Enrichment of rotifers (*Brachionus plicatilis*) with taurine-filled liposomes and taurine-uptake in Ballan wrasse (*Labrus bergylta*) larvae

Thesis for the degree

Master of Science in Aquaculture Biology

Hans Berg Nåvik

Department of Biology

University of Bergen, Norway

May 2018

Alternative front page

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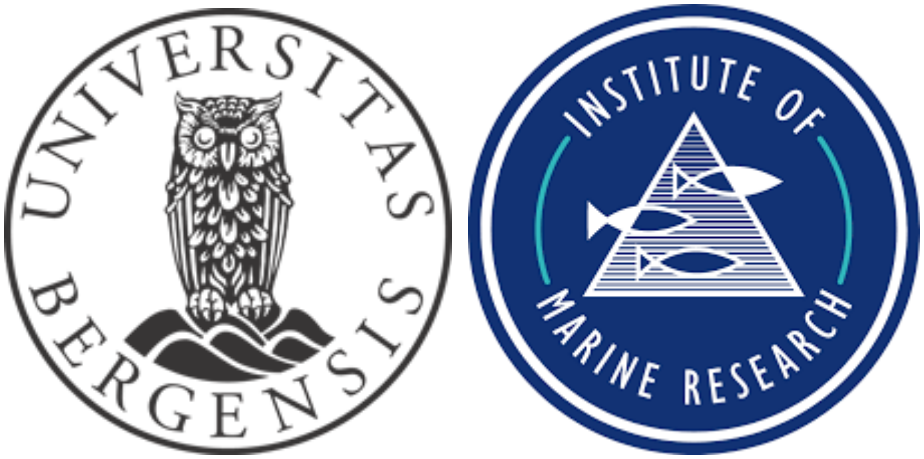

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Last but not least I would like to thank you, the reader. Thank you for reading my thesis, I leave you with a quote from Matthew Hawkyard, which he wrote to me after we discovered high mortality rates in the experimental tanks;

"Welcome to the world of marine fish larvae"

Abstract

Taurine has been reported as a conditionally essential nutrient for several marine fish. Ballan wrasse (*Labrus bergylta*) has become an important species for the Atlantic salmon (*Salmo salar*) industry in Norway. Rotifers are used as start-feed in the intensive production, however taurine concentration has been shown to be nearly absent in conventionally enriched rotifers. As the Ballan wrasse is adapted zooplankton as feed in their larval stages, the taurine concentration found in zooplankton, which is much higher than in rotifers, is assumed closer to the optimum for Ballan wrasse larvae. Therefore, Ballan wrasse is hypothesized to receive too low taurine levels in intensive production.

Liposomes have been used to elevate the concentrations of water-soluble substances, such as taurine in rotifers. Ballan wrasse larvae fed taurine-supplemented rotifers contained significantly higher taurine concentrations than larvae fed both conventionally enriched rotifers, and rotifers enriched with saline water-filled liposomes. This finding indicates that the Ballan wrasse larvae is dependent of taurine through its feed, as they are not likely to be able to biosynthesize taurine in sufficient amounts *in vivo*.

Two experiments were conducted, one investigating the effects of elevated dietary taurine concentration on Ballan wrasse larvae, and a multivariate experiment aimed at investigating the liposome production and enrichment method used in the larval experiment. The larval experiment was affected by production-based difficulties, causing nearly 100 % mortality rates in all tanks, which affected sampling. The larval experiment was concluded at 23 dph, and no significant difference in SL between the treatments were found. Taurine concentration in rotifers fed to the larvae were found to have a significant effect in the taurine concentration in the larval body. Rotifers enriched with taurine had significantly higher taurine concentrations than the control groups. Despite the successful elevation of taurine in the rotifers, the increased concentration observed in taurine-enriched rotifers did not elevate the taurine concentrations enough to cover the assumed required dietary taurine concentration of the larvae, compared to other studies using similar methods.

In conclusion, taurine seems to be an essential nutrient for Ballan wrasse larvae and the current enrichment protocols used in the intensive production of this species do not cover the requirement of taurine. The liposome enrichment method is effective at elevating taurine-levels in rotifers. However, other studies report more successful results in terms of enrichment success of taurine than the present study.

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List of figures, tables and equations

List of abbreviations

AA	Amino acid
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
CDO	Cysteine dioxygenase
CSAD	Cysteine sulfinic acid decarboxylase (EC 4.1.1.29)
DM	Dry mass, in percent of WW of a sample
dph	Days past hatching
DW	Dry weight
FAA	Free amino acid
FDEL	Freeze-dried empty liposomes
GLM	General linear models
2GSH/GSSG	Reduced/ oxidized glutathione
HAA	Hydrolyzed amino acid
IMR	Institute of Marine Research, Norwegian: Havforskningsinstituttet
In vivo	"in the body", endogenous
Nifes	National Institute of Nutrition and Seafood Research. Per 2018, Nifes is a part of IMR
NOK	Norwegian Krone
p	p-value
PH90H	Phospholipon 90H
ppt	Parts per thousand
rpm	Revolutions per minute
SL	Standard length, length from snout to end of notochord
SD	Standard deviation
w v^{-1}	Unit of weight divided by the corresponding unit for volume
WW	Wet weight

1 Introduction

1.1 Salmon farming

In the 1970s the industry of Atlantic salmon (*Salmo salar*) production took shape in Norway. The Norwegian coastline is said to be well suited for salmon production, because of the water temperature and currents. Furthermore, the Norwegian coast is very long, and fish farms may be found along almost the entire coastline. Since industry's beginning, Norway has gradually evolved to be the leading producer of Atlantic salmon. Today, the Norwegian salmon industry is the main producer of salmon to the worldwide market and had a production share of 51 % in 2010 (Asche *et al*, 2013). As well as being an important contributor to the worldwide salmon market, the industry was also important for the Norwegian economy. In 2016 alone, the Norwegian aquaculture industry produced over 1.3 million tons food-fish, of which 93 % was Atlantic salmon, which had a firsthand value of 60 billion NOK. Additionally, in 2016, over seven thousand persons were employed in the Norwegian salmon and rainbow trout (*Oncorhynchus mykiss*) industry (SSB, 2017). As the industry has grown, new problems have arisen, as the number of farms and total biomass has increased. Among these issues is the salmon lice problem.

1.1.1 The salmon lice problem

One of the main problems in the Atlantic salmon (*S. salar*) aquaculture is the salmon louse (*Lepeophtheirus salmonis*) (Costello, 2009; Leclercq *et al*, 2014). The salmon louse is an ectoparasite specialized on salmonid species, such as Atlantic salmon and sea trout (*Salmo trutta* forma *trutta*). Salmon lice have been estimated to cause an enormous economic loss for the aquaculture industry, causing hundreds of millions of Euros in monetary losses annually (Costello, 2009). Furthermore, the increased availability of hosts has caused problems with wild salmonid species, as the lice-pressure has increased. Salmon lice are found naturally on salmon in sea-water, however with the rise of the intensive production of salmon in net-pens, the conditions of the parasite have become increasingly better for growth and transmission (Torrisen *et al*, 2013). Today, infected salmon is treated for salmon lice in several ways, acute actions such as mechanical or chemical delousing is among these. Others include the use of prophylactic means, such as louse-skirts, medicated feed and cleaner-fish. All these measures present their own positive and negative aspects, for example; repeated mechanical treatments can cause negative consequences for the fish in regard to fish welfare (Mattilsynet, 2017). The use of medicaments and chemicals may also cause issues, both for the surrounding ecosystem

(Haya *et al*, 2001) and for future treatments, as the salmon lice may develop resistance towards the different chemicals used (Torrisen *et al*, 2013). Ballan wrasse (*Labrus bergylta*) has been proven to be effective at controlling the amount of louse in a net-pen at a low level. Skiftesvik *et al* (2013) found that the louse prevalence decreased from 9 lice per fish on average to less than one louse per fish on average when Ballan wrasse was present. They also found that each Ballan wrasse individual consumed a minimum of 23 lice per day, and that the cultivated Ballan wrasse were as effective as wild-caught Ballan wrasse (Skiftesvik *et al*., 2013; Leclercq *et al*, 2014). Ballan wrasse has been revealed as an effective means to control salmon lice in the cages, also when compared to other wrasse species.

1.2 Ballan wrasse in aquaculture

The use of cleaner-fish in Norwegian aquaculture is increasing. Each year from 2006 to 2016, the portion of farms utilizing cleaner-fish (of any species) has increased. In 2016, more than 70 % of all producers of salmon in Norway south of Nordland (~65 ° north) used cleaner-fish (Mortensen *et al*, 2017). In 2016, a total of 41.2 million individual cleaner-fish were reported to be used in the salmon industry. Of these it was estimated that 1.5 million were cultivated Ballan wrasse. In 2014 however, the number of cultured Ballan wrasse used were 0.4 million, meaning that the produced number of Ballan wrasse has almost quadrupled in this period. The use of cultivated lumpsucker (*Cyclopterus lumpus*) has also increased in this period, from 3.5 million in 2014 to an estimated 17.5 million in 2016. However, the use of wild-caught cleaner-fish has been relatively steady at around 21 million (Mortensen *et al*, 2017). It is apparent that the use of cleaner-fish is expanding in the Norwegian salmon industry. At the same time, the increase in supply is based in intensive aquaculture.

A shift from wild caught cleaner-fish to farmed fish is important for the industry. Because of the low genetic diversity in Norwegian Ballan wrasse populations (D'Arcy *et al*, 2013) the natural populations are regarded as vulnerable. The reason being that lower genetic diversity increases the populations' susceptibility to "inbreeding depression". This in turn may cause a reduced fitness and a decreased evolutionary potential (Frankham *et al*, 2002). Furthermore, the Ballan wrasse are sequential hermaphrodites, of which it has been suggested that harvesting larger individuals from wild populations may result in an elimination of the less abundant sex (D'Arcy *et al*, 2013; Darwall *et al*, 1992; Sattar *et al*, 2008). As it is important for the salmon aquaculture industry to have access to cleaner-fish, and the fact that the fishery is unable to provide sufficient amounts of Ballan wrasse to meet the demands of

the salmon farms (Skiftesvik *et al*, 2013), an effective production of the species needs to be achieved. Additionally, an effective cultivation of Ballan wrasse would be able to deliver a steady supply of the species, all year round, something the fisheries are not able to do. The Norwegian regulations for fishery of species of the *Labridae* family state that fishery of the species is legal from either the middle or end of July (depending on geographical location) through October (Lovdata, 2005, §34). This regulation states that fishing of these species are limited in time of year, which in turn limits the availability of cleaner-fish from wild-caught sources.

1.2.1 Cultivation of Ballan wrasse

As the species was introduced relatively recent in intensive aquaculture (2009), there are several questions to what the optimal protocol of production is. Several bottlenecks have been identified in the production. Among these, the larval period and weaning seems to be the most limiting periods of production, as high mortality rates occur in these periods (personal communication, Espen Grøtan). The production of Ballan wrasse in Norwegian aquaculture starts with catching brood-stock. Today, the brood-stock fish are locally wild-caught fish, although breeding of brood-stock is something the industry aims to achieve. At the Marine Harvest site in Øygarden, the brood-stock fish are kept in large tanks (~ 100 m^3) and transferred to smaller tanks (9 m^3) during spawning. The eggs are collected on astro-turf mats and put in incubation tanks until hatching. Newly hatched larvae are then collected, counted and transferred to larval tanks (9 or ~25 m^3). As is the case with many marine fish larvae in intensive aquaculture, the larvae are start-fed with rotifers (*Brachionus plicatilis*) before they are weaned onto *Artemia*, as an intermediary feed before the larvae are weaned onto formulated feed. The formulated feed is then increased in pellet-size as the juvenile fish grows, until they are large enough to be used in net-pens to eat salmon lice (*L. salmonis*) of infected Atlantic salmon (*S. salar*). In the last phase at the facility, the fish are kept in on-growing tanks (25 - 100 m^3). The fish is kept at the facility for 12-18 months, from hatching to delivery, depending on how large they are when sold. The largest are up to 60 grams in size, and the minimum size upon delivery is 20 grams, which is used in smolt net-pens.

1.2.2 Rotifers in Ballan wrasse production, and enrichment

Rotifers are used as the start-feeding of choice for many marine fish species in aquaculture. Rotifers are found to contain low amounts of taurine and essential fatty acids, as well as some vitamins and minerals (Hamre *et al*, 2008) when compared to copepods (Karlsen

et al, 2015; van der Meeren *et al*, 2008). Essential fatty acid composition and concentration can be manipulated to a satisfactory degree in rotifers through enrichment (Karlsen *et al* 2015), which is also in accordance to the recommendation by van der Meeren *et al* (2008); that the nutritional composition of rotifers should be altered to be "made in the direction of copepods". However, enrichment of water-soluble substances has proven to be a challenge. At the facility where the present study took place, the rotifers were fed *Chlorella* sp. and enriched with MultiGain® prior to delivery to the larvae, to increase the nutritional value of the rotifers.

