point. Another noteworthy similarity was the high occurrence of nuclei with two or more nucleoli. Lastly, the Cop/FD group now presented vacuoles of lipid origin (droplets) (Figure 3.10E).

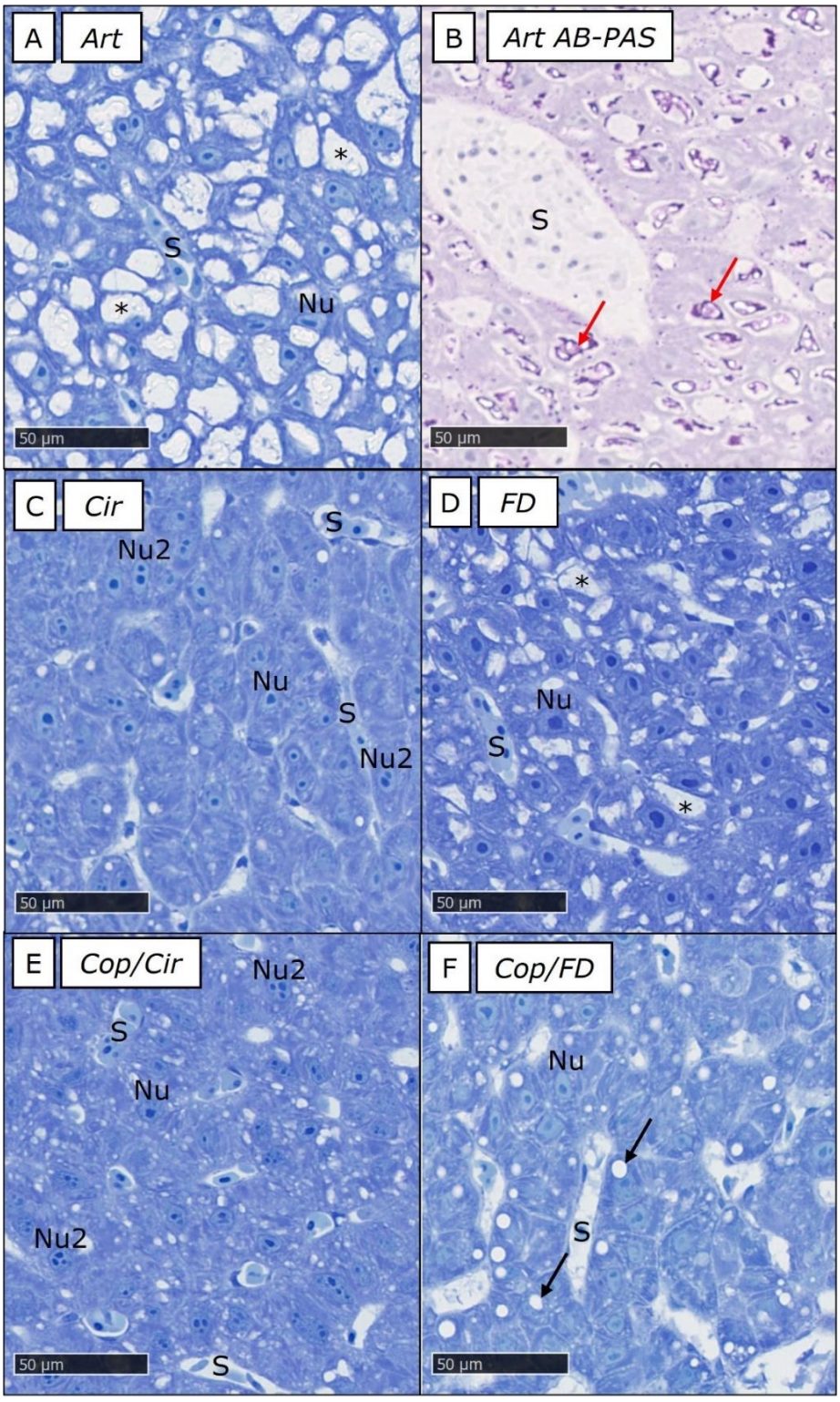


Figure 3.10. Liver structures of lumpfish 21 dph. One larva per treatment group were chosen to represent the overall trend in liver structure among their respective group. A) Art-larva with large and abundant vacuoles (*). B) Art-larva stained with AB-PAS, positive for glycogen content (red arrow) in the vacuoles. C) Cir-larva with an absence of vacuoles and some nuclei with two nucleoli. D) FD-larva with an abundance of vacuoles (*), but not as large as in the Art-larvae. The FD-larvae also seemed to have overall larger nuclei than the other treatment groups. E) Cop/Cir larva with an absence of vacuoles and some nuclei with two nucleoli. F) Cop/FD larva with lipid-like vacuoles (black arrow), as indicated by their droplet form. Scale bars: 50 µm. The sections in A and C-F were stained with TB 0.05%. Abbreviations: Nu=nucleus with one nucleolus, Nu2=nucleus with two nucleoli, S=sinusoid.

The liver had become larger and more elongated towards the ventral side of the buccal cavity by the end of the experiment at 35 dph and constituted a large organ in the fish' body (Figure 3.11A). Still, the liver dominated the left half of the fish and decreased in size towards the centre of the sagittal plane (Figure 3.11B). At the end of the experiment all larvae's liver structures had become similar, with an abundancy of large lipid-like vacuoles in the hepatocytes (Figure 3.12A). This was supported by the AB-PAS staining of the larvae, as there were no longer any PAS-positive contents within the vacuoles (Figure 3.12B).

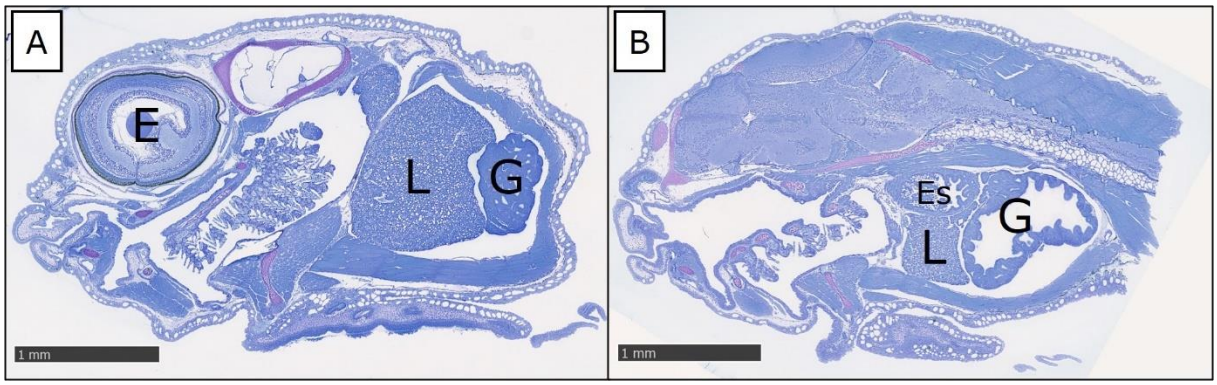


Figure 3.11. Longitudinal sections of lumpfish 35 dph. An Art-larva were chosen to represent all treatment groups. A) The liver had grown and elongated towards the ventral side of the abdominal cavity and constituted a large organ in the fish' body. B) Sectioned past the eye of the fish, the liver still visible under the esophagus. Scale bars: 1mm. The sections were stained with TB 0.05%. Abbreviations: E=eye, Es=esophagus, G=gut, L=liver.

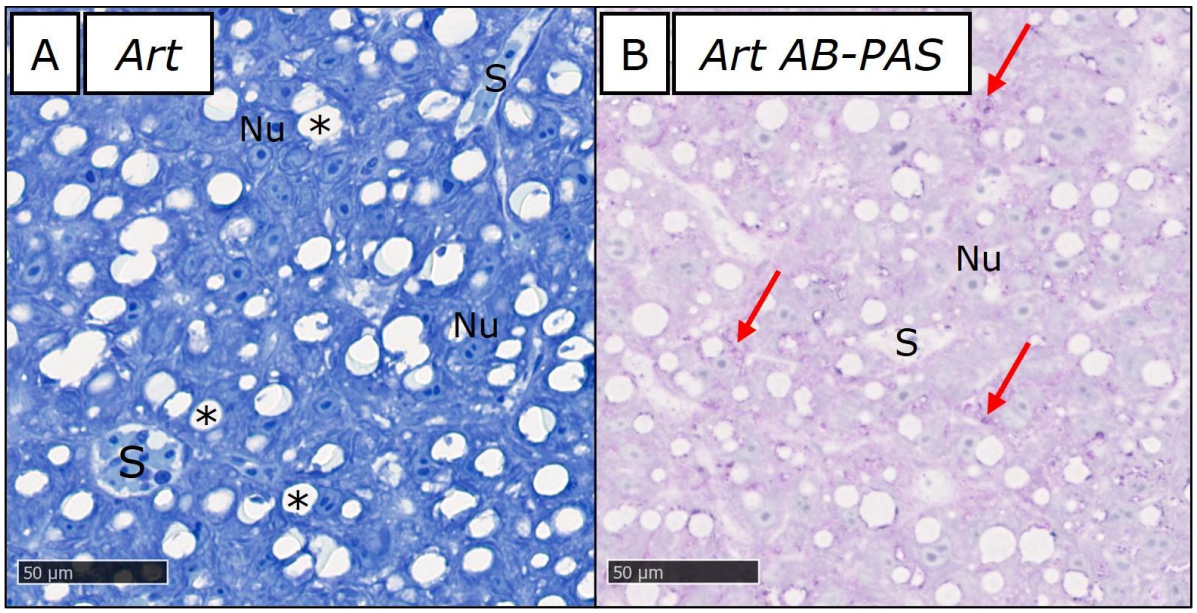


Figure 3.12. Liver structure of lumpfish 35 dph. An Art-larva were chosen to represent all treatment groups. A) All the vacuoles (*) present in the liver resembled lipid droplets and no longer seemed to store glycogen. The section was stained with TB 0.05%. B) AB-PAS staining positive in the hepatocyte cytoplasm (red arrow), but not within the vacuoles, supporting that the vacuoles are likely of lipid origin. Scale bars: 50 µm. Abbreviations: Nu=nucleus, S=sinusoid.

3.3.2 Hepatocyte nucleus size

Hepatocyte nucleus size was the biggest in young larvae and was on average 52.4±3.9 μm^2 at 2 dph (Figure 3.13). A decrease in nucleus size since 2 dph were observed in all larvae at 9 dph. The biggest reduction were observed in FD-larvae, which had the smallest nucleus size of all larvae, but not of statistical signifcance. However by 21 dph, the nucleus size in FD-larvae had increased to become signifcantly bigger than all the other larvae's, which had continued to decrease in size since start. After all larvae had been weaned to formulated diet, there were no longer any significant differences between the larvae at 35 dph. Mean hepatocyte nucleus size for each of the treatment groups is given in Appendix 13, Table A7.

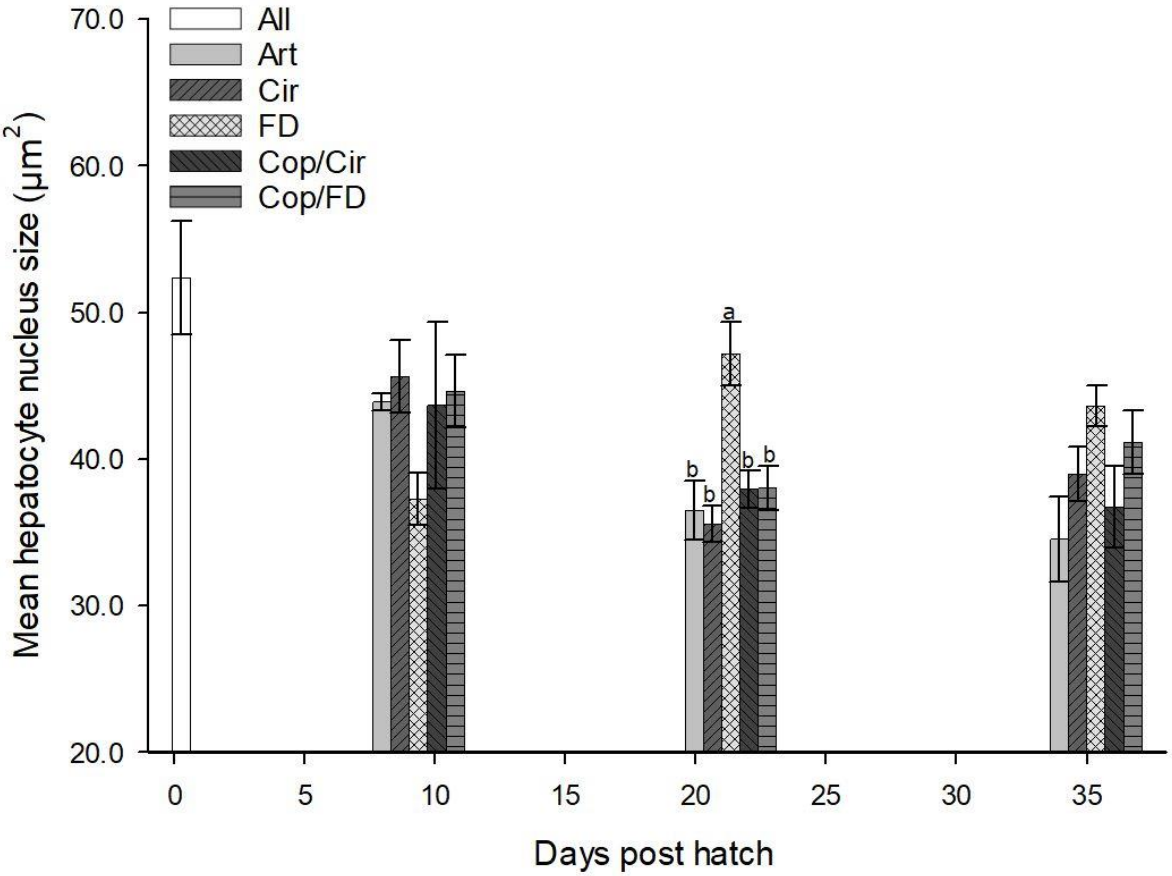


Figure 3.13. Mean hepatocyte nucleus size in lumpfish 2-35 dph. The nucleus size (μm^2) is based upon mean nucleus size in n=5 larvae in total at 2 dph, and n=5 larvae per treatment group for 9, 21 and 35 dph. An average of 264 nuclei were measured per larva. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters. Error bars indicate ± standard error (SE). Note that the vertical axis starts at 20 μm^2.

Mean hepatocyte nucleus size generally decreased with increased SL (Figure 3.14). The main decrease occurred when the lumpfish larvae grew from 5.5 to 7.0 mm. After the fish had become 7.0 mm in length, the hepatocyte nucleus size kept stable between approximately 30.0-50.0 μm^2. Mean SL for each of the treatment groups used for histology and to make correlations are given in Appendix 13, Table A8.

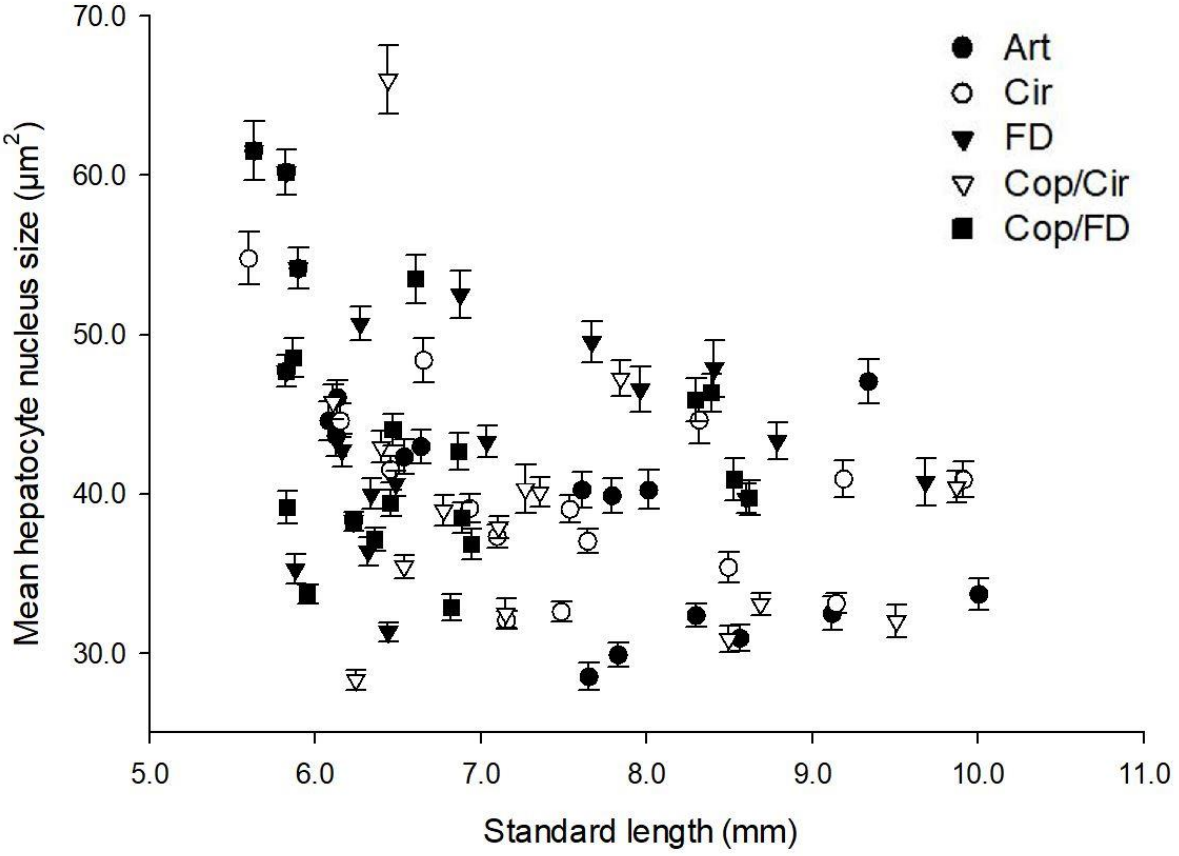


Figure 3.14. Correlation between standard length and mean hepatocyte nucleus size in lumpfish 2-35 dph. Each point represents the mean hepatocyte nucleus size ± standard error (SE) and corresponding SL of an individual larva, with a total of n=20 larvae per treatment group (2-35 dph). Note that the horisontal axis starts at 5.0 mm and the vertical axis at 25.0 μm^2.

