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Effects on growth, survival and bone development from start feeding lumpsucker (*Cyclopterus lumpus*) larvae with *Artemia*, copepods and fomulated feed

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Abstract

Salmon lice (*Lepeoptherius solmonis*) is a major health problem in salmon (*Salmo salar*) production. Reduced lice sensitivity towards several of the chemical treatments have increased the interest in cleaner fish used to biological remove salmon lice. The cleaner fish, lumpsucker (*Cyclopterus lumpus*) is a relatively new aquaculture species and there is little knowledge about the optimal rearing conditions, nutrition and the skeletal development.

The aim of this study was to describe the skeletal development in lumpsucker (*C. lumpus*) and to evaluate growth, survival and skeletal development in larvae fed with three different diets; *Artemia*, copepods (*A. tonsa*) and formulated feed. One group was fed enriched *Artemia*, a second group fed copepods and the third group was fed a combination of *A. tonsa* (2-9 dph) and formulated feed (from 7 dph). At 20 dph all groups were weaned to formulated feed and fed it exclusively from 22-51 dph.

Start feeding with *Artemia* improved larval growth and survival compared to larvae fed copepods and formulated feed. The dry weight, wet weight and standard length were significantly higher already from 6 dph and throughout the experiment. Newly hatched larvae had bone ossification in mouthparts, cleihtrum, vertebrae and suction disc. Earlier onset of skeletal ossification related to age was observed in larvae fed *Artemia***.** Significantly more vertebrae were fully ossified from 10-50 dph and more larvae had ossified pterygiophores of first dorsal and anal fins at 42 dph. In addition, at 21 dph significantly more tail fin rays had ossified. However, the larvae fed copepods had ossification in vertebrae, dorsal, anal and tail fins, urostyle and hypurals at a smaller size. The smallest larva with 80-90 % fully ossified vertebrae was 9.9 mm, over 0,8 mm shorter than larvae fed *Artemia* and formulated feed. The pterygiophores were ossified in larvae fed copepods larger than 8.3 mm, compared to 9,0 and 8,8 mm for larvae fed *Artemia* and formulated feed. The occurrence of severe skeletal anomalies (flat skull and axis deviations) were 1 % at 50 dph. Fusion of two vertebrae were found in less than 11 % of all larvae at 50 dph. Twisted neural arches, a small abbreviation from the normal form was observed in significantly more larvae fed formulated feed. The occurrence was highest in younger larvae and considerably lower at 50 dph.

Sammendrag

Lakselus (*Lepeoptherius salmonis*) er et stort helseproblem i produksjon av laks (*Salmo salar*). Redusert sensitivitet mot flere kjemiske behandlinger har økt interessen for bruk av rensefisk til å fjerne lakselusa. Rensefiskarten rognkjeks (*Cyclopterus lumpus*) er en relativt ny art innenfor akvakultur og det er derfor mangel på kunnskap om optimale oppdrettsforhold, næringsstoffer og larvens skjelettutvikling.

Hensikten med dette studiet var å beskrive skjelettutvikling hos rognkjeks (*C. lumpus*) og evaluere vekst og overlevelse hos larver fôret med tre ulike dietter; *Artemia*, hoppekreps (*Acartia tonsa*) og tørrfôr. En gruppe ble fôret *Artemia*, den andre gruppen ble fôret hoppekreps og den tredje gruppen fikk en kombinasjon av hoppekreps (2-9 dph) og tørrfôr fra 7 dph. Alle grupper ble tilvendt til tørrfôr fra 20 dph og fôret det fra 22 dph. Målinger av vekst, overlevelse og beinanalyser ble brukt til å evaluere effekten fra de ulike startfôringsdiettene. Larver farget med alizarin rød ble brukt til å beskrive skjelettutviklingen og for å evaluere forekomsten av skjelettavvik (anomalier).

Startfôring med *Artemia* resulterte i forbedret vekst og overlevelse sammenlignet med larver fôret med hoppekreps og tørrfôr. Tørrvekt, våtvekt og standard lengde var signifikant høyere allerede fra 6 dph og ut eksperimentet. Dette kan foreslå at DHA og EPA gitt i triglycerider i anriket *Artemia* sikrer tiltrekkelig mengde for normal vekst og overlevelse. De nylig klekte rognkjekslarvene hadde forbeining i munndeler, gjellebue (cleihtrum), ryggvirvler og sugeskive. Skjelettutviklingen relatert til alder startet tidligere i larver fôret *Artemia,* mens larvene fôret copepoder startet forbeiningen i ryggvirvler, rygg-, gatt- og halefinner, urostyle og hypuraler ved mindre størrelser. DHA tilført i fosfolipider i hoppekreps kan ha ført til den tidligere beinutviklingen. Forekomsten av alvorlige beinavvik (anomalier) som flatt skalletak og akseforandringer (lordose, kyfose og skoliose) var 1 % på 50 dph. Sammenvokste ryggvirvler ble funnet i under 11 % av alle larvene på 50 dph, men siden kun to ryggvirvler var sammenvokst vil det trolig ikke påvirke larvens lengde betraktelig.

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Abbreviations

Artemia group/treatment	Larval group fed *Artemia* 2-22 dph
Copepod group/treatment	Larval group fed copepods (*A. tonsa*) 2-22 dph
d°	Days after fertilization • temperature (ºC)
DHA	Docosahexaenoic acid (22:6n-3)
Dph	Days post hatch
DW	Dry weight (mg)
DWI	Daily weight increase
EPA	Eicosapentaenoic acid (20:5n-3)
Formulated feed group/treatment	Larval group fed copepod (*A. tonsa*) 2-9 dph and formulated feed 7-50 dph.
PUFA	Polyunsaturated fatty acid (≥ 20 carbon atoms)
SE	Standard error
SGR	Specific growth rate
SL	Standard length (mm), measured from the tip of the nose to the end of the vertebrae
WW	Wet weight (mg)

1. Introduction

1.1. Use of cleaner fish in salmon aquaculture

Norway is the world's biggest producer of Atlantic salmon (*Salmo salar*) and a large quantity is exported (Liu *et al.*, 2011, FAO, 2017). Today the main health problem in salmon aquaculture is infections from salmon lice. Salmon lice (*Lepeoptherius salmonis*) is an ectoparasite living of skin, blood and mucus of salmonids. The risk of secondary infections and osmotic stress increases when the ectoparasite break the salmonid immune protection against the environment (Grimnes and Jakobsen, 1996). Chemical, physical and biological treatment are used to remove salmon lice. Reduced sensitivity and potential resistance towards several of the chemical treatments used are observed the recent years (Lees *et al.*, 2008, Aaen *et al.*, 2015). This have increased the interest in other possible treatment methods like biological treatment with cleaner fish.

Cleaner fish is a group of fish species that eat dead or infected skin and ectoparasites of the body surface of other fishes. This symbiosis is utilized in salmon farming to remove the lice of salmon. Bjordal (1991) studied cleaning activity in species of wrasse and was the first to report cleaning behaviour in four species of wrasse when exposed to lice infected salmon. The use of cleaner fish increased from 682 000 fish in 2006 to over 37 millions in 2016 (Fiskeridirektoratet, 2017a). In 2016 the industry used 43 % lumpsucker (*Cyclopterus lumpus*), 36 % wrasse species and 21 % unspecified species to remove salmon lice (Fiskeridirektoratet, 2017a). In temperatures under 6 ºC wrasses enters dormancy with low activity and feeding, and perform bad as lice eaters (Kelly *et al.*, 2014). Lumpsucker tolerate colder seawater and is actively feeding during the winter in temperatures down to 3-4 ºC (Imsland *et al.*, 2014, Imsland, 2018).

The production of lumpsucker increased from 431 000 – 15 million fishes from 2012-2016 (Fiskeridirektoratet, 2017b). No standardized production procedure exists today and the information about nutritional requirements, optimal diet and rearing procedure are minimal. Producers often experience high variability in survival and individual larval size (Imsland *et al.*, 2014, Dahle *et al.*, 2017) and high mortality in sea cages is reported (Bornø *et al.*, 2016). The larval growth rate, development and survival can be affected by rearing conditions and nutrition like reported in rearing of other marine fish larvae (Boeuf and Le Bail, 1999, Planas and Cunha, 1999, Downing and Litvak, 2000, Cahu *et al.*, 2003). To succeed with producing a

robust and effective lice eater, knowledge about the requirements for normal larval development is important.

1.2. Optimal feed for lumpsucker larvae

There is little information about the optimal start feed for lumpsucker larvae. Producers have observed a larval acceptance of formulated feed from the start of feeding, but problems with settlement of uneaten feed in fish tanks and high variability in growth and survival is reported (Imsland *et al.*, 2014). In nature lumpsucker larvae live close to seaweed and the main food source is crustaceans associated with the seaweed (Ingólfsson *et al.*, 2002). Ingólfsson *et al.* (2002) observed that lumpsucker larvae fed selectively and ignored smaller and slow-moving prey organisms like rotifers. The main prey organisms they found in the stomach of lumpsucker larva was species from two copepod orders; Harpaticoida and Calanoida.

The start of exogenous feeding is a critical phase for marine fish larvae where optimal feed is important to ensure normal larval development and survival (Yúfera and Darias, 2007). In start feeding of marine fish larvae live and formulated feed are commonly used. Live feed, the natural prey for marine fish larvae is easy digestible and reduces the waste from uneaten feed. Their movement keeps them longer suspended in the water column which give larvae more time to catch them. The nutritional content of the natural feed is optimal for the fish larvae. Live feed is however more expensive compared to formulated feed and knowledge about the cultivation and maintenance of the live feed species are required.

Brine shrimp (*Artemia*) and rotifers (*Brachionus sp.*) are commonly used live feeds in rearing of marine fish larvae. Standardized production procedures make them easy and affordable as larval feeds. Rotifers are used as a first feed for smaller marine fish larvae which cannot eat *Artemia* or dry feed from the start of exogenous feeding. The level of the essential polyunsaturated fatty acids (PUFAs); EPA and DHA in *Artemia* and rotifers are lower than the requirements of marine fish larvae (Evjemo and Olsen, 1997, Léger *et al.*, 1987). Enrichment with marine fish oils with sufficient content of lipids and proteins are important to meet the nutritional requirements. In *Artemia* and rotifers the lipids are stored as energy in triglycerides (Coutteau and Mourente, 1997). *Artemia* catabolize DHA to energy, and when using *Artemia* as live feed a second enrichment procedure is important to ensure sufficient amount of DHA (Evjemo *et al.*, 1997). Storage of *Artemia* at 10 °C before use as feed reduce the catabolism of DHA (Sorgeloos *et al.*, 2001).

DHA and EPA are required for normal fish growth and development (Sargent *et al.*, 1997). DHA is found in cell membranes, brain and nerve tissue of marine fish larvae (Sargent, 1995). Many larvae are not capable of synthesizing sufficient amount of essential fatty acids and are dependent on a supply from feed sources (Geurden *et al.*, 1995). Earlier studies reported a positive effect on growth, survival, stress tolerance and bone ossification when DHA and EPA were supplied in polar lipids (Coutteau *et al.*, 1997, Gisbert *et al.*, 2005, Kjørsvik *et al.*, 2009, Wold *et al.*, 2009). Authors assumed that fatty acids provided in polar lipids (phospholipids) were easier digested and assimilated compared to fatty acids added in neutral lipids. Polar lipids (phospholipids) are important structural and functional components of cell membranes, brain and neural tissue (Tocher and Harvie, 1988)

Copepods are the natural prey for many marine fish larvae including lumpsucker larvae (Ingólfsson *et al.*, 2002). Their nutritional content are considered optimal for marine fish larvae with sufficient amount of both DHA and EPA incorporated in phospholipids (Evjemo *et al.*, 2003, van der Meeren *et al.*, 2008, Evjemo and Olsen, 1997). Start feeding experiments with ballan wrasse, cod and turbot start fed with copepods (*A. tonsa*) had better growth, survival and stress tolerance compared to larvae fed *Artemia* and rotifers (Kjørsvik *et al.*, 2014, Øie *et al.*, 2017, Hamre, 2006, Witt *et al.*, 1984). *Artemia* and rotifers have lower content of phospholipids compared to copepods (Evjemo and Olsen, 1997, Næss *et al.*, 1995). Disadvantages with the use of copepods are that they are difficult and expensive to cultivate compared to *Artemia* and rotifers.

Formulated feed must fulfil several structural and biochemical requirements to satisfy the development of marine fish larvae (Hamre *et al.*, 2013). The disadvantages with formulated feed are the waste settling on the bottom of the tanks from uneaten feed. This increase the organic load and provides more growth substrate for bacteria in the fish tanks (Dahle *et al.*, 2017). Frequently cleaning is important to reduce the amount of organic matter settling in fish tanks.

A pilot study performed by NTNU, SINTEF and a lumpsucker producer (Nordland rensefisk) evaluated if use of copepods (*A. tonsa*) as start feed (2-14 dph) resulted in better growth and survival than formulated feed (Dahle *et al.*, 2017). The larvae fed copepods obtained higher growth and survival. The variation in survival between larvae fed copepods were lower compared to those fed formulated feed. In addition, the bacteria composition in larval tanks fed

copepods was less variable. This may suggest that start feeding with formulated feed is less optimal compared to copepods.

1.3. Skeletal development in marine fish larvae

The fish skeleton supports body posture, development and locomotion, functions as attachment point for muscles, protects organs and cells and is a reservoir for ions (Lall and Lewis-McCrea, 2007). Teleost bones are composed of osteoblasts, osteocytes and bone lining cells, a mineral phase and an organic component of extracellular matrix (Boglione *et al.*, 2013a). Osteoblasts is the bone forming cells. Osteocytes break down bone tissue, an important feature in bone maintenance, repair and remodelling. The bone lining cells cover the bone surfaces and can differentiate into osteogenic cells developing into osteoclasts (Parfitt, 2001). The mineral phase consists mainly of calcium hydroxyapatite salts embedded in the organic matrix of collagen fibers type 1 and 2 (Benjamin and Ralphs, 1991).

In general, bones in advanced teleost species lack the osteoclasts that remodel bones and perform calcium metabolism. Instead calcium is taken up through the gills (Witten, 1997) and the remodelling functions are performed by other cells. Most of the bone remodelling is performed related to growth (Witten and Huysseune, 2009). Teleost bone tissue continues to grow which make the growth-related maintenance possible throughout the fish life. However , if fish have osteoclasts the differentiation takes place in the head kidney in fish (Witten and Huysseune, 2009). These osteoclasts are mono nuclear and smaller compared to multinucleated osteoclasts found in mammals (Boglione *et al.*, 2013a).

Bone formation in fish is described by three mechanisms; endochondral, perichondral and intramembranous ossification (Boglione *et al.*, 2013a). Endochondral ossification consists of several steps replacing a cartilaginous template with bones. Many teleost species lack the endochondral ossification mechanisms, and the cartilage tissue usually found inside bones is replaced by adipose tissue. Perichondral bone ossification are mainly the ossification process found in fish. The perichondrium of a cartilagous template is transformed into a periosteum which secrete bone matrix and have osteoblast-similar functions (Boglione *et al.*, 2013a). During intramembranous ossification bones are formed by osteoblasts differentiated directly from mesenchymal cells without a cartilagous template (Franz-Odendaal, 2011). The mesenchymal cells differentiate into osteoblasts, blood vessels grow into the area with osteoblasts and bone develops. The fish bones formed through intramembranous ossification

are dermal or membrane bones teeth, denticles, cranial dermal bones, scales and fin rays (Boglione *et al.*, 2013a).

The fish skeletal tissue supporting the body posture and providing attachment point for muscles is the notochord. It originates from a fibrous collagen sheath and not from cartilage common in other vertebrates. Secretion of fibrous collagen and formation of epithelial cells (notochordoblasts) forms a fibrous sheath which surround and stiffen the notochord (Boglione *et al.*, 2013a). The vertebral bodies are established by mineralization of the fibrous sheath (Witten and Huysseune, 2009).

The fin bones are composted of a dermal- and an endoskeleton. The dermal skeleton consists of fin rays and the endoskeleton connects the fins to the rest of the fish skeleton (Witten and Huysseune, 2007). In unpaired fins, like dorsal, anal and tail fin the endoskeleton is connected directly to the axial skeleton, while paired fins is connected to girdles. The endoskeleton of anal and dorsal fins consists of pterygiophores, and in the caudal fin the urostyle is the endoskeleton supporting the fin. The paired pectoral fins are connected to a shoulder girdle composed of both endoskeletal and dermal elements.

The lumpsucker skeleton is not well described in literature. Voskoboinikova and Kudryavtseva (2014) describe the bone development in wild sampled lumpsucker eggs and larvae. The shortest larvae with standard length (SL) 4.8 mm obtained from eggs had no bone ossification. Ossification were first observed in free living larvae longer than 5.5 mm. At this size the maxilla, premaxilla, dental, ceratohyal, branchiostegal rays, opercle, preopercle, cleihtrum, pelvis and pelvic fin rays were ossified. Pelvic fins were modified into a suction disc making larvae capable of adhering to hard substrates. In larvae larger than 6.5 mm teeth and the fin rays of second dorsal, anal, and caudal fin were ossified. The rows of teeth increased from one to four rows during larval development. Ossification of nine vertebrae were observed in 5.5 mm large larvae and from 6.5 mm all of them had ossification, except from the two last closest to the urostyle. The total number of vertebrae reported were 27 or 28. Urostyle and hypural bones supporting the caudal fin ossified in larvae longer than 8.1 mm. The epurals also providing caudal fin support ossified in larvae from 13 mm.

1.4. Skeletal anomalies in marine fish larvae

Skeletal anomalies are common in rearing of marine fish larvae (Divanach *et al.*, 1996). They are often found in fins, the spinal column and the head. Absence of the entire fin, fin rays or pterygiophores, decreased size, duplication of fins and other deformities are found in different fish species (Kocour *et al.*, 2006, Georgakopoulou *et al.*, 2010). Spinal anomalies include scoliosis (S-shaped lateral side), lordosis (downward curvature) and kyphosis (upward curvature) and often a combination of these (Divanach *et al.*, 1996). Axis deviation affects the fish body shape and the growth are usually lower compared to fish without spinal anomalies. Other anomalies that occur in the spinal column include fusion, dislocation, compression and shortening of the vertebrae (Boglione *et al.*, 2001). Divanach *et al.* (1996) defined six different anomalies occurring in the head region; Gill cover anomalies, pugheadness, crossbite, lower jaw reduction, ventrally projected hyobranchial skeleton and pike jaw deformity.

Environmental, nutritional and genetic factors can interfere with normal skeletal development in fish. Water temperature, pH and water currents (Hemmer *et al.*, 1990, Divanach *et al.*, 1997, Chatain, 1994, Abdel *et al.*, 2004, Georgakopoulou *et al.*, 2010) and the nutritional content (Cahu *et al.*, 2003, Lall and Lewis-McCrea, 2007) are factors observed to cause anomalies. Boglione *et al.* (2013b) suggest that several factors may cause the same skeletal anomaly and one factor may cause several different anomalies. This makes it difficult to identify the main reason behind a specific skeletal anomaly. However, a high proportion of skeletal anomalies during larval rearing indicate that something is less optimal.

Villeneuve *et al.* (2005) relate the lipid content of feed to fish skeletal development in sea bass. DHA and EPA provided in phospholipids are reported to give better skeletal development and less anomalies in cod (Kjørsvik *et al.*, 2009), ballan wrasse (Øie *et al.*, 2017), seabass (Cahu *et al.*, 2003). Jump and Clarke (1999, as sited in Cahu e*t al.*, 2003) state that the highly unsaturated fatty acids affects specific nuclear receptors involved in regulation of genes during skeletal development under ontogenesis in mammals. DHA and EPA might have the same impact during development of the fish skeleton.

Protein with the optimal amino acid profile is important to support the fast bone development in fish larvae (Cahu *et al.*, 2003). Deficiency of the amino acid tryptophan are reported to increase the occurrence of scoliosis in salmonids (Akiyama *et al.*, 1986). Moderate levels of protein hydrolysates in the diet was reported to decrease the skeletal anomalies in gilthead seabream, while high levels (over 12 % of dry matter) resulted in more skeletal anomalies

(Gisbert *et al.*, 2012). Protein hydrolysates consists of peptide chains with 10-20 amino acids or di- and tri-peptides instead of whole proteins. (Cahu and Infante, 1995) state that a diet with optimal level of protein hydrolysates may accelerate the maturation process of the intestine.

Vitamins and minerals may also affect the skeletal development in fish. Lall and Lewis-McCrea (2007) compared several studies of skeletal anomalies in marine fish larvae and found that suboptimal levels of phosphorus, vitamin A and K can increase the occurrence of skeletal anomalies. Deficiency of phosphorous is reported to interfere with the bone mineralization in rainbow trout (Fontagné *et al.*, 2009), haddock (Roy, 2002) and Atlantic halibut (Roy and Lall, 2003). In haddock, authors suggested that phosphorus deficiency increased the number of osteoclasts performing bone resorption. Phosphorus deficiency was reported to increase number of anomalies in the hemal and neural spines in Atlantic halibut. Elevated levels of vitamin A (>188 ng mg^{-1} DW of the enriched live prey) in the enrichment diets of live feeds (*Artemia* and rotifers) was reported to result in more skeletal deformities in sea bream (Fernández *et al.*, 2008). Fernández *et al.* (2008) suggested that vitamin A accelerate the skeletal development and high levels increase the number of skeletal deformities. Roy (2002) observed deformities in haddock fed diets with deficiency of vitamin K.