Taurine has been identified as a limiting factor in rotifers for optimal larval growth and development, as rotifers have very low concentrations of taurine, especially when compared to copepods (Karlsen *et al*, 2015). Marine fish larvae fed rotifers tend to have a higher proportion of deformed individuals than fish fed mainly copepods (Karlsen *et al*, 2015). Furthermore, growth-rate has been shown to be higher in copepod-fed fish than rotifer-fed fish (Øie *et al*, 2017; Karlsen *et al*, 2015). It has been hypothesized that the positive effects of copepods in comparison to rotifers as fish feed is based of differences in nutritional composition between rotifers and copepods (Hamre *et al*, 2008). Karlsen *et al* (2015) concludes their study with the hypothesis that "Low levels of protein and/ or taurine in rotifers and *Artemia* are most likely the cause of poor growth in larvae fed rotifers".

In spite of the mentioned nutritional deficiencies, rotifers are still widely used in the cultivation of marine fish (Conceição *et al*, 2010), for species where start-feeding with formulated feeds is not possible, such as the Ballan wrasse. The main reason is probably an economic one, as rotifers are relatively cheap to produce, and easy to cultivate and enrich, when compared to copepods (Støttrup, 2002). Liposomes have been used to deliver a range of nutrients, among these free amino acids (FAA) to live-feed in studies on fish larvae nutrition (Barr and Helland, 2007; Hawkyard *et al*, 2015; Hawkyard *et al*, 2016). Essentially, liposomes are artificial cells, built up by phospholipids, which can be filled with desired substances, through rehydration after the liposomes have been freeze-dried. The present study hopes to increase the understanding of the effect of taurine on Ballan wrasse larvae, and verify the hypothesis that taurine is conditionally essential for marine fish larvae.

1.3 Taurine, background

Taurine is the common name for 2-aminoethanesulfonic acid. It is not incorporated in the synthesis of proteins, but rather resides in the free amino acid pool (Hamre *et al*, 2013b). The positive effects of taurine have been studied in mammals for decades (Huxtable, 1992; El-Sayed, 2014). For marine fish on the other hand, these effects have been studied in an increasing frequency in the recent years, and taurine has historically not been viewed as an essential nutrient for optimal growth and development in marine fish (Salze and Davis, 2015). Taurine is found in high concentrations in algae and fish (Huxtable, 1992). Fish meal has high concentrations of taurine, as well as other animal products, in particular marine invertebrate products (Li *et al*, 2009). As mentioned, taurine is found in rotifers only in very low concentrations. Taurine can be biosynthesized *in vivo* from its precursor methionine (Takeuchi *et al*, 2001). In this process methionine is converted to hypotaurine, through cysteine. The enzymes cysteine dioxygenase and cysteine sulfinic acid decarboxylase (CSAD, EC 4.1.1.29) are involved in the biosynthesis of hypotaurine (Gaylord *et al*, 2007). The rate-limiting enzyme for taurine biosynthesis is CSAD (De la Rosa and Stipanuk, 1985). Finally, hypotaurine is oxidized to taurine, through the enzyme hypotaurine dehydrogenase. However, the activity of CSAD has been reported to be varying between species (El-Sayed, 2014; Salze and Davis, 2015). An apparent lack of the CSAD activity has been revealed in the *Labridae, Scombridae* and *Soleidae* families in early studies (Salze and Davis, 2015). Therefore, today taurine is considered an essential free amino acid for several marine fish species. Supplementary taurine may be especially important for larvae and juveniles (Huxtable, 1992), and fish fed plant-based feed, as taurine is found to be absent in non-algae plants (Mæhre *et al*, 2013).

The physiological roles of taurine in marine fish larvae are not completely understood. However, taurine serves an important osmoregulatory function. Additionally, taurine has a role in calcium modulation and functions as an antioxidant, taurine is also involved in neurotransmitter modulation, membrane stabilization and early development of the retina, as well as neural and muscular systems (Pinto *et al*, 2010; Huxtable, 1992). Furthermore, taurine is used for cell volume regulation and bile salt synthesis (Hamre *et al*, 2013b), and may therefore be important for lipid digestion. Huxtable (1992) also argues that taurine has an enantiostatic effect in organisms, a term that is defined as a regulation that "occurs when the effect of change in one chemical or physical property of the internal milieu is opposed by a change in another. Thus the internal milieu is unstable, but the net effect of the flux on a

particular physiological system is stability, or a tendency toward it" (Mangum and Towle, 1977). Furthermore, Huxtable (1992) points out that the developing brain is where the highest concentrations of taurine are found within an organism, and with development this concentration decreases. This pattern has been observed in humans, monkeys, mice, rabbits and rats (Huxtable, 1992). Takeuchi *et al* (2001) showed that taurine concentration in red sea bream (*Pagrus major*) decreased right after hatching. However, to the candidates' knowledge, this has not been examined in the developing brain of other marine fish larvae.

1.4 Nutritional requirements

As stated in Hamre *et al* (2008); "It is important to optimize all these factors (environmental factors, bacterial and viral activity and nutrition) to obtain good culture practices". Considering that the intensive production of Ballan wrasse in an industrial manner is relatively young, the species' dietary requirements and optimal rearing conditions are not fully understood.

There are some studies comparing the effects of different live-feeds on Ballan wrasse (Øie *et al*, 2017; Sørøy, 2012; Almli, 2012; Gagnat, 2012), all of whom found positive effects in larvae fed zooplankton. Taurine deficiency is hypothesized to be an issue in Ballan wrasse production, as taurine has been identified as one of the most plausible reasons to the positive effects observed in marine fish species when fed zooplankton, in comparison to rotifers (Karlsen *et al*, 2015; Almli, 2012; Øie *et al*, 2017). Nonetheless, there are no studies, to the candidates' knowledge, investigating the actual requirements of taurine for the species. Therefore, there is a need to assess the requirements of the species, to provide sufficient amounts of nutrients in the feed of the fish, and thereby increase both productivity and fish welfare at aquaculture facilities cultivating the species.

1.5 Previous scientific work on the effect of taurine on marine fish larvae, and liposomes

Generally, marine fish larvae have been found to lack the ability to biosynthesize taurine (Al-Feky *et al*, 2016; Goto *et al*, 2001; Salze and Davis, 2015; El-Sayed, 2014). Taurine has been shown to cause an increase in growth-rate in northern rock sole (*Lepidopsetta polyxystra*; Hawkyard *et al* 2015), pacific cod larvae (*Gadus microcephalus*; Matsunari *et al* 2005) and Japanese flounder (*Paralichthys olivaceus*; Chen *et al*, 2005) and increase protein retention in the larval stage and metamorphosis rate in Senegalese sole (*Solea senegalensis*; Pinto *et al*, 2010). Katagiri *et al* (2017) reports that taurine improves growth and

shortens development period in Pacific bluefin tuna (*Thunnus orientalis*) and yellowfin tuna (*Thunnus albacares*). Earlier onset of metamorphosis in northern rock sole has also been reported (Hawkyard *et al*, 2014), as well as increased metamorphosis rate in Senegalese sole (Pinto *et al*, 2010). In addition to improved growth, taurine has been shown to improve development, feeding-rate and settling-behavior in Japanese flounder (Chen *et al*, 2005; Takeuchi *et al*, 2001). One study on gilthead seabream did not show direct beneficial effects from elevated levels of dietary taurine (Pinto *et al*, 2013).

Hawkyard *et al* (2015) examined the retention of taurine in liposomes, for up to 1 hour. They reported that the taurine concentration in the liposomes were reduced to 85 % of the initial concentration (liposomes were rehydrated in 10% (w v^{-1}) taurine solution) after 5 minutes spent suspended in seawater, no further decrease in concentration were observed throughout the sampling. Barr and Helland (2007) reported retention of free amino acids in liposomes of 90.9 %, after 2 hours suspended in seawater. The greatest losses occurred in the first 5 minutes. As per the candidate's knowledge, no studies regarding the retention of taurine in rotifers exists at this time. Brown *et al* (1998) showed that rotifers retained ascorbic acid for 24 hours with no significant changes in concentration. Srivastava *et* al (2012) found that concentration of iodine in enriched rotifers declined rapidly, and were nearly absent two hours after enrichment. Furthermore, enrichment of rotifers with liposomes with varying concentrations of taurine is not previously studied, neither has the effect of storage on liposome-enriched rotifers regarding taurine concentration, to the candidates' knowledge.

Several papers have described that for several marine fish species, including *L. bergylta*, live feed type does not have a significant effect on larval survival rates (Koedijk *et al*, 2010; Almli, 2012; Hamre, 2002). Thus, it was not expected that taurine should increase the survival rate of the larvae. However, physiological factors, such as an increased growth rate, was expected to be seen in the larvae, as this has been proven for several marine fish species after increased dietary taurine supplements (Matsunari *et al*, 2005; Pinto *et al*, 2010; Chen *et al*, 2005; Katagiri *et al*, 2017; Conceição *et al,* 2010; Pinto *et al,* (2010)). Based on current literature, increased dietary taurine is expected to increase the growth-rate of the *L. bergylta* larvae.

1.6 Hypothesis and aims of the study

Hypotheses:

- Taurine is an essential nutrient for Ballan wrasse larvae.
- Taurine levels in rotifers fed to Ballan wrasse larvae are too low, which in turn causes a deficiency of taurine in the larvae.
- Enrichment of taurine through liposomes is an efficient way of elevating taurine concentrations in rotifers.

The present study aimed to investigate the effects of elevated dietary taurine levels on Ballan wrasse (*Labrus bergylta*) larvae and establish a functional method for introducing these elevated concentrations of taurine in rotifers, based on the methods developed by Barr and Helland (2007) and described in Hawkyard *et al* (2015). When investigating the effect of taurine on Ballan wrasse larvae; standard length (SL), taurine concentration (whole body) and dry weight (DW) samples were taken at the facility hosting the experiments (Marine Harvest site in Øygarden). For development of an effective protocol for elevating taurine levels in rotifers a multivariate experiment was carried out, to identify significant variables affecting enrichment success and an optimal protocol was determined based on the tested variable values. However, it is important to note that the optimal protocol is only the optimum between the tested values and may change should the different variables change.

2 Materials and methods

2.1 Liposome production

The liposomes produced were either filled with saline water (Empty) or a solution of taurine (Tau). The first trials of liposome production started early April 2017 and lasted until early June 2017. The methods used to produce liposomes were based on the methods described by Barr and Helland (2007) and Hawkyard *et al* (2015). Liposomes were produced using Phospholipon 90H (P90H; Lipoid, Frankfurt, Germany), which is a hydrogenated phosphatidylcholine from soybean with 16:0 (palmitic acid) and 18:0 (stearic acid) as the predominant fatty acids (product technical data). The production of freeze-dried empty liposomes was identical for each trial.