3.3.3 Hepatocyte size

Estimated hepatocyte size in the youngest larvae at 2 dph were 750±63 μm^2 (Figure 3.15). Hepatocyte sizes were stable for all larvae from 0-9 dph except for FD-larvae, which exhibited a significant decrease in size to as low as 535±15 μm^2. After Cop/FD-larvae were weaned early onto formulated diet (10-15 dph), the hepatocyte size had decreased at 21 dph. The Cop/Cir-larvae had also been weaned at this time to cirripeds but had kept stable. The Art-larvae stood out, exhibiting hypertrophy at 21 dph and had a significantly bigger hepatocyte size than both Cir- and Cop/FD-larvae. From 21-35 dph the Art-larvae's hepatocyte size kept stable, whereas the other larvae's hepatocyte sizes increased to the same range as the Art-larvae. At the experiments end at 35 dph, there was found no significant difference between the treatment groups. Mean hepatocyte size for each of the treatment groups is given in Appendix 13, Table A7.

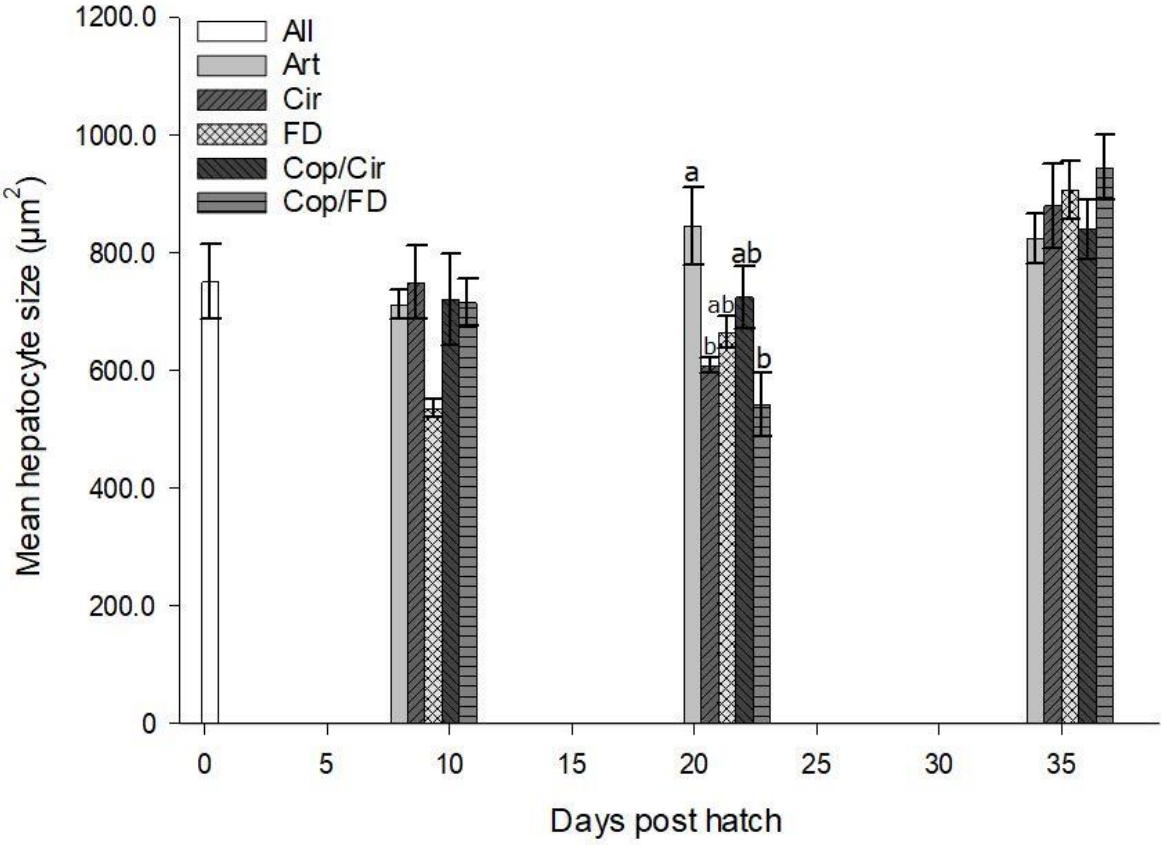


Figure 3.15. Mean hepatocyte size in lumpfish 2-35 dph. Hepatocyte size (μm^2) is based upon mean size in n=5 larvae at 2 dph, and n=5 larvae per treatment for 9, 21 and 35 dph. Hepatocyte size was estimated in each larva by dividing analysed liver area by the number of nuclei. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters. Error bars indicate ± standard error (SE).

The hepatocyte size correlated positively with standard length (r=0.37, r^2=0.13) (Figure 3.16). The hepatocyte size was significantly affected by the SL of the larvae. However, there was no significant differences detected when accounting for the effect of the treatments on this relationship.

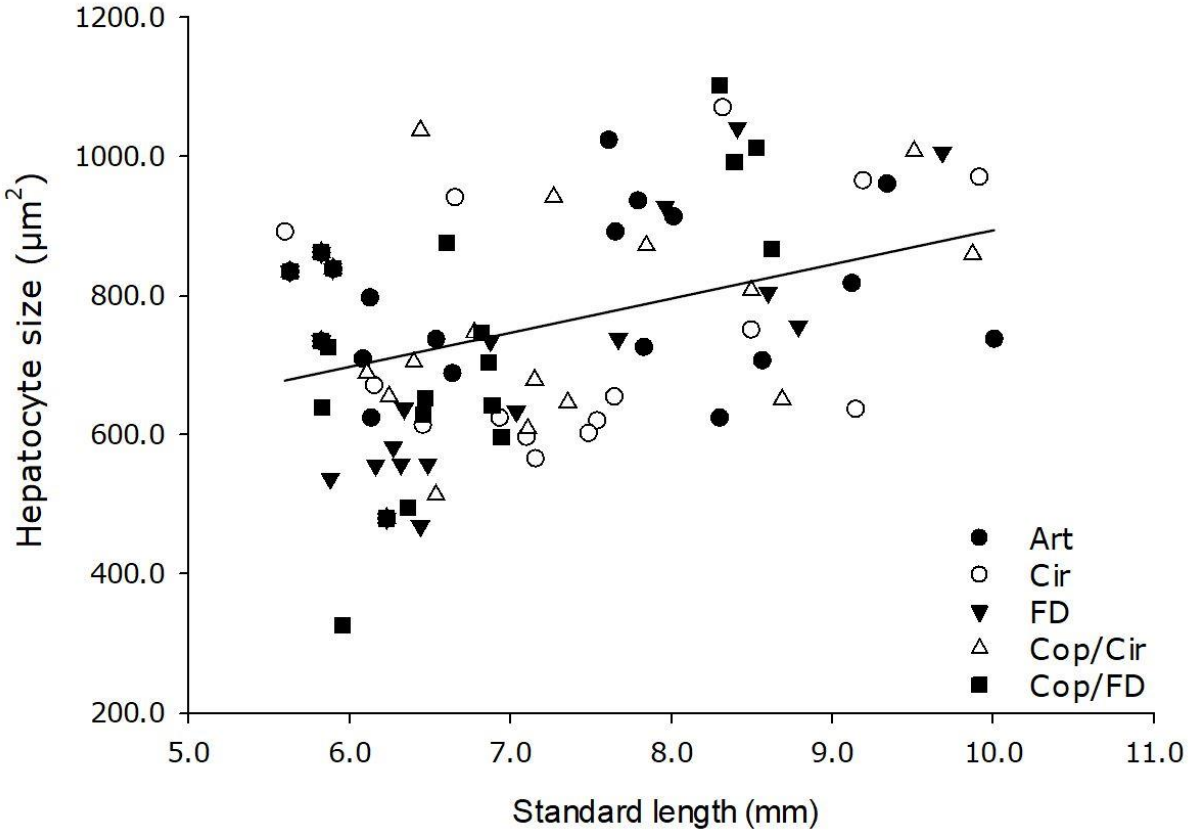


Figure 3.16. Correlation between hepatocyte size and standard length in lumpfish 2-35 dph. Each point represents the hepatocyte size and corresponding SL of an individual larva, with a total of n=20 larvae per treatment group (2-35 dph). Since there was no significant differences between the treatment groups, only the linear relationship for the pooled data is shown. Pearson correlation and r^2 for the pooled data: r= 0.37, r^2=0.13. Note that the horisontal axis starts at 5.0 mm and the verrtical axis at 200.0 µm^2.

3.3.4 Vacuole fraction

Vacuole fraction differed greatly among the treatment groups throughout the start-feeding experiment. Larval vacuole fraction was 15.0±4.5% at 2 dph (Figure 3.17), confirming the variation observed visually among young larvae in Figure 3.8. By 9 dph there were already significant differences, with a significantly larger vacuole fraction in Art-larvae than both larval groups fed copepods at 9 dph, Cop/FD- and Cop/Cir-larvae. The Cir-larvae also had a large vacuole fraction at 9 dph, however, this was due to large variation among the sampled larvae, thus the large standard error. The Art-larvae achieved the largest vacuole fraction of all larvae in the experiment, which was 32.2±1.6% at 21 dph and was significantly larger than all the other larval groups at this time, coinciding with the liver structure shown in Figure 3.10A. Also having large vacuole fractions at 21 dph were the FD- and Cop/FD-larvae, with the FD-larvae's vacuole fraction being significantly larger than the latter. However, differences between these two larval groups had disappeared by the last sampling day, when all larval groups had been fed formulated diet. Cir- and Cop/Cir-larvae's vacuole fraction had also increased after weaning to formulated diet (21-25 dph) and was within the same range as the other larval groups at the experiments end (35 dph). Larvae fed copepods in the beginning (Cop/FD and Cop/Cir) had also increased significantly in vacuole fraction between 21 and 35 dph and was within the same range as the other larvae at the end of experiment (35 dph). This was consistent with Figure 3.12, which showed that all larvae had a similar level of vacuolization at 35 dph. Mean vacuole fraction, as well as the nuclear- and 'other' fraction for each of the treatment groups is given in Appendix 13, Table A9.

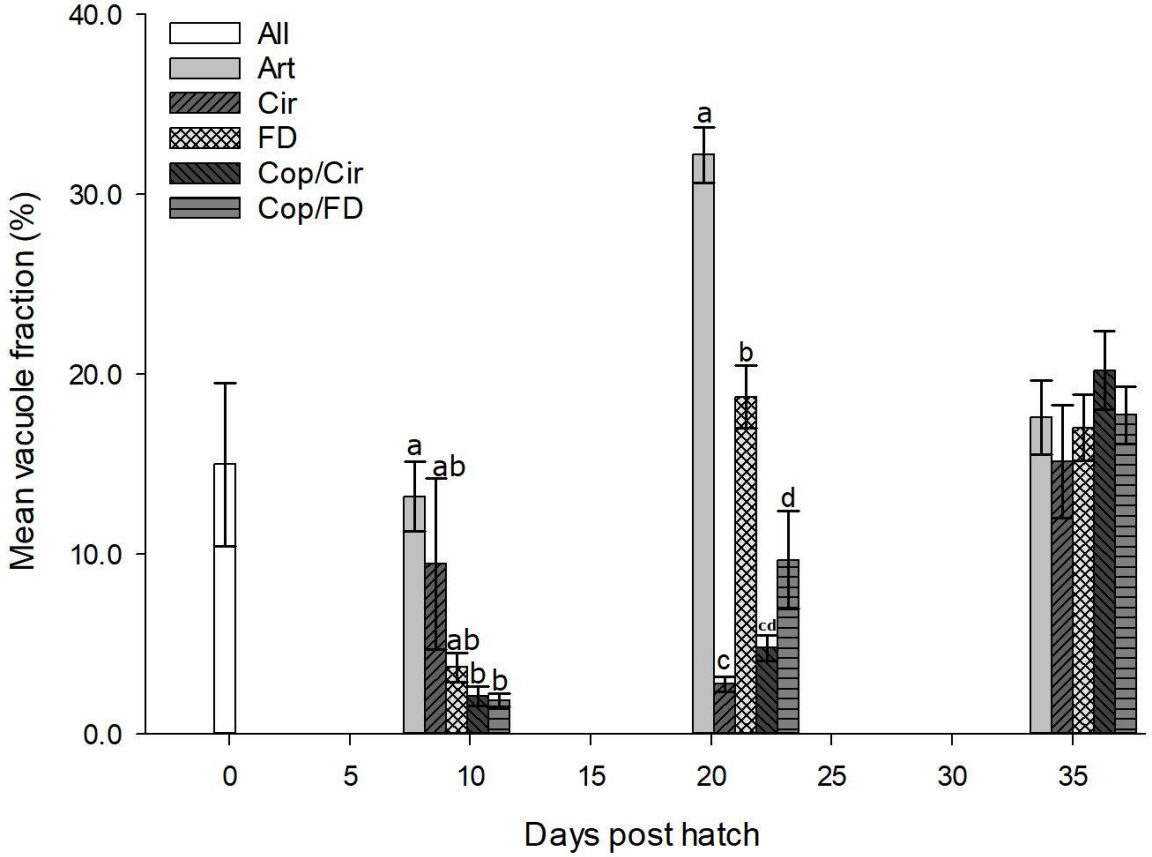


Figure 3.17. Mean vacuole fraction in lumpfish 2-35 dph. Vacuole fraction (%) is based upon mean fraction in n=5 larvae at 2 dph, and n=5 larvae per treatment for 9, 21 and 35 dph. Vacuole fraction was estimated by using a point grid. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters. Error bars indicate ± standard error (SE).

As the fish grew in length, the vacuole fraction generally increased (Figure 3.18). There was large variation in the vacuole fraction among the smallest larvae, as it increased the most when the larvae grew from approximately 5.5 to 8.0 mm in SL. After the fish had become 8.0 mm in length, the vacuole fraction of most larvae was above 10 %.

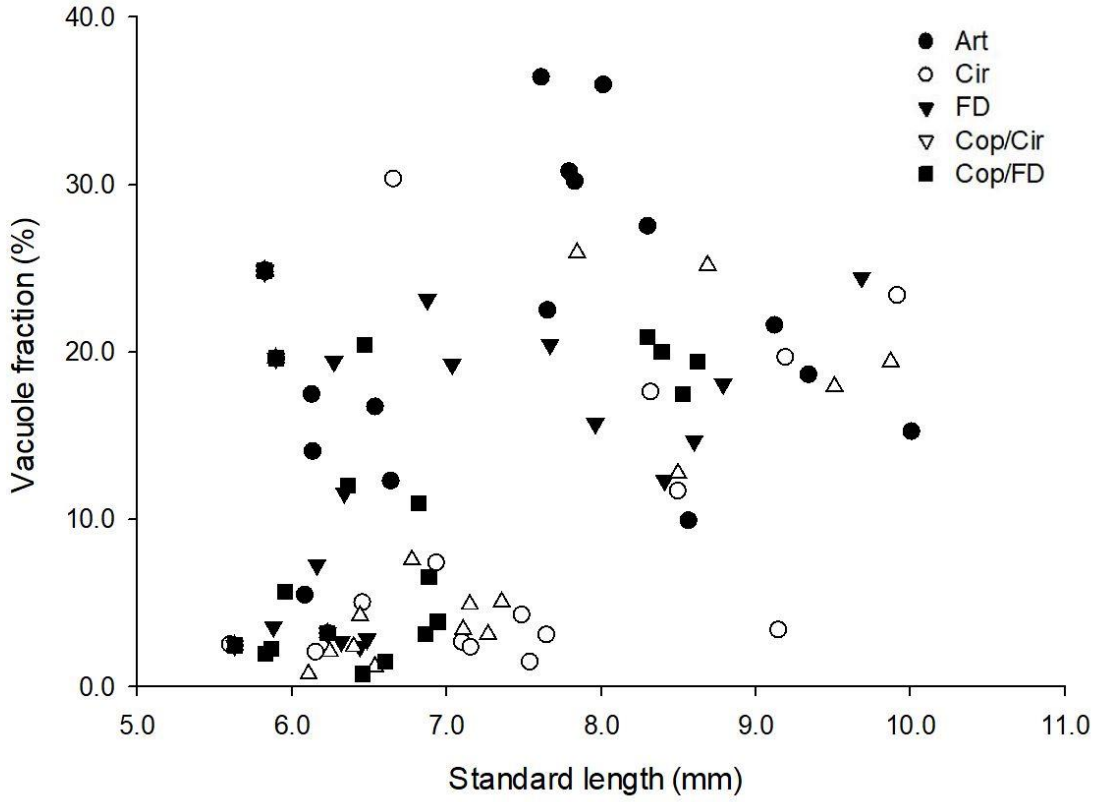


Figure 3.18. Correlation between standard length and vacuole fraction in lumpfish 2-35 dph. Each point represents the vacuole fraction and corresponding SL of an individual larva, with a total of n=20 larvae per treatment group (2-35 dph). Note that the horisontal axis starts at 5.0 mm.

There was a positive correlation between the vacuole fraction and the hepatocyte size for the pooled data (Figure 3.19). The hepatocyte size was significantly affected by the vacuole fraction, meaning an increase in vacuole fraction causes hepatocyte size hypertrophy. There was however no significant differences detected when accounting for the effect of the treatments on this relationship.