Other vitamins and minerals are also suggested to be involved in vertebral skeletal development. Vitamin E may be involved in the defence against free radicals that might damage osteoblasts and stimulate osteoclasts (Lall and Lewis-McCrea, 2007). Darias *et al.* (2010) reported that deficiency or elevated levels of vitamin D increased the occurrence of skeletal deformities in sea bass. They suggested a relation between vitamin D levels and the absorption of Ca^{2+}. Less Ca^{2+} was absorbed in sea bass fed higher levels of vitamin D.

1.5. Aim of study

The aim of this study was to describe the skeletal development in lumpsucker (*Cyclopterus lumpus*) and to evaluate growth, survival, skeletal development and anomalies in larvae fed with three different diets; *Artemia*, *A. tonsa* or formulated feed. These diets represent common start feeding regimes of marine fish larvae.

Lumpsucker larvae were fed the three different diets the first 22 days post hatch (dph). Two groups were fed copepods (*A. tonsa*) and enriched *Artemia* exclusively and the third group was fed a combination of *A. tonsa* (2-9 dph) and formulated feed (7-22 dph). From 20 dph all groups were weaned to formulated feed and fed formulated feed exclusively from 22 dph. The effects of the diets were measured in growth (standard length, dry weight and wet weight), survival, skeletal development (onset of ossification in specific parts) and skeletal anomalies. Bone analysis were performed on alizarin stained larvae.

The hypothesis was that lumpsucker larvae fed *Artemia* and *A. tonsa* would have better growth, survival, bone development and less anomalies compared to larvae fed formulated feed.

The start feeding experiment was in collaboration with two other master students; Joachim Larsen Marthinsen studied the digestive system and Job van Mil studied larval growth.

2. Materials and methods

The start feeding experiment, growth- and bone analysis of lumpsucker (*Cyclopterus lumpus*) was performed at Norwegian University of Science and Technology (NTNU) at Centre of Fisheries and Aquaculture (Sealab). The experiment started on May 2017 and was terminated in the end of June 2017. Growth analysis were conducted parallel with the experiment and bone analysis were performed in the autumn 2017.

2.1. Egg incubation and larval rearing

Wild caught lumpsucker eggs were supplied from Morefish at Tjeldsbergodden. The eggs (fertilized 30.03.17) were incubated in 6,8 °C for 198 d° at Morefish. Before transported to Sealab, they were disinfected with Buffodine. Eggs were incubated in darkness in two family incubators (Fishtech AS) with continuous water flow, moderate aeration and gradually increasing water temperature from 8-10 ºC until hatching at 307 dº.

After hatching (307 dº, 0 dph) the larvae were transferred with water current to tubes connecting the incubators to a holding tank. Larvae was released into a plankton net placed in the holding tank. The plankton net prevented larvae from attaching to the tank walls. The 60 L white plastic holding tank was filled with 10 °C seawater aerated from the bottom. At 2 dph nine 1 L-measuring jugs was used to distribute larvae into nine 160 L black cone-bottomed fish tanks. Larval density in each fish tank calculated when ending the experiment, ranged from 54-77 larvae/L (table A1.1, appendix 1). Fish tanks were filled with 100 L treated seawater (34 ppt) of 10 °C. The seawater treatment consisted of sand filtration, 1 micron filtration (mesh size 1 µm), heating, microbial maturation (Skjermo *et al.*, 1997), degassing and a second 1 micron filtration. A bottom sieve with net size 700 µm was covering the water outlet in all tanks.

The water exchange rate was increased from 5-25 times day^{-1} when necessary depending on the feeding amount (table 2.1). Oxygen levels were measured every third/forth day using an optical dissolved oxygen meter (ProODO, YSI, USA), and kept over 80 % by adjusting aeration added to the tank. The water temperature maintained at 10 ºC was measured daily with a Digitron digital thermometer (2000T Type K, UK). Light regime consisted of 20 hours light using daylight fluorescent tubes (Philips MASTER TL-D 90 Graphica, 18W/965) and 4 hours darkness.

Three 5x5 cm dark grey silicone mats (macaron baking sheets, Clas Ohlson, Norway) were placed in each fish tank to create a larger surface for larvae to attach to (Imsland *et al.*, 2015). Skimmers were installed at the water surface in tanks fed formulated feed. The larval tanks fed live feed were cleaned daily to remove faeces, uneaten food and dead larvae. Since feeding with formulated feed increased the sedimentation of uneaten food, those tanks were cleaned twice daily. A bottom sieve covering the water outlet was cleaned every second day.

2.2. Feeding regime

The start feeding experiment consisted of three feeding regimes with each three replicates (fish tanks), presented in table 2.1. One group was fed *Artemia* from 2-22 dph; first newly hatched and not enriched *Artemia* from 2-4 dph, then a 50/50 combination of newly hatched unenriched and enriched *Artemia* from 5-6 dph and from 7-22 dph enriched *Artemia*. The two other groups were fed copepod, *A. tonsa* from 2-22 dph and 2-8 dph. At 6-8 dph the formulated feed group was weaned to and fed formulated feed (Gemma micro 150) until 22 dph. All groups were weaned to formulated feed (Gemma micro 300) from 20-22 dph and fed exclusively for the rest of the experiment (22-50 dph).

Artemia and *A. tonsa* were fed to larval tanks from 100 L feed reservoirs using peristatic pumps. Feeding was scheduled 6 times/day during the light period (at 14.00, 18.00, 22.00, 03.00, 06.30 and 10.00 o'clock). The feeding amount was increased gradually from 600 000-1 800 000 live prey day^{-1} tank^{-1} (Table A2.1 in Appendix 2). The desired density of live feed in larval tanks per feeding was 1000-3000 live prey L^{-1} feeding^{-1}.

The formulated feed Gemma micro 150 and 300 distributed by Skretting AS was fed with feeding automats (Sterner disk feeder 905) placed over funnels with running water flow. They were programmed to feed desired feeding amount using the program Normatic Web Server, from Normatic AS. The feeding amount (Table A2.1 in Appendix 2) was increased in relation to estimated larval density and growth following recommendations from Skretting.

Table 2.1. *Specification of the start feeding experiment with dates, days post hatch (dph), day degrees (d°), water exchange rate (times tank volume/day), feeding regimes and sampling days for dry weight (DW), wet weight (WW), standard length (SL), mortality, bone analysis and fatty acid analysis.*

Date		**May 2017**																						
		9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Dph		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
d°		300	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	460	470	480	490	500	510	520
Water exchange				5 times/day				10 times/day																
Feeding regime	*Artemia*			*Artemia* (newly hatched)		*Artemia* (newly + enriched)		*Artemia* (enriched with Multigain)																
	Artemia																					Formulated feed (Gemma micro 300)		
	Copepod			Copepods (*A. tonsa*)																				
	Copepod																					Formulated feed (Gemma micro 300)		
	Form. feed			Copepods (*A. tonsa*)																				
	Form. feed							Formulated feed (Gemma micro 150)																
	Form. feed																					Formulated feed (Gemma micro 300)		
Sampling																								
DW WW SL		x		x				x				x			x				x				x	
Bone analysis		x		x				x				x			x				x				x	

Date		**June 2017**															
		1	2	3 - 6	7	8 - 11	12	13 - 15	16	17 - 19	20	21	22	23	24 - 27	28	29
Dph		23	24	25 - 28	29	30 - 33	34	35 - 37	38	39 - 41	42	43	44	45	46 - 49	50	51
d°		530	540	550 - 580	590	600 - 630	640	650 - 670	680	690 - 710	720	730	740	750	760 - 790	800	810
Water exchange		15 times/day						25 times/day									
Feeding regime	*Artemia*																
	Artemia	Formulated feed (Gemma micro 300)															
	Copepod																
	Copepod	Formulated feed (Gemma micro 300)															
	Form. feed																
	Form. feed																
	Form. feed	Formulated feed (Gemma micro 300)															
Sampling																	
DW WW SL			x		x		x		x		x			x		x	x
Bone analysis			x		x		x		x		x			x		x	

2.3. Live feed cultivation

Artemia and microalgae were cultivated during the start feeding experiment. Microalgae was used as feed for copepod (*A. tonsa)*, which was produced and delivered from C-feed and maintained until fed to the lumpsucker larvae.

2.3.1. Cultivation of microalgae

Rhodomonas baltica (Clone NIVA 5/9 Cryptophycea: Pyrenomonadales) was cultivated semi-continuously in two 200 L transparent polycarbonate plastic cylinders filled with seawater. In advance the seawater (34 ppt) was sand filtered, heated to 20-23 °C, filtered through a 1 µm mesh, chlorinated and dechlorinated. The chlorination was performed by use of 0,25 ml chlorate (NaOCl) per litre seawater for minimum 5 hours without aeration. Afterwards the seawater was dechlorinated with 0,03 g sodium thiosulfate pentahydrate ($Na_2S_2O_3$) per litre seawater under heavy aeration for minimum 5 hours. The microalgae were cultivated with constant illumination by 6 fluorescent tubes (Aura t8 ultimate 850 58W) on three sides, and aeration with 1-2 % CO_2 added from the bottom.

Daily, 40-50 % of the microalgae culture was harvested and fed to the copepods, see descriptions in maintenance of copepods. The remaining culture was diluted with treated seawater and 1 ml/L seawater of Conwy medium was added (appendix 3 modified from Walne (1979)).

2.3.2. Maintenance of copepods

A. tonsa (clone DFH.AT1) of stages CI-CIII was delivered from C-feed AS in plastic containers three times during the experiment. The CI-CIII stages are 410-580 µm (Hagemann, 2014). The density in each container was calculated manually and the desired prey density for one day feeding was distributed to holding tanks filled with 100 L of treated seawater (34 ppt and 10 °C). The seawater treatment was the same as for the water in larval fish tanks. At low temperatures copepods have reduced grazing and development slows down (Maren Ranheim Gagnat, C-feed, pers. comm, 10. May 2017). To maintain the low grazing *A. tonsa* was fed 6 L microalgae (*R. baltica*) per 100 L holding tank. The density of fed microalgae was not measured. 50 % of the water was flushed out and renewed with fresh seawater daily. The content of one holding tank was fed to larval tanks daily. The bottom outlet was covered with a 100 µm mesh sized filter.

2.3.3. Cultivation of *Artemia*

Artemia cysts (EG ® INVE Aquaculture, Belgium) were hydrated in fresh water (450-500 g cysts, 4,9 L water, 15-25 ºC) with heavy aeration and decapsulated according to (Sorgeloos *et al.*, 1977). The decapsulated cysts were stored at 4 ºC for maximum 7 days and hatched in 60 L cylindrical tanks filled with seawater (34 ppt). Aeration was added at the bottom and water temperature was kept at 25-28 ºC. After 24 hours, unhatched cysts were removed by turning of aeration for 5 minutes and flushing out sediments through the bottom valve for ½ second. The newly hatched *Artemia* was concentrated in a sieve and transferred to a new 60 L tank filled with seawater with the same temperature and heavy aeration. Enrichment with 10 g MultiGain medium (produced by Biomar) per 60 L seawater was added two times over 24 hours. After enrichment, *Artemia* was washed gently for 10-15 minutes in an *Artemia*-washer, concentrated in a sieve and transferred to a feeding tank filled with 55 L seawater (32 ppt and 10°C). The holding tanks had stagnant water flow and heavy aeration added at the bottom. *Artemia* were fed to larvae over a period of 24 hours. The size of *Artemia* nauplii instar 2 (6-8 h) is 500-1000 µm (Léger *et al.*, 1987).

2.4. Sampling

Sampling for dry weight (DW), wet weight (WW), standard length (SL) and bone analysis was sixteen times at regular intervals during the experiment (table 2.1). Sampling size was increased from 15-150 larva/treatment from 0-51 dph presented in table 2.2. All larvae were sampled randomly from larval tanks using plastic cups or with a small hand net, anesthetized with tricaine methane sulfonate (MS-222 Finquel®, Agent Chemical Laboratories Inc., USA) and rinsed in distilled water before further analysis.

Table 2.2. *Sampling size (larva/tank) for growth, bone and fatty acid analysis. At 0 and 2 dph sampling size are the total number taken before larvae were distributed to larval tanks.*

Days post hatch	Sample size (larva/tank)	
	Growth	Bone analysis
0, 2	16	15
6, 10, 13, 17, 21, 24, 29 34, 38	5	15
42, 45	10	30
50	10	50
51	16	-

2.5. Growth and survival

Larval standard length (SL) was measured from the tip of the mouth to the end of the notochord (Figure 2.1). Larvae were photographed using a stereomicroscope (Leica MZ 7.5, Germany) equipped with a camera (Axiocam Erc 5s, Carl Zeiss Microscopy GmbH, Germany) and software Zen 2.3 for windows. Standard length was measured from pictures using the Windows software ImargeJ (version 1.8.0_112).

After photographing, larvae were dried with wipes to remove excess water, placed in pre-weighed tin capsules and wet weight (WW) was measured with an ultra-microbalance weight (Mettler Toledo UMX2). For measurement of dry weight (DW) the tin capsules with larvae were dried in 60 ºC for minimum 48 hours, before weighed.

The specific growth rate (SGR) and percentage of daily weight increase (% DWI) was calculated according to Ricker (1958) presented in equation 2.1 and 2.2, where DW1 and DW2 are dry weight at time t1 and t2.

$$SGR = \frac{\ln DW2 - \ln DW1}{t_2 - t_1} \quad [2.1]$$

$$\% \, DWI = \left(e^{(SGR)} - 1\right) * 100 \quad [2.2]$$

Larval survival was calculated based on daily registration of dead larvae from 3-50 dph and counting of remaining larvae when ending the experiment. Numbers were corrected for sampled larvae for growth and bone analysis.

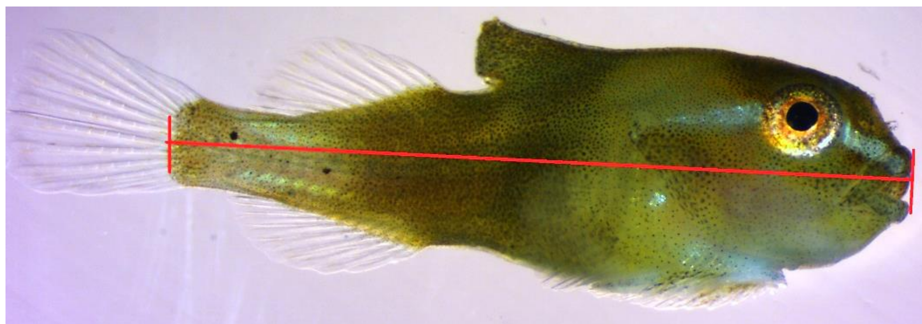


Figure 2.1. *Standard length measurement on a lumpsucker larvae photographed with a stereo Lupe equipped with a camera.*

2.6. Skeletal analyses

Skeletal analyses were not performed on all larva sampled. Ten larvae/tank at five sampling times (0, 10, 21, 34 and 42 dph) and 50 larvae/tank at the end of the experiment (50 dph) were analysed.

Anesthetized and rinsed larvae were fixated in 4 % paraformaldehyde (PFA) in phosphate buffered saline (pH 7.4, Apotekproduksjon AS; Norway), and stored at 4 °C. Larvae were stained with alizarin red according to Kjørsvik *et al.* (2009).

The bone staining procedure (appendix 5) contained five steps; (1) rehydration, (2) bleaching, (3) clearing, (4) staining and (5) preservation. Rehydration in solutions of decreasing amount of ethanol (95 %, 50 % and 15 %) to distilled water for 0.5-1 hour in each solution depending on larval size. A transparent plastic well tray of different sizes (2x6 or 4x4 wells) depending on the number of and size of larvae, were used to separate larvae sampled from different tanks when staining. After rehydration the larvae were stored in a sodium borate buffer until the next day if necessary. Then bleached in 3% hydrogen peroxide in 1% potassium hydroxide (ratio 1:9) under strong light. The bleaching process was monitored and stopped when all pigmentation was removed. Larvae under 10 mm were bleached after 1-2 hours, while larvae from 10-20 mm used up to 6 hours. Clearing with a trypsin buffer to almost transparent took 20 hours for the smallest larvae (>10 mm). The time was prolonged to 2-4 days and buffer was renewed daily for larger larvae (10-20 mm). Clearing was difficult on the fattest larvae sampled at 50 dph and a stronger solution of the trypsin buffer was used to shorten the time. The trypsin buffer did not remove the stomach content. Starving before the last sampling at 50 dph would probably reduced the stomach content. After clearing the bones were stained with Alizarin for 1-2 days depending on larval size.

The stained larvae were preserved in 40 % glycerol in 1 % KOH for 2 days and photographed under a stereomicroscope (Leica MZ75, Germany) equipped with a camera (Nikon Digital Sight DS-5M L1, Japan). Analysis of larvae were performed using the stereomicroscope and on pictures. ImageJ (version 1.8.0_112) software for windows was used to measure standard length (Figure 2.2) and counting numbers of ossified vertebrae segments and fin rays.

Photographed and analysed larvae were preserved in 70 % glycerol in 1 % KOH for 1-2 days and then 100 % glycerol in 4 °C.

Figure 2.2. *Left: Standard length measurement of bone stained lumpsucker larvae. Right: Bone nomenclature in tail region. VS = vertebra segment, HA = hemal arch, NA = neural arch, U = urostyle, HY = hypurals and FR = fin rays (of tail fin).*

2.6.1. Ossification of vertebrae

Vertebrae segments were classified as partly ossified (P), compact (C), transparent (T) and fully ossified (FO), depending on the intensity and fill of colour in each segment (Figure 2.3). Transparent segments had no visible bone ossification, partly ossified segments had sign of ossification but were not filling the whole segment. In compact segments did the colour fill the whole segment but without full intensity. Fully ossified vertebrae had full intensity of colour, filling the whole segment. Neural and hemal arches, urostyle and hypurals were classified as ossified if any sign of ossification was observed (Figure 2.2).

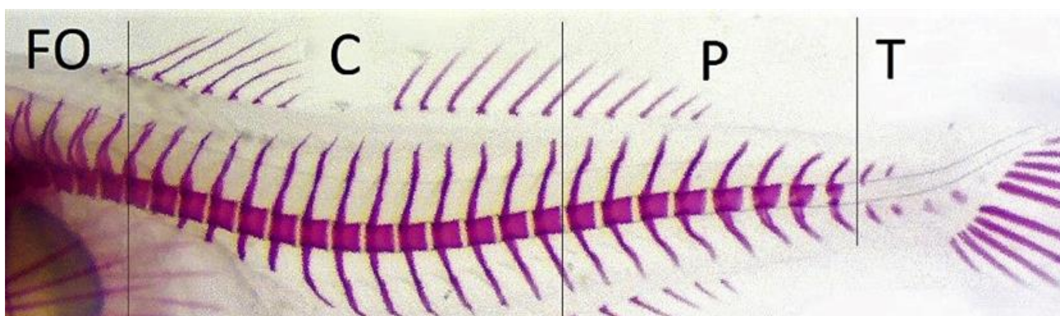


Figure 2.3. *Classification of vertebrae segments in lumpsucker larvae based on saturation of colour. FO = fully ossified, C = compact, P = partly ossified and T = transparent.*

2.6.2. Ossification of fins

Dorsal fins and anal fin were scored 0-3 depending on the ossification (Figure 2.4). Score 0 equalled no ossification, 1 = partly ossification of dorsal/anal fin rays, 2 = ossification of

dorsal/anal fin rays, but not of pterygiophores, 3 = ossification of all dorsal/anal fin rays and pterygiophores. The tail fin was evaluated by counting number of ossified fin rays.