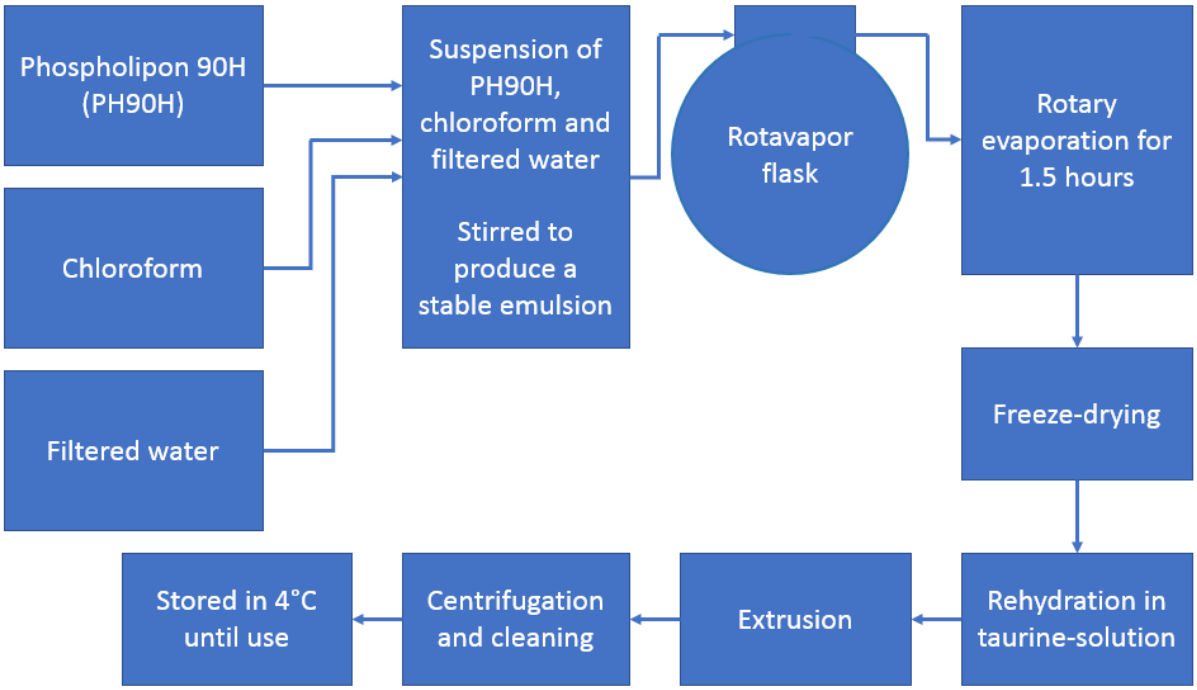

Figure 2.1.1: *Flow-chart of the liposome production. Schematic overview of the extrusion process is presented in figure 2.1.2.*

The liposome production consisted of several steps, presented in figure 2.1.1, starting with the production of freeze-dried empty liposomes (FDEL). All work with organic solvents was performed in a fume hood. The first step was to dissolve P90H in chloroform; 37.5 mg of P90H was added to 750 mL chloroform (Sigma Aldrich) and stirred using a magnet stirrer until the P90H was dissolved. Thereafter, 750 mL of filtered water (Millipore, Milli-Q) was added to the solution under stirring. The solution then started to thicken and obtained a white

color. A sonicator (VibraCell^{TM} Sonics & Materials INC, Danbury, CT, USA) was used to dissolve the water, chloroform and P90H solution. The sonicator was used at 60-70 amplitude for 15 minutes, together with the magnet stirrer, until a stable emulsion was attained. The next step was to transfer the stable emulsion to a rotavapor-flask (round-bottomed receiving flask) and start the evaporation process. This process used a rotary evaporator (Rotavapor R-124, BÜCHI) to evaporate all chloroform from the solution, using vacuum and temperature manipulation, leaving only the phospholipids and water. The temperature was set to 60 °C and the rotation of the rotavapor flask to 110-125 rpm (revolutions per minute). The vacuum pressure was unknown since there was no accurate method to control or measure it. Nitrogen gas (N_2) was also pumped into the rotavapor flask to control the vacuum pressure.

The evaporation process was broken down into several steps and lasted for 1.5 hours. During the first 25-30 minutes most of the chloroform had evaporated. This was followed by an increase of emulsion volume as foaming started to occur. During this time, it became important to reduce the vacuum pressure in the system, either by reducing the vacuum pump, or increasing the N_2 influx, to contain the solution in the rotavapor-flask, and keep it from spewing into the instrument. Throughout the remaining 1 hour of the evaporation process, evaporating from the solution gradually decreased until no evaporation occurred. After 1.5 hours in the evaporator, liposomes filled with and suspended in water had been formed, and the solution was transferred to small, flat, plastic containers. These were marked and weighed and then frozen to -20 °C. The liposomes were then freeze-dried for at least 72 hours, until freeze-dried empty liposomes (FDEL) were ready to be rehydrated. The freeze-drying process removes any last traces of chloroform (Hawkyard *et al*, 2016).

The rehydration-process was the next step in the liposome production. The liposomes were filled with a solution of taurine (6 % or 10 % (w v^{-1}) in the first and second initial trial, respectively) and 3.5 % (w v^{-1}) NaCl (Natrium-chloride) was added to match core osmolality of the liposomes to the osmolality of the seawater which would later be used in the rotifer enrichment. Then, 100 mg FDEL ml^{-1} was rehydrated in the preheated (60 °C) taurine-NaCl solution and stirred for 15 minutes, using a magnet-stirrer. After a stable emulsion of rehydrated liposomes was attained, the emulsion was extruded using a 22 gauge (inner diameter; 0.41 mm) smooth-flow tapered tip (Henke Sass Wolf, Tullingen, Germany). The extrusion was important to obtain the correct liposome sizes. A peristaltic pump was used for the extrusion, and the process lasted until all of the liposomes had passed the needle and had

been transferred to 45 ml centrifugation-tubes (Superclear^{TM}, VWR®) to ensure all the liposomes were extruded.

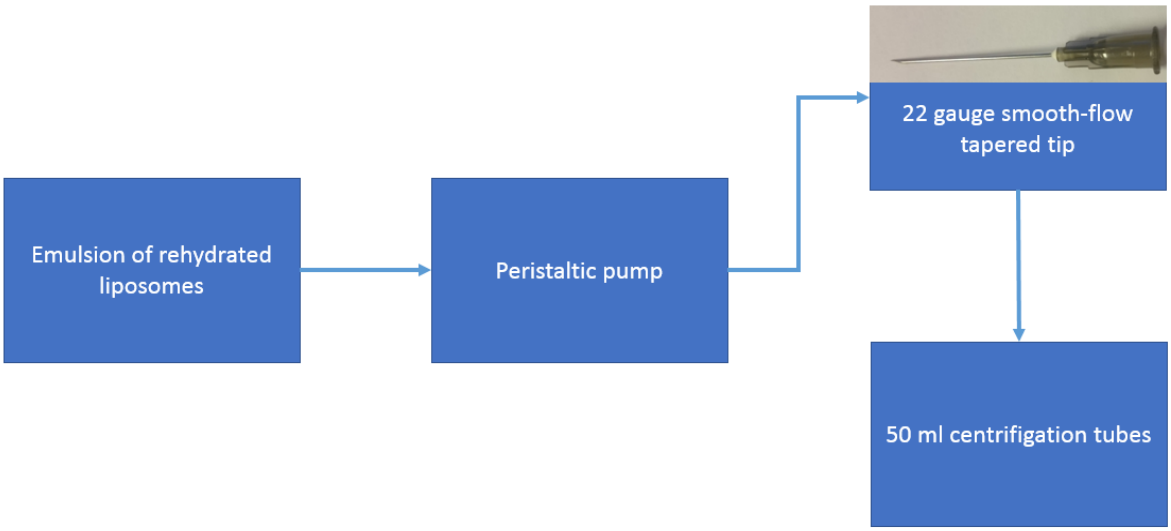

Figure 2.1.2: *Flow-chart of the extrusion process. Blue lines represent tubing.*

After the extrusion was finished, the tubes were centrifuged for 15 minutes at 3500 rpm, to produce a liposome pellet. The supernatant was removed and replaced with filtered water with a NaCl concentration of 3.5 % (w v^{-1}). This process was repeated 3 additional times. The taurine-filled liposomes were then stored in a refrigerator (4 °C) until used. Saline liposomes, or "Empty" liposomes, were also produced using the same method, with the exception that no taurine was added to the rehydration medium. The same method as the one described in this segment was used to produce liposomes for the larval and rotifer experiments, except the larval experiments' liposomes were rehydrated in 0 or 6 % (w v^{-1}) taurine solution, while liposomes used in the rotifer experiments were rehydrated in either 3, 6 or 9 % (w v^{-1}) taurine solution. Other factors varied in the rotifer experiment as well, which is presented in chapter 2.2.2.

2.2 Enrichment of Rotifers

2.2.1 Rotifer enrichment during preliminary tests

Prior to the enrichment of the rotifers (*B. plicatilis*), they were continuously fed *Chlorella sp.* (1.8 ml million rotifers^{-1} day^{-1}). Enrichment of rotifers was done at the production facility, in their live-feed department. Four buckets were added seawater from the facility, as well as liposomes (250 mg L^{-1}) and rotifers (500 000 L^{-1}). First, liposomes with 6 and 10 % (w v^{-1}) taurine were tested. Aeration was added, and the buckets were left undisturbed for 1 hour. Following this, rotifers were placed in a sieve (75 µm mesh-size) and rinsed with clean seawater. Then the sieve was dried with tissue-paper from the bottom of the sieve, to the rotifers presented itself as a matt mass in the sieve and were transferred to cryotubes using a spatula. The cryotubes were then put on ice and taken to the lab at Nifes to conduct analysis.

2.2.2 Multivariate experiment with optimization of taurine enrichment in rotifers

For the rotifer enrichment experiment, the enrichment duration varied between the different trials. Study design is presented in table 2.2.1. Enrichment durations of 0.5, 1 and 1.5 hours were used, the amount of added liposomes (200, 250 and 300 mg L^{-1}) and concentration of taurine in the liposomes (3, 6 and 9 % (w v^{-1}) taurine) also varied. The different traits were given codes based on the numerical value of the trait, see table 2.2.2. All combinations of 0 and 1 were tested, for example; one tested rotifer enrichment was 0, 0, 1. This code represents a taurine concentration in the liposomes of 3 % (w v^{-1}), a liposome concentration in the enrichment water of 200 mg liposomes L^{-1}, and an enrichment period of 1.5 hours. In addition to these tests, 9 additional tests were carried out with the following code: 0.5, 0.5, 0.5. These samples were used to determine the retention of taurine in the rotifers when stored at 4 °C and provide a center-point for the experiment. Three samples of the center-point group were collected on a sieve immediately after the enrichment-period (1 hour) and constituted the center-point, three samples were stored in 4 °C for 3 hours before collection, and the last three were stored for 21 hours in 4 °C. The durations and temperatures were chosen based on how these variables were during the larval experiment (3, 9, 15 and 21 hours after enrichment, the rotifers were fed to the larvae) and due to practical reasons; collecting after 0, 3 and 21 hours while stored in 4 °C were chosen for the study design.

Table 2.2.1: *Experimental design of the multivariate experiment. See table 2.2.2 for variable codes. The design was made with regards to a multivariate approach to the enrichment-process, where different variables are tested for their contribution to enrichment success. This way, it is possible to optimize the enrichment process.*

Treatment	Taurine (% w v^{-1})	Liposomes (mg L^{-1})	Enrichment time
1	0	0	0
2	0	0	1
3	0	1	0
4	0	1	1
5	1	0	0
6	1	0	1
7	1	1	0
8	1	1	1
9 - 11	0.5	0.5	0.5

Table 2.2.2: *Variable codes used in the multivariate experiment*.

Variable	0	0,5	1
% (w v^{-1}) taurine	3	6	9
mg liposomes L^{-1} enrichment water	200	250	300
Enrichment time	0,5	1	1,5

After collecting the rotifers in a sieve (75 µm mesh-size), the rotifers were placed in marked 1,8 mL cryotubes and stored in the on-site freezer (-20 °C), until they were transported by car on ice to Nifes (approximately 1 hour drive) and stored in -20 °C at Nifes until analyzed. The analyses performed on the samples were dry weight (at 104 °C) and the hydrolyzed amino acid method used for all taurine-content analyzes in this paper, described in its own section.

2.2.3 Rotifer enrichment during larval experiment

Rotifers were enriched at the Marine Harvest facility for production of Ballan wrasse, Øygarden, at their live-feed-department. Rotifers were enriched in 10-liter buckets with a density of 500 rotifers ml^{-1}, with either the control treatment ("Empty" liposomes; 250 mg liposome phospholipid L^{-1}) or the Tau treatment (250 mg taurine-filled liposomes L^{-1}). Since the experiment consisted of an estimated equal number of larvae used in each treatment, 50 % of the enriched rotifers were enriched with the control treatment, and the other 50 % were enriched with the Tau treatment. Furthermore, an additional treatment (MultiGain) was added to the experiment. The MultiGain treatment consisted of the same enrichment used on the facility in its normal production (MultiGain®, Biomar, Myre, Norway), and was conducted by the employees at the facility. Every third day antibiotics (Florfenicol, up to 0.8 g L^{-1}) were added to the enrichment during the larval experiment. The MultiGain treatment rotifers were enriched with 0.2 g MultiGain® per million rotifers per day. Rotifers used for the Empty and Tau treatments were collected from the same batch of unenriched rotifers as the ones used in the MultiGain treatment. The enrichment process is presented in figure 2.2.2.