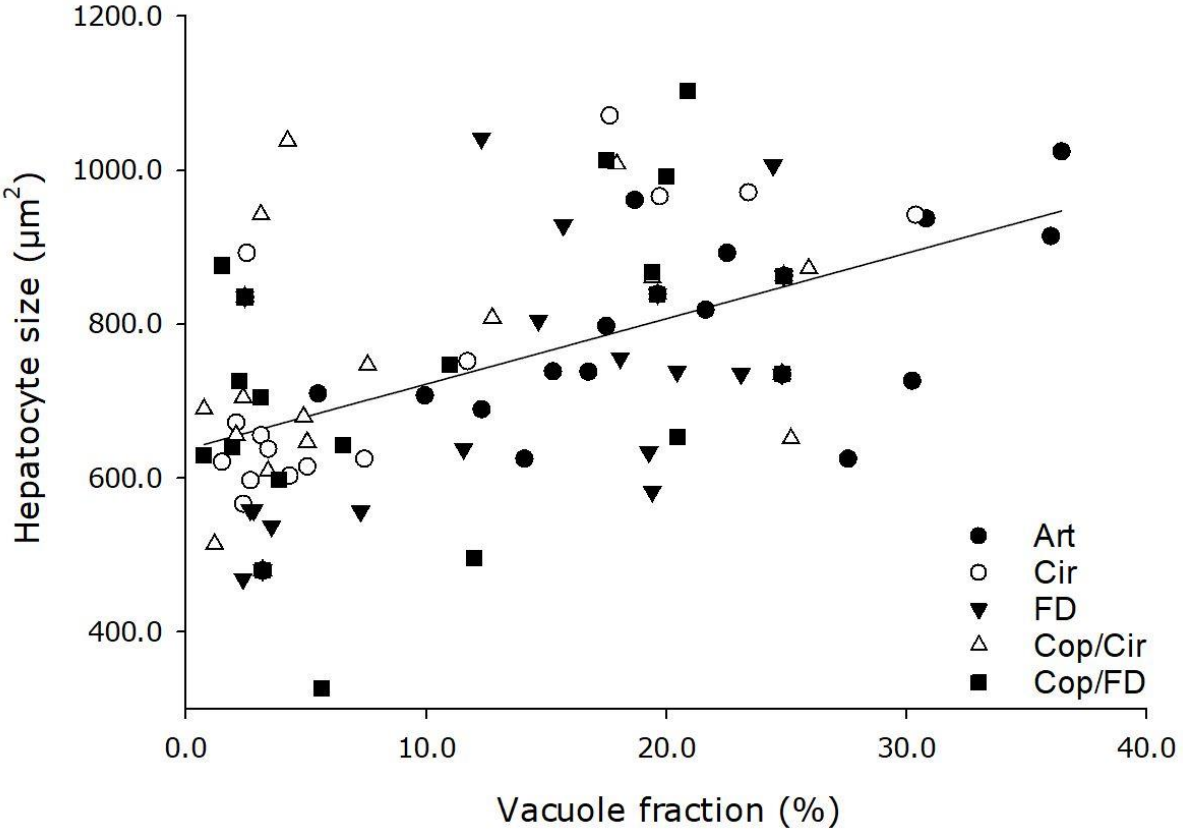


Figure 3.19 Correlation between vacuole fraction and hepatocyte size in lumpfish 2-35 dph. Each point represents the hepatocyte size and corresponding vacuole fraction of an individual larva, with a total of n=20 larvae per treatment group (2-35 dph). Since there was no significant differences between the treatment groups, only the linear relationship for the pooled data is shown Pearson correlation and r^2 for the pooled data: r= 0.54, r^2=0.29. Note that the y-axis at 300.0 µm^2.

3.3.5 Hepatocyte nuclei types

The proportion of hepatocyte nuclei with one nucleolus or nuclei with two or more nucleoli differed over the experimental period. From here on, these two nuclei types will be referred to as Nuclei 1 and Nuclei 2+, respectively. At 2 dph, Nuclei 1 were clearly the most abundant, making up as much as 99±0 % of all nuclei (Figure 3.20). By 9 dph the proportion had shifted and was approximately 80 % Nuclei 1 and 20 % Nuclei 2+ for all treatment groups, yet Nuclei 1 was still the most abundant type. The FD-larvae had a significantly higher proportion of Nuclei 1 than the Cir- and Cop/Cir-larvae at 21 dph. At the experimental end (35 dph) it was however the Art-larvae that had the highest proportion of Nuclei 1, differing significantly from that of the Cir- and FD-larvae. In contrast, the Cir- and FD-larvae had a significantly higher proportion of Nuclei 2+ than the Art-larvae. Mean proportions of the two nuclei types for each of the treatment groups are given in Appendix 13, Table A10.

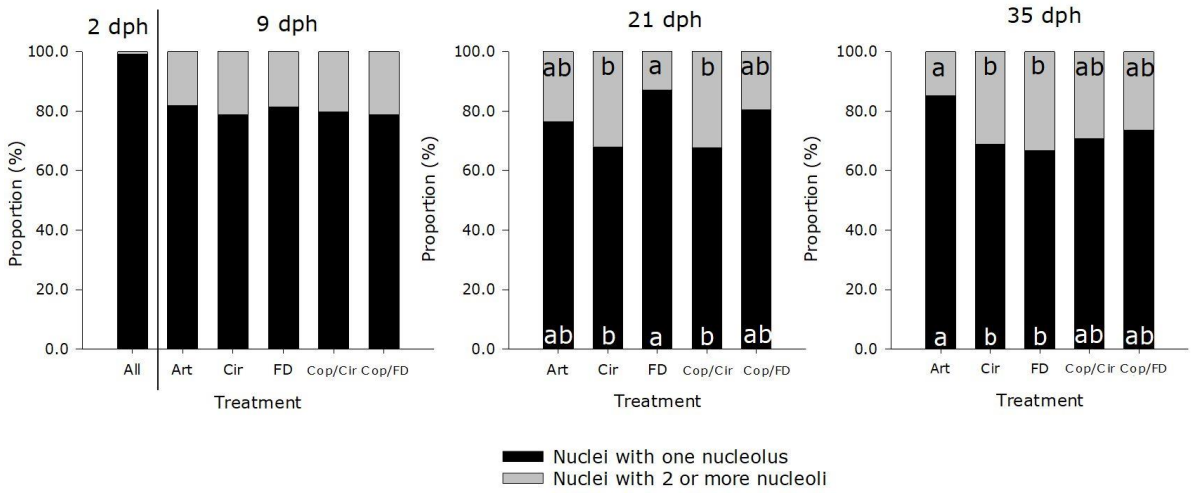


Figure 3.20. Mean proportion of hepatocyte nuclei types in lumpfish 2-35 dph. All hepatocyte nuclei measured for the histological analysis were categorized as either having one nucleolus (Nuclei 1) or two or more nucleoli (Nuclei 2+). The total number of nuclei measured and respective category were thus used to get the proportion of the nuclei types. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters.

4 Discussion

4.1 Start-feeding regimes effects on larval growth and survival

The feeding-regime using enriched *Artemia* nauplii generally gave the highest growth and survival in the experiment. However, Cir-larvae increased to a similar DW and SL to Art-larvae after weaning to formulated diet towards the experiments end. Both start-feeding regimes starting off with copepods, Cop/Cir and Cop/FD, were not as successful, with the latter treatment group having the highest mortality by the experiments end. Similarly, the larval diet consisting solely of formulated diet for FD-larvae also yielded a lower growth and survival than the diets using *Artemia* and cirripeds. It should however be noted that all larval groups had a high survival rate at the experiments end, ranging from 86-95 %, with only the Cop/FD-larvae having a significantly lower survival rate than the Art-larvae.

The feeding-regime using enriched *Artemia* nauplii gave the highest growth and survival in the experiment. Additionally, Art-larvae were significantly heavier and longer than larvae from all the other treatment groups at 21 dph, before weaning to formulated diet. Art-larvae also had the highest survival throughout the experiment but was at the experimental end only significantly higher than Cop/FD-larvae's survival rate. These findings confirm hypothesis nr. 1, stating that lumpfish fed *Artemia* will have better growth and survival than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof. This is also consistent with previous studies in lumpfish, where feeding *Artemia* improved larval growth and survival compared to larvae fed copepods and formulated feed (Hanssen, 2018; Marthinsen, 2018; Rian, 2019).

The essential fatty acids DHA and EPA must be found in phospholipids (polar lipids) rather than neutral lipids for proper development of several marine fish species, as they have been shown to be more available and effectively utilized by the larvae (Watanabe *et al.*, 1989; Fontagné *et al.*, 1998; Cahu, Zambonino Infante & Barbosa, 2003; Evjemo, Reitan & Olsen, 2003; Gisbert *et al.*, 2005; Kjørsvik *et al.*, 2009; Wold *et al.*, 2009). *Artemia* do have a higher lipid content than copepods after enrichment (van der Meeren *et al.*, 2008), and likely also cirripeds, as they are said to have a similar nutrient composition as copepods (Planktonic AS, 2022). However, the lipids found in *Artemia* are approximately 60-70 % neutral lipids, meaning the majority of DHA and EPA are not found in the polar lipids (Hamre *et al.*, 2013; Øie *et al.*, 2017). It would therefore be natural to assume that *Artemia* would result in reduced growth compared to the copepod *A. tonsa*, which in contrast have a polar lipid fraction at approximately 70 % (Øie *et al.*, 2017). This was however not the case in the present study. It could therefore be reasonable to assume that the higher lipid content in the *Artemia* in general are of greater importance than whether the HUFAs are in the neutral or polar lipid fraction. Furthermore, lumpfish hatch from demersal eggs and at a more developed stage than many of the larvae that require the HUFAs in the polar lipid fraction. Marthinsen (2018) showed that the lumpfish larvae had a well-developed digestive system already at hatching, which is not the case in pelagic species like Atlantic cod, Atlantic halibut (*Hippoglossus hippoglossus*) nor white seabream (*Diplodus sargus*) (Luizi *et al.*, 1999; Ortiz-Delgado *et al.*, 2003; Wold *et al.*, 2008). As a result, it appears that the lumpfish is more effective in digesting and using HUFAs in neutral lipids compared to the aforementioned species in the early life stages. This is also supported by the fact

that Marthinsen (2018) observed gastric glands in the lumpfish' stomach already at 10 dph, which indicates an increased digestive capacity (Tanaka, 1971). To emphasize, this is considerably earlier than the formation of gastric glands in the pelagic species Atlantic halibut, which does not occur before 60 dph (Luizi *et al.*, 1999).

Furthermore, an adequate supply of proteins from the diet is also essential, especially in fish larvae which develop so rapidly compared to adult fish (De Silva & Anderson, 1995). The copepod *A. tonsa* have nearly twice the amount of protein compared to *Artemia*, at approximately 680 mg g^{-1} and 350 mg g^{-1}, respectively (Øie *et al.*, 2017). The cirriped *S. balanoides* also contain about the same amount of protein as the copepod (Planktonic AS, 2022). This fact, together with the higher proportion of polar lipids in copepods and likely also in cirripeds, indicated that these live feeds would have satisfied the fish larvae's nutritional requirements better than the *Artemia*. As this was not the case in the present study, the amount of protein in *Artemia* was likely sufficient in meeting the lumpfish larvae's needs. Nevertheless, it should be noted that the nutritional composition of the cirripeds is still largely undocumented in the literature and results should be interpreted with caution.

Not only must the nutritional quality of the feed be adequate, but the size and the fish' attraction to the feed is of great importance if the feed is to be used successfully. *Artemia* was the largest feed offered to the lumpfish, at about 800 µm (FAO, 1996), which is over twice the size of the other live feeds, cirripeds at 350 µm (CryoPlankton data sheet, user's manual from Planktonic AS) and the largest copepods at 394 µm (C-FEED AS, 2014). This could suggest that the *Artemia's* success might not only be due to its higher fraction of lipids, but also its bigger size. Furthermore, *Artemia* has a vibrant orange color, perhaps making it easier to spot for the lumpfish. *Artemia* is also relatively slow and do not avoid predatory attacks by fishes (Margulies, 1989; Trager, Achituv & Genin, 1994), as it avoids most natural predators simply by inhabiting hypersaline environments (Van Stappen, 1996). Thus, a non-evasive slow live prey like *Artemia* could be advantageous for lumpfish, as it prefers to use its suction disc and feed passively to conserve its energy (Killen, Brown & Gamperl, 2007).

Art-larvae had a high DWI in the live feed period. In contrast, there was a decrease in DWI at the last interval when the Art-larvae were weaned to formulated diet. Weaning to formulated diet is known to cause mortality in lumpfish (Powell *et al.*, 2018), as seen in pelagic fish species as well (Bengtson, Lydon & Ainley, 1999; Hamlin & Kling, 2001). Most fish larvae lack a functional stomach and have a lower ability do digest and assimilate nutrients from formulated diet compared to adult fish (Govoni, Boehlert & Watanabe, 1986). This also seems likely for the lumpfish, as Marthinsen (2018) showed that digestive system morphogenesis was not completed before 34 dph. The transition in feed size could also have been a contributor to the lowered growth, as the Art-larvae went from 800 µm long *Artemia* to 150-300 µm formulated diet pellets, which is in contrast to the natural progression towards larger prey as the fish grow and achieves an increased gape size (Schael, Rudstam & Post, 1991). Furthermore, formulated diets movements is restricted to those of the water currents and could therefore end up aggregating on top of the water or settle on the tank floor (D'Abramo, 2002; Conceição *et al.*, 2010). The formulated diet might also not stimulate the larvae's hunting instinct the same ways as live prey (D'Abramo, 2002; Conceição *et al.*, 2010). This could suggest that the lumpfish were forced to switch to active foraging mode and swim to tank floor or the water surface to feed, as opposed to its preferred passive feeding mode (Killen, Brown & Gamperl, 2007). This implies that more energy was allocated towards swimming to the feed rather than

growth. Having to swim for the feed seem undesirable for lumpfish larvae, as a study by Hvas *et al.* (2018) also found them to have relatively low aerobic scope and poor critical swimming speed.

Cir-larvae had the second-best growth among the treatment groups at the experimental end. However, Cir-larvae grew poorly before they were weaned to formulated diet. Additionally, Cir-larvae also had a lower survival than Art-larvae throughout the entire experimental period, although not significantly different. Therefore, hypothesis nr. 2 stating that lumpfish fed cirripeds will have better growth and survival than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof, is only partly true, as the Cir-larvae's growth was quite similar to the FD-, Cop/Cir- and Cop/FD-larvae before weaning to formulated diet at 21 dph. After weaning, Cir-larvae had a higher DWI than Art-larvae and was no longer significantly different in DW at neither 29 dph nor 34 dph. Similarly, Cir-larvae experienced increased growth in SL and was in the same range as the Art-larvae at 29 and 34 dph. The larger sample of SL (750 fish per treatment group) at the experimental end did however show a different result than 34 dph, with the Cir-larvae being significantly shorter than the Art-larvae. There was however found no significant difference within treatments in the predicted length of the larvae at 35 dph (based on 34 dph larvae and SGR) versus the actual SL found at 35 dph in the large sample, indicating that there were no sampling bias present. Nonetheless, Cir-larvae had a higher DWI in the interval 21-34 dph than Art-larvae. This implies that there were some positive carry-over effects when larvae were weaned from cirripeds to formulated feed, for instance it could have affected the larvae's ability to digest and assimilate formulated feed. In addition, the transition in feed size was perhaps also less stressful for the Cir-larvae, as they transitioned from 350 µm cirripeds to a similar sized feed, 150/300 µm formulated diet pellets.

The Cop/Cir-larvae were also fed cirripeds, but after a period with copepods first. The Cop/Cir-larvae grew similarly to the Cop/FD-larvae in the period between 2-9 dph. However, the survival rate in the Cop/FD-larvae tended to be lower than the Cop/Cir-larvae's between 2-9 dph even though both groups were fed copepods at this time. The reason for this is not known, but it could be speculated that it was due to genetic variation, since the lumpfish eggs were from eight different females. After weaning to cirripeds for the Cop/Cir-larvae they outgrew the Cop/FD-larvae and had a significantly higher DW and SL at 21 dph than the latter. Although the Cop/Cir-larvae were weaned from cirripeds to formulated diet at the same time as the Cir-larvae were, the rapid growth pattern observed in Cir-larvae did not appear. This suggests that the copepod period first was the reason for the lowered growth in this group. Additionally, Cop/Cir-larvae were weaned two times to a new feed type as opposed to the other treatment groups which were only weaned once. This could therefore also have been a contributing factor to the lower growth and survival observed in this group. The Cop/FD-larvae on the other hand were weaned from copepods to formulated diet directly and was the treatment group with the overall lowest growth. Additionally, the survival rate dropped after weaning to formulated diet and continued to drop and was by the experiments end significantly lower than the Art-larvae's. Based on these findings in both treatment groups fed copepods at the beginning at the experiment, Cop/Cir- and Cop/FD-larvae, copepods are not a live feed suited for lumpfish.

Copepods supposedly have an optimal nutrient profile for marine fish larvae (Hamre *et al.*, 2013), and are believed to be an attractive live prey because of their typical zigzag movement (FAO, 1996). In the wild, lumpfish has also been observed to feed on different genera of copepods (Ingólfsson & Kristjánsson, 2002). Calanoid copepods was in fact the

second most abundant feed item found in the lumpfish larvae's stomach and habitat (seaweed) (Ingólfsson & Kristjánsson, 2002). Despite this, the nauplii of the calanoid copepod *A. tonsa* do not seem to be a fitting live prey for lumpfish in aquaculture based on the present study, and previous studies with lumpfish (Hanssen, 2018; Marthinsen, 2018; Rian, 2019). For instance, the cirripeds fed after the copepod period in Cop/Cir-larvae did not induce the same growth spurt as seen in the Cir-larvae when weaning to formulated diet, suggesting that the negative effects of the copepod period prior had affected further growth. The copepods were also the smallest live feed in this experiment, ranging between 193-394 µm in length. This further supports that a larger live prey seems more fitting for the lumpfish, as perhaps the small transparent copepods were harder to spot. The copepods were also likely harder to catch, as they are known to produce strong escape jumps in response to predators (Gemmell *et al.*, 2012), which could indicate that the lumpfish had to allocate more energy towards catching prey than growth, which contradicts the principles of optimal foraging theory (Werner & Hall, 1974) and the lumpfish' preferred passive feeding mode (Killen, Brown & Gamperl, 2007). Based on this, it may therefore be of interest to at least try larger copepods and perhaps other species/genera if future experiments are to further test copepods as a live feed option for lumpfish. A suggestion could be to try harpacticoid copepods, as these were more abundant than calanoids in the wild lumpfish' stomach and habitat (Ingólfsson & Kristjánsson, 2002).