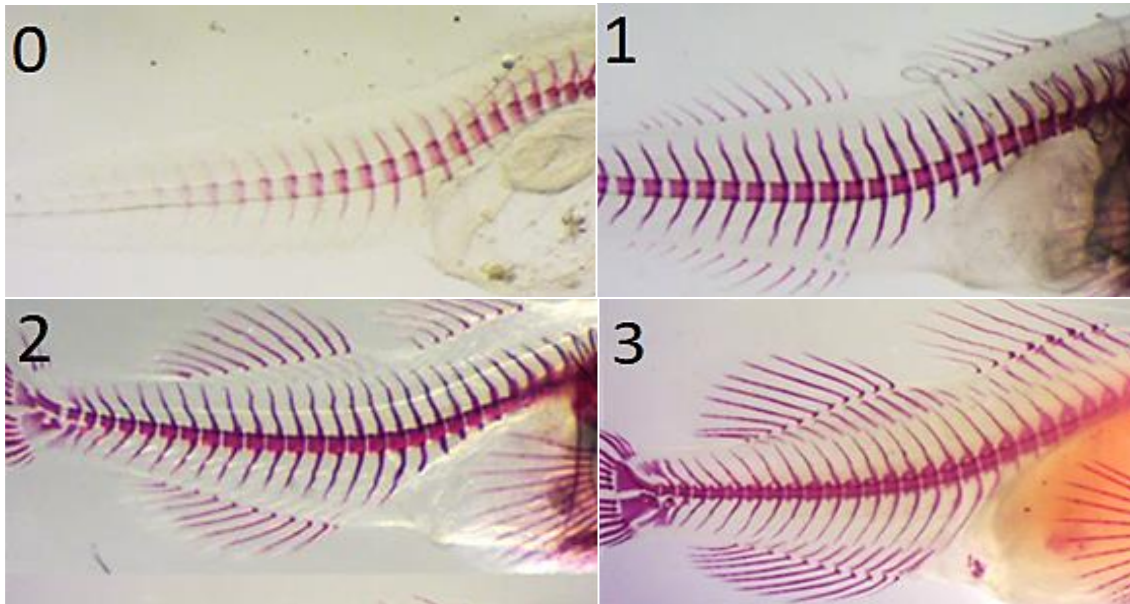


Figure 2.4. *Scoring of dorsal and anal fin rays (0-3) based on ossification. Scores for all fins are indicated on left side of pictures. Score 0 = no ossification, 1 = partly ossification of dorsal/anal fin rays, 2 = ossification of dorsal/anal fin rays, but not of pterygiophores, 3 = ossification of all dorsal/anal fin rays and pterygiophores.*

2.6.3. Ossification of other parts

Suction disc, mouthparts (premaxillare, maxillare, dentale and the hyorid arch), cleithrum, urostyle and hypurals where evaluated as ossified when the first sign of ossification was observed.

Ossification of urostyle and hypurals related to size was visualized in a table, derived from (Sæle *et al.*, 2004, Sørøy, 2012). Light grey area marked SL of the smallest larva when the first sign of ossification occurred in a specific bone to SL of the largest larva without ossification. Dark grey area marked which SL all larva had ossification in that bone.

2.6.4. Skeletal anomalies

An anomaly is defined as “a deviation from the normal shape of a body part or an organ” (Heiberg, 2009). Analysis of skeletal anomalies were performed on the pictures of bone stained larvae.

Head, mouthparts, vertebrae segments, hemal and neural arches, urostyle, hypurals, dorsal fins, anal fin and tail fin were analysed for skeletal anomalies. Number of larvae with anomalies and number of anomalies per larvae were counted, and the frequency of larvae with anomalies was calculated.

These definitions derived from Boglione *et al.* (2001) were used to identify anomalies observed:

- Axis deviation:
    - Lordosis: Inward curvature of the spine
    - Kyphosis: Outward curvature of the spine
    - Scoliosis: Sideways “S” or “C” shaped curvature of the spine
- Fused vertebrae: two or more vertebral segments are fused and might share the same neural and/or hemal arch.
- Abnormal neural or hemal arches: twisted arches (arches/spines form a spiral at apex)
- Abnormal hypurals
- Abnormal fins: dorsal, anal or tail fin rays or pterygiophores
- Flat skull: skull flat on dorsal side
- Abnormal jaw: shorter upper jaw compared to the lower jaw

2.7. Statistical analysis of data

Data were first tested for normality using a Shapiro-Wilk-test and homogeneity of variance with Levene’s test. Normal distributed data were tested for significant differences between groups using a one-way ANOVA. The Welch test was used to determine significant differences, followed by post hoc tests; Student-Newman-Keuls used for homogenous data and Dunnett T3 for non-homogenous data. Not normally distributed data were tested with a non-parametric test, and to compare groups an independent samples Kruskal-Wallis test (2-sided, asymptotic significances) was used. This test included pairwise comparison and adjustment of significance values. Percentage data were arcsine transformed before statistical analysis. A Persson linear correlation test were used to check for correlation between data.

All statistical analysis was performed with IBM SPSS Statistics v19.0 (SPSS Inc., Chicago, USA) for Windows and significance level of $p = 0.05$ was used for all tests. Tables and graphs were made with Microsoft Office Excel 2016.

3. Results

3.1. Larval development

Lumpsucker larvae hatched with a big yolk sac, open mouth and pigmented eyes (Figure 3.1). Yellow-brown pigmentation with dark dots were observed in the head and the trunk, but with variations between individual larvae. The tail region was transparent with fields of light beige pigmentation. No fins were developed at 0 dph. Already from hatching larvae had a suction disc on the underside of the body which they used to attach to surfaces in the fish tank. The ability to attach to surfaces was even observed in sedated, fixated and dead larvae.

From 6 dph fin rays of two dorsal fins, tail fin, anal fin and pectoral fins were developed. The first dorsal fin was half covered with pigmented skin, while the covering of the second dorsal fin, anal fin and tail fin were transparent skin. The yolk sac was no longer visible under the increased pigmentation in the head. Approximately ¼ of the trunk was pigmented.

At 10 dph the whole first dorsal fin was covered with pigmented skin. Start of flexion was observed in all larvae from 10 dph. Light brown and spotted pigmentation was visible from the tail region to start of the tail fin. The head was darker pigmentated with a light and shiny horizontal stripe from behind the eyes and over the nose. A larger suction disc with the same pigmentation as the head was developed. The mouth was also pigmented at 10 dph.

At 21 dph the whole larval body was pigmented, except from fin rays of the second dorsal, tail and anal fin. All the fin rays had grown in length. Larger brown spots were observed in the larval trunk with a red colour in the lower half. The vertebrae were visible in the larval trunk up to 21 dph, but at 29 dph it was covered by darker pigmentation. At 29 dph the fin rays were longer and the horizontal stripe was lighter and shinier. The larval head and trunk had grown in width.

From 42 dph the larvae had a green-brown head and trunk, while the tail part had developed some lighter spots. At the end of the experiment (50 dph) larvae had a wider trunk and lighter pigmentation with some darker spots. Their body shape began to resemble an adult fish with wider body shape and a suction disc covering ¼ of the underside of the body.

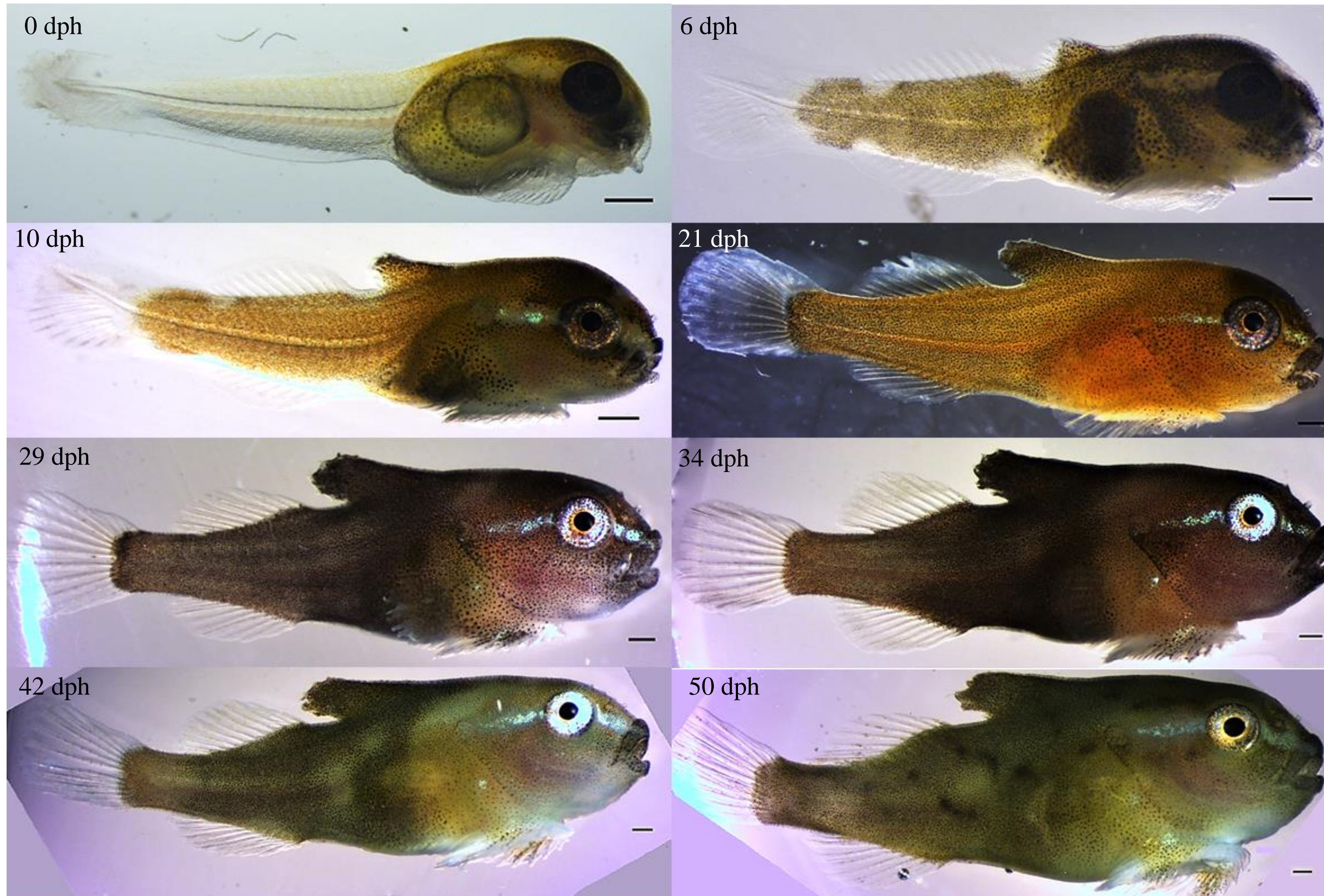


Figure 3.1. *Development from 0-50 dph of lumpsucker larvae fed Artemia. Larval age and standard length; 0 dph 6.0 mm, 6 dph 6.9 mm, 10 dph 7.2 mm, 21 dph 8.4 mm, 29 dph 9.8 mm, 34 dph 11.1 mm, 42 dph 12.2 mm and 50 dph 13.6 mm.*

The variation in larval pigmentation observed in the copepod treatment at 38-45 dph is illustrated in Figure 3.2. Larvae from the other groups had less variations in colour. The majority of *Artemia* and formulated feed fed larvae were dark grey-green. Larva 1 in Figure 3.2 was yellow with brown spots in the head and start of the trunk and smaller black spots scattered around the body. Larva 2 was light grey with a tint of blue and scattered brown spots of different sizes. The third larva was red with small dark spots. Larva 4 had dark pigmentation with lighter brown spots and a pink area under the eyes. All larvae had the light, shimmering stripe from behind the eyes and over the nose. The variation between individual larva decreased and at 50 dph less differences were observed.

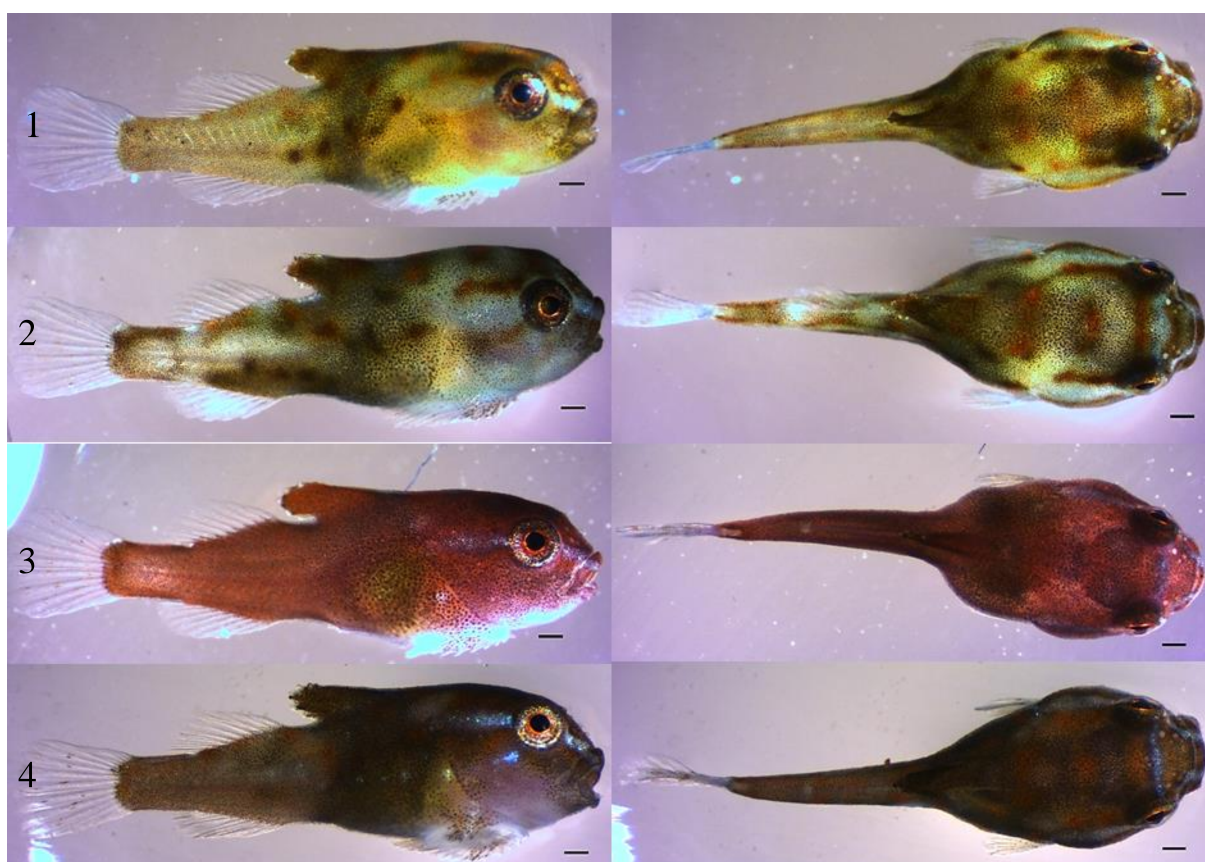


Figure 3.2. *Examples of different pigmentation observed in larvae from the copepod group, at 38-45 dph. Age and standard length (mm) of larvae: 1: 38 dph and 9.2 mm, 2: 38 dph and 9.8 mm, 3: 38 dph and 9.8 mm, and 4: 42 dph and 9.8 mm. The black bar is 500 µm*

3.2. Growth

3.2.1. Dry- and wet weight

Mean larval dry and wet weight were significantly higher for larvae fed *Artemia* from 6-51 dph (Figure 3.3). The difference between larvae fed *Artemia* and the other groups fed copepod and formulated feed increased at 2-22 dph. When one of the groups fed copepods were weaned to formulated feed (7-9 dph), the growth decreased and dry- and wet weight were significantly lower than the other groups. The larval group still fed copepods increased their weight until weaning to formulated feed started at 20 dph. Weaning to formulated feed resulted in a slowdown of in weight increase in the two groups fed live feeds. Larvae weaned from *Artemia* had a lower weight increase from 21-24 dph compared to earlier. The period with slow growth was lasted to 29 dph for the larvae fed copepods. The rest of the formulated feed period had all groups a steady increase in dry and wet weight.

At the end of the experiment (51 dph) the larvae fed *Artemia* (22.7 mg) were significantly higher and twice the dry weight of larvae fed copepods and formulated feed (11.2 and 11.9 mg). The wet weights at 50 dph were 108, 63 and 64 mg for larval groups fed *Artemia*, copepods and formulated feed.

The Pearson correlation test showed a positive linear correlation between wet weight and dry weight in all groups (Figure 3.4), where the dry weight increase was 0.14-0.15 times the wet weight.

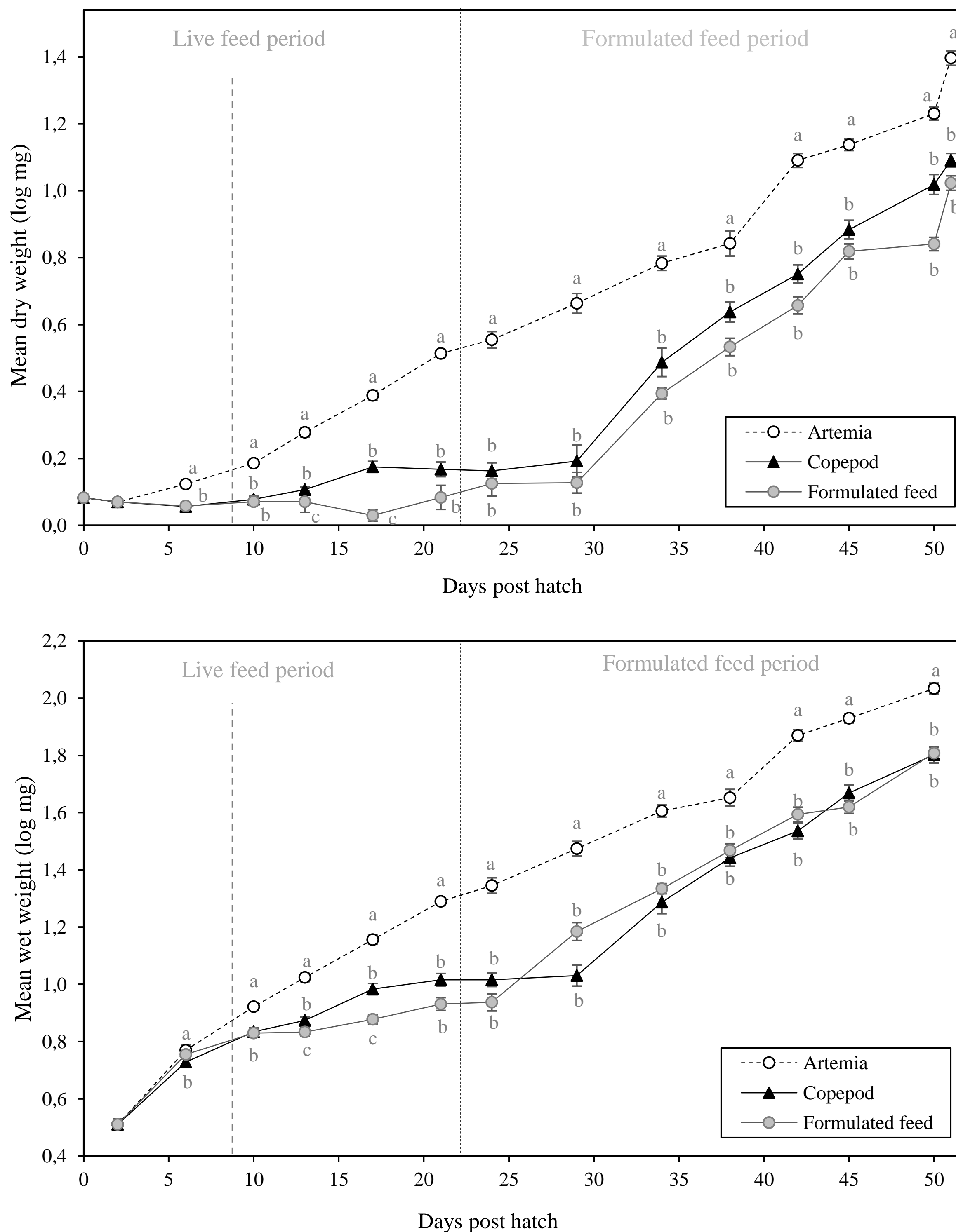


Figure 3.3. *Mean larval dry weight (log mg treatment^{-1}) from 0-51 dph and mean larval wet weight (log mg treatment^{-1}) from 0-50 dph. Sampling size; n = 16 treatment^{-1} at 0 and 2 dph, n = 15 treatment^{-1} at 6, 10, 13, 17, 21, 24, 29, 34 and 38 dph, n = 30 treatment^{-1} at 42, 45 and 50 dph, and n = 48 treatment^{-1} at 51 dph (only DW). First dotted line at 9 dph marks the transfer from feeding copepod to formulated feed for the formulated feed group. Second dotted line at 22 dph, marks the end of live feed period for Artemia sp. and copepod treatments. All treatments had formulated feed diets from 22 dph. Standard error (±) are represented by error bars. Letters indicate significant differences between treatments.*