Rotifers were washed to exclude excess foodstuffs and debris for at least 2 hours before being transferred to the enrichment buckets. The washing was conducted by the workers at the facility. An appropriate volume of unenriched rotifers was taken from the washer so that 4.5 million rotifers were extracted from the washer for each bucket (based on the estimated density of rotifers in the washer). The rotifers were placed in the buckets, and water (35 ppt NaCl) was added, until a total volume of 9 L. Aeration was turned on to produce a stirring effect, and each bucket had the same air pressure in the aeration. Following this, the enrichment was added. Empty or Tau liposome enrichment (250 mg L^{-1}) were then added. Then the rotifers were enriched for 1 hour. The liposome enrichment procedure is equal to that described by Hawkyard *et al* (2015), except the use of buckets instead of cones.

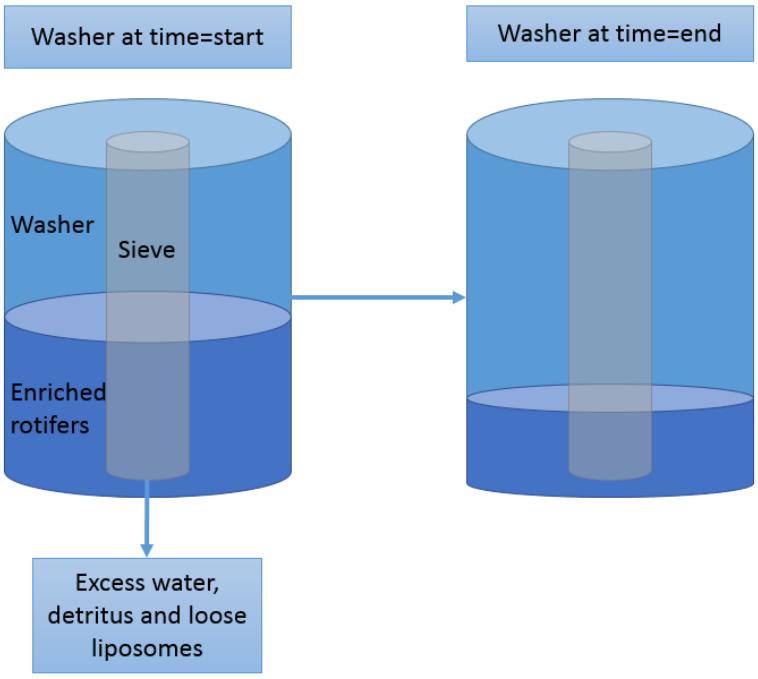

Figure 2.2.1: *Principle of the washing process. The sieve contains the rotifers in the washer, while excess water and other smaller objects pass the sieve. The remaining water has a higher concentration of rotifers, allowing for a smaller container to store them in.*

After 1 hour of enrichment, the rotifers were transferred to a washer to reduce the amount of water in the buckets to approximately 7 liters (in total for each treatment), presented in figure 2.2.1. This was done to be able to store the buckets in a cooler-tank (4 °C). Furthermore, the washing removed leftover liposomes, while containing the rotifers in the washer. What was left was one bucket for each treatment, these were then transferred to the cooler-tank, and aeration was added to the buckets. This process was done once each day during the larval experiment.

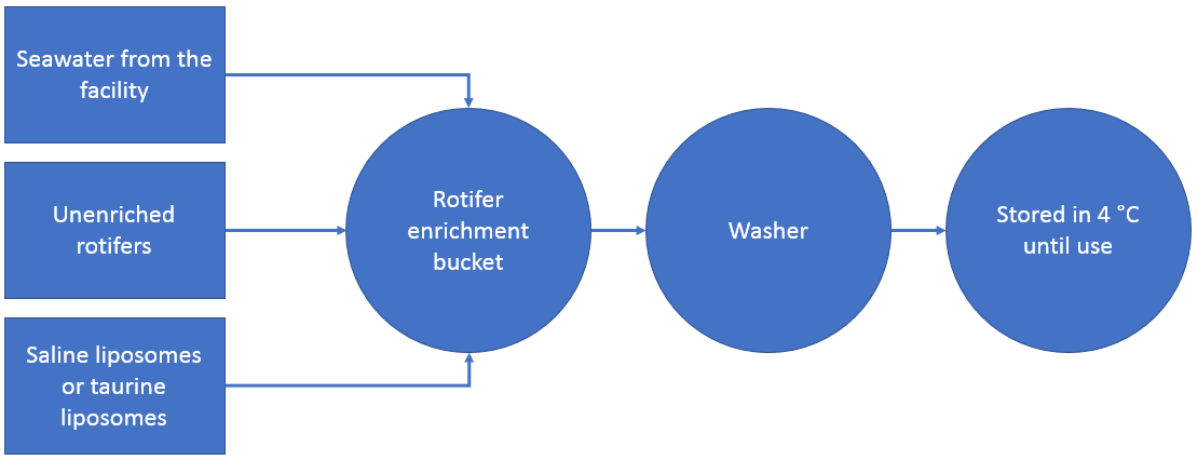

Figure 2.2.2: *Overview of the enrichment process.*

2.2.4 Commercial enrichment protocol

Rotifers at the facility were fed *Chlorella* sp. (1.8 ml per million rotifers per day) continuously. Prior to enrichment of MultiGain® the rotifers were washed in a washer for at least 2 hours, until debris and leftover feed were removed from the rotifer containing water. After washing, the rotifers were enriched with MultiGain® (0.2 g per million rotifers) for 1.5 hours, after which the enriched rotifers were stored in holding tanks in 4 °C until delivery to the larvae. During the last half hour of enrichment, a wide spectrum fungicide and bactericide (Pyceze®; Novartis, Oslo, Norway) (0.3 ml L^{-1}) was added to the enrichment water.

2.3 Larval experiment

Larvae were fed rotifers enriched with Empty or Tau liposomes or with the enrichment diets used by the commercial production facility (MultiGain®). The larval experiment started 10. October 2017 on Marine Harvest facility for producing Ballan wrasse, Øygarden, Hordaland, Norway, and lasted until 23 dph (days past hatching). The tanks were washed and disinfected with FPC (Arrow FPC with foam, Arrow) and Perfectoxid (Perfectoxid with foam, Aco Hygiene) prior to the start of the experiment. Surface-sieves (315 µm mesh size), as well as feeding pumps, clay pump and a tubing-system was installed in the tanks prior to the transfer of larvae. The rotifer pumps were calibrated to ensure each tank received the correct number of rotifers during automated feeding. The clay was pumped directly into the water line, ensuring the tanks received the same amount of clay. An overview of input and output from the tanks is presented in figure 2.3.1. Sampling containers were prepared for each tank, and the influx of water was calibrated to approximately 0.9 L^{-min}. In total; 9 tanks were used in the experiment, these were marked numerically, where tanks 1 through 3 was used for the Tau treatment group, tanks 4 through 6 was used for the MultiGain treatment group and tanks 14 through 16 was used for the Empty treatment group, see figure 2.3.2. There were two waterlines in the experimental room containing the tanks, with eight tanks on each line. All tanks except for tank 6 were on the same waterline, see figure 2.3.3. However, the two waterlines collected the water from the same exterior waterline, meaning the origin of the water was always identical for each tank.

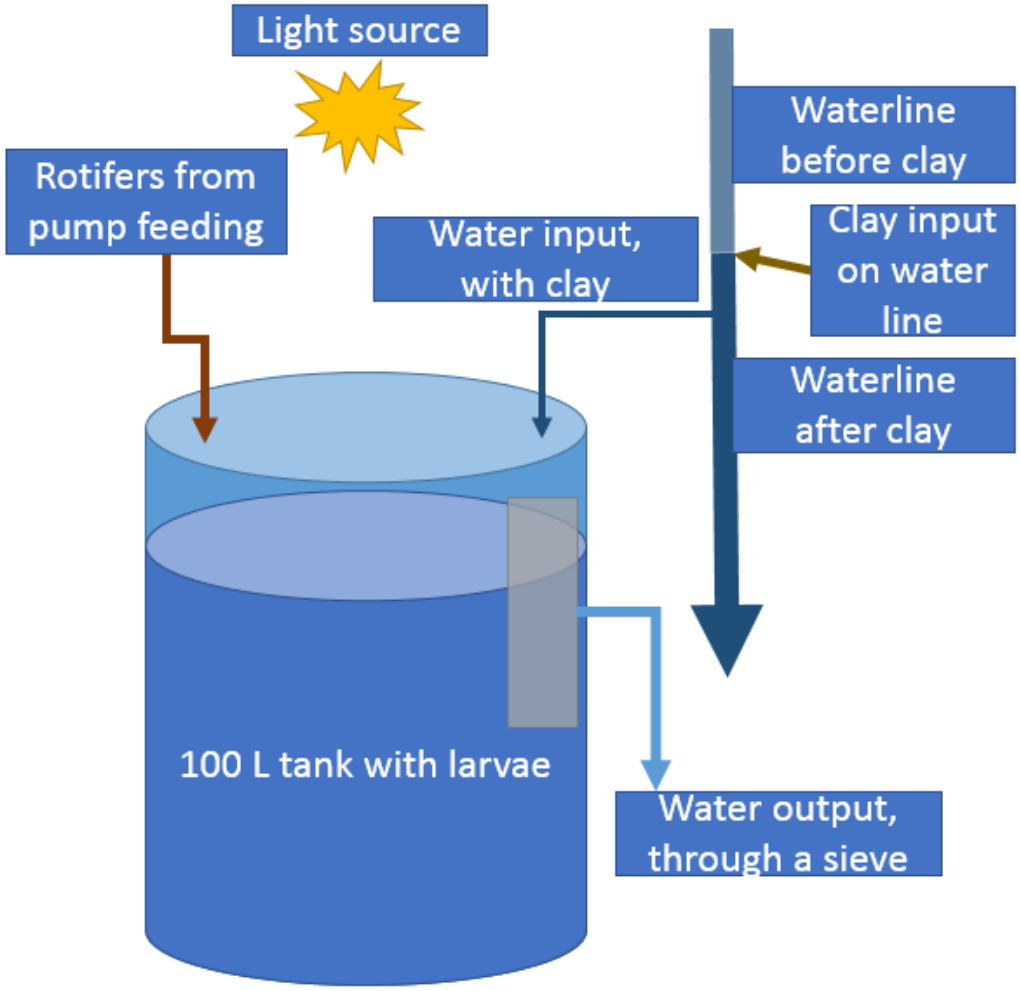

Figure 2.3.1: *Overview of input and output for each tank. In addition to this, handfeeding of the tanks' respective treatment was conducted, a schematic overview of this is presented in figure 2.3.2. Waterline inlet and clay are presented in figure 2.3.3. Each tank had identical designs*.

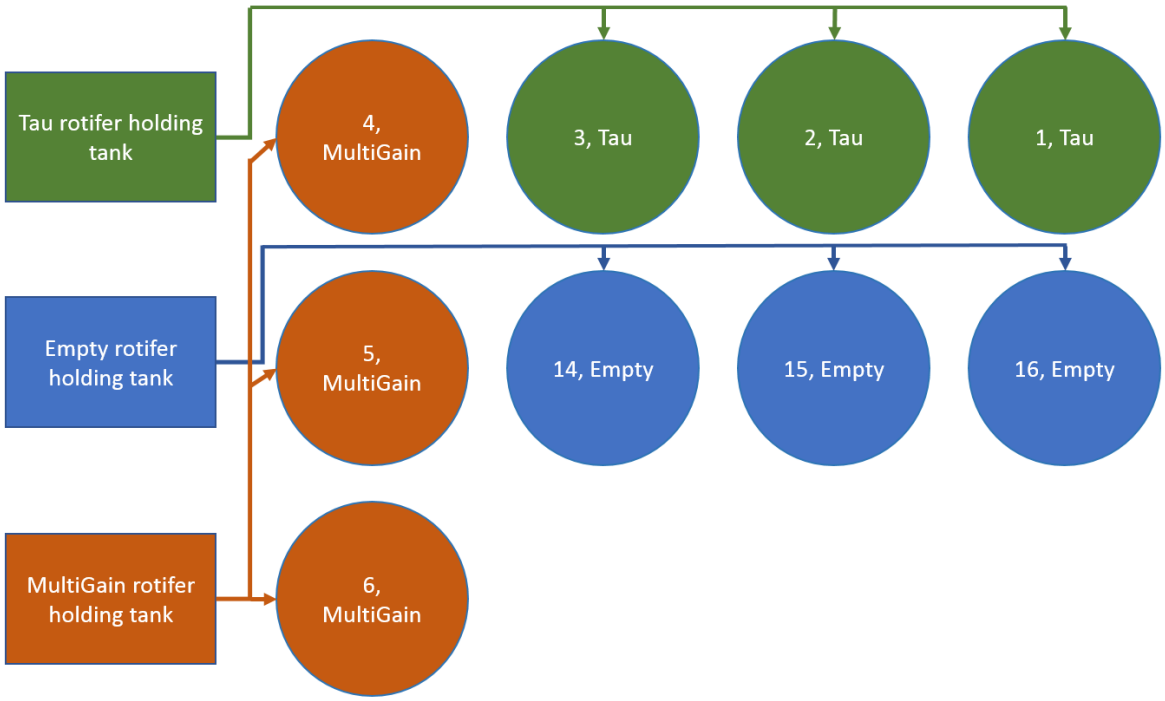

Figure 2.3.2: *Overview of experimental design and hand-feeding regime. Green indicates Tau treatment; blue indicates Empty treatment and orange indicates MultiGain treatment*.