FD-larvae showed a similar growth to that of Cop/FD-larvae. FD-larvae's diet started off with the smallest formulated diet pellets at 100-200 µm, which also was the smallest feed type in the entire experiment. Thus, the feed size could have been a factor for this lowered growth as well. It is also known that microdiets are prone to leaching of important nutrients like free amino acids (Kvåle *et al.*, 2006; Nordgreen, Tonheim & Hamre, 2009; Hamre *et al.*, 2013) which could indicate that the lumpfish' protein requirements were not fulfilled with this diet. Furthermore, microdiets can results in poor tank hygiene and it has therefore been suggested that the period with the smallest formulated diet may be desirable to avoid (Dahle *et al.*, 2017). As mentioned earlier, the formulated diet could also have been a little attractive feed item because of its lack of movement in contrast to live prey (D'Abramo, 2002). Under the assumption that lumpfish thrive better of larger feeds, it could be beneficial to test larger formulated diet pellets in the future.

Based on the present findings, *Artemia* seems like the most appropriate live feed for lumpfish due to its size, lack of evasion instinct and likely also met the fish' dietary requirements better than the other live feeds. Copepods is not a suitable live feed for the relatively well-developed lumpfish and seem more fit to smaller pelagic larvae. Furthermore, introduction to formulated diet later in the regime after a live feed period seems like the best option, which concurs better with the differentiation of the lumpfish' digestive system (Marthinsen, 2018) and tank hygiene (Dahle *et al.*, 2017). Lastly, the results also point to cirripeds as a viable live feed option for lumpfish, as the positive carry-over effect to formulated diet yielded a growth similar to the larvae fed *Artemia* by the experiments end. Based on the good result of both *Artemia* and cirripeds, it could therefore be proposed that a start-feeding regime where both *Artemia* and cirripeds are offered to the lumpfish larvae before weaning to formulated diet could be optimal. It is however advisable to document the cirripeds nutritional contents before they are utilized as a live feed option. Future research in start-feeding of lumpfish should in general also aim to identify the lumpfish' nutritional requirements further, as well as explore using larger prey and formulated diet pellets.

4.2 Start-feeding regimes effects on nutritional status – liver histology

In newly hatched fish, liver hepatocytes develop rapidly and are generally described as differentiated (Hoehne-Reitan & Kjørsvik, 2004). Furthermore, the timing of liver differentiation reflects the developmental status of the fish at hatching (Hoehne-Reitan & Kjørsvik, 2004). The present study found that the lumpfish liver was fully differentiated and containing glycogen-like vacuoles at 2 dph, which agrees with Marthinsen (2018) findings in newly hatched lumpfish larvae. However, vacuoles were not present in all lumpfish larvae at 2 dph. This could suggest that they had metabolized these energy stores already in order to maintain nutritional homeostasis, as they perhaps hatched earlier than what was regarded as 0 dph (50 % of larvae has hatched) in the present study. Based on this, it may be beneficial to introduce feed already at 0 dph to prevent mortality due to asynchronous hatching. Furthermore, it should be noted that these variations also could have been linked to genetic variation, as the lumpfish eggs were from eight different females.

According to Marthinsen (2018), the lumpfish's ability to store and mobilize nutrients in its liver indicate that they can survive periods with food scarcity. In an aquaculture setting there is however food in abundance, and mortality in the present study is likely not linked to a lack of food available. The high survival rate in the current study (>86%) and other lumpfish studies (Dahle *et al.*, 2017; Hanssen, 2018; Marthinsen, 2018; Rian, 2019) might therefore rather be explained by the lumpfish hatching at a comparably more developed stage than the other cultured cleaner fish, the ballan wrasse, where the survival has been as low as 12-16 % already at 13-15 dph (Berg, 2012; Romundstad, 2015).

Gisbert, Ortiz-Delgado & Sarasquete (2008) reviewed nutritional biomarkers in early life stages of fish and found that suboptimally fed larvae rapidly depleted their liver's glycogen stores and endogenous lipids. At 9 dph, the absence of vacuoles in all treatment groups except Art-larvae could therefore have been an indication of that, meaning that the other treatment groups feeding regimes might not have been adequate for the lumpfish at this time. This also coincides with the general growth of the larvae, as this was the time that Art-larvae grew faster than the other treatment groups. When the liver's energy stores are depleted, the larvae will have to rely on catabolism of muscle protein to survive (Gisbert, Ortiz-Delgado & Sarasquete, 2008). This could have been the case at this time in the present study, as it was also found that the size of the red muscle was significant larger in Art-larvae than Cop/FD- and Cop/Cir-larvae (Lindskog, 2021). Thus, the larvae fed copepods could have resorted to using their muscle proteins to maintain nutritional homeostasis, as muscle proteins have been found to be the main energy source in starved young larvae (Catalán, 2003; Gisbert, Ortiz-Delgado & Sarasquete, 2008).

At 21 dph the Art-larvae had a significantly higher vacuole fraction than all other treatments, and as these larvae still had the highest growth at this time, it would suggest that a high vacuole fraction in lumpfish is indicative of high nutritional status. Increasing hepatocyte size have been attributed to an increase in storage of glycogen and lipids in many fish species (Segner *et al.*, 1994; Ostaszewska & Sysa, 2004), and this has also been seen in lumpfish and ballan wrasse (Romundstad, 2015; Marthinsen, 2018). This coincides with the findings in lumpfish as an increased vacuole fraction seem to correlate positively with hepatocyte size. Large hepatocytes have also been linked to a deficiency of EFAs in the diet (Watanabe *et al.*, 1989). This would not seem to be the case in the present study, as the significantly larger hepatocyte size in Art-larvae than in Cir- and Cop/FD-larvae at

21 dph was at the same time as the Art-larvae had the best growth. The higher vacuole fraction in Art-larvae before weaning to formulated did however not result in a higher growth after weaning compared to Cir-larvae with a significantly lower vacuole fraction at 21 dph. This indicates that the increased growth in Cir-larvae after weaning was not decided by its previous energy stores, and that the effect was more likely caused by an increased ability to digest and assimilate the nutrients in the formulated diet. Nonetheless, the vacuole fraction in the liver should be interpreted with caution, as it does not describe whether the vacuoles were of lipid or glycogen origin.

The hepatocyte nucleus size has often been used as an indicator of nutritional condition in fish larvae (Lazo, Darias & Gisbert, 2011), as it has been assumed that a larger nucleus could indicate a higher metabolic activity (Ghadially, 1997). Marthinsen (2018) showed that hepatocyte nucleus size was larger in lumpfish larvae fed *Artemia* compared to larvae fed copepods and formulated diet throughout the entire experiment, which agrees with the highest growth in the larvae fed *Artemia* in that study. A study by Wold *et al.* (2009) also showed that Atlantic cod larvae with the highest growth also had larger hepatocyte nuclei. These findings did not concur with the findings of the present study, as the larval group with the highest growth, Art-larvae, had smaller hepatocyte nuclei at 21 and 35 dph than most larval groups with lower growth. The hepatocyte nucleus size also correlated negatively with SL for all larval groups. The hepatocyte nucleus size was actually the largest in 2 dph larvae and seemingly decreased before stabilising in the present study. Strüssmann & Takashima (1990) showed a similar pattern for the first-feeding pejerrey larvae, where the nuclei of fasted larvae shrank gradually and obtained its lowest value before starvation death. However, the decreasing pattern observed in lumpfish is likely not due to starvation as some of the lowest hepatocyte nucleus sizes throughout the experiment were in Art-larvae, which in contrast were the larvae that had the best growth and survival throughout the experiment. Perhaps the slightly lower DWIs observed at 2-9 dph in this study compared to the lumpfish in the study by Marthinsen (2018) could have been a contributing factor to the decreasing pattern in nucleus size. Nonetheless, the reason behind these contrasting findings in lumpfish is not clear.

Interestingly, as much as 99 % of the nuclei categorized in 2 dph larvae were Nuclei 1. Thereafter, the proportion of Nuclei 2+ increased and there were significant differences in the proportions among the feeding regimes at 21 and 35 dph, where the proportions of Nuclei 1 and Nuclei 2+ were approximately 70-80 % and 20-30 %, respectively. In the literature, hepatocyte nucleoli in fish are mostly denoted as either prominent or present (Rodrigues & Fanta, 1998; Romundstad, 2015; Marthinsen, 2018). Data on number of hepatocyte nucleoli, as well as number of nucleoli in other tissues in fish is lacking in general, with most articles available focusing on nucleoli in oocytes (Yamamoto, 1956; Rastogi, 1968; Thiry & Poncin, 2005). The nucleolus produces ribosomes, which serves as the site for protein synthesis in the cell (Farley *et al.*, 2015). It could therefore be beneficial to have multiple nucleoli in the hepatocytes, and for the proportions of Nuclei 2+ to increase from hatching, as the liver is one of the most important organs for making protein (Rodrigues, Saturnino & Fernandes, 2017). This could perhaps also explain the positive carry-over effect seen in Cir-larvae, as they were found to have high proportions of Nuclei 2+ at 21 and 35 dph, thus indicating that the effect was caused by an increased ability to assimilate protein.

The different feeding regimes clearly affected the histomorphological organization of the liver throughout the start-feeding experiment. The fact that no significant differences between treatment groups at 35 dph was found in neither hepatocyte nucleus size,

hepatocyte size nor vacuole fraction point towards the liver accurately reflecting the feeding conditions as all larvae was fed formulated diets at this time. More importantly, it suggests that the effects of suboptimal feeding can be reversed in lumpfish larvae, at least to a certain point, as was also seen by Marthinsen (2018). Hypothesis nr. 3 and 4 stating that lumpfish fed *Artemia* and cirripeds will have larger hepatocytes, hepatocyte nuclei and vacuole fractions than the larvae fed regimes consisting of copepods, formulated feed or the combination thereof, was however not the case in the present study, as larger characteristics and a higher vacuole fraction was not always synonymous with higher growth and survival. The present findings might therefore suggest that hepatocyte size and vacuole fraction might be more suited as nutritional condition indices in lumpfish than hepatocyte nuclei size since these findings was more consistent with the growth and survival of the larvae.

5 Conclusions

The present study demonstrated that the use of enriched *Artemia* nauplii gave the best growth and survival in lumpfish larvae throughout the experiment. Furthermore, it demonstrated that the relatively new live feed cryopreserved cirripeds could be an option in start-feeding of lumpfish. The larvae fed cirripeds grew poorly in the beginning of the experiment, however they showed a higher growth than larvae fed *Artemia* when weaned to formulated diet, and thus resulted in larvae of similar sizes at the end of the experiment. As the cirripeds clearly had a positive carry-over effect when weaned to formulated diet, it could be reasonable to suggest that a feeding regime where one combines both enriched *Artemia* and cirriped nauplii before the fish is weaned to formulated diet could be a viable option in start-feeding regimes for lumpfish larvae. Feeding regimes starting off with copepods had negative effects on the lumpfish' growth, regardless of whether the fish was weaned to cirripeds after or directly onto formulated diet. The conclusion from this study is that copepods is likely not a suitable live feed for lumpfish larvae. The feeding regime introducing the lumpfish to formulated diet directly without a live feed period prior also seems unsuitable, as it resulted in lower growth.

The lumpfish livers histomorphological organization was clearly affected by the different feeding regimes throughout the start-feeding experiment. The present findings suggests that hepatocyte size and vacuole fraction might be more suited as nutritional condition indices in lumpfish than hepatocyte nuclei size since these findings was more consistent with the growth and survival of the larvae.

The findings of this study provide valuable information in finding the optimal feeding regimes for lumpfish larviculture, as it demonstrates how the lumpfish' growth and survival is affected by composing feeding regimes of different live feeds and formulated diet. Furthermore, it provides contrasting findings in the use of different histological biomarkers in the liver compared to previous studies, highlighting the importance of correct interpretation of these and how accurately they relate to the fish' nutritional condition. However, the findings and conclusions made from the histology of the liver do come from a rather small population and should therefore be interpreted with caution.

Future research in start-feeding of lumpfish should aim to further identify the lumpfish' nutritional requirements and how different live feeds fulfils these. There is also an apparent need for more documentation of the cirripeds nutritional composition, as the literature on them as live feed organisms is lacking. Furthermore, the feeding-regimes long term effects remains to be discovered. This would be essential to disclose, as the goal of farming lumpfish is for it to be an effective cleaner fish in relation to sea lice in Atlantic salmon aquaculture.

References

Akiyoshi, H. & Inoue, A. (2004) Comparative Histological Study of Teleost Livers in Relation to Phylogeny, *Zoological Science*, 21(8), pp. 841-850.

Albers, C. S., Kattner, G. & Hagen, W. (1996) The compositions of wax esters, triacylglycerols and phospholipids in Arctic and Antarctic copepods: evidence of energetic adaptations, *Marine Chemistry*, 55(3), pp. 347-358.

Barrett, L. T., Oppedal, F., Robinson, N. & Dempster, T. (2020) Prevention not cure: a review of methods to avoid sea lice infestations in salmon aquaculture, *Reviews in Aquaculture*, 12(4), pp. 2527-2543.

Benfey, T. J. & Methven, D. A. (1986) Pilot-scale Rearing of Larval and Juvenile lumpfish (*Cyclopterus lumpus* L.), with some Notes on Early Development, *Aquaculture*, 56(3), pp. 301-306.

Bengtson, D. A., Lydon, L. & Ainley, J. D. (1999) Green-Water Rearing and Delayed Weaning Improve Growth and Survival of Summer Flounder, *North American Journal of Aquaculture*, 61(3), pp. 239-242.

Berg, M. (2012) *The effect of different live prey types on growth and muscle development, in ballan wrasse (Labrus bergylta, Ascanius 1767) larvae*. Master's Thesis, Department of Biology, NTNU. 57p.

Bjordal, Å. (1991) Wrasse as cleaner-fish for farmed salmon, *Progress in Underwater Science*, 16, pp. 17-28.

Blacker, R. W. (1983) Pelagic records of the lumpsucker, *Cyclopterus lumpus* L., *Journal of Fish Biology*, 23(4), pp. 405-417.

Blanco Gonzalez, E. & de Boer, F. (2017) The development of the Norwegian wrasse fishery and the use of wrasses as cleaner fish in the salmon aquaculture industry, *Fisheries Science*, 83(5), pp. 661-670.

Braden, L. M., Monaghan, S. J. & Fast, M. D. (2020) Salmon immunological defence and interplay with the modulatory capabilities of its ectoparasite *Lepeophtheirus salmonis, Parasite Immunology*, 42(8), pp. 1-16.

Brown, J. A. (1986) The development of feeding behaviour in the lumpfish, *Cyclopterus lumpus*, *Journal of Fish Biology*, 29(sA), pp. 171-178.

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J. & Bostick, K. (2010) Chemical use in salmon aquaculture: A review of current practices and possible environmental effects, *Aquaculture*, 306(1), pp. 7-23.

C-FEED AS (2014) C-FEED Product specification. Available at: http://www.cfeed.no/wp-content/uploads/2014/12/C-Feed-Product-Specification-20141126.pdf.

Cahu, C. L., Zambonino Infante, J. L. & Barbosa, V. (2003) Effect of dietary phospholipid level and phospholipid:neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet, *British Journal of Nutrition*, 90(1), pp. 21-28.

Catalán, I. A. (2003) *Condition Indices and their Relationship with Environmental Factors in Fish Larvae*. Doctoral Thesis, Universitat de Barcelona. 265p.

Conceição, L. E. C., Yúfera, M., Makridis, P., Morais, S. & Dinis, M. T. (2010) Live feeds for early stages of fish rearing, *Aquaculture research*, 41(5), pp. 613-640.

Costello, M. J. (2006) Ecology of sea lice parasitic on farmed and wild fish, *Trends in Parasitology*, 22(10), pp. 475-483.

D'Abramo, L. R. (2002) Challenges in Developing Successful Formulated Feed for Culture of Larval Fish and Crustaceans, *Avances en Nutrición Acuícola VI. Memorias del VI Simposium Internacional de Nutrición Acuícola* 3, pp. 143-151.

Dahle, S. W., Hagemann, A., Attramadal, K. J. K., Kjørsvik, E. & Bardal, T. (2017) *Vannkvalitet og startfôring av rognkjeks - Effekt av fôring med levende copepoder i tidlige faser (FHF prosjekt nr 901174)*. (OC2017 A-029): SINTEF Ocean AS. Available at: http://hdl.handle.net/11250/2443379.