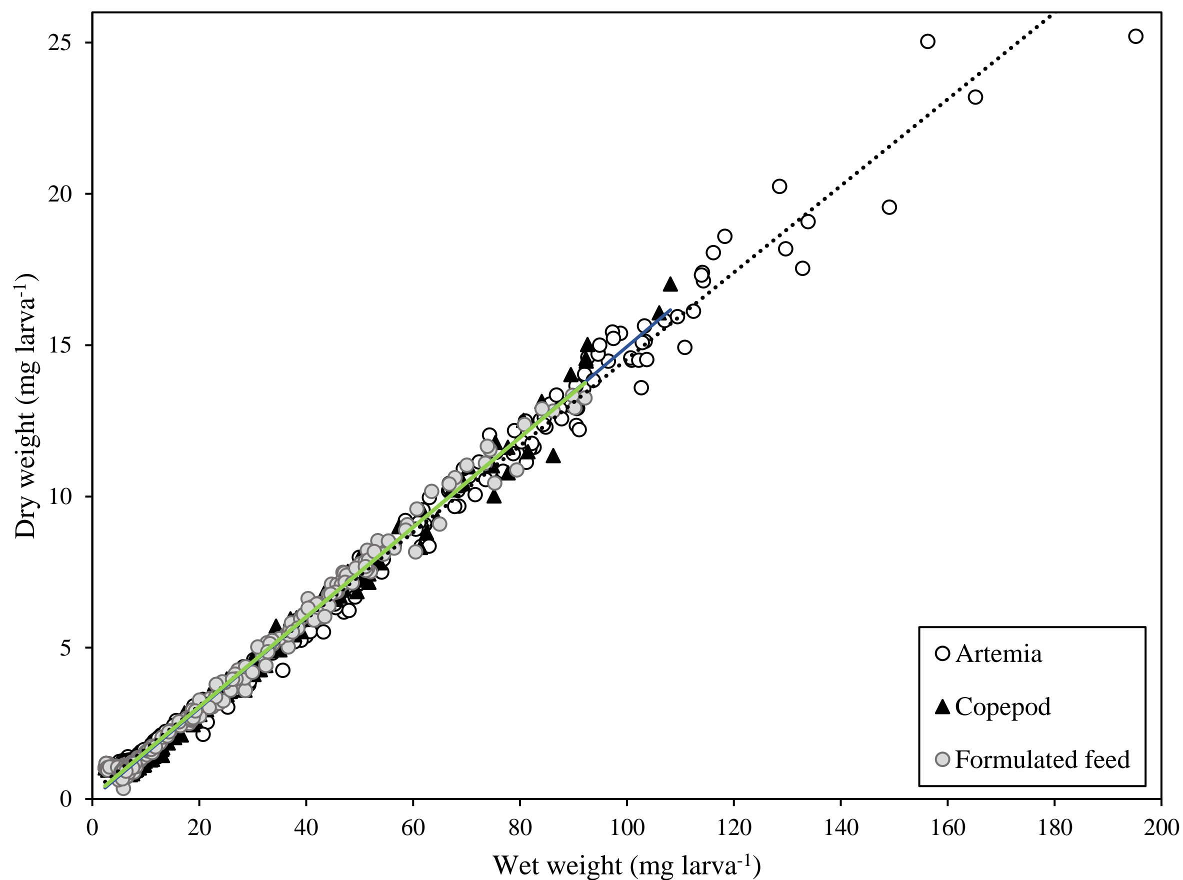


Figure 3.4. *Correlation of larval dry weight (mg larva^{-1}) and wet weight (mg larva^{-1}). Black dotted line is the DW and WW correlation for Artemia fed larvae, blue for copepod fed larvae and green for formulated feed fed larvae. Pearson point-biserial linear correlation coefficients, R_{pb}^2 = 0,995 (Artemia), 0,995 (Copepod) and 0,996 (formulated feed) and the correlation was significant for all groups.*

3.2.2. Daily weight increase

The daily weight increase (% DWI) based on dry weight was negative before exogenous feeding started at 2 dph (Figure 3.5). From 2-21 dph, the DWI was significantly higher for larvae fed *Artemia* compared to the groups fed copepods and formulated feed. Larvae fed *Artemia* increased their dry weight 5.5 % per day, while the other larvae groups grew 1.1 and 0.6 %. After weaning to formulated feed for all groups the DWI was 6.9-7.9 % and no significant difference between the groups was found. The group weaned to formulated feed at 9 dph had the highest DWI for this period. The trend for the whole feeding period (2-50 dph) was higher DWI for the *Artemia* larval group (6,3 %) than for the copepod group (4.9 %) and the formulated feed group (5.0 %). However, no significant difference between the groups was found (p=0.053).

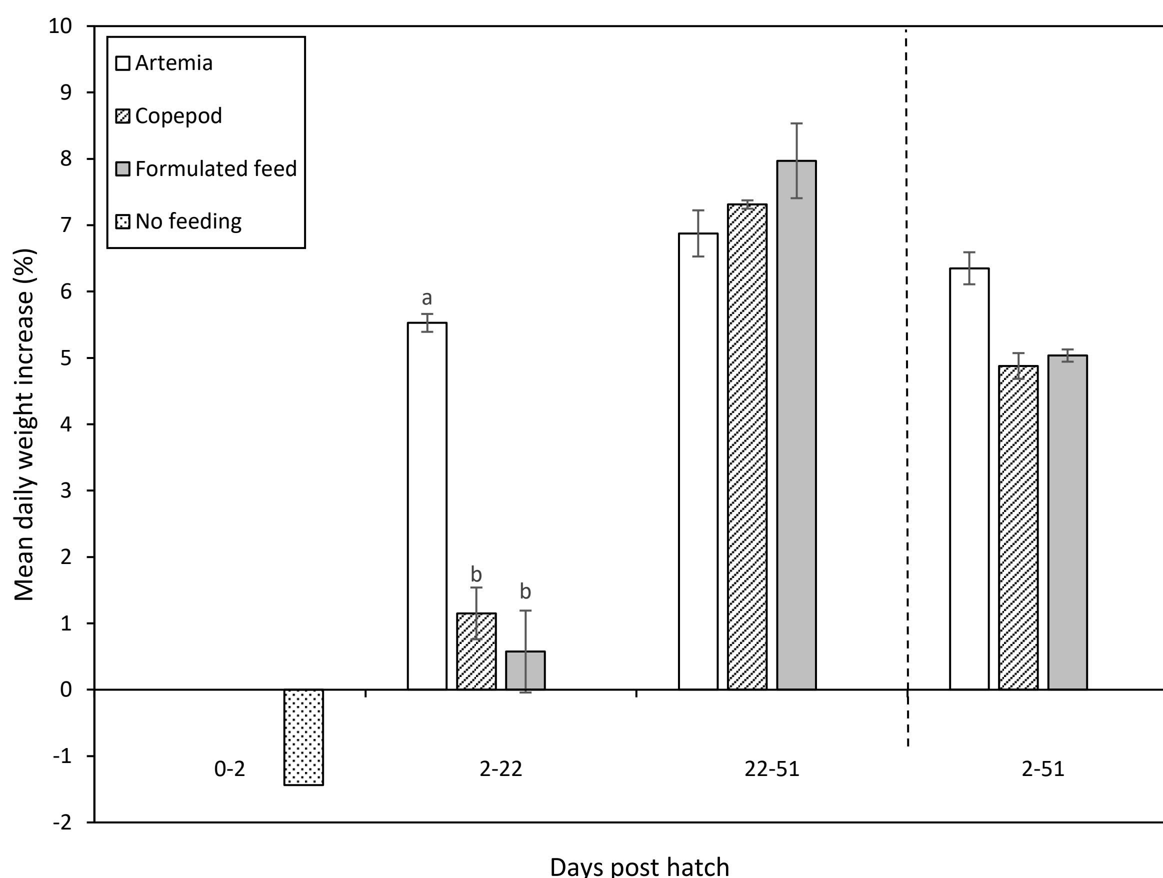


Figure 3.5. *Mean daily weight increase (%, treatment^{-1}) for time intervals representing the different feeding periods; 0-2 dph: before feeding started, 2-22 dph: live feed period, but 7-22 dph: one group fed formulated feed, 22-51 dph: formulated feed period for all groups and 2-51 dph: feeding period. Sampling size; n = 16 at 0 and 2 dph, n = 15 treatment^{-1} at 6, 10, 13, 17, 21, 24, 29, 34 and 38 dph, n = 30 treatment^{-1} at 42, 45 and 50 dph, and n = 48 treatment^{-1} at 51 dph (only DW). Standard error (±) are represented by error bars. Letters indicate significant differences between treatments.*

3.2.3. Standard length

Before feeding started, the newly hatched lumpsucker larvae had a mean standard length of 6.4 mm (Figure 3.6). Already from 6 dph the larvae fed *Artemia* had significantly higher standard length compared to the other two groups. The difference between the *Artemia* fed group and the larvae fed copepods and formulated feed continued throughout the experiment.

Both the groups weaned from copepods to formulated feed (9 and 22 dph) had a following period with slower increase in standard length. During this period the standard length was slower for the group weaned to formulated feed compared to both the other two groups.

At the end of the experiment the *Artemia* group had a mean standard length of 13.7 mm, significantly longer than the copepod and formulated feed groups; 11.2 and 11.8 mm. Significant difference between the copepod and the formulated feed group were also found at 50 dph.

The Pearson correlation test showed a significant and positive linear correlation between larval dry weight and standard length in all groups (Figure 3.7). The maximum size obtained for larvae from *Artemia* group had standard length 16.1 mm and dry weight 25.2 mg and for the formulated feed group 13 mm and 13.3 mg. The heaviest larvae from copepod group was 16.1 mg and 12.6 mm long, while the longest was 12.9 mm long and weighted 12.5 mg.

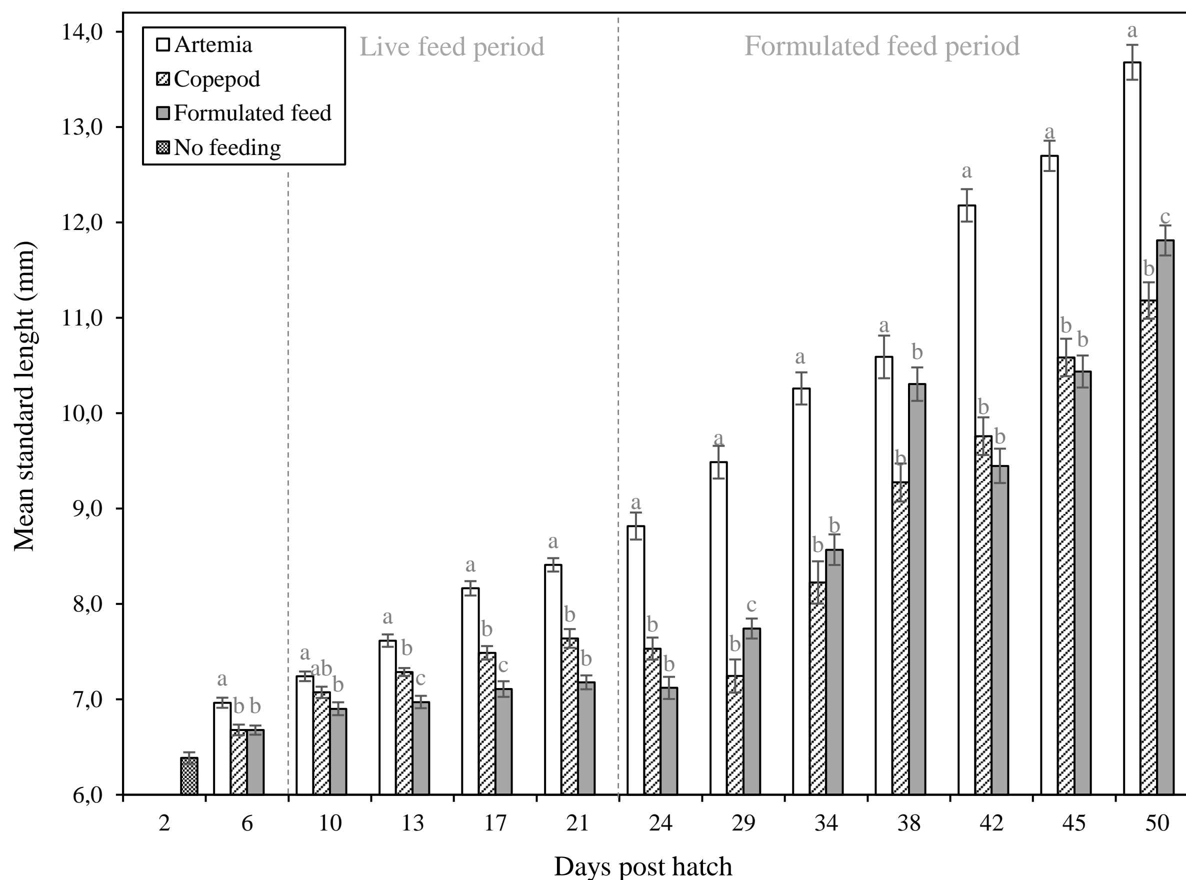


Figure 3.6. *Mean standard length (mm treatment^{-1}) at 2, 6, 10, 13, 17, 21, 24, 29, 34, 38, 42, 45 and 50 dph. Sampling size; n = 16 treatment^{-1} at 2 dph, n = 15 treatment^{-1} at 6, 10, 13, 17, 21, 24, 29, 34 and 38 dph, and n = 30 treatment^{-1} at 42, 45 and 50 dph. Letters indicate significant differences between treatments and standard error are represented with ± error bars. The first dotted line indicate the end of feeding copepods to formulated feed group, and the second dotted line are the end of live feed period forArtemia and copepod groups.*

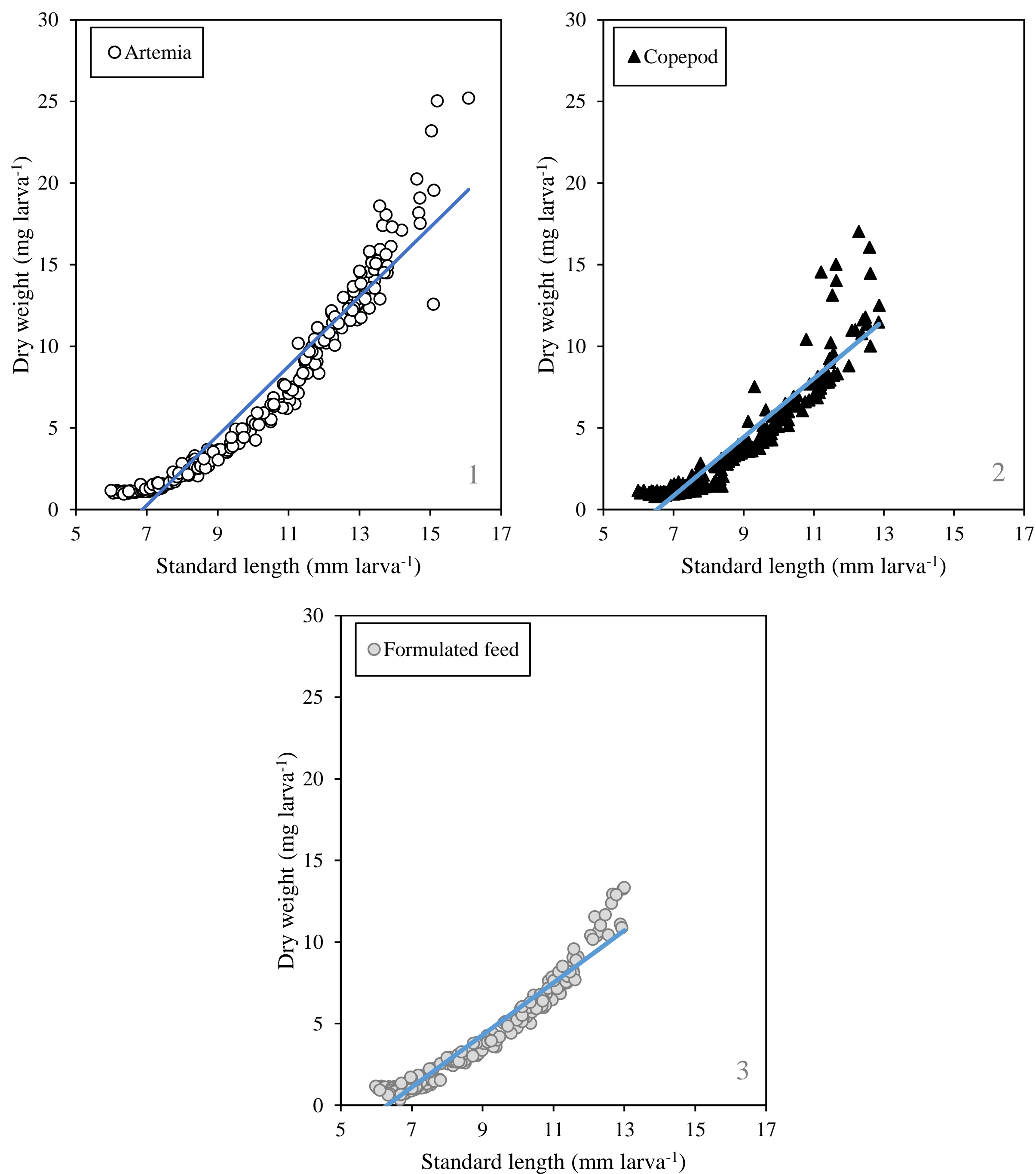


Figure 3.7. *Correlation between dry weight (mg larva^{-1}) and standard length (mm larva^{-1}). 1: larvae fed Artemia, 2: larvae fed copepods and 3: larvae fed formulated feed. Blue lines are the linear correlation lines. Pearson point-biserial linear correlation coefficient, r_{pb}^2=0,965 (Artemia* sp.*), 0,935 (copepod) and 0,974 (formulated feed) and the correlation was significant for all groups.*

3.3. Survival

For all groups the mean survival was over 79 % during the experiment (Table 3.1). Larvae from the *Artemia* group had significantly higher survival than larvae fed formulated feed 34-51 dph. The lowest survival was observed for the copepod fed larvae, but the high variation between the three tanks in this treatment gave no significant differences from the other treatments. At the last sampling day, the survival of the larvae fed *Artemia* was significantly higher than both the other groups.

The variation in survival between larval tanks was from 34 dph higher in the copepod and formulated feed larval groups. There was 0 % variation in survival between the three tanks fed *Artemia.*

After weaning to formulated feed, a sudden increase in mortality was experienced from 27-34 dph in copepod and formulated feed fed larval groups. At the same time heavy growth of bacterial threads appeared in those tanks. The bacteria threads were first observed in a copepod fed tank (tank 10). From 31-33 dph the mortality was 10-31 % higher than in the two other tanks from the copepod treatment. Several larvae got tangled into bacteria treads and suffocated and an increased number of larvae were found dead on the bottom. Water exchange was increased more at this point to try to reduce the bacteria. Different amount of bacteria threads was observed in larval tanks fed copepod and formulated feed the following days.

Table 3.1. *Mean survival (%) ± standard error (SE) per treatment on 21, 34, 42 and 51 dph from daily measurements at 3-51 dph. Sampling size n=3 tanks treatment^{-1}. Significant differences are indicated by different letters.*

Dph	**Mean survival (%) ± SE**		
	Artemia	**Copepod**	**Formulated feed**
21	100 ± 0	100 ± 0	99 ± 0
34	99 ± 0[a]	83 ± 7[ab]	89 ± 2[b]
42	99 ± 0[a]	80 ± 7[ab]	87 ± 2[b]
51	97 ± 0[a]	79 ± 6[b]	83 ± 3[b]

3.4. Skeletal development

Figure 3.8 present the age-related skeletal development of the larval group fed with *Artemia* showed the with highest growth. Newly hatched lumpsucker larvae had ossification in mouthparts (maxilla, premaxilla and dentale), suction disc, opercle, cleihtrum, the first 19-20 vertebrae with the associated neural and hemal arches. At 10 dph, the fin rays of all fins (dorsal, pectoral, anal and tail fins) were ossified. Larval skull/cranium and branchiostegal rays were also ossified. Mouthparts had more ossification and teeth were observed in the dental bones. The end of the spine, the urostyle bone had a bend upwards and flexion had started.

First sign of ossification in neural and hemal arches were seen in newly hatched larvae (0 dph). The hemal and neural arches of compact, partly and fully ossified vertebrae were ossified. Larvae had 28 neural arches, the same amount as vertebrae. The number of hemal arches was 17 of normal length. Close to the stomach larvae had 3-5 shorter hemal arches, which not covered the stomach region (Figure 3.8). From 10-42 dph many of the hemal and neural arches were not straight and had one/two bends (larvae 2-5, Figure 3.8). This were seen in larvae up to 11 mm, while larvae over 12 mm had straight arches without the bends.

The urostyle and both hypural bones were ossified from 21 dph in larvae with SL 7-8 mm. More of the fin rays were ossified in all fins. From 10-21 dph, the fin rays have grown longer. First ossification of pterygiophores of dorsal and anal fins were observed at 34 dph. Four radials of the pectoral fins were visible under the branchiostegal rays. The pterygiophores of dorsal and anal fins grew longer and in number from 42-50 dph. At 50 dph the neural and hemal arches were straight and met the end of the pterygiophores, meaning that bones covered the entire trunk. Hypural supporting the tail fin were fully ossified in larvae over 12 mm.

High variation was found between number of fully ossified vertebrae in individual larvae (Figure 3.10). The smallest larvae with all 28 vertebrae fully ossified was 11.2 mm, while the biggest larvae without all vertebrae ossified was 16 mm.