The larvae originated from 8 different tanks of spawning brood stock, whose eggs were collected on specialized Astroturf mats overnight, which were placed in an incubator. In the incubators, the eggs were treated with Pyceze® and formalin (Aquacen, Cenavisa S.L.). The eggs stayed in the incubators with constant water-flow for 10 days until they hatched. After hatching, a 250 L conical collecting tank was connected to the incubator with tubing and the sieve (315 µm mesh size) by the outlet of the incubator was removed and aeration was added to the collecting tank. The collecting tank was connected to the incubator for approximately 24 hours, until all larvae in the incubator had been transferred to the collecting tank. Then the collecting tank was moved closer to the experimental tanks, aerated and added 75 ml Pyceze®. For one hour, the collecting tank was minimally disturbed to let the Pyceze® disinfect the larvae. Aeration made the larvae distribute throughout the tank and the density of larvae was estimated by taking out aliquots and multiplying upwards.

After one hour of disinfecting, 10 000 larvae were taken from the collecting tank using a measuring container, and placed gently in the experimental tanks. For each transfer, the density of larvae in the measuring container was estimated using aliquots and multiplying, to ensure the number of larvae was as close to 10 000 as practically possible. Since all larvae originated from the 8 tanks of brood-stock, not all individuals had the same parent

individuals. However, since the incubator and collecting tank was under constant stirring and mixing, it is reasonable to assume each tank had approximately the same number of larvae from each parent wrasse.

Clay was prepared by dissolving 1 kg of clay (type K-148, Alt for keramikk AS) in 50 L seawater using a handheld mixer. Then the dissolved clay was transferred to a conical tank (250 L) and diluted further in seawater (35 ppt, 12°C), with aeration to ensure sedimentation was minimal. Additionally, 75 ml Pyceze® was added to the tank to disinfect the clay. Clay was pumped onto the waterline at a velocity of 10 L h^{-1}, and a new batch of clay was prepared each day.

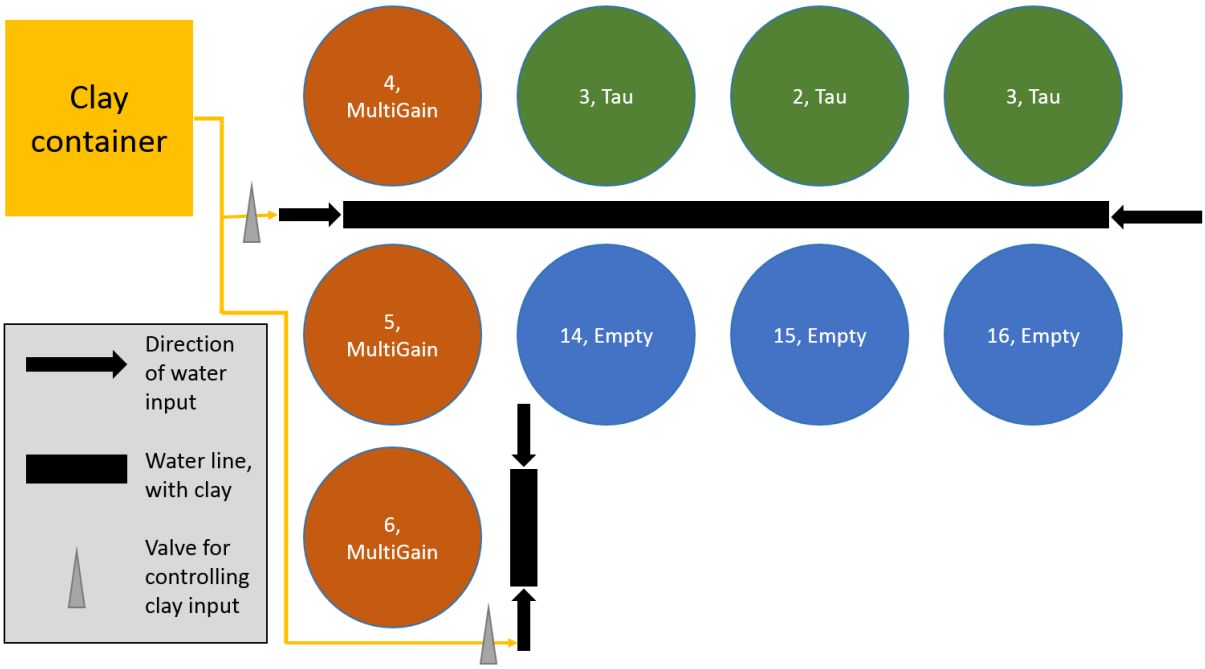

Figure 2.3.3: *Overview of clay and water inlets, with water being added to the water line from two directions.*

During the first three days, the larvae were not disturbed in any way. Day four, the clay pump was started (10 L h^{-1}), and after the clay had spread throughout the tank, light was turned on above each tank. Clay was used since it has been showed that the presence of clay is favorable for the survival and growth for marine fish larvae (Attramadal *et al*, 2012). Enrichment of rotifers was conducted, and rotifers for the MultiGain group were added to a separate bucket in the cooler-tank. Rotifers used in the MultiGain group were collected from a separate cooler-tank (4 °C) the facility normally uses to store enriched rotifers. The feeding regime used in the experiment was the same as in the facility, with feeding every 6 hours, starting at 14:00 (14:00, 20:00, 02:00 and 08:00). The first feeding was conducted at 4 dph

(13. September 2017), at 14:00. The 20:00 and 02:00 feedings were done through pump feeding, and all tanks received the MultiGain® enriched rotifers during these feedings, to ensure the larvae could cover their dietary needs for nutrients. The 14:00 and 08:00 feedings were done by handfeeding, using small containers.

Starting day 6 (15.09.2017), the following regime was repeated daily until the end of the experiment; handfeeding 08:00, enrichment of rotifers started 10:00, tending the tanks 10:00, transfer the enriched rotifers to cooler-tank 11:00, handfeeding 14:00. During the time the rotifers were being enriched; the sedimented clay, foodstuffs and debris were carefully collected using a bottom-brush, in such a manner that minimal stirring of debris into the water-column occurred. A siphon was used to extract the collected matter into a sieve (315 µm mesh size), which contained the dead larvae, but not debris. The larvae were then transferred to a measuring container and stirred to produce an equal distribution in the container. Aliquots were taken to estimate the number of dead larvae. The bottom-brush and siphon were thoroughly rinsed with water between each tank, and disinfected after the last tank, using Perfectoxid. This process started with the MultiGain treatment tanks (tanks 4, 5 and 6), followed by the Empty treatment (tanks 14, 15 and 16) and lastly the Tau treatment tanks (tanks 1, 2 and 3), this regime was used to reduce the probability of transferring unwanted substances between the treatment-groups, particularly taurine-filled liposomes. The same regime was used to measure the oxygen-content and temperature in the tanks, using a handheld probe (Handy Polaris 2, Oxyguard). Dietary taurine concentrations delivered to the tanks were calculated using equation 2.3.1.

Equation 2.3.1: *Estimated dietary taurine equation. As the Empty and Tau treatments were co-fed MultiGain®-enriched rotifers, the dietary taurine had to be estimated. The larvae in these treatments were fed 50 % treatment rotifers and 50 % MultiGain rotifers. MultiGain group larvae were fed 100 % MultiGain®-enriched rotifers. [Taurine] represents taurine concentration in mg g^{-1} DW, MG represents MultiGain and rot represents rotifers.*

$$Estimated\ dietary\ taurine = ([Taurine]\ MG\ rot + [Taurine]Treatment\ rot)/2$$

Abiotic factors such as temperature, salinity and O_2 saturation were not regulated by the candidate, as the rig of tanks were connected to the facility waterline. However, these three factors were controlled daily. Temperatures were recorded to be very stable between the tanks; with the highest deviation between two tanks at the same day were 0.2 °C. The temperature of the tank water varied however, from 1 dph to 10 dph, the temperature was between 12.7 and 13.5 °C. From 11 dph to 15 dph, the temperature was gradually increased to 17.5 °C. From 16 dph to 23 dph, the temperature varied from 16.3 to 17.1 °C. Salinity was constant at 35 ppt (parts per thousand). Oxygen saturation was stable in the tanks throughout the experiment; however, there were some differences between the tanks. The lowest recorded oxygen saturation was in tank 1, at 1 dph with 85 % saturation. Only one measurement after this one was below 90 %, which was in tank 3, at 17 dph. Other than these observations, the oxygen saturation levels were between 92 and 98 %. Dissolved oxygen levels were between 7.1 and 8.8 mg L^{-1}.

2.4 Measurements

Samples for standard length (SL) analyzes were taken at 1, 9, 14, 19 and 23 dph. Samples for dry-weight analyzes were taken at 1 and 23 dph.

2.4.1 Standard length

At 1 dph, newly hatched larvae were sampled from the collecting tank, photographed for length analyzes, rinsed in freshwater before being transferred to marked cryotubes (Cryotube™ vials, Thermo Fischer Scientific, Nunc AS, Roskilde, Denmark) and put on dry-ice, for dry-weight and taurine content analyzes. Samples for SL and dry weight analyzes were sampled from each tank using a measuring container by collecting water from the tank and extracting larvae from the container, this was done until approximately 30 larvae were sampled. The larvae were photographed using a camera-phone (Sony Xperia Z3) under a loupe and transferred to cryotubes after being rinsed in freshwater and stored in liquid nitrogen. The photographed larvae were then analyzed for SL using ImageJ (ImageJ 1.51k, National Institutes of Health, USA) by measuring the length from the tip of the larvae's snout to the end of the notochord. Paper with known distances (mm-scale) was used to calibrate the measuring. Figure 4.4.1 presents an example of the SL measurements.

2.4.2 Dry weight

Larval samples were counted and washed in freshwater, to remove excess NaCl and other components in the water, to be able to calculate the mean DW larvae^{-1} in the sample. The samples were dried at 104 °C, after filtration using cotton-pieces and vacuum. However, the filtration process ruined the samples, and no comparative results were possible to be attained from the samples. This was due to a misunderstanding between the laboratory technicians and the candidate, as the samples were not supposed to be filtered. Instead the following routine should have been applied to the process; the sampling tubes should have been weighed before sampling, and then the samples should be dried in 104 °C, then re-weighed. This way, it would be possible to calculate the dry-weight of the larvae sample, by subtracting the pre-sampling tubes' weight from the final weight (after drying), then total DW of the sample would be known. And since the number of larvae in each sample was known, the mean weight could be calculated.

2.4.3 Total amino acids

Total amino acids were analyzed according to Cohen *et al* (1989) to give a measure of the taurine concentration in liposomes, rotifers and larvae. In this method, subsamples were hydrolyzed in 6 M HCl for 22 hours at 110 °, before analyzing using the Waters HPLC (high performance liquid chromatography) system (Pico Tag). An in-depth description of the method is presented in appendix 1. The initial analytic tests were conducted by the workers at Nifes. However, for the samples taken from the rotifer and larval experiments in the present study, the analysis was conducted by the candidate. Before that could be done, the candidate had to be approved for the hydrolyzed amino-acid (HAA) method. The candidate was approved at Nifes in ISO 13903:2005 at 26. October 2017. The method is used to determine the total content of amino-acids, except for cysteine and tryptophan, in specific types of matter, including feedstuffs and organic tissue. For taurine, the measuring range of the method is 0.6-5 mg g^{-1} sample. It is important to specify that, for personal safety and the safety of those nearby; all handling with HCl (Hydrochloric acid) was done in fume hoods, with protective gloves, clothing and glasses. Gloves were used throughout the process, as well as a laboratory coat and protective glasses.

2.5 Statistical analysis

Raw data were treated in Microsoft Excel 2013 (Microsoft Corp., Redmond, WA), and Statistica ver. 13 (Statsoft Inc., Tulsa, OK) was used to conduct the statistical analysis. Graph Pad Prism 7 ver. 7.03 (GraphPad Software, Inc., San Diego, CA) was used to make graphs. When ANOVA analysis was used, data were checked for homogenous variance by Levene's test to check if the data were compatible with ANOVA analysis, which it was if p-value > 0.05 in the Levene's test (following p-values will be presented as "p"). The Unequal N HSD and Tukey HSD post hoc test was used to identify differences between means.