De Silva, S. S. & Anderson, T. A. (1995) Larval nutrition and growth, in Verreth, J. (ed.) *Fish Nutrition in Aquaculture.* London, UK: Chapman & Hall, pp. 143-150.
Dhont, J. & Van Stappen, G. (2003) Biology, Tank Production and Nutritional Value of *Artemia*, in Støttrup, J. G. & McEvoy, L. A. (ed.) *Live Feeds in Marine Aquaculture.* Iowa, USA: Blackwell Publishing, pp. 65-121.
Evjemo, J. O., Reitan, K. I. & Olsen, Y. (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus L*.) with special emphasis on the nutritional value, *Aquaculture*, 227(1), pp. 191-210.
FAO (1996) *Manual on the Production and Use of Live Food for Aquaculture*. (FAO Fisheries Technical Paper 361). Rome, Italy: FAO.
FAO (2020) *The State of World Fisheries and Aquaculture 2020. Sustainability in action.* Rome, Italy.
Farley, K. I., Surovtseva, Y., Merkel, J. & Baserga, S. J. (2015) Determinants of Mammalian Nucleolar Architecture, *Chromosoma*, 124(3), pp. 323-331.
Feller, S. E. (2008) Acyl chain conformations in phospholipid bilayers: a comparative study of docosahexaenoic acid and saturated fatty acids, *Chemistry and Physics of Lipids*, 153(1), pp. 76-80.
FHF (2019) *Program rensefisk: Optimalisert startfôring av rensefisk (STARTRENS)*. Available at: https://www.fhf.no/prosjekter/prosjektbasen/901561/
Finstad, B., Bjørn, P. A., Grimnes, A. & Hvidsten, N. A. (2000) Laboratory and field investigations of salmon lice [*Lepeophtheirus salmonis* (Krøyer)] infestation on Atlantic salmon (*Salmo salar* L.) post-smolts, *Aquaculture Research*, 31(11), pp. 795-803.
Fontagné, S., Geurden, I., Escaffre, A.-M. & Bergot, P. (1998) Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio L.*) larvae, *Aquaculture*, 161(1), pp. 213-223.
Garcia de Leaniz, C., Rabadan, C. R., Barrento, S. I., Stringwell, R., Howes, P. N., Whittaker, B. A., Minett, J. F., Smith, R. G., Pooley, C. L., Overland, B. J., Biddiscombe, L., Lloyd, R., Consuegra, S., Maddocks, J. K., Deacon, P. T. J., Jennings, B. T., Rey Planellas, S., Deakin, A., Moore, A. I., Phillips, D., Bardera, G., Castanheira, M. F., Scolamacchia, M., Clarke, N., Parker, O., Avizienius, J., Johnstone, M. & Pavlidis, M. (2021) Addressing the welfare needs of farmed lumpfish: Knowledge gaps, challenges and solutions, *Reviews in Aquaculture*, 00, pp. 1-17.
Gemmell, B. J., Jiang, H., Strickler, J. R. & Buskey, E. J. (2012) Plankton reach new heights in effort to avoid predators, *Proceedings of The Royal Society B*, 279(1739), pp. 2786-2792.
Ghadially, F. (1997) *Ultrastructural pathology of the cell and matrix*. 4th edn. Massachusetts, USA: Butterworth-Heinemann.
Gisbert, E., Villeneuve, L., Zambonino-Infante, J. L., Quazuguel, P. & Cahu, C. L. (2005) Dietary Phospholipids Are More Efficient Than Neutral Lipids for Long-Chain Polyunsaturated Fatty Acid Supply in European Sea Bass *Dicentrarchus labrax* Larval Development, *Lipids*, 40(6), pp. 609-618.
Gisbert, E., Ortiz-Delgado, J. B. & Sarasquete, C. (2008) Nutritional cellular biomarkers in early life stages of fish, *Histology and Histopathology*, 23(12), pp. 1525-1539.
Govoni, J. J., Boehlert, G. W. & Watanabe, Y. (1986) The physiology of digestion in fish larvae, *Environmental Biology of Fishes*, 16(1-3), pp. 59-77.
Hamilton, R. M., K. (2004) *Monitoring and Quantifying Weed Cover Using a Dot-Grid Sampling Technique* Salt Lake City, USA: Remote Sensing Applications Center.
Hamlin, H. J. & Kling, L. J. (2001) The culture and early weaning of larval haddock (*Melanogrammus aeglefinus*) using a microparticulate diet, *Aquaculture*, 201(1), pp. 61-72.
Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L. E. C. & Izquierdo, M. (2013) Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing, *Reviews in Aquaculture*, 5(Suppl. 1), pp. S26-S58.

Hanssen, J. T. (2018) *Effects on growth, survival and bone development from start feeding lumpsucker (Cyclopterus lumpus) larvae with Artemia, copepods and fomulated feed*. Master's Thesis, Department of Biology, NTNU. 69p.
Hemmingsen, W., MacKenzie, K., Sagerup, K., Remen, M., Bloch-Hansen, K. & Dagbjartarson Imsland, A. K. (2020) *Caligus elongatus* and other sea lice of the genus *Caligus* as parasites of farmed salmonids: A review, *Aquaculture*, 522, pp. 735160.
Hoehne-Reitan, K. & Kjørsvik, E. (2004) Functional Development of the Liver and Exocrine Pancreas in Teleost Fish, *American Fisheries Society*, 40, pp. 9-36.
Houde, E. D. & Schekter, R. C. (1981) Growth rates, rations and cohort consumption of marine fish larvae in relation to prey concentrations, *Rapp. P. -v. Réun. Cons. int. Explor. Mer*, 178, pp. 441-453.
Hunter, J. R. (1981) Feeding Ecology and Predation of Marine Fish Larvae, in Lasker, R. (ed.) *Marine Fish larvae : Morphology, Ecology, and Relation to Fisheries.* Seattle, USA: Washington Sea Grant Program, pp. 34-77.
Hvas, M., Folkedal, O., Imsland, A. & Oppedal, F. (2018) Metabolic rates, swimming capabilities, thermal niche and stress response of the lumpfish, *Cyclopterus lumpus*, *Biology Open*, 7(9).
Imsland, A. K. D., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Elvegård, T. A., Urskog, T. C., Hanssen, A. & Mikalsen, B. (2018a) Effects of different feeding frequencies on growth, cataract development and histopathology of lumpfish (*Cyclopterus lumpus* L.), *Aquaculture*, 501.
Imsland, A. K. D., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Elvegård, T. A., Urskog, T. C. & Mikalsen, B. (2018b) Effects of three commercial diets on growth, cataract development and histopathology of lumpfish (*Cyclopterus lumpus* L.), *Aquaculture Research*, 49(9), pp. 3131-3141.
Ingólfsson, A. & Kristjánsson, B. K. (2002) Diet of Juvenile Lumpsucker *Cyclopterus lumpus* (Cyclopteridae) in Floating Seaweed: Effects of Ontogeny and Prey Availability, *Copeia*, 2002(2), pp. 472-476.
Johnson, S. C., Treasurer, J. W., Bravo, S., Nagasawa, K. & Kabata, Z. (2004) A Review of the Impact of Parasitic Copepods on Marine Aquaculture, *Zoological Studies*, 43(2), pp. 229-243.
Jonassen, T. M., Lein, I. & Nytrø, A. V. (2018) Hatchery management of lumpfish, in Treasurer, J. (ed.) *Cleaner Fish Biology and Aquaculture Applications.* Sheffield, UK: 5M Publishing Ltd., pp. 114-136.
Killen, S. S., Brown, J. A. & Gamperl, A. K. (2007) The effect of prey density on foraging mode selection in juvenile lumpfish: balancing food intake with the metabolic cost of foraging, *Journal of Animal Ecology*, 76(4), pp. 814-825.
Kjørsvik, E., Pittman, K. & Pavlov, D. (2004) From Fertilisation to the End of Metamorphosis - Functional Development, in Moksness, E., Kjørsvik, E. & Olsen, Y. (ed.) *Culture of Cold-Water Marine Fish.* Oxford, UK: Blackwell Publishing Ltd, pp. 204-278.
Kjørsvik, E., Olsen, C., Wold, P.-A., Hoehne-Reitan, K., Cahu, C. L., Rainuzzo, J., Olsen, A. I., Øie, G. & Olsen, Y. (2009) Comparison of dietary phospholipids and neutral lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*), *Aquaculture*, 294(3), pp. 246-255.
Kvåle, A., Yúfera, M., Nygård, E., Aursland, K., Harboe, T. & Hamre, K. (2006) Leaching properties of three different micropaticulate diets and preference of the diets in cod (*Gadus morhua* L.) larvae, *Aquaculture*, 251(2), pp. 402-415.
Lazo, J. P., Darias, M. J. & Gisbert, E. (2011) Ontogeny of the digestive tract, in Holt, J. G. (ed.) *Larval Fish Nutrition.* Chichester, UK: Wiley-Blackwell, pp. 5-46.
Lindskog, M. S. (2021) *Muscle growth and development in lumpfish (Cyclopterus lumpus) larvae in relation to start-feeding diets (Artemia, cirripedia, copepod (Acartia tonsa) and formulated diet)*. Master's Thesis, Department of Biology, NTNU. 69p.
Liu, Y. & Bjelland, H. v. (2014) Estimating costs of sea lice control strategy in Norway, *Preventive Veterinary Medicine*, 117(3-4), pp. 469-477.

López, D. A. L., Boris A., Pham, C. K. & Isidro, E. J. (2012) Potency of Barnacle in Aquaculture Industry, in Muchlisin, Z. (ed.) *Aquaculture.* 7th edn. InTech, pp. 295-316.
Luizi, F. S., Gara, B., Shields, R. J. & Bromage, N. R. (1999) Further description of the development of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia* prey, *Aquaculture*, 176(1), pp. 101-116.
Margulies, D. (1989) Size-Specific Vulnerability of Predation and Sensory System Development of White Seabass, *Atractoscion nobilis*, larvae, *Fishery Bulletin* 87(3), pp. 537-552.
Marthinsen, J. L. (2018) *Ontogeny of the digestive system in lumpfish (Cyclopterus lumpus L.) larvae in relation to growth and start feeding diet* Master's Thesis, Department of Biology, NTNU. 96p.
Merchie, G. (1996) *Use of nauplii and meta-nauplii*. (Manual on the Production and Use of Live Food for Aquaculture.). Rome: FAO.
Nordgreen, A., Tonheim, S. & Hamre, K. (2009) Protein quality of larval feed with increased concentration of hydrolysed protein: effects of heat treatment and leaching, *Aquaculture Nutrition*, 15(5), pp. 525-536.
Norwegian Directorate of Fisheries (2018) *Nøkkeltall fra norsk havbruksnæring 2018*. Available at: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Statistiske-publikasjoner/Noekkeltall-for-norsk-havbruksnaering.
Norwegian Directorate of Fisheries (2020) *Nøkkeltall fra norsk havbruksnæring 2020*. Available at: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Statistiske-publikasjoner/Noekkeltall-for-norsk-havbruksnaering.
Norwegian Directorate of Fisheries (2021) *Utsett av rensefisk 1998-2020*. Available at: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Rensefisk.
Norwegian Institute of Marine Research (2021) *Risikorapport norsk fiskeoppdrett 2021 - risikovurdering*. (Rapport fra havforskningen 2021-8). Available at: https://www.hi.no/hi/nettrapporter/rapport-fra-havforskningen-2021-8.
Nytrø, A. V., Vikingstad, E., Foss, A., Hangstad, T. A., Reynolds, P., Eliassen, G., Elvegård, T. A., Falk-Petersen, I.-B. & Imsland, A. K. (2014) The effect of temperature and fish size on growth of juvenile lumpfish (*Cyclopterus lumpus* L.), *Aquaculture*, 434, pp. 296-302.
Olsen, Y., van der Meeren, T. & Reitan, K. I. (2004) First feeding technology, in Moksness, E., Kjørsvik, E. & Olsen, Y. (ed.) *Culture of Cold-Water Marine Fish.* Oxford, UK: Blackwell Publishing Ltd., pp. 279-336.
Ortiz-Delgado, J. B., Darias, M. J., Cañavate, J. P., Yúfera, M. & Sarasquete, C. (2003) Organogenesis of the digestive tract in the white seabream, *Diplodus sargus*. Histological and histochemical approaches, *Histology and Histopathology*, 18, pp. 1141-1154.
Ostaszewska, T. E. & Sysa, P. (2004) Development of hepatocytes in nase (*Chondrostoma nasus* (L.)) larvae following hatch, *Archives of Polish Fisheries*, 12(2), pp. 151-161.
Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K. & Stien, L. H. (2019) Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review, *Reviews in Aquaculture*, 11(4), pp. 1398-1417.
Planktonic AS (2022) *The world's best and easiest live feed - CryoPlankton*. Available at: https://planktonic.no/.
Powell, A., Treasurer, J. W., Pooley, C. L., Keay, A. J., Lloyd, R., Imsland, A. K. & Garcia de Leaniz, C. (2018) Use of lumpfish for sea-lice control in salmon farming: challenges and opportunities, *Reviews in Aquaculture*, 10(3), pp. 683-702.
Rastogi, R. K. (1968) Studies on the Fish Oogenesis I. Histomorphological and cytochemical changes in the oocyte nucleus of *Amphipnous cuchia* (Ham.), *CYTOLOGIA*, 33(3-4), pp. 357-369.

Rian, S. T. (2019) *Start-feeding of lumpfish (Cyclopterus lumpus L.) larvae with Artemia and copepods, focusing on growth effects, survival, live prey selection and larval robustness*. Master's Thesis, Department of Biology, NTNU. 74p.
Rodrigues, E. d. L. & Fanta, E. (1998) Liver histopathology of the fish brachydanio rerio hamilton-buchman after acute exposure to sublethal levels of the organophosphate dimethoate 500, *Revista Brasileira De Zoologia*, 15(2), pp. 441-450.
Rodrigues, R. A., Saturnino, K. C. & Fernandes, C. E. (2017) Liver histology and histomorphometry in hybrid sorubim (*Pseudoplatystoma reticulatum* × *Pseudoplatystoma corruscans*) reared on intensive fish farming, *Aquaculture research*, 48(9), pp. 5083-5093.
Romundstad, M. (2015) *Effects of different live feed on ballan wrasse (Labrus bergylta) larval hepatocyte and enterocyte development*. Master's Thesis, Department of Biology, NTNU. 52p.
Rueness, E., Berg, P. R., Gulla, S., Halvorsen, K., Järnegren, J., Malmstrøm, M., Mo, T. A., Rimstad, E., de Boer, H., Eldegard, K., Hindar, K., Hole, L. R., Kausrud, K., Kirkendall, L., Måren, I., Nilsen, E. B., Thorstad, E. B., Nielsen, A. & Velle, G. (2019) *Assessment of the risk to Norwegian biodiversity from import of wrasses and other cleaner fish for use in aquaculture*. (15). Oslo, Norway: Norwegian Scientific Committee for Food and Environment (VKM).
Rønnestad, I., Tonheim, S. K., Fyhn, H. J., Rojas-García, C. R., Kamisaka, Y., Koven, W., Finn, R. N., Terjesen, B. F., Barr, Y. & Conceição, L. E. C. (2003) The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings, *Aquaculture*, 227(1), pp. 147-164.
Salte, R., Syvertsen, C., Kjønnøy, M. & Fonnum, F. (1987) Fatal Acetylcholinesterase Inhibition in Salmonids Subjected to a Routine Organophosphate Treatment, *Aquaculture*, 61(3), pp. 173-179.
Sargent, J., Bell, G., McEvoy, L., Tocher, D. & Estevez, A. (1999a) Recent developments in the essential fatty acid nutrition of fish, *Aquaculture*, 177(1), pp. 191-199.
Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D. (1999b) Lipid nutrition of marine fish during early development: current status and future directions, *Aquaculture*, 179(1), pp. 217-229.
Sargent, J. R., McEvoy, L. A. & Bell, J. G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds, *Aquaculture*, 155(1), pp. 117-127.
Sayer, M. D. J. & Reader, J. P. (1996) Exposure of goldsinny, rock cook and corkwing wrasse to low temperature and low salinity: survival, blood physiology and seasonal variation, *Journal of Fish Biology*, 49(1), pp. 41-63.
Schael, D. M., Rudstam, L. G. & Post, J. R. (1991) Gape Limitation and Prey Selection in Larval Yellow Perch (*Perca flavescens*), Freshwater Drum (*Aplodinotus grunniens*), and Black Crappie (*Pomoxis nigromaculatus*), *Canadian Journal of Fisheries and Aquatic Sciences*, 48(10), pp. 1919-1925.
Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nature Methods*, 9(7), pp. 671-675.
Segner, H., Storch, V., Reinecke, M., Kloas, W. & Hanke, W. (1994) The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus*, *Marine biology*, 119(3), pp. 471-486.
Skiftesvik, A. B., Bjelland, R. M., Durif, C. M. F., Johansen, I. S. & Browman, H. I. (2013) Delousing of Atlantic salmon (*Salmo salar*) by cultured vs. wild ballan wrasse (*Labrus bergylta*), *Aquaculture*, 402-403, pp. 113-118.
Skretting (2021a) *GEMMA Micro 300*. Available at: https://zebrafish.skrettingusa.com/products/gemma-micro-300 (2021).
Skretting (2021b) *GEMMA Micro 150*. Available at: https://zebrafish.skrettingusa.com/products/gemma-micro-150 (2021).
Sorgeloos, P., Dhert, P. & Candreva, P. (2001) Use of the brine shrimp, *Artemia* spp., in marine fish larviculture, *Aquaculture*, 200(1), pp. 147-159.