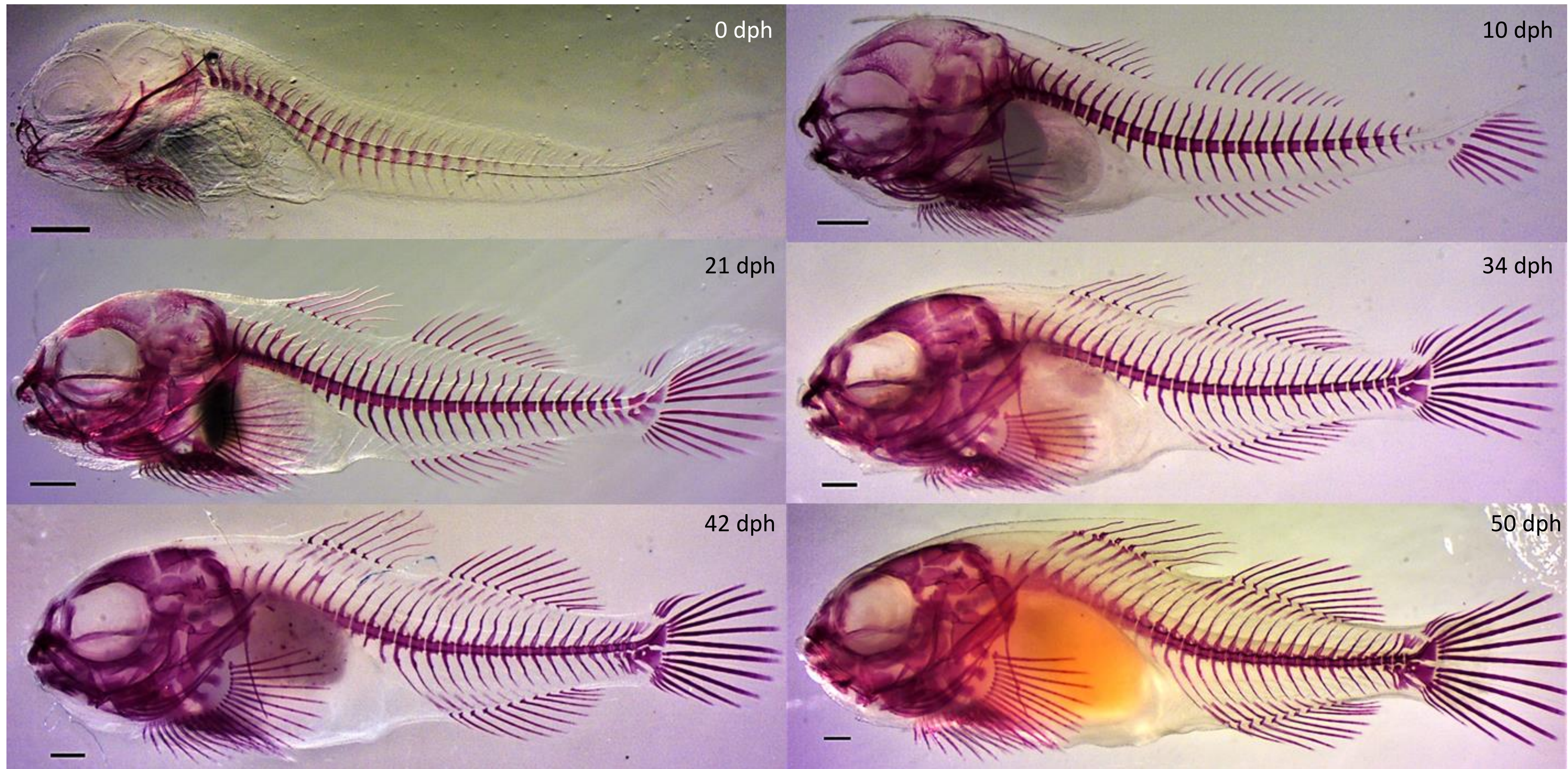


Figure 3.8. *Visualisation of bone development from 0-50 dph of lumpsucker larvae fed Artemia sp. Larval standard length: 0 dph 5.5 mm, 10 dph 6.4 mm, 21 dph 7.0 mm, 34 dph 8.9 mm, 42 dph 9.4 mm, 50 dph 11.5 mm. Black bars equal 500 µm.*

3.5. Bone ossification in specific parts

There was difference between the larval treatments when comparing onset of ossification in vertebrae, tail, anal and dorsal fins, urostyle and hypurals. The different feeding effect on skeletal development were found related to both age and size.

3.5.1. Suction disc

Newly hatched larvae had ossification in pelvis, spine ray and soft rays of the suction disc (Larvae 1 in Figure 3.9). Most part of the development of the suction disc happened the first 10 days post hatching. The pelvis was more ossified and the spine and soft rays were longer and wider. From 10-21 dph the pelvis ossified more, the rays elongated, and the size of the suction disc increased. The spine and soft rays were larger and more curved. After 21 dph were the changes in ossification small, and comparison with the larvae from 50 dph indicated that the suction disc was complete already from 21 dph.

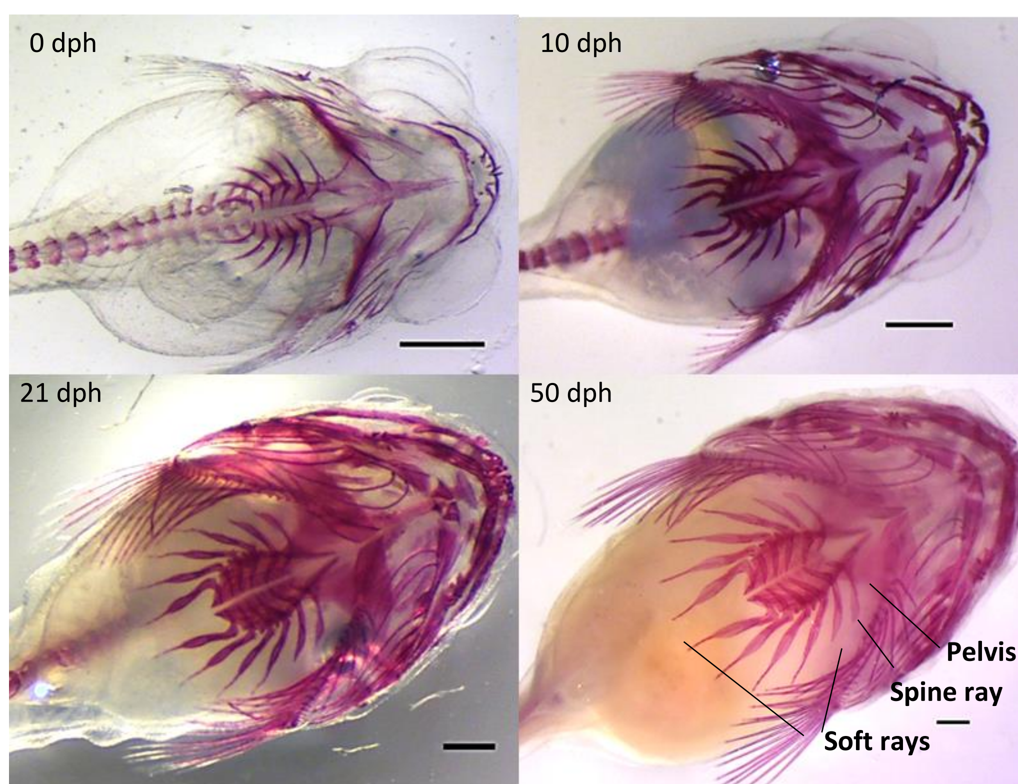


Figure 3.9. *Visualization of bone ossification on the ventral side of lumpsucker larvae with the suction disc. Larval standard length at 0 dph 5.7 mm, 10 dph 6.5 mm, 21 dph 8.9 mm and 50 dph 14.2 mm. Nomenclature defined by Voskoboinikova and Kudryavtseva (2014) are presented on larvae from 50 dph. Black bars equal 500 µm.*

3.5.2. Vertebrae

Newly hatched larvae had an average of 5.1 transparent and 20.7 partly ossified vertebrae (Table 3.2). At 10 dph the larvae fed *Artemia* had significantly higher number of fully ossified vertebrae and the difference from the other two groups continued throughout the experiment.

All larval groups had a mean total number of 28 vertebras from 10 dph, and over 60 % of the vertebrae were compact ossified. The first fully ossified vertebra was observed in larvae fed *Artemia* at 10 dph, while the other groups had none. One larva from the formulated feed group had only 27 vertebrae at 21 dph. Significantly less compact ossified vertebrae were found in larvae fed formulated feed from 21-34 dph.

The increase in fully ossified vertebrae was low until 42 dph for all larval groups. At 42 dph over 56 % of vertebrae of the *Artemia* larvae were fully ossified. The other two groups had less than 14 % fully ossified vertebrae at 42 dph. The increase in number of fully ossified vertebrae was high for the copepod and formulated feed group from 42-50 dph and at 50 dph over 73 % of vertebrae were fully ossified. *Artemia* fed larvae had at the same time over 96 % of the vertebrae fully ossified.

Larvae from the copepod group had a higher amount of fully ossified vertebrae at a smaller size compared to the other treatments (Figure 3.10). The smallest larvae with 80-90 % of the vertebrae fully ossified was 9.9 mm compared to 11.4 and 10.7 mm for *Artemia* and formulated feed fed larval groups. Over 90 % fully ossified vertebrae were observed in 1,4 mm shorter larvae than from the other groups. The difference in length between the groups was smaller in larvae with all 28 vertebrae fully ossified. A higher amount of the larvae fed *Artemia* had over 90 % fully ossified vertebrae than the other two groups. However, those larvae had standard lengths over 14 mm, larger larvae than found in the other groups.

Table 3.2. *Mean number of vertebrae (larva^{-1}) classified depending on the degree of ossification with mean standard length (mm) ± standard error (SE) per treatment. Transparent = no visible bone ossification, partly = ossification starting at the arches root but not filling the whole vertebrae, compact = ossification in the whole vertebrae but not fully saturated colour, and fully ossified = fully saturated colour filling the whole vertebrae. The total number of vertebrae is the sum of transparent, partly, compact and fully ossified vertebrae. Sampling size; n = 10-150 treatment^{-1} at 0, 10, 21, 34, 42 and 50 dph. Significant differences between treatments are indicated by different letters.*

Dph	Treatment	N	Mean SL (mm) ± SE	Number of vertebrae (larva^{-1}) ± SE				
				Transparent	Partly	Compact	Fully ossified	Total
0	No feeding	10	5.4 ± 0,1	5.1 ± 0.4	20.7 ± 0.2	0	0	25,8 ± 0,5
10	*Artemia*	21	6.7 ± 0,1	1.9 ± 0.1	7.2 ± 0.5^{a}	18.9 ± 0,5	0,8 ± 0,1^{a}	28,0 ± 0,1
	Copepod	21	6.3 ± 0,1	1.7 ± 0.2	8.7 ± 0.1^{b}	17.6 ± 0,1	0^{b}	28,0 ± 0,0
	Form. feed	21	6.2 ± 0,0	1.6 ± 0.1	9.2 ± 0.2^{b}	17.2 ± 0,0	0^{b}	28,0 ± 0,0
21	*Artemia*	30	8.2 ± 0,1^{a}	0^{a}	5.0 ± 0.5^{a}	19.2 ± 0,3^{a}	3,8 ± 0,2^{a}	28,0 ± 0,0
	Copepod	30	6.9 ± 0,1^{b}	0^{a}	7.4 ± 0.4^{b}	18.6 ± 0,7^{a}	1,9 ± 0,5^{b}	28,0 ± 0,0
	Form. feed	30	6.4 ± 0,1^{c}	1.0 ± 0.0^{b}	10.3 ± 0.5^{c}	15.1 ± 0,8^{b}	1,5 ± 0,2^{b}	27,9 ± 0,1
34	*Artemia*	30	9.8 ± 0,1	0^{a}	0.4 ± 0.2^{a}	22.8 ± 0,2^{a}	4,7 ± 0,4^{a}	28,0 ± 0,0
	Copepod	30	8.4 ± 0,1	0.1 ± 0.1^{ab}	6.2 ± 1.2^{b}	19.2 ± 1,0^{b}	2,4 ± 0,4^{b}	28,0 ± 0,0
	Form. feed	30	8.2 ± 0,1	0.3 ± 0.1^{b}	8.8 ± 0.8^{b}	16.9 ± 0,8^{c}	2,0 ± 0,2^{b}	28,0 ± 0,0
42	*Artemia*	30	11.9 ± 0,2	0	0^{a}	12.1 ± 1,7^{a}	15,8 ± 1,7^{a}	28,0 ± 0,0
	Copepod	30	9.6 ± 0,2	0	1.0 ± 0.1^{a}	23.2 ± 0.1^{b}	3.8 ± 0.1^{b}	28.0 ± 0.0
	Form. feed	30	10.1 ± 0.2	0	2.6 ± 0.2^{b}	22.2 ± 0.3^{b}	3.2 ± 0.0^{b}	28.0 ± 0.0
50	*Artemia*	150	13.6 ± 0.1^{a}	0	0^{a}	0.9 ± 0.2^{a}	27.1 ± 0.1^{a}	28.0 ± 0.0
	Copepod	150	11.4 ± 0.1^{b}	0	0.4 ± 0.1^{b}	5.1 ± 1.4^{b}	22.4 ± 1.5^{b}	28.0 ± 0.0
	Form. feed	150	11.6 ± 0.1^{c}	0	0.6 ± 0.2^{b}	6.6 ± 0.5^{c}	20.7 ± 0.6^{c}	27.9 ± 0.1

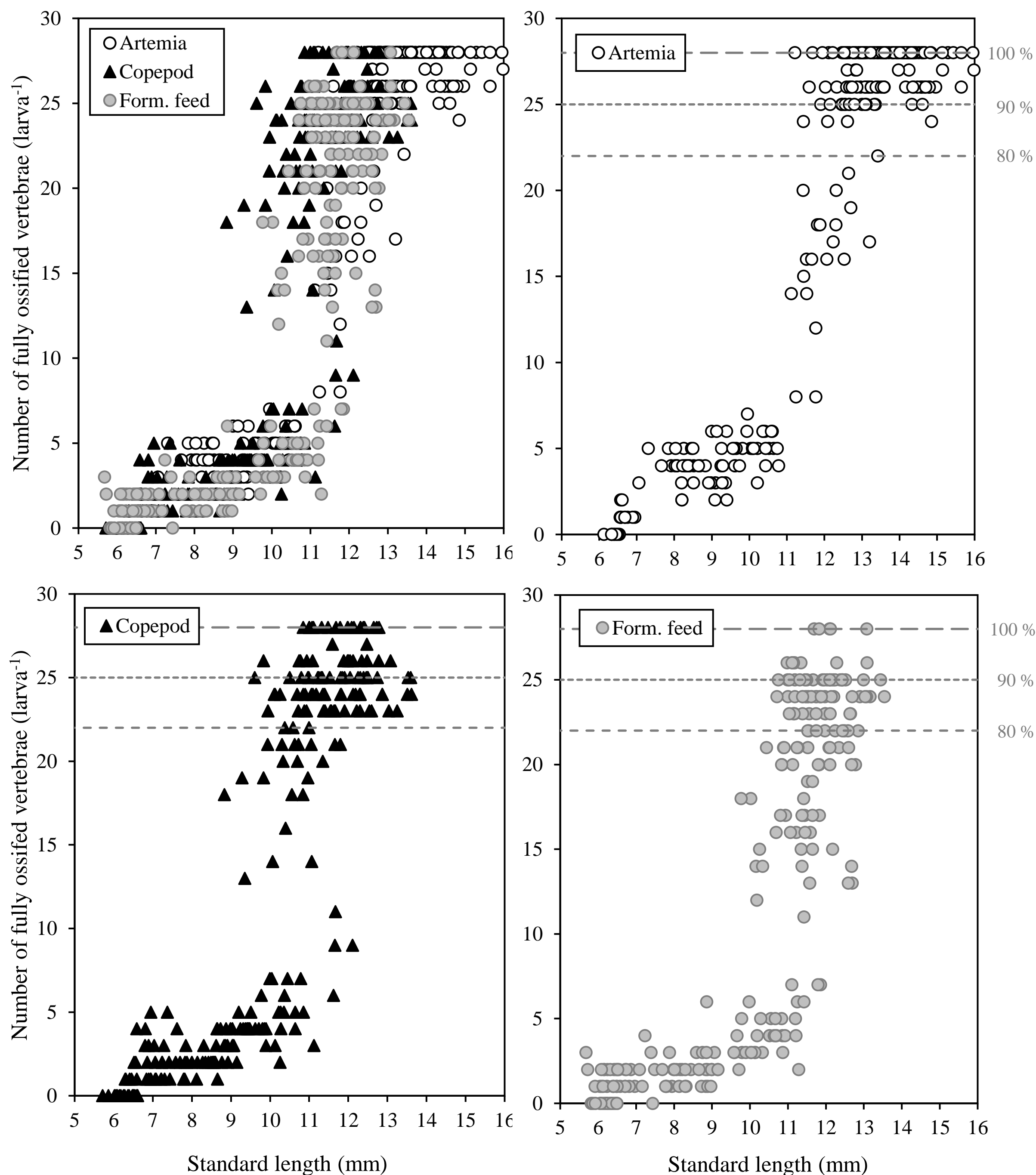


Figure 3.10. *Number of fully ossified vertebrae (larva^{-1}) plotted with larval standard length (mm). Total number of vertebrae per larva was 28. Larvae was sampled at 0, 10, 21, 34, 42 and 50 dph, and sampling size, n = 10 treatment^{-1} at 0 dph, n = 21 treatment^{-1} at 10 dph, n = 30 treatment^{-1} day^{-1} at 21, 34 and 42, and n = 150 treatment^{-1} at 50 dph. The grey lines are marked with the percentage of vertebrae fully ossified e.g. 80 % = 80 % of the all vertebrae in a larva is fully ossified.*

3.5.3. Dorsal fins and anal fin

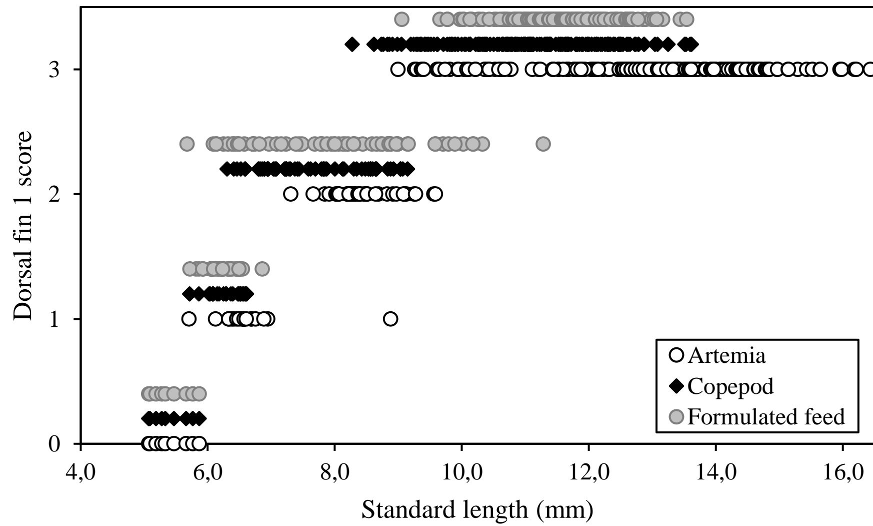
No ossification was observed in the anal and dorsal fins in larvae from 0 dph. From 10 dph all larvae had ossification in the fin rays and was classified with score 1 (Table 3.4). Score 2 was obtained from 21 dph, and significantly more larvae from the *Artemia* treatment had score 2 in the second dorsal fin than from the formulated feed treatment. The highest difference between the treatments was seen in the anal fin, where 97 % *Artemia*, 50 % copepod and 17 % formulated feed fed larvae had score 2. However no significant difference was found due to variation between larval tanks and only three replicates (fish tanks) per treatment. Ossification in the pterygiophores (score 3) was from 34 dph, and at 42 dph in 100 % of the larvae fed *Artemia.* The number of larvae fed formulated feed with score 3 in first dorsal and anal fin were significantly lower than larvae fed *Artemia* At the last sampling day (50 dph) over 99 % of the larvae from all treatment had ossification in pterygiophores.

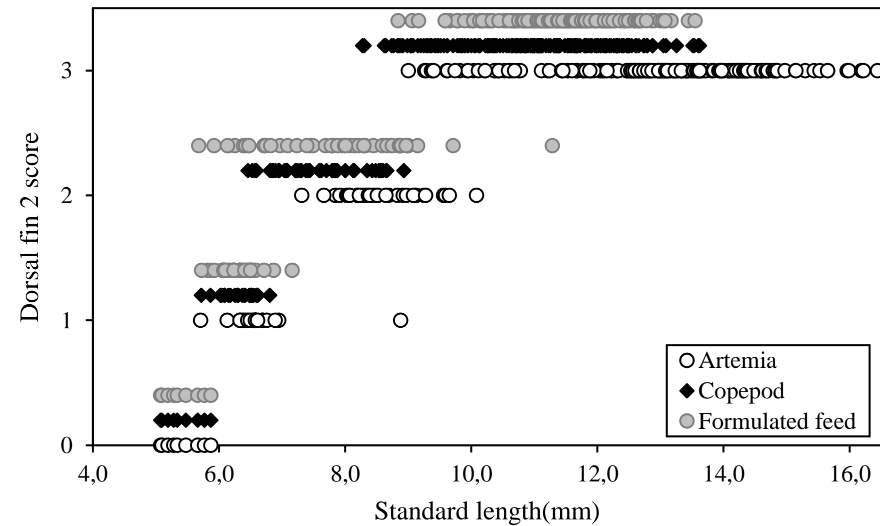
The development of anal and both dorsal fin related to standard length was similar. Larvae with standard length 5-6 mm had score 0 and score 1 were observed in 5.7-7.7 mm larvae except from one larvae of 8.8 mm (Figure 3.11). From 5.7-11.3 mm larvae had score 2, score 3 was reached from 8.3 mm and in all larvae over 11.3 mm.