2.5.1 Rotifer experiment

Multiple regression was performed to identify significant variables. After the significant variables were identified, another multiple regression was performed, using the significant variables. This was then repeated once more, by excluding insignificant variables, until all variables tested were significant. Appendix 2 presents an in-depth description of the multivariate regression. From the output, a regression line can be expressed as follows;

Equation 2.5.1: *Regression equation, where Y= predicted value in mg g^{-1} DW, a= intercept, b_n= coefficient of variable or interaction term, x_n= independent variable n, $x_{n1}x_{n2}$= interaction term between two variables, $x_{n1}x_{n2}x_{n3}$= interaction term between all variables and u= standard error of equation.*

$$Y = a + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1x_2 + b_5x_1x_3 + b_6x_2x_3 + b_7x_1x_2x_3 + u$$

One-Way ANOVA was used to check if storage duration in 4 °C had a significant effect on taurine concentration in rotifers, as well as investigating significant difference in taurine concentration between groups, based on the concentration of taurine in the liposome rehydration medium.

2.5.2 Larval experiment

In the larval experiment, Nested design ANOVA test were used in the analysis of the SL-values at day 23. SL at day 23 was chosen as the dependent variable, while tank and treatment were chosen as categorical factors, with the tank factor nested in the treatment factor. Following the Levene's test, Post-hoc tests were conducted using the Unequal N HSD model, since not all samples had an equal number (n) of data points. One-Way ANOVA was used to test if age, in dph, had a significant effect on SL. Repeated measures ANOVA analysis was also conducted, to test if the treatments had significant effects on each stage the larval length were measured separately (9, 14, 19 and 23 dph). Samples for SL analysis at the start of the experiment were taken from the collecting tank and were therefore equal for all treatments. For this analysis, the lengths at the different days were chosen as the dependent variables, while treatment was chosen as the categorical variable. The repeated measurement factor was selected to have 4 levels, as there were 4 different measurements for each tank, linear regression was used when Levene's test for homogeneity of variance is failed. Linear regression was conducted to test if the concentration of taurine in rotifers had a significant effect on the concentration of taurine in the larvae. Taurine content in larvae was chosen as the dependent variable, and taurine content in rotifers were chosen as the continuous regressor. GLM (general linear models) analysis was used to test if treatment had a significant effect on larval whole-body taurine concentration.

3 Results

3.1 Rotifer experiment

The rotifer experiment was successful in terms of identifying significant variables, impacting enrichment success. The multiple regression results from the rotifer enrichment samples showed that the concentration of taurine in the liposomes used to enrich the rotifers, had the highest impact on the enrichment success ($b = 0.082$, $p < 0.0001$), however it was the only variable or interaction term that were proven to be significant. Center point (Table 3.1.1; sample number 9-11) was used to estimate tank variation.

Table 3.1.1: *Experimental design and results from rotifer experiment. Mean with SD are presented in for samples 9-11, DW taurine concentration values are presented.*

Sample number	**Concentration of taurine in liposomes (g L^{-1})**	**Concentration of liposomes (mg L^{-1})**	**Enrichment duration (h)**	**Taurine in rotifers (mg g^{-1})**
1	30	200	0.5	0.315
2	30	300	0.5	0.472
3	30	200	1.5	0.297
4	30	300	1.5	0.385
5	90	200	0.5	0.867
6	90	300	0.5	0.797
7	90	200	1.5	0.708
8	90	300	1.5	1.073
9-11	60	250	1.0	0.554 ± 0.016

Table 3.1.2: *Output from the multiple regression. A higher b value describes a steeper slope for the variable. [Taurine] represents the concentration of taurine in liposomes. SD of estimate = 0.105, $p < 0.001$, Adjusted R^2: 0.812.*

Coefficient	**b-value**	**p-value**
(Intercept)	(0.104)	(0.231)
[Taurine]	0.082	<0.001

Table 3.1.2 can be used to express a regression equation for the experiment, as the b-values of the different variables expresses the contribution the variable have on enrichment success. The equation is expressed in equation 3.1.1. As only the taurine concentration in liposomes was identified as significant.

Equation 3.1.1: *Regression equation for the multivariate experiment. Based on equation 2.5.1.*

$$[Tau]_{rotifer} = \left(0.082 * [Tau]_{liposome}\right)$$

The discovered optimal procedure for enriching rotifers with taurine, based on the tested values for the different variables, was as follows: a taurine concentration of 9 % (w v^{-1}) in the liposomes, at least 200 mg liposomes L^{-1} enrichment water and enrichment duration of at least 0.5 hours. However, one could increase the concentration of taurine in liposomes, enrichment duration and the amount of liposomes in the enrichment water, which may change the optimal procedure. An estimation of the concentration of taurine in rotifers enriched using the discovered optimal procedure is possible to obtain using the regression equation.

Equation 3.1.2: *Estimated concentration of taurine in rotifers using the "optimal" procedure, based on the current variable values, and equation 3.1.1.*

$$[Tau]_{rotifer} = (0.082 * 9) \pm 0.101$$

$$[Tau]_{rotifer} = 0.842 \pm 0.101 \frac{mg}{g} DW$$

The concentrations of taurine was chosen based on the crystallization effect taurine may have in liposomes at concentrations above 6 % (w v^{-1}) when stored in 4 °C (personal communication, Matthew Hawkyard). Therefore, the "optimal" procedure discovered in this multivariate experiment may not be optimal, as the regression equation determined in equation 3.1.2, is linear. Increased taurine concentrations, or changes in other parameters, may provide a more accurate optimal procedure. The suggested optimum in the present study is based on the values presented in table 3.1.1.

By grouping the results by the significant variable, figure 3.1.1 presents how the increasing values of this factor affect the taurine concentration (mg g^{-1} DW) in the samples. Note however that the low (3% (w v^{-1}) taurine) and the high group (9 % (w v^{-1}) taurine) presented in this graph are affected by varying variable values. The low and high groups had samples with a concentration of liposomes in the enrichment bucket of either 200 or 300 mg L^{-1}, and the enrichment duration was either 0.5 or 1.5 hours. While for the medium (6 % (w v^{-1}) taurine) group, these variables were constant at 250 mg L^{-1} liposomes and 1 hour enrichment duration. Even though variance of the variable values exists, all samples were treated equally, by following the same protocol, apart from the variable values. The medium group was included in the experiments to investigate the effects storage has on the taurine retention in rotifers, and to provide a center-point for the regression. The high taurine group had significantly higher taurine concentrations then both other groups, however the low and medium taurine groups were not significantly different from each other. (One-Way ANOVA: $p < 0.001$, Levene's test: $p = 0.170$, Tukey HSD: high-low; $p < 0.001$, high-medium; $p = 0.014$, low-medium; $p = 0.116$).

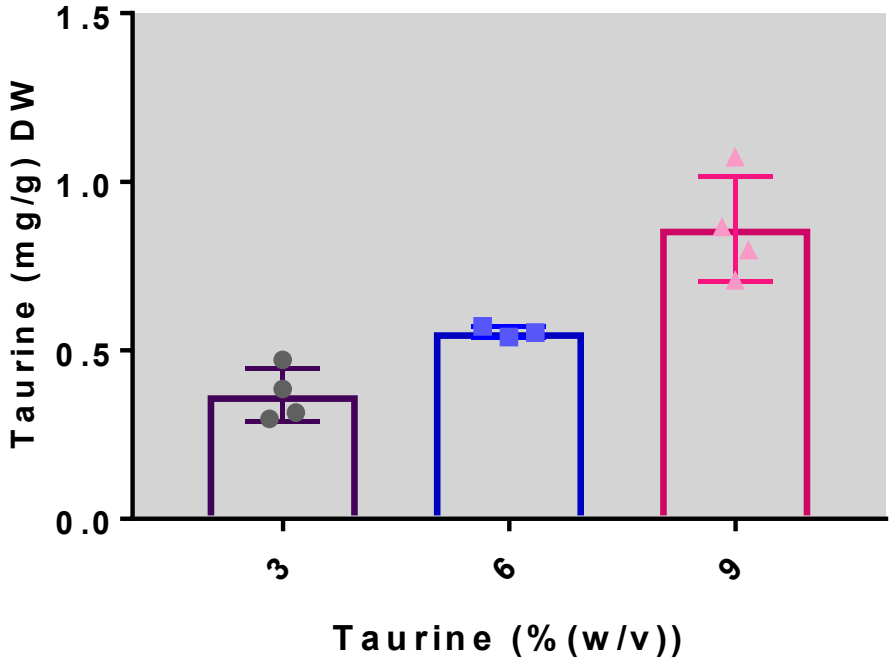

Figure 3.1.1: *Column graph (means and SD) presenting taurine concentration (mg g^{-1} DW, n=4 for low and high groups and n=3 for medium group) in rotifers as a function of taurine concentration in liposomes. Means were 0.367 mg g^{-1} DW in the low group, 0.554 mg g^{-1} DW in the medium group and 0.861 mg g^{-1} DW in the high group.*

3.2 Effect of storage

Rotifers were found to have a high retention rate of taurine, when enriched with the liposome method. An experiment with the medium group was conducted to assess the effect of storage on the taurine levels in the rotifers. Enriched rotifers were stored in 4 °C for up to 21 hours. There was not found to be a significant effect of hours stored and taurine content of the rotifers (Levene's test; p = 0.120, ANOVA; p = 0.750). However, this may be due to the relatively short duration of storage tested. 21 hours at maximum were used as that is normally the maximum of hours rotifers are held in cooling tanks after enrichment at the facility and for the experiments described in this paper.

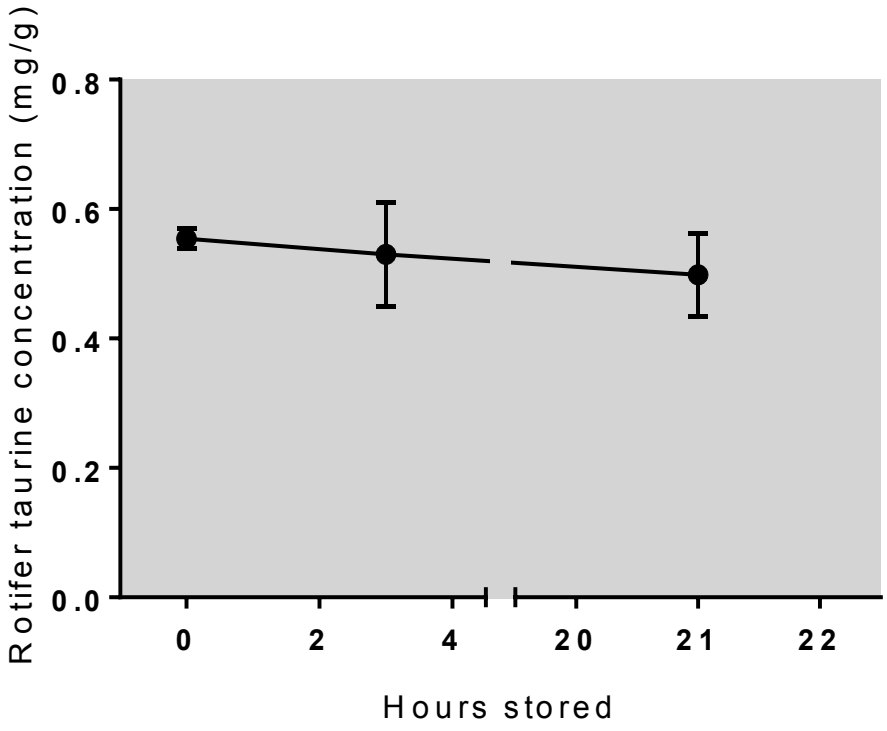

Figure 3.2.1: *Taurine concentration (mg g^{-1} WW, mean ± SD, n=3) in enriched rotifers during storage. Enriched rotifers were stored in 4 °C for 0, 3 and 21 hours after enrichment. Each data-point on the graph is based on 3 samples with 2 parallels. Significant correlation between storage duration and taurine concentration in the rotifers were not identified.*

3.3 Larval experiment

The larval experiment lasted until 23 dph, before the *Artemia* phase of the productions was started, and was conducted at Marine Harvest cleaner-fish facility in Øygarden, Norway in September 2017.