Strachan, F. & Kennedy, C. J. (2021) The environmental fate and effects of anti-sea lice chemotherapeutants used in salmon aquaculture, *Aquaculture*, 544, pp. 737079.
Strüssmann, C. A. & Takashima, F. (1990) Hepatocyte nuclear size and nutritional condition of larval pejerrey, *Odontesthes bonariensis* (Cuvier et Valenciennes), *Journal of Fish Biology*, 36(1), pp. 59-65.
Støttrup, J. G. (2003) Production and Nutritional Value of Copepods, in Støttrup, J. G. & McEvoy, L. A. (ed.) *Live Feeds in Marine Aquaculture.* Blackwell Sciene Ltd., pp. 145-205.
Tanaka, M. (1971) Studies on the Structure and Function of the Digestive System in Teleost Larvae -III. Development of the Digestive System during Postlarval Stage, *Japanese Journal of Ichtyology*, 18(4), pp. 164-174.
Thiry, M. & Poncin, P. (2005) Morphological changes of the nucleolus during oogenesis in oviparous teleost fish, *Barbus barbus* (L.), *Journal of Structural Biology*, 152(1), pp. 1-13.
Thorstad, E. B. & Finstad, B. (2018) *Impacts of salmon lice emanating from salmon farms on wild Atlantic salmon and sea trout*. (NINA Report 1449). Trondheim, Norway: Norwegian Institute for Nature Research. Available at: https://brage.nina.no/nina-xmlui/bitstream/handle/11250/2475746/1449.pdf?sequence=1&isAllowed=y.
Tocher, D. R. (2003) Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish, *Reviews in Fisheries Science*, 11(2), pp. 107-184.
Tocher, D. R. (2010) Fatty acid requirements in ontogeny of marine and freshwater fish, *Aquaculture Research*, 41(5), pp. 717-732.
Tonheim, S. K., Nordgreen, A., Høgøy, I., Hamre, K. & Rønnestad, I. (2007) *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients, *Aquaculture*, 262(2), pp. 426-435.
Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O. T., Nilsen, F., Horsberg, T. & Jackson, D. (2013) Salmon lice - impact on wild salmonids and salmon aquaculture, *Journal of Fish Diseases*, 36(3), pp. 171-194.
Trager, G., Achituv, Y. & Genin, A. (1994) Effects of prey escape ability, flow speed, and predator feeding mode on zooplankton capture by barnacles, *Marine Biology*, 120(2), pp. 251-259.
Treasurer, J. (2018) An introduction to sea lice and the rise of cleaner fish, in Treasurer, J. (ed.) *Cleaner Fish Biology and Aquaculture Applications.* Sheffield, UK: 5M Publishing Ltd., pp. 3-25.
Treasurer, J. W. (2002) A review of potential pathogens of sea lice and the application of cleaner fish in biological control, *Pest Management Science*, 58(12), pp. 546-558.
van der Meeren, T., Olsen, R. E., Hamre, K. & Fyhn, H. J. (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish, *Aquaculture*, 274(2), pp. 375-397.
Van Stappen, G. (1996) *Introduction, biology and ecology of Artemia*. (Manual on the Production and Use of Live Food for Aquaculture). Rome, Italy: FAO.
Watanabe, T. (1982) Lipid nutrition in fish, *Comparative Biochemistry and Physiology*, 73(1), pp. 3-15.
Watanabe, T., Thongrod, S., Takeuchi, T., Satoh, S., Kubota, S. S., Fujimaki, Y. & Cho, C. Y. (1989) Effect of Dietary n-6 and n-3 Fatty Acids on Growth, Fatty Acid Composition and Histological Changes of White Fish *Coregonus lavaretus maraena*, *Nippon Suisan Gakkaishi*, 55(11), pp. 1977-1982.
Werner, E. E. & Hall, D. J. (1974) Optimal Foraging and the Size Selection of Prey by the Bluegill Sunfish (*Lepomis Macrochirus*), *Ecology* 55(5), pp. 1042-1052.
Wilson, R. P. (2002) Amino Acids and Proteins, in Halver, J. E. & Hardy, R. W. (ed.) *Fish Nutrition.* 3rd edn. California, USA: Academic Press, pp. 144-181.
Wold, P.-A., Hoehne-Reitan, K., Rainuzzo, J. & Kjørsvik, E. (2008) Allometric growth and functional development of the gut in developing cod *Gadus morhua* L. larvae, *Journal of Fish Biology*, 72(7), pp. 1637-1658.
Wold, P. A., Hoehne-Reitan, K., Cahu, C. L., Infante, J. Z., Rainuzzo, J. & Kjørsvik, E. (2009) Comparison of dietary phospholipids and neutral lipids: effects on gut,

liver and pancreas histology in Atlantic cod (*Gadus morha* L.) larvae, *Aquaculture Nutrition*, 15(1), pp. 73-84.
Yamamoto, K. (1956) Studies on the Formation of Fish Eggs : II. Changes in the Nucleus of the Oocyte of *Liopsetta obscura*, With Special Reference to the Activity of the Nucleolus, *Journal of the Faculty of Science, Hokkaido University*, 12(3), pp. 375-390.
Zambonino Infante, J. L., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J. & Cahu, C. L. (2008) Ontogeny and physiology of the digestive system of marine fish larvae, *Feeding and Digestive Functions of Fishes*, pp. 281-348.
Øie, G., Galloway, T., Sørøy, M., Holmvaag Hansen, M., Norheim, I. A., Halseth, C. K., Almli, M., Berg, M., Gagnat, M. R., Wold, P. A., Attramadal, K., Hagemann, A., Evjemo, J. O. & Kjørsvik, E. (2017) Effect of cultivated copepods (*Acartia tonsa)* in first-feeding of Atlantic cod (*Gadus morhua*) and ballan wrasse (*Labrus bergylta*) larvae, *Aquaculture Nutrition*, 23(1), pp. 3-17.
Aaen, S. M., Helgesen, K. O., Bakke, M. J., Kaur, K. & Horsberg, T. E. (2015) Drug resistance in sea lice: a threat to salmonid aquaculture, *Trends in Parasitology*, 31(2), pp. 72-81.

Appendices

Appendix 1. Water exchange rates and outlet mesh sizes

Table A1. Water exchange rates and outlet mesh sizes in the start-feeding experiment of lumpfish 0-35 dph. The values are given for each treatment group. Water exchange rates were either 300, 600, 1200 or 2400 %, whereas there were three different outlet mesh sizes: 100, 350 and 750 µm.

	Art		Cir		FD	
Dph	**Water exchange rate (%)**	**Mesh size (µm)**	**Water exchange rate (%)**	**Mesh size (µm)**	**Water exchange rate (%)**	**Mesh size (µm)**
0	2400	750	1200	350	2400	750
1	2400	750	1200	350	2400	750
2	2400	750	1200	350	2400	750
3	2400	750	1200	350	2400	750
4	2400	750	1200	350	2400	750
5	2400	750	1200	350	2400	750
6	2400	750	1200	350	2400	750
7	2400	750	1200	350	2400	750
8	2400	750	1200	350	2400	750
9	2400	750	1200	350	2400	750
10	2400	750	1200	350	2400	750
11	2400	750	1200	350	2400	750
12	2400	750	1200	350	2400	750
13	2400	750	1200	350	2400	750
14	2400	750	1200	350	2400	750
15	2400	750	1200	350	2400	750
16	2400	750	1200	350	2400	750
17	2400	750	1200	350	2400	750
18	2400	750	1200	350	2400	750
19	2400	750	1200	350	2400	750
20	2400	750	1200	350	2400	750
21	2400	750	2400	350	2400	750
22	2400	750	2400	350	2400	750
23	2400	750	2400	350	2400	750
24	2400	750	2400	350	2400	750
25	2400	750	2400	350	2400	750
26	2400	750	2400	750	2400	750
27	2400	750	2400	750	2400	750
28	2400	750	2400	750	2400	750
29	2400	750	2400	750	2400	750
30	2400	750	2400	750	2400	750
31	2400	750	2400	750	2400	750
32	2400	750	2400	750	2400	750
33	2400	750	2400	750	2400	750
34	2400	750	2400	750	2400	750
35	2400	750	2400	750	2400	750

Table A1 continued. Water exchange rates and outlet mesh sizes in the start-feeding experiment of lumpfish 0-35 dph. The values are given for each treatment group. Water exchange rates were either 300, 600, 1200 or 2400 %, whereas there were three different outlet mesh sizes: 100, 350 and 750 µm.

	Cop/Cir		Cop/FD	
Dph	**Water exchange rate (%)**	**Mesh size (µm)**	**Water exchange rate (%)**	**Mesh size (µm)**
0	300	100	300	100
1	300	100	300	100
2	300	100	300	100
3	300	100	300	100
4	300	100	300	100
5	300	100	300	100
6	300	100	300	100
7	300	100	300	100
8	300	100	300	100
9	300	100	300	100
10	300	100	300	100
11	300	100	300	100
12	600	100	600	100
13	600	100	600	100
14	1200	100	1200	100
15	1200	100	1200	100
16	1200	100	1200	100
17	1200	350	1200	350
18	1200	350	1200	350
19	1200	350	1200	350
20	1200	350	1200	350
21	2400	350	2400	350
22	2400	350	2400	350
23	2400	350	2400	350
24	2400	350	2400	350
25	2400	350	2400	350
26	2400	750	2400	750
27	2400	750	2400	750
28	2400	750	2400	750
29	2400	750	2400	750
30	2400	750	2400	750
31	2400	750	2400	750
32	2400	750	2400	750
33	2400	750	2400	750
34	2400	750	2400	750
35	2400	750	2400	750

Appendix 2. Rearing tank set up

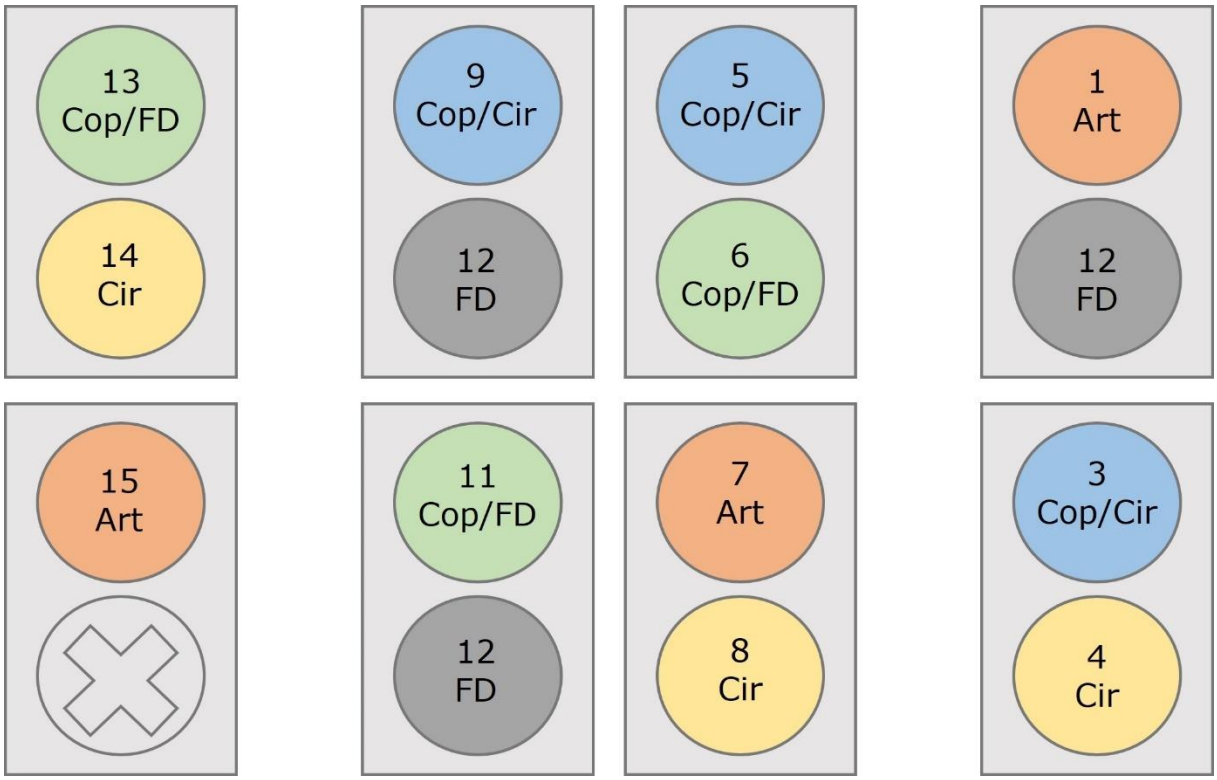


Figure A1. Rearing tank set up in the start-feeding experiment of lumpfish (0-35 dph). Each of the five treatments were replicated in three tanks, in total 15 tanks. The tanks were randomized to avoid bias. The tank crossed over was in use at the time of the experiment, but for another experiment with lumpfish larvae.

Appendix 3. Feeding amounts of live feed and formulated diet

Table A2. Feeding amounts of live feed and formulated diet in the start-feeding experiment of lumpfish 0-35 dph. Amounts of live feed (individuals/mL) and formulated diet (gram/day) are given for the five different feeding regimes. In the FD columns the number of feedings can be interpreted, as the feed automat drops 1g feed per second. E.g., 12g-2s mean the automat dropped 12 g in total that day for 2s each time, indicating 6 feedings. Overlapping feeds indicate a weaning period.

Dph	Art		Cir		FD
	Art (ind/mL)	FD (g/day)	Cir (ind/mL)	FD (g/day)	FD (g/day)
2	9		18		4-1s
3	10		24		4-1s
4	11		30		4-1s
5	15		30		6-1s
6	17		42		6-1s
7	18		42		12-1s
8	19		42		12-1s
9	23		54		24-2s
10	25		54		24-2s
11	27		60		24-2s
12	29		60		24-2s
13	32		60		24-2s
14	34		72		24-2s
15	37		72		30-2s
16	40		78		30-2s
17	43		78		30-2s
18	46		78		30-2s
19	50		78		30-2s
20	81		78		30-2s
21	70	6-1s	60	6-1s	30-2s
22	57	6-1s	54	6-1s	30-2s
23	41	12-1s	24	12-1s	30-2s
24	22	12-1s	18	12-1s	30-2s
25		24-2s	12	24-2s	30-2s
26		24-2s		24-2s	30-2s
27		30-2s		30-2s	30-2s
28		30-2s		30-2s	30-2s
29		24-1s		24-1s	24-1s
30		24-1s		24-1s	24-1s
31		24-1s		24-1s	24-1s
32		24-1s		24-1s	24-1s
33		24-1s		24-1s	24-1s
34		24-1s		24-1s	24-1s
35		24-1s		24-1s	24-1s

Table A2 continued. Feeding amounts of live feed and formulated diet in the start-feeding experiment of lumpfish 0-35 dph. Amounts of live feed (ind/mL) and formulated diet (g/day) are given for the five different feeding regimes. In the FD columns the number of feedings can be interpreted, as the feed automat drops 1g feed per second. E.g., 12g-2s mean the automat dropped 12 g in total that day for 2s each time, indicating 6 feedings. Overlapping feeds indicate a weaning period.

Dph	Cop/Cir			Cop/FD	
	Cop (ind/mL)	Cir (ind/mL)	FD (g/day)	Cop (ind/mL)	FD (g/day)
2	30			30	
3	30			30	
4	50			50	
5	50			50	
6	50			50	
7	70			70	
8	70			70	
9	90			90	
10	70	54		70	6-1s
11	70	60		70	6-1s
12	50	60		50	12-1s
13	30	60		30	12-1s
14	30	72		30	24-2s
15	20	72		20	24-2s
16	10	78		10	30-2s
17		78			30-2s
18		78			30-2s
19		78			30-2s
20		78			30-2s
21		60	6-1s		30-2s
22		54	6-1s		30-2s
23		24	12-1s		30-2s
24		18	12-1s		30-2s
25		12	24-2s		30-2s
26			24-2s		30-2s
27			30-2s		30-2s
28			30-2s		30-2s
29			24-1s		24-1s
30			24-1s		24-1s
31			24-1s		24-1s
32			24-1s		24-1s
33			24-1s		24-1s
34			24-1s		24-1s
35			24-1s		24-1s

Appendix 4. Artemia protocol

The following work protocol for *Artemia* (*A. franciscana*) production was made in accordance with the manufacturer's recommendations: INVE Aquaculture, Thailand (EG ® INVE Aquaculture) for the cysts and BioMar AS, Norway (LARVIVA Multigain) for the enrichment.

PROTOCOL FOR Artemia HATCHING, HARVESTING AND ENRICHMENT – STARTRENS 2020

- *Prepare two incubation cones, screw aeration caps, leave valves open, fill them with **60 L of sea water, plug in heaters and start aerations** (Do this daily - the day before the incubation)*
- *Label the cones and note which tanks you use*
- *NB: Prepare the needed Artemia 2 days before they are going to be fed to the fish larvae*

INCUBATING *Artemia* CYSTS

1. Take out the pre-weighed bag containing Artemia-cysts from the fridge, **see to that you take the correct one**
2. Check water temperature in the cone first (25-29°C), make sure the aeration and heater are on. Then pour the cysts into the cone
3. Gently rinse the cysts into the water, they should not stick on the sides
4. It will take about 24h for the hatching to be complete

HARVESTING *Artemia* NAUPLII

1. **Unplug the heater**
2. Close the bottom valve, THEN close the aeration (which is at the top)
3. Let the system settle for about 5 min
4. Prepare the *Artemia* separator: on a nicely levelled surface, put strainers at the outlet
5. **Remove the heater**
6. Unscrew the aeration-cap on the bottom of the cone
7. **Open and close the valve on the tank quickly** to flush out unhatched cysts at the bottom, if there are any
8. Place a bucket underneath and slowly empty the cone water into the bucket, remember to close the valve when the bucket is full. Don't empty the tank too fast, as high pressure/speed will damage/kill the *Artemia*
9. Take the water+*Artemia* and pour gently into the separator to separate hatched nauplii from empty shells/unhatched cysts
10. Take the strainer containing the separated *Artemia* and rinse it with clean seawater into the second cone with clean seawater (60L, 25-29°C, aerated)

PROTOCOL FOR Artemia HATCHING, HARVESTING AND ENRICHMENT – STARTRENS 2020

11. Continue to empty the cone until it's about 5 cm of water left. This last water you can just flush out (mostly unhatched cysts and are not easily trapped by the magnets)
12. Tip the separator slightly to flush out the last *Artemia* in it and put them in the second cone as well
13. Clean the cone and other equipment (buckets, separator, floor) with warm water and get it ready for use again if needed (60L water, 25-29°C). The aeration-cap at the bottom needs to be rinsed as well before you screw it back on. Make sure the **gasket (round rubber band)** is in place!