Larvae from the copepod treatment reached the fin ossification score 3 in smaller larvae compared to the other treatments, but the difference between the larval treatments was not significant. The size interval with fin development from score 2 to 3 was also smaller for the copepod fed larvae, which means they had a faster fin development. From 8.3-9.1 mm the fins developed to score 3, while fins in larvae fed *Artemia* and formulated feed were 9.0-10.6 and 8.8-11.3 mm.

Table 3.4. *Mean percentage of larva with first dorsal, second dorsal and anal fin ray scores and larval mean standard length (mm) ± standard error (SE) for the treatments. Sampling days; 10, 21, 34, 42 and 50 dph, and sampling size, n = 21-150 treatment^{-1}. Fin ray score 0 = no ossification, 1 = ossification of less than half of the length of dorsal/anal fin rays, 2 = ossification in over half the length of dorsal/anal fin rays, but not pterygiophores and 3 = ossification of all dorsal/anal fin rays and pterygiophores. "-" = 0 % larvae with that particular score. Significant difference between the treatments is indicated by different letters.*

Dph	Treatment	Mean SL (mm) ± SE	n	Dorsal fin 1 score				Dorsal fin 2 score				Anal fin score			
				0	1	2	3	0	1	2	3	0	1	2	3
10	*Artemia*	6.7 ± 0.1	21	-	100	-	-	-	100	-	-	-	100	-	-
	Copepods	6.3 ± 0.1	21	-	100	-	-	-	100	-	-	-	100	-	-
	Form. feed	6.2 ± 0.0	21	-	100	-	-	-	100	-	-	-	100	-	-
21	*Artemia*	8.2 ± 0.1[a]	30	-	3[a]	97	-	-	3[a]	97[a]	-	-	3	97	-
	Copepods	6.9 ± 0.1[b]	30	-	7[ab]	93	-	-	13[ab]	87[ab]	-	-	50	50	-
	Form. feed	6.4 ± 0.1[c]	30	-	33[b]	67	-	-	53[b]	47[b]	-	-	83	17	-
34	*Artemia*	9.8 ± 0.1	30	-	-	20	80	-	-	27	73	-	-	43	57
	Copepods	8.4 ± 0.1	30	-	-	80	20	-	-	70	30	-	-	87	13
	Form. feed	8.2 ± 0.1	30	-	-	97	3	-	-	97	3	-	-	100	-
42	*Artemia*	11.9 ± 0.2	30	-	-	-	100[a]	-	-	-	100	-	-	-	100[a]
	Copepods	9.6 ± 0.2	30	-	-	17	83[ab]	-	-	3	97	-	-	17	83[ab]
	Form. feed	10.1 ± 0.2	30	-	-	50	53[b]	-	-	20	80	-	-	30	70[b]
50	*Artemia*	13.6 ± 0.1[a]	150	-	-	-	100	-	-	-	100	-	-	-	100
	Copepods	11.4 ± 0.1[b]	150	-	-	1	99	-	-	-	100	-	-	-	100
	Form. feed	11.6 ± 0.1[c]	150	-	-	1	99	-	-	1	99	-	-	1	99





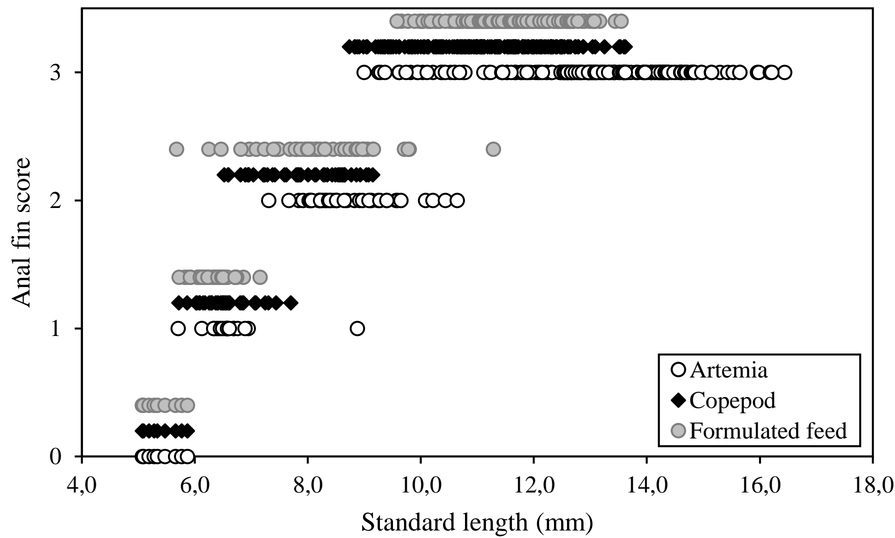


Figure 3.11. *Fin ray score of first dorsal, second dorsal and anal fin in individual larva with standard length (mm). Larvae sampled at 0, 10, 21, 34, 42 and 50 dph, and sampling size, n = 10 treatment^{-1} at 0 dph, n = 21 treatment^{-1} at 10 dph, n = 30 treatment^{-1} day^{-1} at 21, 34 and 42, and n = 150 treatment^{-1} at 50 dph. Fin ray score 0 = no ossification, 1 = ossification of less than half of the length of dorsal/anal fin rays, 2 = ossification in over half the length of dorsal/anal fin rays, but not pterygiophores and 3 = ossification of all dorsal/anal fin rays and pterygiophores. Scoring for different treatments are lifted to see differences better.*

3.5.4. Tail (caudal) fin and tail region

Newly hatched larvae had no ossification in the tail fin rays, urostyle or hypural bones. The average number of tail fin rays were 9-10 at 10 dph and increased with age to 14-15 rays (Figure 3.12). Larvae fed *Artemia* had significantly more ossified fin rays than larvae from the other treatments from 21-50 dph. At 21 dph the average number of fin rays of *Artemia* fed larvae was 15 ossified fin rays, while larvae fed copepods and formulated feed had 14 and 12. The tail fin of *Artemia* fed larvae was fully developed from 21 dph, since the number of tail fin rays not continued to increase. For the other treatments was the fully developed tail fin observed at 34 dph. The maximum number of fin rays at 50 dph was 18 for the larvae fed *Artemia*, while max 17 rays was observed in larvae from the other two treatments.

The size- related ossification of tail fin rays showed a shift where larvae under 6.5-7.0 mm had under 15 ossified rays and bigger larvae had over 15. From larval size 5.5-7.0 mm most of the ossification of tail fin rays happened. The number of tail fin rays in individual larvae varied from 14-18 in larvae over 7 mm. Ossification in over 15 tail fin rays was observed in 0.3 mm smaller larvae fed copepods compared to the other treatments (Figure 2.13).

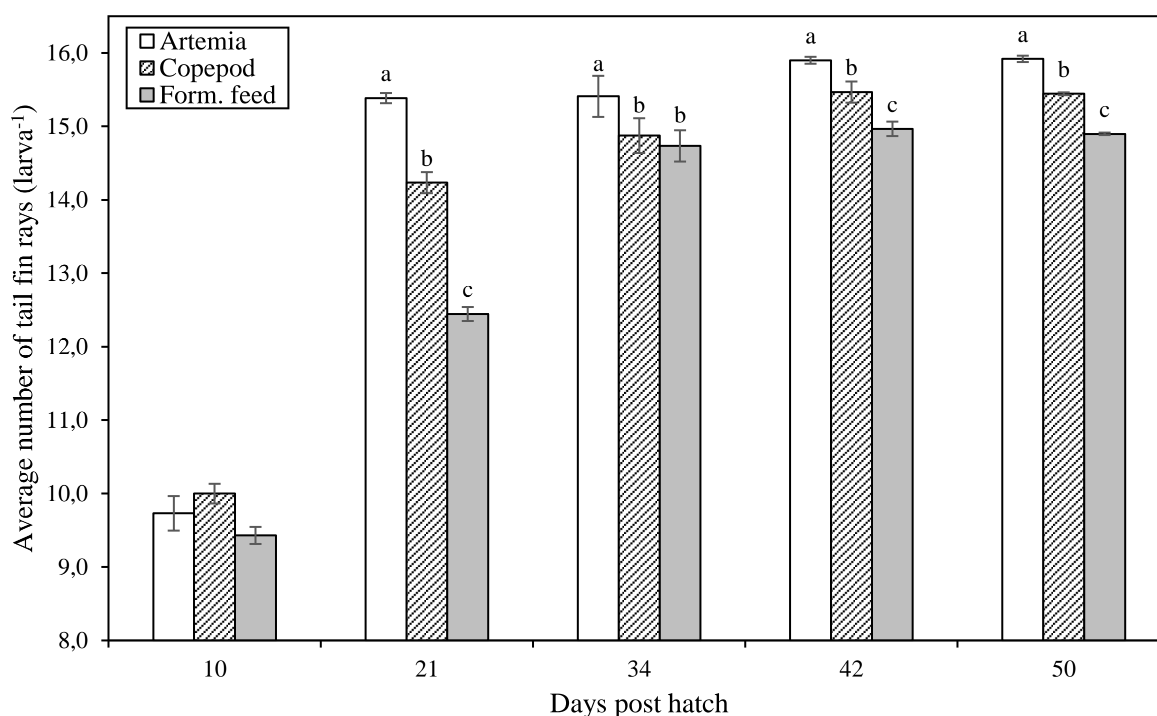


Figure 3.12. *Mean number of ossified tail fin rays ± standard error (SE) larva^{-1} treatment^{-1}. Larvae sampled at 0, 10, 21, 34, 42 and 50 dph, and sampling size, n = 10 treatment^{-1} at 0 dph, n = 21 treatment^{-1} at 10 dph, n = 30 treatment^{-1} day^{-1} at 21, 34 and 42, and n = 150 treatment^{-1} at 50 dph. Error bars represent ± SE and significant differences between treatments are indicated by different letters.*

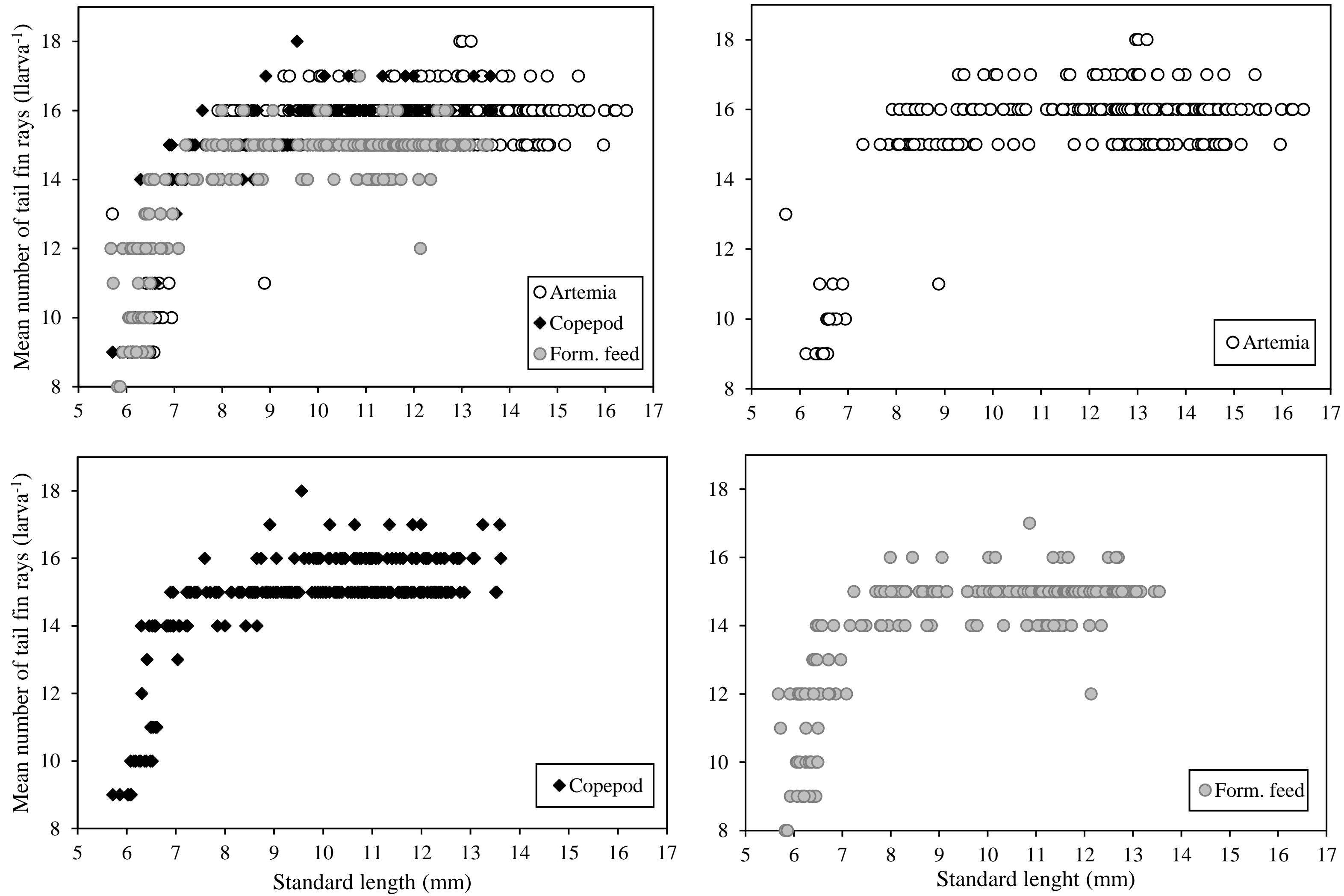


Figure 3.13. *Mean number of ossified tail fin rays related to size, standard length (mm) larva^{-1} treatment^{-1}. Top-left: all treatments, top-right: Artemia* sp.*, lower-left: copepod and lower-right: formulated feed. Sampling size, n=21-150 larva treatment^{-1} on 0, 10, 21, 34, 42 and 50 dph. Larva from 0 dph had no ossified tail fin rays.*

Ossification of the urostyle was first observed in all larvae from all treatment over 9.0 mm (Table 3.6). The first sign of ossification in a 6.5 mm long larva fed copepod, while the first larvae fed *Artemia* and formulated feed were 7.5 and 7.0 mm. Hypurals started to ossify in all larvae over 7.5 mm fed *Artemia* and in 6.5 mm larvae fed copepod and formulated feed.

Table 3.6. *Bone ossification of bones in tail region (urostyle, hypurals and tail fin rays) and presence of flexion related to size, standard length (mm). Light grey represents the shortest larva observed with sign of ossification, and dark grey is the size where all larvae had ossification in that specific part. All larvae over 9.5 mm had ossification in urostyle, hypurals, tail fin rays and presence of flexion.*

	Treatment	**Standard length (mm)**									
		5.0	**5.5**	**6,0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**	**9.5**
Urostyle	*Artemia*										
	Copepod										
	Form. feed										
Lower hypural	*Artemia*										
	Copepod										
	Form. feed										
Upper hypural	*Artemia*										
	Copepod										
	Form. feed										

3.6. Skeletal anomalies

The skeletal anomalies found in lumpsucker larvae were twisted neural arches, flat head, abnormal vertebrae and axis deviations (Table 3.7 and Figure 3.14), while abnormal jaw was not observed in any larva. Other anomalies observed were abnormal fin rays or pterygiophores presented in larva d and e in Figure 3.14. None of the anomalies were observed in larvae at 10 dph. From 21-50 dph there was only significant difference between the treatments in occurrence of twisted neural arches.

Table 3.7. *Mean percentage of larva with anomalies (%) ± standard error (SE); flat head, abnormal vertebrae segments, axis deviations (lordosis, kyphosis and scoliosis) and other deformities (fins). Sampling size, n=30 larvae treatment^{-1} at 21, 34 and 42 dph, and n=150 larvae treatment^{-1} at 50 dph.*

Dph	Treatment	Mean percentage of larvae with anomalies (%) ± SE				
		Abnormal skull	Abnormal vertebrae	Axis deviations	Other anomalies	Twisted neural arches
21	*Artemia*	10 ± 5	-	-	-	7 ± 3^{a}
	Copepod	13 ± 7	-	-	-	40 ± 5^{b}
	Form. feed	11 ± 9	-	-	-	71 ± 8^{c}
34	*Artemia*	-	3 ± 3	-	-	33 ± 11^{a}
	Copepod	7 ± 3	3 ± 3	-	-	83 ± 10^{b}
	Form. feed	13 ± 7	3 ± 3	-	-	83 ± 14^{b}
42	*Artemia*	-	7 ± 3	-	-	20 ± 5^{a}
	Copepod	3 ± 3	-	-	-	43 ± 3^{a}
	Form. feed	3 ± 3	-	-	3 ± 3	90 ± 5^{b}
50	*Artemia*	-	6 ± 0	1 ± 0	1 ± 1	11 ± 3^{a}
	Copepod	1 ± 1	11 ± 2	1 ± 0	1 ± 1	17 ± 5^{a}
	Form. feed	-	7 ± 1	1 ± 0	1 ± 1	32 ± 4^{b}

3.6.1. Abnormal skull

Larvae observed with a flat head had a flatter dorsal side of the skull compared to the average skull shape (larva c in Figure 3.14). The highest number of larvae with flat head was observed at 21-34 dph. At 42-50 dph decreased the occurrence of flat head and under 3 % were found to have the anomality the last two sampling days. No significant difference between the treatments were found due to high variations between tanks.

3.6.2. Abnormal vertebrae and axis deviations

Two types of abnormal vertebrae were observed; (1) two vertebrae fused without a separation between (larva b, Figure 3.14) and (2) vertebrae with different position compared to the rest of the spine (larva a, Figure 3.14). Fused vertebrae were only observed in the two last vertebrae of the caudal part of the spine. No significant difference was found between the treatments. The occurrence was highest on 50 dph when 6, 11 and 7 % of the larvae fed *Artemia*, copepod and formulated feed had abnormal vertebras.

Axis deviations; lordosis, kyphosis and scoliosis were only found in 1 % of larvae at 50 dph from all treatments (Table 3.7). This equalled in total five larvae affected from all treatments. One larva fed formulated feed had both lordosis and kyphosis (larva f in Figure 3.14). Lordosis alone was found in one larva fed *Artemia* and one larva fed formulated feed. Kyphosis was observed alone in one larva fed copepod and one other larva from the same treatment had scoliosis. No significant difference was found between the treatments regarding axis deviations.

3.6.3. Twisted neural arches

Twisted neural arched were observed in larvae from 21-50 dph (Table 3.7 and larva g in Figure 3.14). The same spiral at apex were also found in the hemal arches. Significantly less percentage of larvae from the *Artemia* group had twisted neural arches at 21-34 dph. The last sampling day, the occurrence of twisted neural arches decreased for all treatments. Larvae fed formulated feed had significantly more twisted neural arches than the other treatments at 50 dph. The number of twisted arches per individual larvae also decreased at 50 dph, where the average number per larvae was under 1 for all treatments (Figure 3.14). The same was seen in the *Artemia* and copepod larval groups at 42 dph, where the mean number of twisted neural arched per larvae was under 2.