3.3.1 Standard length of larvae

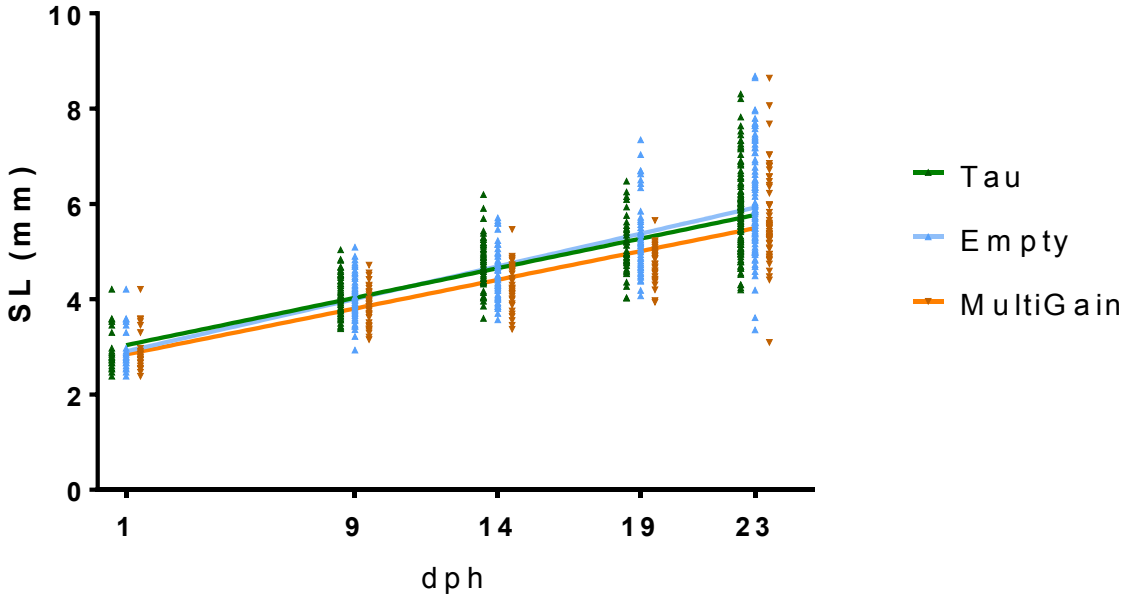

Figure 3.3.1: *Standard lengths of the larvae during the course of the experiment.*

L. bergylta larvae were measured to a mean SL of 2.96 ± 0.45 mm at 1 dph. At 23 dph, mean SL varied from 5.48 to 6.90 mm, figure 3.3.1. The highest mean SL was found in tank 14, in which the larvae were treated with Empty rotifers. However, the lowest mean value of SL was also found in the same treatment-group, in tank 15. Age had a significant effect on SL (One-Way ANOVA: $p < 0.001$). Repeated measures ANOVA showed that treatment did not have a significant effect on SL ($p = 0.090$), however the Levene's test was violated in samples from 19 dph (Levene's test: 19 dph; $p = 0.003$, for all other ages; $p > 0.420$). Unequal N HSD post hoc analysis of the repeated measures ANOVA analysis revealed that there were significant differences between some treatments at given larval ages (appendix 5). There were no significant differences between the treatments at 9 dph (Levene's test: $p = 0.421$, post hoc: $p > 0.853$ between all treatments). At 14 dph, there was significant differences in SL between Tau and both other groups, but not between MultiGain and Empty (Levene's test: $p = 0.720$, post-hoc: Tau-Empty; $p = 0.001$, Tau-MultiGain; $p = 0.017$, Empty-MultiGain; $p = 0.399$). At 19 dph, Empty larvae had significantly different SL value compared to the MultiGain group (post-hoc: $p = 0.013$), no other significant differences were found, however the Levene's test showed that ANOVA analysis was not compatible with these observations. Linear regression was therefore conducted for these samples; which revealed that there was a significant difference between MultiGain and both other groups

(Levene's test: p = 0.492, GLM: p < 0.001, Unequal N HSD: MultiGain-Tau; p < 0.001, MultiGain-Empty; p = 0.019). At 23 dph no significant difference between the treatments were found (Levene's test: p = 0.529, post hoc between all treatments: p > 0.247). Nested design ANOVA showed that there was a significant variation between treatments or tanks of the same treatment (Levene's test: p= 0.549, ANOVA: p = 0.000). Unequal N HSD post-hoc analysis revealed that tank 14 had a significantly higher SL-value than all other tanks, except for tank 3 (for all tanks except for tank 3: p < 0.001, tank 3: p= 0.339). Furthermore, tank 3 was significantly different from tank 1 (p= 0.003), 2 (p= 0.032) and 15 (p= 0.003). However, overall no significant difference between the treatments were found (Levene's test; p= 0.175, One-way ANOVA: p= 0.112).

3.3.2 Registrations of dead larvae during tending of the tanks

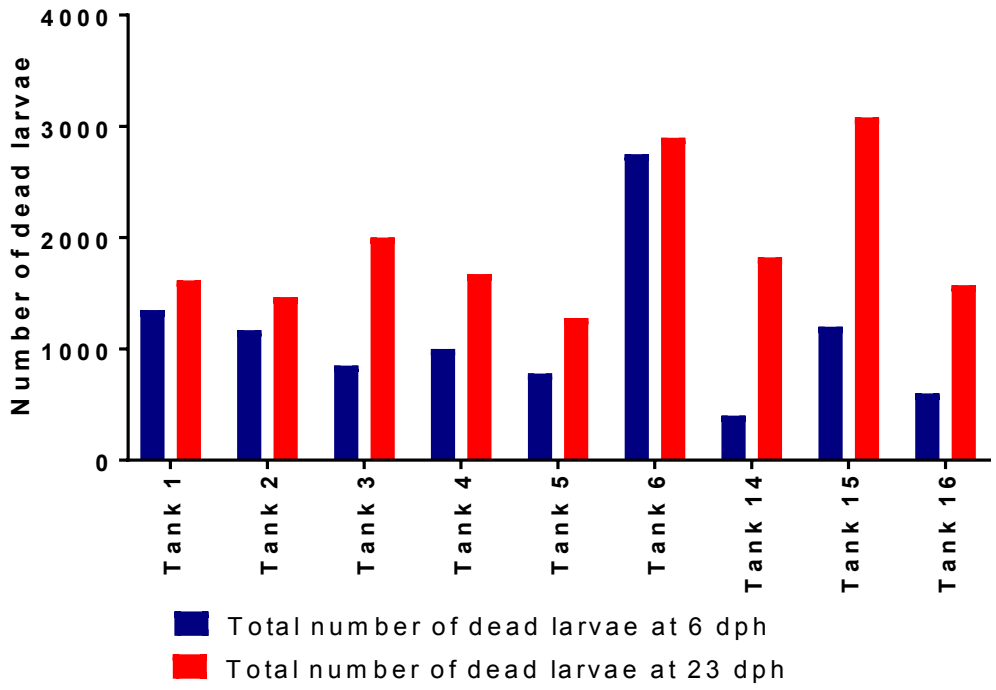

Figure 3.3.2: *Number of dead larvae counted out during tending of the tanks at beginning of tank tending (day 6) and at the end of the experiment (day 23). Tank 6 was discontinued at day 14. Each tank had an estimated identical initial population, n = 10 000.*

The tank with the highest observation of total registered dead larvae at 23 dph was tank 15 (3081 dead), and the lowest was tank 5 (1279 dead), figure 3.3.2. However, at the end of the experiment, it was evident that the registered number of dead larvae was wrong, as there were estimated 10 000 individuals in each tank at the beginning of the experiment, and

less than 100 individuals in each tank at the end of the experiment. Therefore, the number of registered dead larvae is not completely comparable to the real situation, as the highest registration of total dead larvae was at 3081 dead individuals (tank 15). At 6 dph, when the tending of the tanks started, tank 6 presented the highest number of dead larvae (2750 dead), while the lowest observation was in tank 14 (400 dead).

3.3.3 Survival-rates of larvae according to the number of larvae counted into and out of the tanks

Close to 100 % mortality was registered in all tanks by the end of the experiment, see table 3.3.1. At 14 dph, only 7 larvae were still alive in tank 6 (MultiGain treatment), and the tank were therefore discontinued at this date.

Table 3.3.1: *Survival-rates of larvae for each treatment at 23 dph. Each tank had an estimated initial population of 10 000 individuals.*

Treatment	Number of living larvae at 23 dph	Mean per tank	Survival-rate (%)
Tau	179	59.7 ± 30.5	0.597
MultiGain	77	25.7 ± 25.5	0.257
Empty	188	62.7 ± 28.2	0.627

ANOVA analysis showed that there was no significant difference in the number of living individuals between the treatments (Levene's test: $p = 0.917$, One-Way ANOVA: $p = 0.278$). The Empty group exhibited the highest survival-rate among the treatments, while the MultiGain group was found to have the lowest, however this was found to be an insignificant difference. All tanks had lower than 1 % surviving individuals. The tank with the highest number of surviving individuals was tank 14, with 95 (0.95 %) surviving individuals.

3.3.4 Concentrations of taurine in liposomes and rotifers

Taurine concentrations in rotifers from the larval experiment are presented in table 3.3.2. As with the larvae observations, the highest concentration of taurine in the rotifers analyzed was found in the Tau group (0.948 ± 0.160 mg g^{-1} DW). Furthermore, the Empty rotifer group had the lowest concentration of taurine (0.107 ± 0.010 mg g^{-1} DW), contrary to

the observations of the larvae samples, where the Empty larvae group had higher concentrations of taurine than the MultiGain larvae group. The MultiGain rotifers showed a higher variance between the parallels than the other treatment groups relative to the concentration value. As shown in table 3.3.2, there exist some degree of variance of taurine enrichment success, which leads to an uneven deliverance of taurine to the larvae, in other words; not a standardized amount of taurine was offered to the larvae each day.

Taurine concentrations in liposomes decreased approximately to 50 percent from the first measurement to the last (4 dph and 19 dph). Taurine liposomes were produced the day prior to the larval experiment; therefore, the measurements were taken when the liposomes were 5 and 20 days old, respectively.

Table 3.3.2: *Taurine concentrations (mg g^{-1} DW, means with SD) of the rotifer groups and liposomes used. Rotifers were taken from the same batch of unenriched rotifers prior to their treatment enrichment. Samples were collected directly after enrichment was finished (1 hour).*

Dph of larvae	Tau liposomes	Rotifers enriched with Tau liposomes	Rotifers enriched with empty liposomes	Rotifers enriched with MultiGain®
4	156 ± 4.425	1.062 ± 0.021	(0.187 ± 0.092)*	0.149 ± 0.007
9	-	0.719 ± 0.006	0.106 ± 0.009	0.182 ± 0.087
14	-	1.055 ± 0.034	0.117 ± 0.026	0.201 ± 0.007
19	79.1 ± 0.667	0.956 ± 0.020	0.098 ± 0.009	0.096 ± 0.025
Means:	118	0.948 ± 0.160	0.107 ± 0.010	0.157 ± 0.046
Estimated dietary taurine**		0.553	0.132	0.157

*Brackets and *: Unenriched rotifers, which the Empty treatment was fed the first five days. **: The Empty and Tau treatments were co-fed conventionally enriched rotifers, equal to those in the MultiGain treatment, the dietary taurine in these treatments are calculated following equation 2.3.1.*

3.3.5 Taurine concentrations of larvae

Some tanks did not have enough surviving individuals to perform a total amino-acid analysis (tanks 1, 5 and 6). As n varies between the treatments, it was not possible to perform ANOVA analysis on the samples. Therefore, GLM (General linear models) analysis was performed instead. The highest concentrations of taurine were found in the Tau-treatment group (tanks 1, 2 and 3), as expected, figure 3.3.3. The lowest concentration of taurine was found in the MultiGain group, however; this group had only 1 parallel from 1 tank (tank 4), which in turn lowers the significance of this observation. All larval samples analyzed contained a lower concentration of taurine at 23 dph than 1 dph (0.619 mg g^{-1} taurine WW).