1ST ENRICHMENT

1. Take the enrichment (Larviva Multigain) out from the fridge
2. Measure **10g** of the enrichment (Larviva Multigain) in a measuring cup with a spoon
3. Fill ¼ of the measuring cup (with the enrichment in) with lukewarm water
4. Stir with a hand-mixer for 3 minutes
5. Remove as much foam as possible with a spoon
6. Rinse the foam which are still around the measuring cup
7. Take it to the other room and empty it into the cone with **the newly hatched *Artemia* nauplii,** rinse the rest with seawater

2ND ENRICHMENT

1. See 1st enrichment. Do this early in the morning.

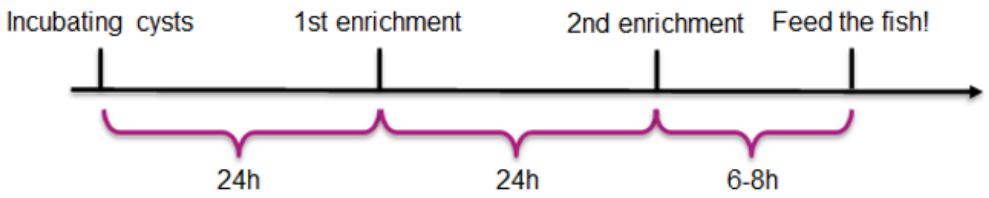
HARVESTING FOR FEEDING THE LARVAE (AFTERNOON AFTER 2ND ENRICHMENT)

1. Prepare two buckets, **first bucket** be marked at a level which indicates for example 5 L
2. Add a small volume of clean seawater (ca 2 L) into the **first bucket** and put it on aeration
3. Add clean sea water to about half in the **second bucket**
4. In the cone with enriched *Artemia,* close the valve at the bottom, the aeration and unplug the heater
5. Wait for about 3 minutes for the heater to cool down, then take it out
6. Put a strainer in the second bucket and slide the bucket under the cone, open the cone to **gently** pour the enriched *Artemia* into the bucket while holding the strainer (the enriched *Artemia* will be soaked in the water but retained in the strainer)
7. Rinse the *Artemia* into the **first bucket**

PROTOCOL FOR Artemia HATCHING, HARVESTING AND ENRICHMENT – STARTRENS 2020

8. Fill the bucket with clean seawater to the known volume (5 L), increase the aeration to distribute the *Artemia* and take 5 samples of 1 ml each to estimate the density under microscope
9. After estimating the density in for example 5 L **(average density in 1 ml x 5000 ml)**, you will be able to estimate the volume to add in each tank for feeding

TIMELINE



Appendix 5. Cirripedia protocol

The following work protocol for cirriped (*S. balanoides*) production was given by the manufacturer: Planktonic AS (CryoPlankton Large):

PLANKTONIC

Procedure for thawing, washing and revitalization of CryoPlankton

Easy method for thawing, washing and revitalization

Step	To do	Description	Notice
1	Prepare water for revitalization	Decide how much CryoPlankton is needed. Prepare the revitalization water. The water amount should be a minimum of 50 litres per kg CryoPlankton. Fill seawater in the tank and add ice. Use aeration to thaw the ice.	Water temperature < 5°C, salinity 24-36 psu, aeration.
2	Prepare water for thawing	Fill the thawing tank with seawater. The water amount should be a minimum of 25 litres per kg CryoPlankton.	Water temperature 5-12°C (no need for cooling), salinity of 24-36 psu, aeration.
3	Weigh out CryoPlankton	Find a scale, gloves, net, safety glasses and a container to transport the plankton in. Tare the container on the scale, collect the plankton and weigh out the wanted amount. Use safety equipment and protect arms and legs with clothes. Read the material safety data sheet before handling liquid nitrogen.	CryoPlankton that are collected from the thermos should not be transferred back to the thermos. CryoPlankton will not survive the extreme changes in temperature.
4	Thaw CryoPlankton	Add the plankton in the thawing tank with aeration.	The time between taking the plankton out of the thermos until it is transferred to the seawater should not exceed 5 minutes. The vitality of the nauplii could be affected.

Versjon nr	Godkjent av
1	03.07.18 Maria Bergvik

5	Wash CryoPlankton	The plankton can be transferred to the net when the plankton is thawed. Rince with sea water for 5 minutes.	The cryo protection agaent is important to remove because it is toxic for the fish larvae.
6	Revitalize CryoPlankton	Transfer the plankton to the revitalization tank with cold sea water and aeration. CryoPlankton is revitalized after 6 hours and ready to feed to the fish larvae.	

Versjon nr	Godkjent av
1	03.07.18 Maria Bergvik

Appendix 6. Copepod protocol

The following work protocol for copepod (*A. tonsa*) production was made in accordance with the manufacturer's recommendations: C-FEED AS, Norway.

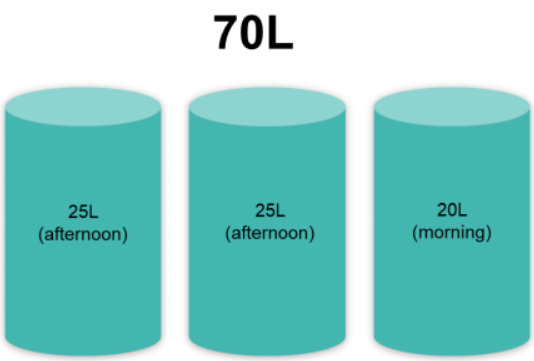
COPEPODS PROTOCOL

*The seawater is warm, to cool it, let it run for a few minutes before using.

AFTERNOON

Harvesting of *Acartia*

1. Check how many litres to filter into each of the 3 buckets (right number for each day will be posted on the wall).
2. Fill all the buckets with some seawater and put aeration before putting the copepods. Filter the right amount into the buckets.
3. When done with filtration, fill up the 3 buckets as show below:



Feeding of *Acartia*

4. Feed the big reservoir with around 20L of microalgae and the "morning" bucket with around 1.5 L.
5. Fill the big reservoir back to 900 L.
6. Around 15 min before feeding to the fish tanks (15:45), feed the "afternoon" buckets with around 2.5 L each. Transport them to CodTech lab with aeration.

MORNING

7. Feed the "morning" bucket with around 2 L of microalgae.
8. Put aeration in the tank to be transported (can use 2 of the microalgae containers with the attached aeration).
9. Transport the bucket to CodTech Lab.
10. Wait around 15 minutes from feeding microalgae, before filling the feeding reservoir to the fish tanks.

Filling the feeding reservoirs

	17h	7h
Normal treatment	8 L/tank	3.3 L/tank
Mix treatment	2.5 L/tank	1.0 L/tank

Appendix 7. Embedding procedure using Technovit ® 7100

Component 1:	Basic resin (monomer), 500 mL	hydroxyethylmethacrylate (HEMA)
Component 2:	Activator (powder), 5 pkg. of 1 g	benzol peroxide
Component 3:	Hardener II (herder), 40 mL	

DEHYDRATIRION FLUIDS:

I.	3 parts distilled water + 1 part basic resin (HEMA)
II.	2 parts distilled water + 2 parts basic resin (HEMA)

PFA-fixated whole lumpfish were immersed in the first dehydration fluid I for 3 hours at 4 °C, then immersed in dehydration fluid II for 2 hours at room temperature.

INFILTRATION FLUID:

50 mL basic resin + 0.5 g activator

The infiltration fluid was made by dissolving the powder in the solution using a magnet stirrer. Dehydrated lumpfish were immersed in the infiltration fluid and placed on a digital shaker (ROCKER 3D digital, IKA ®, USA) overnight. Infiltration fluid was stored at 4 °C for up to 2 weeks.

POLYMERIZATION FLUID:

1,5 mL infiltration fluid + 100 µL hardener II (per fish)

As the polymerization process happens quickly, the infiltration fluid and hardener were mixed and used immediately. The samples were placed and oriented in individual molds for embedment and filled up partially. A block holder (an adapter holding the sample on the microtome) was placed on top of the mold and the rest of the mold were filled through a hole in the centre of the holder. The embedment was complete after 40-120 minutes in room temperature.

Figure A2. Lumpfish embedded in resin on a block holder. A 21 dph Art-larva embedded in resin. The larva has been cut behind the anus in the transversal plane prior to embedment.

Appendix 8. Staining protocols for TB and AB-PAS

Toluidine blue (TB) 0.05%

1. Stain the section with TB 0.05 % in saturated borate buffer for 30 seconds.
2. Rinse with distilled water to remove excess stain from the section.
3. Dry on heating table at 75 % for at least 10 minutes before mounting with coverslide.

Alcian Blue-Periodic acid Schiff (AB-PAS)

1. Immerse the section in Alcian blue solution for 5 minutes.
2. Immerse the section in a beaker filled with tap water for 3 minutes while letting the tap water run to continuously change out the water. Rinse the section in distilled water.
3. Immerse the section in periodic acid 0.5 % for 10 minutes.
4. Repeat step 2.
5. Immerse the section in Schiff's reagent for 15 minutes.
6. Repeat step 2.
7. Immerse the section in Mayers Hematoxylin and Eosin staining for 5 minutes.
8. Repeat step 2.
9. Dip the section four times quickly in 1% HCl in 70 % ethanol.
10. Repeat step 2.
11. Dry on heating table at 75 % for at least 10 minutes before mounting with coverslide.

Appendix 9. Mean dry weight per tank

Table A3. Mean dry weight per tank in lumpfish 2-34 dph. The mean dry weight (DW) for each tank for the different treatment groups, with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean DW±SE (mg/larva)	Total *N*
2	All	-	0.91 ± 0.05	15
9	Art	1	1.04 ± 0.06	5
		7	1.17 ± 0.10	5
		15	0.98 ± 0.04	5
	Cir	4	0.80 ± 0.02	5
		8	0.73 ± 0.11	5
		14	1.01 ± 0.07	5
	FD	2	0.90 ± 0.06	5
		10	0.98 ± 0.03	5
		12	0.80 ± 0.07	5
	Cop/Cir	3	1.05 ± 0.08	5
		5	1.04 ± 0.07	5
		9	0.86 ± 0.06	5
	Cop/FD	6	0.92 ± 0.05	5
		11	1.02 ± 0.04	5
		13	0.93 ± 0.11	5
15	Art	1	1.73 ± 0.13	5
		7	1.68 ± 0.13	5
		15	1.93 ± 0.09	5
	Cir	4	1.17 ± 0.08	5
		8	1.16 ± 0.09	5
		14	1.29 ± 0.08	6
	FD	2	0.83 ± 0.06	5
		10	0.90 ± 0.09	5
		12	1.16 ± 0.12	5
	Cop/Cir	3	1.13 ± 0.07	5
		5	1.24 ± 0.05	5
		9	1.13 ± 0.06	5
	Cop/FD	6	1.07 ± 0.09	5
		11	1.14 ± 0.08	5
		13	1.09 ± 0.09	5
21	Art	1	3.03 ± 0.12	10
		7	2.59 ± 0.12	10
		15	2.59 ± 0.09	10
	Cir	4	1.90 ± 0.12	10
		8	1.90 ± 0.15	10
		14	1.73 ± 0.09	10
	FD	2	1.36 ± 0.09	10
		10	1.41 ± 0.07	10
		12	1.65 ± 0.11	10
	Cop/Cir	3	1.96 ± 0.14	10
		5	1.90 ± 0.07	10
		9	1.51 ± 0.05	10
	Cop/FD	6	0.93 ± 0.05	10
		11	1.38 ± 0.09	10
		13	1.18 ± 0.14	10

Table A3 continued. Mean dry weight per tank in lumpfish 2-34 dph. The mean dry weight (DW) for each tank for the different treatment groups, with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean DW±SE (mg/larva)	Total *N*
29	Art	1	2.92 ± 0.25	15
		7	3.89 ± 0.20	16
		15	3.66 ± 0.21	15
	Cir	4	2.97 ± 0.24	15
		8	3.11 ± 0.23	15
		14	2.97 ± 0.21	15
	FD	2	2.27 ± 0.21	15
		10	2.42 ± 0.16	15
		12	2.57 ± 0.11	15
	Cop/Cir	3	2.12 ± 0.28	15
		5	2.19 ± 0.15	15
		9	2.60 ± 0.17	15
	Cop/FD	6	2.27 ± 0.17	15
		11	2.10 ± 0.13	15
		13	2.18 ± 0.19	15
34	Art	1	4.35 ± 0.24	15
		7	4.11 ± 0.17	15
		15	4.82 ± 0.25	15
	Cir	4	4.70 ± 0.26	15
		8	4.38 ± 0.38	15
		14	4.12 ± 0.38	15
	FD	2	3.54 ± 0.23	15
		10	2.96 ± 0.19	15
		12	3.53 ± 0.17	15
	Cop/Cir	3	3.86 ± 0.27	15
		5	3.12 ± 0.29	15
		9	3.16 ± 0.30	15
	Cop/FD	6	2.85 ± 0.23	15
		11	2.96 ± 0.24	15
		13	3.23 ± 0.21	15

Appendix 10. Mean daily weight increase per tank

Table A4. Mean daily weight increase per tank in lumpfish 2-34 dph. The mean daily weight increase (DWI) is shown for each tank for the different treatment groups at specific time intervals (dph), with total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean DWI±SE (mg/larva)	Total *N*
2-9	Art	1	1.97	5
		7	3.73	5
		15	1.10	5
	Cir	4	-1.75	5
		8	-3.02	5
		14	1.59	5
	FD	2	-0.10	5
		10	1.12	5
		12	-1.40	5
	Cop/Cir	3	2.10	5
		5	2.05	5
		9	-0.65	5
	Cop/FD	6	0.27	5
		11	1.66	5
		13	0.34	5
9-21	Art	1	9.35	5
		7	6.85	5
		15	8.48	5
	Cir	4	7.47	5
		8	8.31	5
		14	4.58	6
	FD	2	3.54	5
		10	3.10	5
		12	5.97	5
	Cop/Cir	3	5.37	5
		5	5.14	5
		9	4.75	5
	Cop/FD	6	0.04	5
		11	2.56	5
		13	2.01	5
21-34	Art	1	2.82	10
		7	3.63	10
		15	4.87	10
	Cir	4	7.23	10
		8	6.62	10
		14	6.90	10
	FD	2	7.60	10
		10	5.87	10
		12	6.05	10
	Cop/Cir	3	5.34	10
		5	3.87	10
		9	5.86	10
	Cop/FD	6	9.03	10
		11	6.06	10
		13	8.07	10

Table A4 continued. Mean daily weight increase per tank in lumpfish 2-34 dph. The mean daily weight increase (DWI) is shown for each tank for the different treatment groups at specific time intervals (dph), with total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean DWI±SE (mg/larva)	Total *N*
2-34	Art	1	5.03	15
		7	4.85	16
		15	5.36	15
	Cir	4	5.28	15
		8	5.05	15
		14	4.85	15
	FD	2	4.35	15
		10	3.77	15
		12	4.35	15
	Cop/Cir	3	4.64	15
		5	3.94	15
		9	3.99	15
	Cop/FD	6	3.65	15
		11	3.77	15
		13	4.05	15

Appendix 11. Mean standard length per tank

Table A5. Mean standard length per tank in lumpfish 2-34 dph. The mean standard length (SL) is shown for each tank for the different treatment groups, with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean SL±SE (mm/larva)	Total *N*
2	All	-	5.94 ± 0.10	15
9	Art	1	6.39 ± 0.26	5
		7	6.78 ± 0.16	5
		15	6.62 ± 0.07	5
	Cir	4	6.60 ± 0.05	5
		8	6.25 ± 0.21	5
		14	6.46 ± 0.12	5
	FD	2	6.49 ± 0.11	5
		10	6.61 ± 0.07	5
		12	6.34 ± 0.13	5
	Cop/Cir	3	6.61 ± 0.10	5
		5	6.40 ± 0.15	5
		9	6.50 ± 0.08	5
	Cop/FD	6	6.65 ± 0.14	5
		11	6.44 ± 0.08	5
		13	6.61 ± 0.15	5
15	Art	1	7.87 ± 0.13	5
		7	7.51 ± 0.11	5
		15	7.79 ± 0.07	5
	Cir	4	7.08 ± 0.08	5
		8	6.80 ± 0.39	5
		14	7.37 ± 0.15	6
	FD	2	6.59 ± 0.16	5
		10	6.77 ± 0.12	5
		12	7.05 ± 0.15	5
	Cop/Cir	3	7.24 ± 0.19	5
		5	7.40 ± 0.14	5
		9	7.16 ± 0.07	5
	Cop/FD	6	7.04 ± 0.04	5
		11	7.04 ± 0.16	5
		13	7.14 ± 0.12	5
21	Art	1	8.62 ± 0.08	10
		7	8.12 ± 0.11	10
		15	8.28 ± 0.09	10
	Cir	4	7.79 ± 0.16	10
		8	7.80 ± 0.15	10
		14	7.68 ± 0.10	10
	FD	2	7.37 ± 0.11	10
		10	7.41 ± 0.10	10
		12	7.46 ± 0.09	10
	Cop/Cir	3	7.85 ± 0.08	10
		5	7.91 ± 0.08	10
		9	7.57 ± 0.07	10
	Cop/FD	6	6.94 ± 0.07	10
		11	7.27 ± 0.11	10
		13	7.24 ± 0.15	10

Table A5 continued. Mean standard length per tank in lumpfish 2-34 dph. The mean standard length (SL) is shown for each tank for the different treatment groups, with respective standard errors (SE) and total number of sampled larvae (Total *N*).