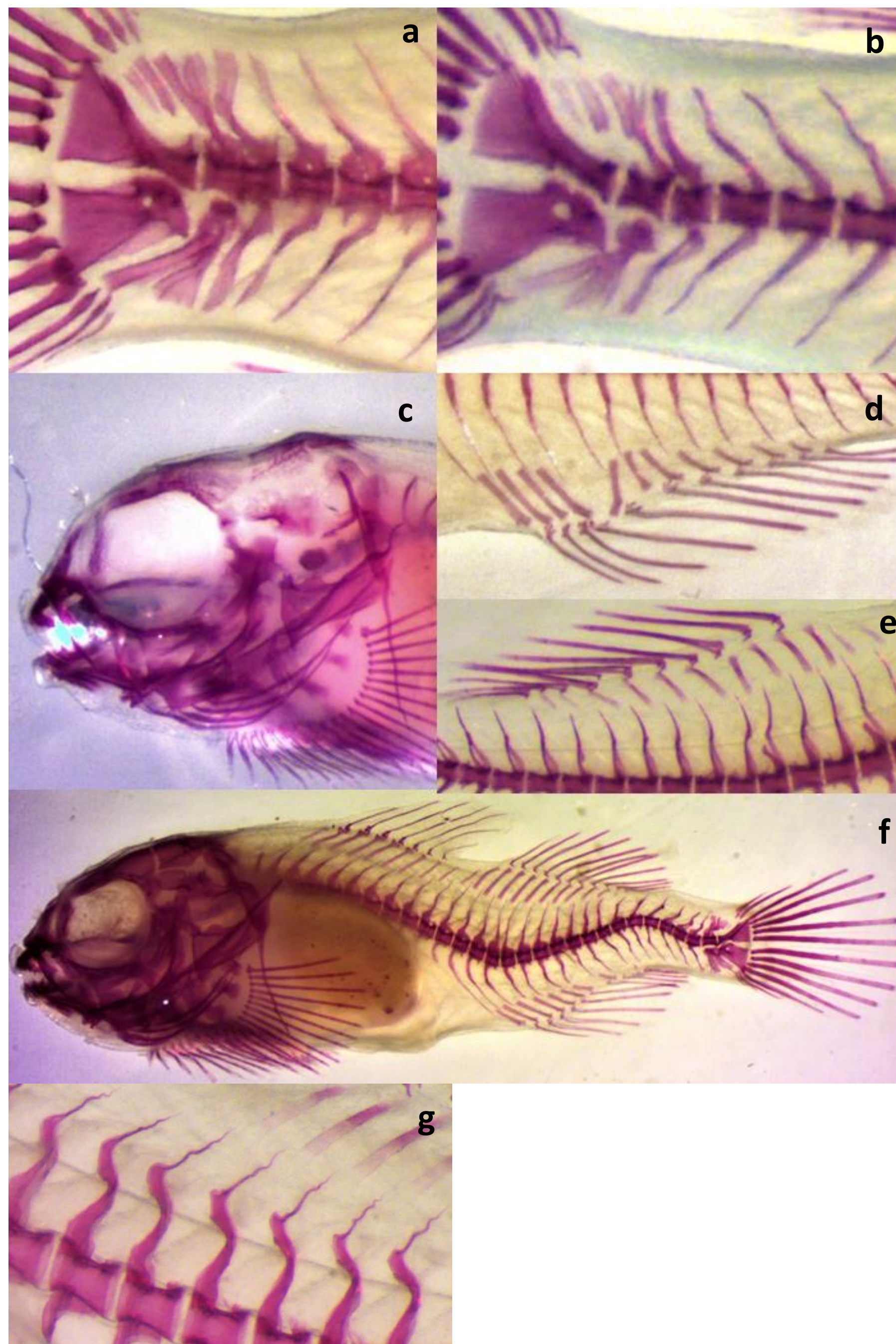


Figure 3.14. *Examples of observed skeletal anomalies in larvae from 10-51 dph. a) Fused two last vertebrae, b) abnormal position of the second last vertebrae, c) flat skull on dorsal side, d) pterygiophore of anal fin orientated wrong way, e) abnormal position of pterygiophore of second dorsal fin, f) lordosis and kyphosis and g) twisted neural arches.*

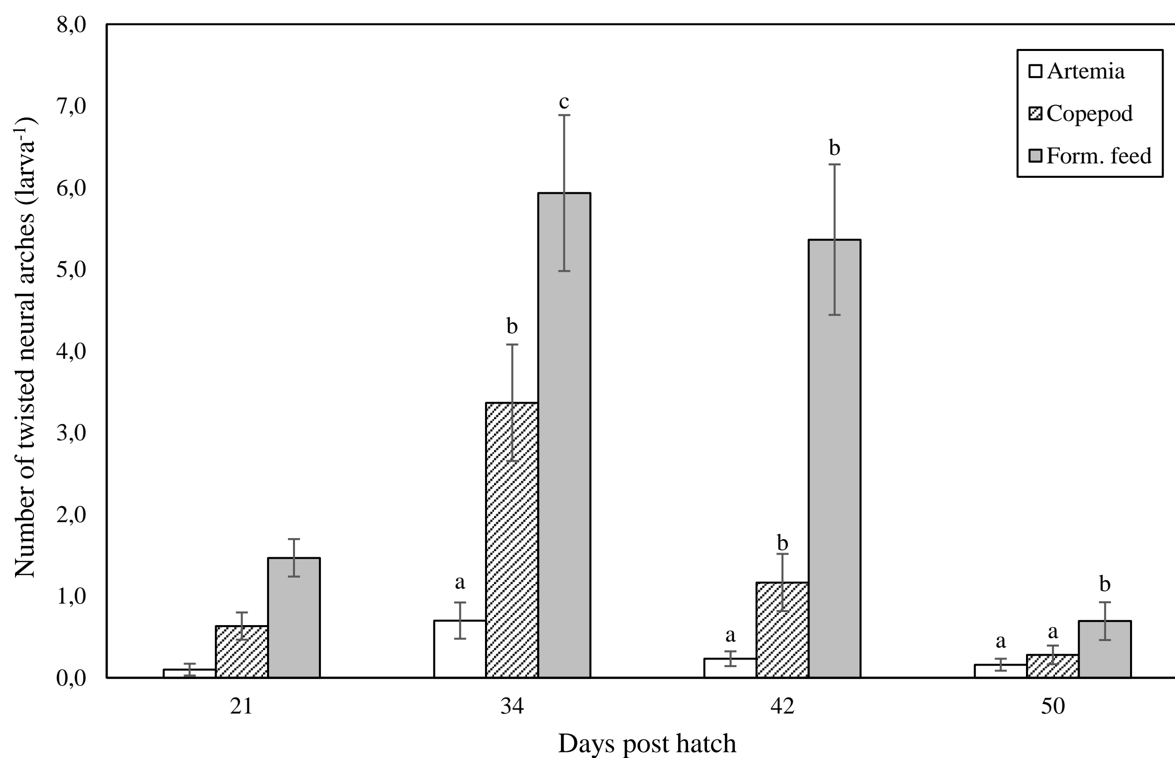


Figure 3.15. *Mean number of twisted neural arches (larva^{-1} treatment^{-1}) at 21, 34, 42 and 50 dph. Sampling size, n=30 larva treatment^{-1} at 21-42 dph and n=150 larva treatment^{-1} at 50 dph. Error bars represent ± SE and significant differences between treatment are marked with different letters.*

4. Discussion

4.1. The effects from different start feeding diets on growth and survival

The growth and survival were improved by feeding with enriched *Artemia* compared to copepods and formulated feed. Significantly higher dry weight, wet weight and standard length were observed already from 6 dph and throughout the experiment. Larvae fed *Artemia* in the present study grew almost twice as much as larvae start fed with copepods (*A. tonsa*) or formulated feed during a pilot study (Dahle *et al.*, 2017). However, feeding larvae copepods and formulated feed gave similar growth in both studies.

Feeding Cryoplankton resulted in higher growth rate and survival of lumpsucker larvae compared to feeding formulated feed (Schaer, 2017). Cryoplankton is wild caught marine copepod species which is stored frozen and thawed in seawater before feeding to fish larvae. The dry weight of larvae fed *Artemia* in the present study was 43-57 % higher compared to larvae fed Cryoplankton. The sizes of larvae fed copepods and Cryoplankton were similar in both studies.

Better growth when start feeding with copepods compared to *Artemia* and rotifers as seen in cod (Wold *et al.*, 2009, Hansen, 2011), ballan wrasse (Dahle *et al.*, 2014) and European seabass (Gisbert *et al.*, 2005, Rajkumar and Kumaraguru vasagam, 2006) larvae was not observed for lumpsucker larvae. Common for these species is their small newly hatched larvae. The dry weight of newly hatched larvae of ballan wrasse and cod were 0.05-0.06 mg (Hansen, 2011, Gagnat, 2012) while lumpsucker larvae was 1 mg. In general, the growth rate is higher for species with small larvae post hatching (Duarte and Alcaraz, 1989). Both cod and ballan wrasse larvae had higher growth rate (DWI > 10 %) over a period of 60 days (Hansen, 2011, Gagnat, 2012). High growth rate are related to high larval requirements of the essential PUFAs, DHA and EPA (Olsen *et al.*, 2007). Lumpsucker larvae with lower growth rate (DWI 4.9-6.4 %) may not have the same requirements regarding DHA and EPA. They might either ingest sufficient amount from triglycerides directly or they elongate and desaturate them from shorter n-3 fatty acids. The ability to elongate fatty acids is reported in freshwater fish species (Bell *et al.*, 1994). Freshwater fish larvae eat invertebrates with low levels of DHA and they fulfil the requirements by elongating sufficient amount of DHA from EPA.

In general, the lipids content of small fish eggs is lower than in larger eggs (Kjørsvik *et al.*, 2004), which result in a yolk sac with less energy. A small yolk sac makes larvae less resistant against starvation (Duarte and Alcaraz, 1989). For example, cod lay small, pelagic fish eggs with a high water content (Fraser *et al.*, 1988). The cod larvae probably have smaller yolk sac compared to lumpsucker larvae from bigger, not pelagic eggs. In addition, the development of feeding apparatus, respiratory system and tail are prioritized over the digestive system in the small fish larvae (Osse and Van den Boogaart, 1995). They usually have a shorter development time in eggs from fertilization to hatching and hatch with a less developed digestive system (Kjørsvik *et al.*, 1991). Larvae from larger fish eggs usually have longer development time from fertilization to hatching (Kjørsvik *et al.*, 2004), and may hatch with a more developed digestive system. Feeding with easy digestible copepods might be more important for the small larvae with less developed digestive systems.

Calculation of feeding density of *A. tonsa* and *Artemia* was based on number of feed organisms. The dry weight of *A. tonsa* stages C1-C3 is 0.3-0.7 µg C depending on the food ingestion (Berggreen *et al.*, 1988). *Artemia franciscana* fed microalgae for 1 and 2 days had dry weight 0.77 and 0.95 µg C (Evjemo and Olsen, 1999). This means that the *Artemia* had higher biomass compared to the same number of copepods (C1-C3). Eating one Artemia provided more biomass compared to one *A. tonsa*. The different biomass would be reduced with an older, larger copepodite stage or by increasing the number of copepod per litre. Due to the mouth size of newly hatched lumpsucker larvae using a larger copepodite stage like C4 of 690 µm and 1.06 µg C (Hagemann, 2014) might be better.

The length of *Artemia* and *A. tonsa* were also different. *A. franciscana* grown 1-2 days on microalgae was 840-1100 µm (Evjemo and Olsen, 1999) and *A. tonsa* stage C1-C3 was 410-580 µm (Hagemann, 2014). The measured mouth width of newly hatched lumpsucker larva in the present study was ~0,8 mm (Mil, 2017), which mean that they can eat bigger organism than copepod stages C1-C3. Studies on other fish species have shown larval selection for bigger prey organisms over the smaller (Olsen *et al.*, 2000) which may suggest that the bigger prey are more beneficial for larvae.

The total lipid content is higher in enriched *Artemia* compared to *A. tonsa*. However, 73.7 % of the total lipid is phospholipids in *A. tonsa* while in *Artemia* only 36.0 % is phospholipids (Øie *et al.*, 2017). The improvement of lumpsucker larval growth from feeding with *Artemia* suggest

that a high lipid content probably is more beneficial. The level of phospholipids in enriched *Artemia* may be enough for lumpsucker larvae.

Ingólfsson *et al.* (2002) reported that the natural prey for lumpsucker larvae was mainly crustaceans living close to seaweed. Natural prey organisms may stimulate feeding behaviour in larvae stronger compared to less suited prey (Olsen *et al.*, 2000). *Artemia* is orange-red and might be easier to detect than transparent *A. tonsa.* Only the eaten microalgae are visible through the stomach of *A. tonsa*. In water temperatures under 25 °C *Artemia* reduce the metabolism and swim slower (Sorgeloos *et al.*, 2001). This may make *Artemia* easier to catch than *A. tonsa* for lumpsucker larvae.

Both groups weaned from copepods to formulated feed had low growth the days after the weaning period. Reduced growth was also observed in both cod and ballan wrasse after weaning from copepods to formulated feed (Koedijk *et al.*, 2010, Hansen, 2011, Gagnat, 2012). Authors suggested that larvae had more trouble switching from a high-quality copepod diet to formulated feed with lower quality. In addition, copepods and formulated feed have very different movement in the water. Copepods are suspended in the water column longer than formulated feed which provide larvae more time to catch them. Prolonging the weaning period might reduce the low growth after weaning. This provide more time for larvae to get used to the new feed while they still eat copepods their used to.

The lower growth after weaning to formulated feed was not observed for larvae fed *Artemia* Their bigger larval size when changing diet probably made it easier to accept the new feed. Other studies also reported that larvae fed *Artemia* and rotifers had less trouble excepting the transition to formulated feed (Koedijk *et al.*, 2010, Hansen, 2011, Gagnat, 2012). Larvae used to eat copepods may need a longer adaption period than larvae fed other live feeds. The different nutritional content, appearance and swimming activity of *Artemia* and rotifers might make the transition to formulated feed easier than from copepods.

The sampling method for growth analysis during the experiment may been less optimal. Larvae attached to tank walls was difficult to catch gently. Collecting larva from silicon mats was faster compared to the tank walls and a higher number of larva was sampled from there. When ending the experiment (51 dph) the sampling was performed more randomly distributed, and a high increase in dry weight was seen from 50-51 dph. This suggest that growth during the experiment may been higher if the sampling was performed more randomly.

Larvae fed copepod in the present study appeared to have more variating and colourful pigmentation than larvae fed *Artemia* and copepods. Schaer (2017) reported also different pigmentation between larvae fed Cryoplankton and formulated feed. Larvae fed Cryoplankton were red/brown while formulated feed larvae were pale white/beige. In halibut, feeding larvae copepods are reported to result in more larvae with normal dorsal pigmentation during metamorphose (Shields *et al.*, 1999, Næss *et al.*, 1995). Over 40 % of the larvae fed enriched *Artemia* were without any pigmentation. Reitan *et al.* (1994) reports that DHA is required for normal pigmentation in turbot larvae. Shields *et al.* (1999) also suggests that the high content of carotenoid in copepods also affect the pigmentation. The pigmentation in lumpsucker larvae might also be affected by the feed content of DHA and/or carotenoids. Which may explain the more colourful pigmentation in larvae fed copepods.

Mean survival of over 79 % experienced for all groups is higher compared to other cultivated species (Dahle *et al.*, 2014, Øie *et al.*, 2014). Other studies also reports high survival in reared lumpsucker larvae (Dahle *et al.*, 2017, Schaer, 2017). Feeding with *Artemia* gave higher survival and less variations between larval tanks compared to copepod and formulated feed. Another study start feeding lumpsucker with live feed (Cryoplankton) resulted also in high survival and less variations between fish tanks compared to feeding a micro diet of dry feed (Schaer, 2017). The mortality in the tanks fed dry feed varied from 10-33 %. In the present study the variation between copepod tanks was highest (6%) and between formulated feed tanks 3 % at the last sampling day. The last sampling day in the present study was at 810 d° where feeding with *Artemia* gave 2 % higher survival than with Cryoplankton (Schaer, 2017).

Decrease in mortality in tanks fed copepod and formulated feed occurred 10 days after weaning to formulated feed. All tanks were fed the same amount of formulated feed. Tanks weaned from formulated feed and copepods had smaller larvae and were therefore probably overfed compared to larvae earlier fed *Artemia* Overfeeding resulted in more uneaten feed settling on the bottom of fish tanks, which are reported to increase the content of total organic carbon and the growth substrate for bacteria (Dahle *et al.*, 2017). Threads of bacteria were found in those tanks weaned for copepods and formulated feed. Schaer (2017) reported a higher total amount of bacteria in lumpsucker larval tanks fed formulated feed than tanks fed live feed. Fish larvae are reported to lack a fully developed immune system (Zapata *et al.*, 2006) and bacteria can cause harmful infections. Dahle *et al.* (2017) recommended to reduce the microbial variability and instability between fish tanks to get a more stable juvenile production. Higher water exchange rate, maturation and recirculation of water and more cleaning could make the bacterial

community more stable (Skjermo *et al.*, 1997, Salvesen *et al.*, 1999, Attramadal *et al.*, 2012b, Attramadal *et al.*, 2012a).

4.2. Skeletal development and the effects from different start feeding diets

The skeletal development of lumpsucker larvae was allometric where the bones related to feeding, locomotion and respiration ossified first. Larvae with allometric growth prioritize the development of organs essential for survival and postpone the development of other less important organs (Osse and Van den Boogaart, 1995). In newly hatched lumpsucker larvae ossification in bones of the mouthparts, suction disc and gill arches were observed. The mouthparts are important for start of exogenous feeding, the suction disc can be related to locomotion and ossification in gill arches are important for the respiration.

Suction disc is a modified pelvic fin which makes larvae capable of attaching to hard substrates already at hatching. Since the ossification is prioritized already before hatching lumpsucker larvae may be dependent of attaching to surfaces to survive. The suction disc is also found in other species and it consists of the subpelvic process, one spine, five soft rays and a triangular bone called basipterygium (Kido, 1988).

The ossification of dorsal, pectoral, anal and tail fin was not prioritized before hatching, but at 10 dph all fins had started ossification. Fin development of is related to an increased swimming capacity making larvae able to catch prey more efficient. The pectoral fins are used during slow swimming while the involvement of the tail fin accelerated the movement (Blake, 2004).

The relationship between the fish body length and the development of specific organs is common in fish (Osse and Van den Boogaart, 1995). At a specific length the growth rate and development of an organ slows down and enters a period of slower development with a lower growth rate. The growth often shift from positive allometric growth to isometric growth (Osse and Van den Boogaart, 1995). From larval length 8-9 mm a change in growth rate for specific organs of the digestive system and of external body parts was reported (Marthinsen, 2018, Mil, 2017). In the digestive system the fundus was developed, stomach enlarged the volume and the pyloric caceca were observed (Marthinsen, 2018). Head length, height and width, mouth width, dry weight, wet weight and the total length was reported to shift from positively allometric to isometric growth (Mil, 2017) During bone development, the urostyle and the pterygiophores of all fins were the bones ossifying at the same length (9 mm). However, all the other bones analysed started ossification at shorter lengths.

Ossification was observed in the same parts in newly hatched larvae in the present study and larvae of same standard length from Voskoboinikova and Kudryavtseva (2014). In the present study the ossification of fins was observed in smaller larvae. All larvae longer than 5.9 mm had ossified dorsal, tail, anal and pectoral fin rays. Voskoboinikova and Kudryavtseva (2014) first observed ossification in the tail, second dorsal and anal fin rays from 6.5 mm. The first dorsal and pectoral fin rays ossified from 8.1 mm. However, only wild caught larvae of some length were analysed and the fin ossification in larvae between 6.5-8.1 mm are not reported. The maximum number of tail fin rays observed was 17, while Voskoboinikova and Kudryavtseva (2014) observed max 15 rays. Urostyle and hypurals, the bones providing the tail fin support were ossified in smaller larvae during the present study.

The average number of vertebrae with ossification at hatching (SL 5.4 mm) was 21. The 5.5 mm large wild-caught larvae had nine visible vertebrae, all without ossification (Voskoboinikova and Kudryavtseva, 2014). From 6.5 mm Voskoboinikova and Kudryavtseva (2014) observed ossification in all vertebrae except from the two last. They reported in total 27-28 vertebrae in all larvae analysed. In the present study all larvae had 28 vertebrae except from one with 27. From 6.5 mm all vertebrae were ossified except from the 1-2 last.

All vertebrae were compact or fully ossified in larvae over ~11 mm. Larvae with all vertebrae fully ossified was observed at the last sampling day. Vertebrae classified as compact were possibly fully ossified. During the bone staining the alizarin solution was renewed one time. Some of the vertebrae in larvae larger than 11 mm was classified as compact due to not full saturation of colour. If the dye of alizarin red in the staining solution was used up the vertebrae may not achieve full saturation. For the biggest larvae a second renewing or a stronger alizarin solution was possibly necessary.

Start feeding with *Artemia* resulted in an earlier bone development related to age than larvae fed copepods and formulated feed. The earlier ossification might be explained by the high growth rate and that the onset of ossification is promoted at specific larval sizes. The development of specific organs if often determined by larval length (Osse and Van den Boogaart, 1995). Wold *et al.* (2008) suggested that when comparing larval development, the same size and not age should be used.

The onset of ossification in the vertebrae, dorsal, anal and tail fin, hypurals and urostyle was observed in smaller larvae fed copepods compared to *Artemia* and formulated feed. This suggest that feeding with copepods have a beneficial effect on the skeletal development of lumpsucker

larvae. The benefits may be explained bu the higher content of the essential DHA and EPA in phospholipids (Øie *et al.*, 2017). Kjørsvik *et al.* (2009) reported that during vertebrae development cod larvae were more sensitive to the source of fatty acids provided and not the amount. Authors suggested that PUFAs provided in phospholipids gave faster size-related ossification of the vertebrae. However, large larval variations and no significant differences was reported. In the present study did feeding with copepods result in earlier onset on size-related vertebrae ossification compared to larvae fed *Artemia.* The PUFAs provided in the polar lipid fraction in copepods might be more beneficial than in the neutral lipid fraction in *Artemia.* In mammals, PUFAs are reported to effect nuclear receptors involved in gene expression and ontogenetic skeletal development (Jump and Clarke, 1999, as sited in Cahu e*t al.*, 2003). The high content and readily available PUFAs might accelerate the skeletal development by acting on the genes involved in skeletal development.