Dph	Treatment	Tank nr.	Mean SL±SE (mm/larva)	Total *N*
29	Art	1	8.51 ± 0.20	15
		7	9.29 ± 0.13	16
		15	9.04 ± 0.16	15
	Cir	4	8.54 ± 0.20	15
		8	8.51 ± 0.19	15
		14	8.43 ± 0.19	15
	FD	2	7.70 ± 0.26	15
		10	8.01 ± 0.16	15
		12	8.13 ± 0.13	15
	Cop/Cir	3	7.62 ± 0.27	15
		5	7.70 ± 0.15	15
		9	8.08 ± 0.15	15
	Cop/FD	6	7.80 ± 0.18	15
		11	7.66 ± 0.15	15
		13	7.71 ± 0.19	15
34	Art	1	9.26 ± 0.13	15
		7	9.19 ± 0.11	15
		15	9.54 ± 0.15	15
	Cir	4	9.25 ± 0.15	15
		8	9.14 ± 0.25	15
		14	9.04 ± 0.26	15
	FD	2	8.57 ± 0.17	15
		10	8.23 ± 0.17	15
		12	8.76 ± 0.14	15
	Cop/Cir	3	8.78 ± 0.19	15
		5	8.22 ± 0.21	15
		9	8.29 ± 0.24	15
	Cop/FD	6	8.09 ± 0.18	15
		11	8.28 ± 0.19	15
		13	8.46 ± 0.15	15
35	Art	1	9.29 ± 0.04	250
		7	9.25 ± 0.04	250
		15	9.34 ± 0.04	250
	Cir	4	9.05 ± 0.04	250
		8	8.79 ± 0.05	250
		14	8.95 ± 0.04	250
	FD	2	8.67 ± 0.04	250
		10	8.39 ± 0.04	250
		12	8.55 ± 0.03	250
	Cop/Cir	3	8.70 ± 0.05	250
		5	8.34 ± 0.05	250
		9	8.46 ± 0.04	250
	Cop/FD	6	8.17 ± 0.04	250
		11	8.54 ± 0.04	250
		13	8.42 ± 0.04	250

Appendix 12. Number of larva per tank (survival)

Table A6. Number of lumpfish larvae 2-35 dph. Estimated number of larvae alive per tank for each of the treatment group from 2-35 days post hatch (dph). The survival estimates are based on number of sampled larvae, registered mortality throughout the experiment and remaining larvae at the experiments end.

Dph	Art			Cir			FD		
	Tank nr.			Tank nr.			Tank nr.		
	1	7	15	4	8	14	2	10	12
2	6663	7017	4361	6359	6220	6774	9314	5923	5680
3	6610	7000	4361	6114	6182	6774	9276	5897	5655
4	6610	7000	4341	6114	6178	6503	9267	5875	5544
5	6594	6999	4341	6092	6175	6380	9264	5863	5538
6	6591	6995	4338	6083	6174	6297	9258	5859	5524
7	6590	6992	4335	6080	6171	6282	9251	5851	5519
8	6586	6990	4332	6077	6169	6261	9249	5850	5517
9	6572	6971	4330	6067	6165	6255	9230	5848	5515
10	6567	6971	4324	6063	6154	6243	9224	5848	5512
11	6565	6963	4324	6061	6150	6228	9217	5842	5510
12	6565	6958	4323	6061	6142	6217	9209	5832	5509
13	6564	6958	4323	6056	6140	6217	9204	5819	5506
14	6562	6953	4321	6054	6138	6216	9199	5804	5489
15	6562	6947	4321	6050	6135	6215	9192	5793	5476
16	6559	6940	4317	6040	6133	6214	9165	5732	5459
17	6557	6933	4312	6035	6132	6212	9149	5732	5442
18	6554	6927	4306	6030	6128	6207	9109	5715	5423
19	6552	6925	4304	6028	6128	6207	9091	5701	5421
20	6550	6923	4296	6023	6126	6205	9082	5662	5409
21	6528	6920	4290	6018	6118	6203	9073	5632	5389
22	6520	6920	4282	5988	6099	6189	9066	5596	5370
23	6516	6909	4278	5983	6093	6184	9057	5543	5353
24	6493	6888	4263	5981	6087	6176	9047	5531	5352
25	6465	6861	4243	5973	6078	6169	9038	5514	5351
26	6434	6831	4231	5969	6057	6150	9030	5463	5326
27	6401	6819	4215	5963	6048	6134	9026	5424	5323
28	6389	6791	4207	5954	6011	6127	9019	5386	5313
29	6383	6778	4204	5948	5992	6122	9013	5295	5310
30	6378	6764	4196	5934	5977	6108	9004	5236	5299
31	6378	6748	4193	5930	5973	6100	8996	5184	5298
32	6363	6725	4183	5925	5968	6096	8983	5115	5295
33	6357	6705	4178	5912	5945	6086	8967	5079	5295
34	6351	6695	4172	5908	5942	6080	8961	5046	5295
35	6347	6686	4171	5904	5937	6075	8958	5028	5292

Table A6 continued. Number of lumpfish larvae 2-35 dph. Estimated number of larvae alive per tank for each of the treatment groups from 2-35 days post hatch (dph). The survival estimates are based on number of sampled larvae, registered mortality throughout the experiment and remaining larvae at the experiments end.

Dph	Cop/Cir			Cop/FD		
	Tank nr.			Tank nr.		
	3	5	9	6	11	13
2	8536	11424	8892	6044	6982	3570
3	8438	11412	8881	5993	6982	3570
4	8438	11412	8881	5993	6831	3545
5	8391	11406	8869	5911	6805	3483
6	8381	11396	8865	5876	6777	3432
7	8378	11394	8844	5863	6740	3411
8	8369	11392	8828	5854	6732	3409
9	8357	11380	8776	5813	6709	3378
10	8355	11373	8759	5788	6686	3355
11	8351	11365	8743	5781	6681	3353
12	8350	11351	8736	5776	6678	3350
13	8256	11293	8719	5768	6648	3347
14	8240	11243	8711	5761	6630	3347
15	8222	11200	8704	5752	6601	3347
16	8193	11174	8678	5737	6537	3325
17	8190	11152	8667	5728	6506	3294
18	8181	11124	8648	5722	6498	3286
19	8173	11120	8633	5692	6486	3273
20	8169	11097	8610	5659	6466	3264
21	8165	11076	8594	5606	6448	3260
22	8155	11076	8544	5590	6427	3254
23	8144	11029	8532	5569	6391	3232
24	8140	10988	8526	5520	6364	3219
25	8112	10950	8485	5473	6317	3204
26	8090	10935	8435	5403	6268	3179
27	8073	10883	8327	5347	6224	3161
28	8049	10848	8213	5291	6182	3126
29	8042	10812	8141	5231	6121	3103
30	8019	10754	8092	5176	6112	3090
31	8011	10701	8072	5156	6092	3080
32	8000	10660	8027	5121	6080	3071
33	7984	10615	8002	5103	6078	3068
34	7963	10586	7996	5079	6075	3065
35	7948	10568	7978	5070	6070	3064

Appendix 13. Histological analysis of liver

Table A7. Mean hepatocyte nucleus size and hepatocyte size in lumpfish 2-35 dph. Mean area size (µm²) of hepatocyte nucleus and hepatocyte cell and respective standard error (SE) given for each treatment group from 2-35 days post hatch (dph) and total number of sampled larvae (Total *N*). Signficant differences ($p<0.05$) between treatment groups at a given dph are indicated by different letters.

Dph	Treatment	Mean area size±SE (µm²)		Total *N*
		Nucleus	Cell	
2	All	52.4 ± 3.9	750.3 ± 63.6	5
9	Art	43.9 ± 0.6	712.1 ± 25.4	5
	Cir	45.7 ± 2.5	749.4 ± 62.4	5
	FD	37.3 ± 1.8	535.7 ± 15.4	5
	Cop/Cir	43.7 ± 5.7	720.4 ± 77.2	5
	Cop/FD	44.7 ± 2.5	715.4 ± 39.6	5
21	Art	36.5 ± 2.0^{b}	845.7 ± 65.7^{a}	5
	Cir	35.6 ± 1.2^{b}	608.6 ± 13.1^{b}	5
	FD	47.2 ± 2.1^{a}	665.4 ± 27.5^{ab}	5
	Cop/Cir	38.0 ± 1.3^{b}	724.8 ± 52.7^{ab}	5
	Cop/FD	38.0 ± 1.5^{b}	543.0 ± 54.4^{b}	5
35	Art	34.5 ± 2.9	823.9 ± 42.2	5
	Cir	39.0 ± 1.9	879.8 ± 71.4	5
	FD	43.6 ± 1.4	907.4 ± 49.7	5
	Cop/Cir	36.7 ± 2.8	839.9 ± 51.5	5
	Cop/FD	41.1 ± 2.2	944.7 ± 55.5	5

Table A8. Mean standard length of lumpfish used for histological analysis of the liver 2-35 dph. Mean standard length (SL) and respective standard error (SE) given for each treatment groups from 2-35 days post hatch (dph), and total number of sampled larvae (Total *N*). SL of larvae used for histological analysis of the liver were similar to those sampled for growth. Signficant differences ($p<0.05$) between treatment groups at a given dph are indicated by different letters.

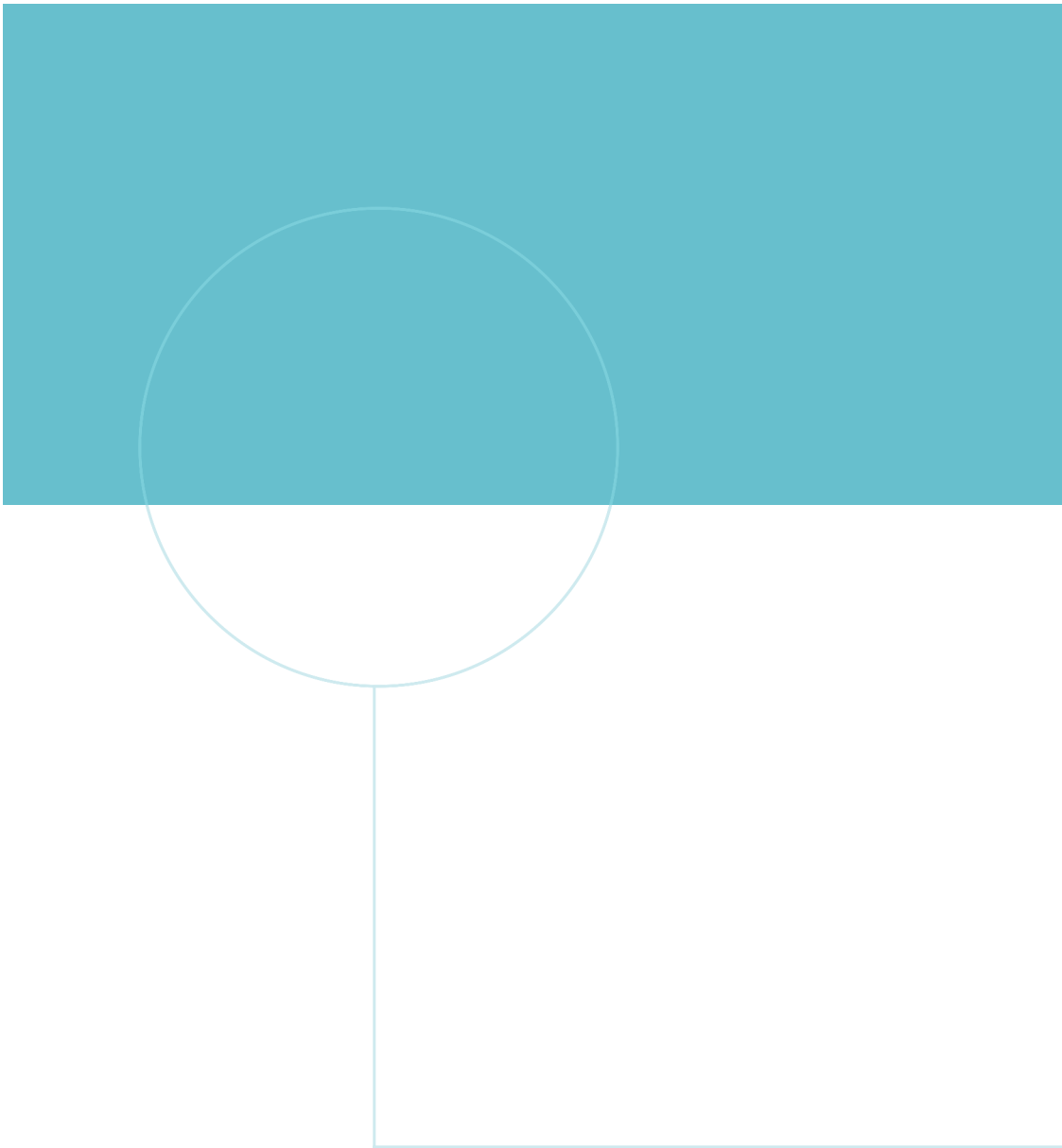
Dph	Treatment	Mean SL±SE (mm)	Total *N*
2	Yolk sac	5.88 ± 0.10	5
9	Art	6.30 ± 0.12	5
	Cir	6.36 ± 0.23	5
	FD	6.26 ± 0.11	5
	Cop/Cir	6.34 ± 0.08	5
	Cop/FD	6.32 ± 0.21	5
21	Art	7.91 ± 0.12a	5
	Cir	7.38 ± 0.11ab	5
	FD	6.84 ± 0.26bc	5
	Cop/Cir	7.13 ± 0.10bc	5
	Cop/FD	6.52 ± 0.18c	5
35	Art	8.93 ± 0.40	5
	Cir	9.01 ± 0.28	5
	FD	8.69 ± 0.28	5
	Cop/Cir	8.88 ± 0.36	5
	Cop/FD	8.13 ± 0.33	5

Table A9. Mean hepatocyte nuclear, vacuole and 'other' area fraction in lumpfish 2-35 dph. The means (%±SE) are based on n=5 sampled larvae in total at 2 dph, and n=5 larvae per treatment for 9, 21 and 35 dph (Total *N*). The nuclear area fraction was estimated as the sum of the hepatocyte nuclear sizes divided by analysed liver area. The vacuole fraction was estimated in the same liver area by using a point grid. The last fraction 'Other' were estimated by subtracting nuclear and vacuole areas and accounts for remaining tissue, such as hepatocyte organelles etc. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters.

Dph	Treatment	Mean surface area fraction±SE (%)			Total *N*
		Nuclei	Vacuoles	Other	
2	All	7.1 ± 0.3	15.0 ± 4.5	78.0 ± 4.3	5
9	Art	6.2 ± 0.3	13.2 ± 1.9^{a}	80.6 ± 1.8^{a}	5
	Cir	6.2 ± 0.3	9.5 ± 4.8^{ab}	84.3 ± 4.5^{ab}	5
	FD	6.7 ± 0.1	3.7 ± 0.8^{ab}	89.6 ± 0.8^{ab}	5
	Cop/Cir	6.1 ± 0.4	2.1 ± 0.5^{b}	91.8 ± 0.6^{b}	5
	Cop/FD	6.2 ± 0.1	1.9 ± 0.4^{b}	91.9 ± 0.4^{b}	5
21	Art	4.4 ± 0.2^{a}	32.2 ± 1.6^{a}	63.4 ± 1.4^{a}	5
	Cir	5.9 ± 0.2^{ab}	2.8 ± 0.4^{c}	91.4 ± 0.3^{c}	5
	FD	7.1 ± 0.4^{b}	18.8 ± 1.7^{b}	74.1 ± 1.9^{b}	5
	Cop/Cir	5.3 ± 0.4^{ab}	4.8 ± 0.7^{cd}	89.9 ± 0.8^{cd}	5
	Cop/FD	7.2 ± 0.7^{b}	9.7 ± 2.7^{d}	82.8 ± 2.9^{d}	5
35	Art	4.2 ± 0.3	17.6 ± 2.1	78.2 ± 1.9	5
	Cir	4.5 ± 0.2	15.2 ± 3.1	80.3 ± 3.0	5
	FD	4.9 ± 0.3	17.0 ± 1.9	78.1 ± 1.8	5
	Cop/Cir	4.4 ± 0.4	20.2 ± 2.2	75.3 ± 2.5	5
	Cop/FD	4.4 ± 0.1	17.8 ± 1.6	77.9 ± 1.6	5

Table A10. Mean proportion of hepatocyte nuclei types in lumpfish 2-35 dph. The mean proportions (%±SE) of hepatocyte nuclei types are based on n=5 sampled larvae in total at 2 dph, and n=5 larvae per treatment for 9, 21 and 35 dph (Total *N*). Nuclei 1 is defined as nuclei with one nucleolus, whereas Nuclei 2+ is nuclei with two or more nucleoli. Signficant differences (p<0.05) between treatment groups at a given dph are indicated by different letters.

Dph	Treatment	Mean nucleolar proportion±SE (%)		Total *N*
		Nuclei 1	Nuclei 2+	
2	All	99.0 ± 0.1	1.0 ± 0.5	5
9	Art	81.7 ± 0.6	18.3 ± 0.9	5
	Cir	78.7 ± 0.4	21.3 ± 0.8	5
	FD	81.4 ± 0.4	18.6 ± 0.6	5
	Cop/Cir	79.7 ± 0.2	20.3 ± 0.4	5
	Cop/FD	78.7 ± 0.7	21.3 ± 1.9	5
21	Art	76.3 ± 0.8^{ab}	23.7 ± 1.5^{ab}	5
	Cir	67.9 ± 0.4^{b}	32.1 ± 0.5^{b}	5
	FD	87.1 ± 0.4^{ab}	12.9 ± 0.8^{a}	5
	Cop/Cir	67.7 ± 0.9^{b}	32.3 ± 1.6^{b}	5
	Cop/FD	80.3 ± 0.3^{ab}	19.7 ± 0.5^{ab}	5
35	Art	85.2 ± 0.4^{a}	14.8 ± 1.2^{a}	5
	Cir	68.7 ± 0.5^{b}	31.3 ± 0.7^{b}	5
	FD	66.8 ± 0.4^{b}	33.2 ± 0.7^{b}	5
	Cop/Cir	70.7 ± 0.9^{ab}	29.3 ± 1.6^{ab}	5
	Cop/FD	73.6 ± 0.7^{ab}	26.4 ± 1.9^{ab}	5



NTNU

Norwegian University of
Science and Technology