The occurrence of abnormal skull, axis deviations and other anomalies at 50 dph was under 1 % for all treatments and there was no significant difference. Abnormal vertebrae were observed in 6 % of larvae fed *Artemia*, 11 % copepod fed larvae and 7 % formulated feed fed larvae, with no significant difference. Witten *et al.* (2005) related the fused vertebrae to alternations in the vertebral growth zones of two vertebrae. The growth zones are separated by the intervertebral space. In two vertebrae with normal development the growth zones produce bones in different directions from the intervertebral space. In fused vertebrae the growth zone cells are pressed into the intervertebral space and replace the notochord tissue with bone and cartilage. Witten *et al.* (2005) reported that vertebrae fusion in salmon (*S. salar*) was related to mechanical pressure from high muscle masses and water currents. Fernández *et al.* (2008) suggested that high levels of vitamin A increased the intramembranous ossification process of vertebral centrums leading to fused vertebrae in sea bream. In halibut more fused and compressed vertebrae was reported when feeding high levels of vitamin A (Lewis-McCrea and Lall, 2010).

Witten and Huysseune (2009) states that the fusion of vertebrae to the urostyle is not an anomaly and a common feature found in other fish species. None of the fused vertebrae observed had fused to the urostyle. However, since only two vertebrae was involved in the fusion the body shape or length of lumpsucker larvae is probably not affected. Boglione *et al.* (2013a) defined vertebral fusion as a severe anomality if it includes many vertebrae (>3) and reduces the vertebral length.

Abnormal skull was mainly observed in larvae from 21 and 34 dph while the occurrence decreased from 42 dph. A flat skull reduces the space for a developing brain, and since the highest number of larvae with this were found at earlier age the anomaly might be lethal. If the development of a flat skull is caused from mechanical injury or alteration of the bone development is uncertain.

Axis deviations (lordosis, kyphosis and scoliosis) were observed in 1 % of the larvae at 50 dph. The change in fish body shape might affect their biological performance resulting in lower growth and negatively impact on the commercial value (Divanach *et al.*, 1996). Different nutritional and environmental factors is reported to cause axis deviations. In Atlantic halibut low phosphorus levels induced scoliosis, deficiency of ascorbic acid gave scoliosis and lordosis and high vitamin A levels resulted in scoliosis (Lewis-McCrea and Lall, 2010). Water temperature and water currents may also affect the development of the vertebral axis (Boglione *et al.*, 2013a). Only 1 % of the lumpsucker larvae at 50 dph had axis deviations (lordosis, kyphosis and scoliosis) which suggest that the feed content of phosphorus, vitamin A and ascorbic acid was sufficient and that no environmental factors caused severe axis deviations.

Twisted arched was the anomaly most frequently observed. Significantly more larvae fed formulated feed had twisted neural arches. The consequences on larval performance from a minor deviation like twisted arches are unknown. In addition, the reduction of number of larva with twisted neural arches at 50 dph when the skeleton was more developed suggest that it might is a stadium before the arches fully develop. In Atlantic halibut, twisted neural and hemal arches (spines) was observed in fish fed low levels of phosphorus (Lewis-McCrea and Lall, 2010). They suggested muscular action to be the cause of alternation in the thin and soft tip of neural and hemal arches.

4.3. Conclusion and future perspectives

Start feeding lumpsucker larvae with *Artemia* the first 22 days resulted in better growth and survival compared to feeding with copepods and formulated feed. The results suggest that *Artemia* is a more optimal start feed for lumpsucker larvae than copepods and formulated feed. Higher growth rate and survival of lumpsucker may provide a more stable and predictable production.

Earlier onset of bone ossification related to age was found in larvae fed *Artemia* Feeding with copepods resulted in ossification in vertebrae, urostyle, hypurals, dorsal, anal and tail fins at a smaller size than the other diets.

The occurrence of skeletal anomalies (flat skull, fused vertebra and axis deviations) was less than 11 % in all larval groups (fusion of vertebrae only involved two vertebrae). At 50 dph, less than 1 % of the larvae had severe anomalies like axis deviations and flat skull. Twisted neural arches was considered a less severe anomaly, since it only caused a small abbreviation from the normal form. At 50 dph, the occurrence was reduced in all larval group and the larvae had very few twisted arches.

Since the fed biomass of *Artemia* and copepods was different in the present study, a feeding experiment with the same live feed biomass should be conducted. In addition, the larval performance during a stress test could be evaluated for the larvae fed *Artemia* and copepods. This might indicate which live feed produces the most robust larva against stress.

The beneficial effects on larval growth and survival from feeding with *Artemia* must also be further evaluated. The enrichment diets, the feeding amount and duration of feeding *Artemia* should be tested to optimize the growth, survival and development of lumpsucker larvae.

It would also be useful to study the long-term effects from start feeding with *Artemia*, and to evaluate the performance of lumpsucker when added into salmon sea cages. Salmon producers are interested in the lice eating ability and performance of lumpsucker larvae in sea cages. If the larvae fed *Artemia* continues to grow and develop faster and survive better in sea cages, both lumpsucker and salmon producers would benefit.

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Appendix 1

Larval density

Table A1. *Number of larva and density (larva/L) in larval tanks calculated at the end of the experiment. Number of larvae in each tank were calculated when ending the experiment and corrected for number of sampled and dead larvae during the experiment. Tank number 8 was not used.*

Tank number	Feeding regime	Number of larvae	Density (larvae/L)
1	*Artemia*	7531	75,3
2	*Artemia*	6491	64,9
3	Copepod	6956	69,6
4	Formulated feed	6459	64,6
5	Formulated feed	6602	66,0
6	Copepod	7725	77,3
7	Formulated feed	6439	64,4
9	*Artemia*	5625	56,3
10	Copepod	5431	54,3

Appendix 2

Feeding amount formulated feed, *Artemia* and *A. tonsa*

Table A2.1 *Feeding amount for tanks fed formulated feed, with dph, feeding times (s), feeding frequency (times/day) and feeding amount (g/day) from 6-51 dph.*

Dph	Feeding time (sec)	Feeding frequency (times/day)	Feeding amount (g/day)
6	1	20	12,2
7	1	20	12,2
8 - 35	2	40	50,4
36 - 40	2	30	37,8
41 - 43	2	35	44,1
44 - 48	2	40	50,4
49 - 51	2	60	75,6

Table A2.2. *Feeding density in larval tanks per feeding, feeding frequency (times/day) and the feeding amount (number of live prey/tank/day) of Artemia and A. tonsa from 2-22 dph.*

Dph	Density (#live prey/L/feeding)	Feeding frequency (times/day)	Feeding amount (#live prey/tank/day)
2	1000	6	600 000
3 - 6	1500	6	900 000
7 - 22	3000	6	1 800 000

Appendix 3

Conwy Medium

Tabell A3. *Content in Conwy medium used to feed microalgae, modified from (Walne, 1979). 1ml Conwy medium was used per litre seawater.*

Sodium Nitrate ($NaNO_3$)	100,0 g/L
Boric Acid (H_3BO_3)	33,6 g/L
EDTA disodium salt (Na_2 EDTA)	45,0 g/L
Sodium Phosphate, monobasic ($NaH_2 PO_4 \bullet H_2O$)	20,0 g/L
Ferric Chloride, 6-hydrate ($FeCl_3 \bullet 6H_2O$)	1,3 g/L
Manganous Chloride, 4-hydrate ($MnCl_2 \bullet 4H_2O$)	0,36 g/L
Zinc Chloride ($ZnCl_2$)	0,021 g/L
Cobalt Chloride, 6-hydrate ($CoCl_2 \bullet 6H_2O$)	0,02 g/L
Ammonium Molybdate, 4-hydrate ($(NH_4)_6MO_7O_{24} \bullet 4H_2O$)	0,009 g/L
Copper Sulphate ($CuSO_4 \bullet 5H_2O$)	0,02 g/L
Cyanocobalamin (Vitamin B_{12})	0,005 mg/L
Thiamine, HCl (Vitamin B_1)	0,1 mg/L
Destilled water (to make 1 L Conwy medium)	1000 ml/L

Appendix 4

Daily weight increase

Table A4. *Daily weight increase (%) per larval tank calculated from dry weight.*

Days post hatch	Treatment	Tank number	Daily weight increase (%)
0-2	-	-	-0,014
2-21	*Artemia*	1	0,051
	Artemia	2	0,055
	Copepod	3	0,006
	Formulated feed	4	0,010
	Formulated feed	5	-0,009
	Copepod	6	0,007
	Formulated feed	7	0,016
	Artemia	9	0,056
	Copepod	10	0,021
21-51	*Artemia*	1	0,063
	Artemia	2	0,062
	Copepod	3	0,070
	Formulated feed	4	0,071
	Formulated feed	5	0,089
	Copepod	6	0,070
	Formulated feed	7	0,069
	Artemia	9	0,074
	Copepod	10	0,072
2-51	*Artemia*	1	0,058
	Artemia	2	0,059
	Copepod	3	0,045
	Formulated feed	4	0,048
	Formulated feed	5	0,051
	Copepod	6	0,046
	Formulated feed	7	0,049
	Artemia	9	0,067
	Copepod	10	0,052

Appendix 5

Bone staining procedure

Modified bone staining procedure after Balon 1985 (modified from Taylor 1967 and Dingerkus & Uhler 1977).

Larval standard length	<10mm	10-20 mm
1. Fixation		
- fix in 10% neutral formalin		
- rinse in distilled water	2 x 5’	2 x 10’
2. Rehydration and bleaching		
- 95% ethanol	2 x 30’	2 x 1h
- 50% ethanol	30’	1h
- 15% ethanol	30’	1h
- Distilled water	30’	1h
- Sodium borate buffer (working solution)	Skip	Skip or store
- Bleach in 1:9 3% H_2O_2:1% KOH under strong light	1-2 h	~ 6 h
3. Clearing		
- Clear in trypsin buffer (to almost transparent)	20 h	2-4 days (renew solution daily)
4. Staining		
- Stain bones in Alizarin working solution	20 h	2 days
5. Preservation		
- Rinse in distilled water	5 min	10 min
- Rinse in 1 % KOH	2x	2x
- 40 % glycerol in 1 % KOH	2 days	2 days
- Take pictures		
- 70 % glycerol in 1 % KOH	2-24 h	1 day
- Store in 100 % glycerol		

Appendix 6

Dry weight, wet weight and standard length

Table A6. *Mean larval dry weight (mg/larva), wet weight (mg/larva) and standard length (mm/larva) ± standard error (SE) per tank at sampling days; 0, 2, 6, 10, 13, 17, 21, 24, 29, 34, 38, 42, 45, 50, 51 dph. Sampling size, n =16 at 0-2 dph, and 5-16 per tank 6-51 dph. Wet weight and standard length were not measured at 0 and 51 dph.*

Dph	Treatment	Tank	N	Dry weight (mg/larva)		Wet weight (mg/larva)		Standard length (mm/larva)	
				Mean	SE	Mean	SE	Mean	SE
0	-	-	16	1,1020	0,0143	-	-	-	-
2	-	-	16	1,0674	0,0126	3,1338	0,1409	6,3856	0,0595
6	*Artemia*	1	5	1,1743	0,0622	5,5523	0,6026	6,9958	0,1000
		2	5	1,2322	0,0502	5,8188	0,3165	7,0176	0,0629
		9	5	1,2254	0,0320	6,3042	0,3699	6,8802	0,0950
	Copepod	3	5	1,0574	0,0098	5,3482	0,2914	6,7546	0,1306
		6	5	1,0379	0,0281	5,3349	0,1916	6,5852	0,0606
		10	5	1,0192	0,0461	5,3794	0,2431	6,7045	0,0250
	Form. feed	4	5	1,0658	0,0157	5,8674	0,0878	6,6470	0,1106
		5	5	1,0254	0,0335	5,7482	0,2042	6,7106	0,0696
		7	5	1,0359	0,0139	5,4547	0,2031	6,6752	0,0579
10	*Artemia*	1	5	1,4254	0,0280	8,6514	0,1915	7,3090	0,0289
		2	5	1,3702	0,0591	8,0362	0,4962	7,1694	0,1137
		9	5	1,4015	0,0540	8,3725	0,3554	7,2466	0,0849
	Copepod	3	5	1,0727	0,0536	7,2105	0,4044	7,2040	0,0836
		6	5	1,1154	0,0294	6,9200	0,2390	7,0668	0,0744
		10	5	1,0822	0,0481	6,3542	0,4553	6,9508	0,1102
	Form. feed	4	5	1,0647	0,0507	6,6975	0,3726	6,8578	0,1131
		5	5	1,0838	0,0487	6,8896	0,4783	7,0194	0,119
		7	5	1,0705	0,0430	6,6859	0,4120	6,8278	0,0999
13	*Artemia*	1	5	1,5702	0,0745	9,6536	0,5131	7,4890	0,0933
		2	5	1,7082	0,1019	10,4480	0,6202	7,5612	0,1291
		9	5	1,9112	0,0746	11,6084	0,5245	7,7978	0,0478
	Copepod	3	5	1,1756	0,0308	7,7774	0,2037	7,2506	0,0772
		6	5	1,1616	0,0396	7,3856	0,4555	7,318	0,07
		10	5	1,1614	0,0418	7,2976	0,3135	7,291	0,0676
	Form. feed	4	5	1,0586	0,0390	7,2462	0,3603	7,0056	0,0644
		5	5	0,7892	0,1099	5,9308	0,1372	6,6858	0,0427
		7	5	1,0816	0,0479	7,2654	0,3922	7,225	0,0498
17	*Artemia*	1	5	2,1152	0,1493	13,3300	0,8712	8,0806	0,1227
		2	5	2,2472	0,1442	14,6362	0,9238	8,153	0,1428
		9	5	2,3306	0,1292	14,9812	1,0115	8,2592	0,1117
	Copepod	3	5	1,2672	0,1226	8,8658	1,0834	7,4556	0,1642
		6	5	1,2950	0,0491	9,1080	0,3439	7,372	0,0744
		10	5	1,5292	0,0338	10,9080	0,2822	7,6356	0,0747
	Form. feed	4	5	1,0806	0,0810	7,2976	0,4689	7,0168	0,1511
		5	5	1,1464	0,0753	7,9590	0,5026	7,3122	0,0842

		7	5	1,0882	0,0852	7,3874	0,5917	6,9952	0,1344
21	*Artemia*	1	5	2,8038	0,1497	18,7824	1,0392	8,298	0,1495
		2	5	3,0526	0,1609	20,0410	0,9495	8,5146	0,1261
		9	5	3,0770	0,0637	19,6286	0,3464	8,4188	0,0357
	Copepod	3	5	1,2026	0,0751	9,6476	0,7412	7,5106	0,108
		6	5	1,2300	0,0393	9,3450	0,2597	7,4766	0,0824
		10	5	1,5896	0,1726	12,1140	1,3750	7,9296	0,2065
	Form. feed	4	5	1,3026	0,1499	8,8364	0,6888	7,2618	0,1083
		5	5	0,9010	0,1070	7,1726	0,4775	6,9628	0,1139
		7	5	1,4444	0,1536	9,5992	0,7777	7,3108	0,094
24	*Artemia*	1	5	3,3376	0,4307	21,8600	3,1167	8,7798	0,2991
		2	5	2,8758	0,1632	19,7258	1,4205	8,5686	0,1591
		9	5	3,5970	0,3234	24,8768	2,2138	9,1032	0,1911
	Copepod	3	5	1,4300	0,2073	10,9430	1,5180	7,643	0,2778
		6	5	1,1256	0,0558	9,1678	0,5098	7,2174	0,0544
		10	5	1,4232	0,0630	11,0416	0,5710	7,7358	0,0997
	Form. feed	4	5	1,4366	0,1562	9,7156	1,0288	7,3536	0,1404
		5	5	0,8784	0,1410	6,7734	0,8489	6,6542	0,1288
		7	5	1,3536	0,1074	9,4734	0,7196	7,3524	0,1409
29	*Artemia*	1	5	4,3746	0,4360	30,1576	2,4866	9,548	0,2395
		2	5	4,3482	0,7146	30,2492	4,4361	9,5484	0,3846
		9	5	3,8802	0,3600	29,0650	2,6164	9,3634	0,233
	Copepod	3	5	1,4048	0,4266	10,4596	2,5736	7,1828	0,3654
		6	5	1,0138	0,0352	8,5386	0,3577	6,9402	0,1978
		10	5	1,8400	0,3493	13,2248	1,8411	7,6104	0,2321
	Form. feed	4	5	2,3534	0,1460	15,9828	1,0596	7,8400	0,1745
		5	5	2,0754	0,3204	14,4160	2,4141	7,6252	0,3947
		7	5	2,3482	0,2130	15,4918	1,3086	7,7626	0,2006
34	*Artemia*	1	5	5,9584	1,4367	43,9958	4,3175	10,639	0,3036
		2	5	4,8666	0,3822	35,5176	3,0438	9,7902	0,2627
		9	5	5,7904	0,2134	41,4738	2,2497	10,351	0,1425
	Copepod	3	5	1,9668	0,1697	14,4658	1,5546	7,6044	0,2081
		6	5	2,3986	0,3538	16,7454	2,3736	7,9082	0,3323
		10	5	4,0338	0,1539	26,9128	1,1795	9,1642	0,1294
	Form. feed	4	5	3,0330	0,1982	21,3452	1,6893	8,5452	0,1486
		5	5	3,1742	0,2971	21,4372	1,9255	8,4500	0,1954
		7	5	3,1322	0,1869	22,0026	1,5088	8,7130	0,1773
38	*Artemia*	1	5	7,2386	0,4851	50,4564	3,2886	11,094	0,2412
		2	5	5,6124	0,9096	41,0668	5,2003	10,197	0,4739
		9	5	6,1812	0,7685	43,2096	5,0772	10,48	0,288
	Copepod	3	5	3,5010	0,4466	25,1164	3,1917	9,0186	0,3551
		6	5	3,7472	0,1316	26,6950	0,5291	9,2476	0,088
		10	5	4,6214	0,8337	31,1972	5,1347	9,557	0,4398
	Form. feed	4	5	3,7432	0,4091	26,8306	2,6367	9,1254	0,2550
		5	5	4,4640	0,7094	31,7376	4,4459	9,6552	0,4061
		7	5	4,2268	0,1991	29,4682	1,2027	9,5644	0,1229
42	*Artemia*	1	10	10,5190	1,0464	69,2249	6,5600	12,005	0,336
		2	10	11,0648	0,8474	74,0421	5,4323	12,175	0,2415
		9	10	12,1351	0,9810	78,9734	6,1864	12,359	0,2879
	Copepod	3	10	4,9759	0,5824	33,5764	3,8799	9,711	0,3575

		6	10	4,9263	0,5348	31,2176	3,5159	9,4093	0,3287
		10	10	5,5355	0,5289	38,4745	3,7447	10,159	0,2937
	Form. feed	4	10	5,2912	0,5588	35,2214	3,5779	9,8809	0,3659
		5	10	6,6880	0,5134	43,8399	3,2619	10,711	0,2132
		7	10	6,6435	0,4514	38,6847	2,9511	10,324	0,2799
45	*Artemia*	1	10	12,4184	0,8050	84,5013	5,4450	12,718	0,2609
		2	10	11,3977	0,8155	79,5149	5,8531	12,394	0,2759
		9	10	13,7289	0,9113	91,0799	5,6828	12,985	0,2546
	Copepod	3	10	5,6155	0,6019	38,2026	4,2057	10,1000	0,3135
		6	10	6,9765	0,6931	46,8704	5,3347	10,588	0,3146
		10	10	8,3498	0,8706	54,8289	5,8037	11,066	0,3198
	Form. feed	4	10	6,5733	0,2152	43,5314	1,5968	10,646	0,1162
		5	10	6,6410	0,6400	42,8297	4,0010	10,614	0,3011
		7	10	5,7533	0,6442	38,7526	4,6169	10,052	0,3571
50	*Artemia*	1	10	13,1619	0,9460	90,8448	6,1693	13,0353	0,2736
		2	10	14,8847	0,5883	102,6982	4,1072	13,4739	0,1730
		9	10	18,4941	1,4967	130,6207	11,1748	14,5296	0,2803
	Copepod	3	10	7,2877	0,5800	50,9655	4,2724	10,9112	0,2485
		6	10	8,4511	0,8464	56,8269	5,9019	11,3154	0,3716
		10	10	12,8339	1,1499	82,4471	7,1377	11,3200	0,3416
	Form. feed	4	10	10,2996	0,7360	68,3792	5,1457	11,9391	0,2294
		5	10	10,0479	0,6556	66,0492	4,3842	12,1024	0,1727
		7	10	8,5120	0,7685	58,2835	5,4162	11,3939	0,3361
51	*Artemia*	1	16	18,7041	0,9183	-	-	-	-
		2	16	19,4913	1,9246	-	-	-	-
		9	16	28,6699	1,7950	-	-	-	-
	Copepod	3	16	9,8466	0,7133	-	-	-	-
		6	16	9,9869	0,7936	-	-	-	-
		10	16	13,7808	0,6515	-	-	-	-
	Form. feed	4	16	11,0111	0,8577	-	-	-	-
		5	16	13,1506	0,7822	-	-	-	-
		7	16	11,5961	0,9907	-	-	-	-