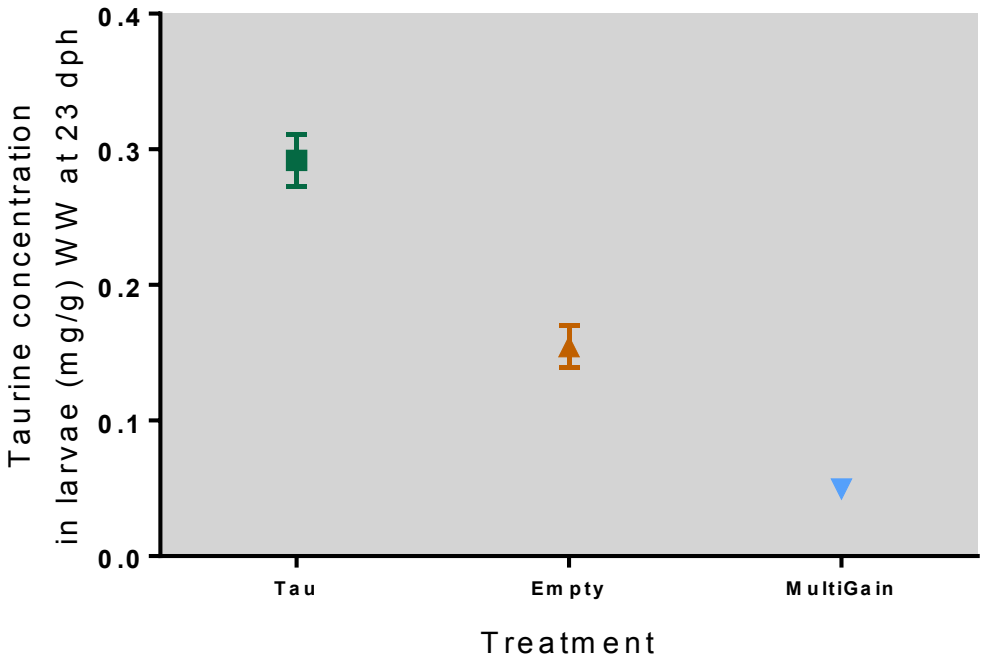

Figure 3.3.3: *Concentrations of taurine in larvae (means with SD, whole body, WW) in the different treatments at 23 dph.* Data points varied, Tau (n=2), Empty (n=3) and MultiGain (n=1).

GLM analysis revealed that treatment had a significant effect on taurine concentrations in the larvae (p = 0.003). Furthermore, concentration of dietary taurine in rotifers was found to have a significant effect on concentration of taurine in larvae, linear regression analysis was used to examine the significance of this correlation (R^2=0.761, p= 0.023).

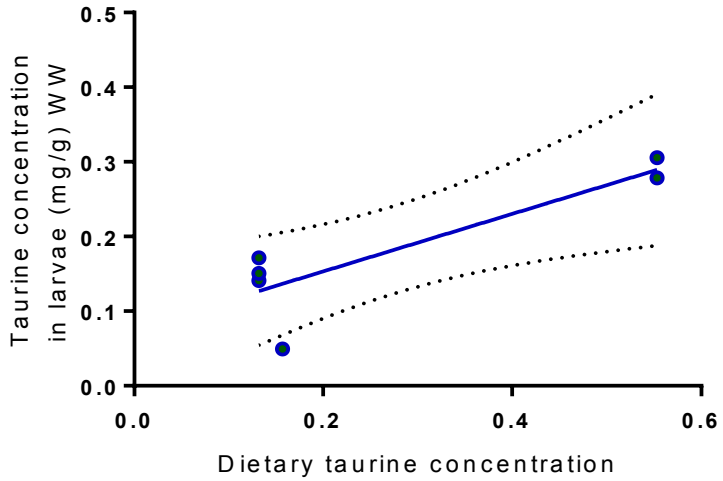

Figure 3.3.4: *Taurine concentration in larvae (WW) as a function of taurine concentration of the rotifers (DW) fed to the larvae. Regression line and 95 % confidence intervals are also added to the graph (R^2 = 0.761, p= 0.023). Dietary taurine concentration is presented in mg g^{-1} DW. Regression line is presented in equation 3.3.1.*

Equation 3.3.1: *Regression line used in figure 3.3.4.*

$$[Tau]_{larvae} = (0.385 * Tau\ rotifer) + 0.076$$

4 Discussion

The purpose of the study was to examine the effect of increasing taurine concentrations in rotifers on Ballan wrasse larvae (*L. bergylta*) and establish an effective method of elevating taurine concentrations in rotifers. The rotifers were enriched with taurine-filled liposomes (Tau). One control group was exclusively fed rotifers enriched with MultiGain® (MultiGain), and another control group was fed rotifers enriched with liposomes filled with saline water (Empty). The groups fed liposome-enriched rotifers were co-fed rotifers enriched with MultiGain® (50/50), to cover other dietary needs. This experiment was affected by outside factors, which caused the experiment to fail. Furthermore, a multivariate experiment was carried out to identify factors that affect enrichment success, in terms of taurine concentration. In addition to this, an experiment studying the effect of storage of taurine-enriched rotifers, up to 21 hours, on the concentration of taurine, was conducted.

4.1 Production problems during the larvae experiment

The facility that provided tanks and larvae for the experiment experienced problems with their larvae. These problems may shed light on what happened to the larvae used in this experiment, since the water for the facilities production and in the experimental tanks originated from the same waterline and the larvae were from the same egg batch. This segment is based on a meeting between the candidate and the biological supervisor at the facility, Espen Grøtan.

The facility experienced a poor production cycle with the same batch of larvae that was used in this paper (September 2017 group). The facility has estimated that approximately 15 million larvae hatched from the eggs, in total. As of 06.02.2018; 2-300 000 individuals had survived, making the survivability of the batch at that point somewhere between 1.33 and 2 percent. This number is much lower than the aim of the facility, which was to have approximately 1.5 million larvae at this time (10 % survival). The group presented two “high-mortality peaks”, which was during the rotifer-phase (15-20 dph) and weaning (55-65 dph). In addition to these peaks, it was suspected that a large portion of the mortality happened during the first couple of days after being transferred from the incubators to the larval tanks. This was happening before registrations of the dead larvae had begun, making it difficult to prove, as no data is present. Some of the dead larvae will most likely sink to the bottom of the tank and then be collected when tending begins, but it is also suspected that a large portion of the

larvae that dies before tending of the tanks begin dissipating in the water and gets flushed out of the tank or becomes a part of the bottom sedimentation.

The facility strongly suspects that there was a bacterial problem that caused the low survival-rates for the facility's production, but also for the larvae used in this study. Two factors differ from this group and previous, more successful groups. First, the mechanical filters used at the facility were faulty during the time this group was started. Dirty water was overflowing the filters, which in turn reduced the effectiveness of the UV-filters, as debris in the water creates "safe areas" for bacteria to hide behind. Second, the production protocol for rotifers was changed prior to the start of the larvae group. This was a small upscale of the intensity of the rotifers in the tanks, and not something the facility expected would cause bacterial issues. The change constituted of an increase in rotifer concentration in the rotifer tanks and increased aeration, in addition to adding flock –filters in the rotifer holding tanks. Earlier the facility has had problems after changing the rotifer protocol, which is why this factor cannot be excluded. However, the earlier problems came because of a more substantial protocol change, that time they changed the enrichment product to Or-One (instead of *Chlorella* sp. and MultiGain®). This happened in 2015, and the facility identified symptoms related to bacterial problems (overeating and alive undigested rotifers in the larvae's intestine). These symptoms were also identified in the September 2017 group, which the larvae used in the present study was a part of.

The brood-fish may have influenced the egg- and larvae quality. Both production- and nutritional problems may cause a decreased egg- and larvae quality (Kjørsvik *et al*, 1990). However, the facility does not suspect this is the main reason for the poor survival-rates, but it can be a contributing factor. In March 2017, the facility experienced brood-fish related issues after using the brood-fish tanks to collect eggs, in addition to the normal spawning tanks. The facility suspects that problems caused by poor water-quality control in the brood-fish tanks were transferred to the other group of eggs, which originated from the spawning tanks. Furthermore, the brood-fish in the brood-fish tanks were older. The fish in the spawning tanks were another group of fish, and had been used in 2016, whereas the "older" fish had been light-manipulated to spawn in March instead of November. In May 2017, the facility brought in newly caught brood-fish, of which they had more success. This may indicate that older fish that has been at the facility for years yield a lower quality of eggs.

It is immensely difficult to identify which factors have caused the main problems in for the larvae, as there are a lot of different impacts each factor may have. As many marine fish larvae are under-developed upon hatching, it is a known fact that most marine fish larvae do not handle stress and varying environments particularly well. This is a result of their life-strategy. Probably, each of the mentioned factors has had some degree of impact on the larvae's poor survival rate.

4.2 Rotifers as live prey

Rotifers are commonly used as the start-feed of choice in intensive Ballan wrasse production, as well as for many other marine fish species (Conceição *et al*, 2010). For Ballan wrasse in nature, on the other hand; zooplankton (mainly copepods) are the main source of exogenous feed, and the larvae are therefore assumed to be adapted to the nutrients found in zooplankton. The taurine profile of copepods is assumed to be optimal for Ballan wrasse larvae (van der Meeren *et al*, 2008; Hamre *et al,* 2008), and is vastly different from that found in rotifers, as rotifers are found to be lacking in taurine (Mæhre *et al*, 2013). Several papers have described the taurine content of rotifers as not detectable (Almli, 2012; Øie *et al*, 2017), and the same studies identified the concentration of taurine in copepods as double the amount of that in *Artemia*, which is used as live-feed until weaning on formulated diets. Zooplankton has been reported to have taurine concentrations of approximately 7 mg g^{-1} DW, see table 4.2.1 (Karlsen *et al*, 2015; Mæhre *et al*, 2013). As the taurine analysis methods used in van der Meeren *et al* (2008) differs from the present study, values obtained from Karlsen *et al* (2015) and Mæhre *et al* (2013) are more comparable to those found in the present study.

Karlsen *et al* (2015) demonstrated that enriched rotifers' taurine levels were 0.09 ± 0.04 - 0.10 ± 0.04 mg g^{-1} DW, which is similar to the levels found in MultiGain®-enriched rotifers in the present study (0.16 ± 0.05 mg g^{-1} DW). In comparison, unenriched *Artemia* were found to consist of 3.63 ± 0.13 – 5.01 mg g^{-1} DW taurine, and zooplankton 6.26 ± 0.75 – 7.88 ± 0.50 mg g^{-1} DW (Karlsen *et al*, 2015). Mæhre *et al* (2013) assessed taurine concentrations in rotifers enriched with different commercially available products (MultiGain®, Ori-Green and *Chlorella* sp.), and all samples proved to have non-detectable concentrations of taurine, however, zooplankton was found to contain 7.7 ± 0.6 mg g^{-1} DW. Unenriched rotifers used in the present study had a taurine concentration of 0.187 mg g^{-1} DW. From these findings it seems apparent that the rotifers are mostly devoid of taurine. Rotifers enriched with the Tau treatment contained nearly ten times higher taurine concentrations

(0.948 ± 0.160 mg g^{-1} DW) than the MultiGain rotifers in the present study; however, the Tau rotifers were still not close to the levels found in zooplankton or *Artemia*. Unenriched rotifers had a low concentration of taurine (0.187 mg g^{-1} DW); and no significant differences were found between the MultiGain, Empty and unenriched rotifer groups (linear regression: p = 0.176). In comparison, rotifers enriched using the discovered "optimum" protocol from the multivariate experiment had a taurine level of 1.073 ± 0.001 mg g^{-1} DW. Rotifers are enriched with commercial products to cover a range of dietary needs for marine fish larvae, it is clear however that conventional enrichment products do not focus on taurine levels obtained in rotifers from enrichment products. The hypothesis in this study is that the current taurine levels in enriched rotifers are too low to cover the requirements of taurine for Ballan wrasse larvae, as taurine is hypothesized to be an essential nutrient for the species.

Karlsen *et al* (2015) fed Atlantic cod (*G. morhua*) larvae with either zooplankton or rotifers and found that the larval concentrations of taurine were dependent on live-feed fed to the larvae. The same relationship was identified in the present study, as the concentration of taurine in the rotifers were found to have a significant effect on taurine levels in larvae, see figure 3.3.4. Atlantic cod larvae fed rotifers had a taurine concentration of 0.084 ± 0.017 mg g^{-1} WW at 20 dph, while zooplankton-fed larvae contained 1.45 ± 0.035 mg g^{-1} WW taurine at 20 dph (values modified from Karlsen *et al* (2015) to be comparable to data from the present study). Ballan wrasse larvae in the present study fed taurine-enriched rotifers contained 0.292 ± 0.019 mg g^{-1} WW taurine (n=2), whilst larvae fed MultiGain-enriched rotifers contained 0.050 mg g^{-1} WW taurine (n=1) and the Empty group; 0.154 ± 0.016 mg g^{-1} WW taurine (n=3), at 23 dph. While the zooplankton-fed cod larvae (Karlsen *et al*, 2015) contained higher values of taurine than the Tau group Ballan wrasse larvae in the present study, both these groups had higher concentrations than larvae fed conventionally enriched rotifers. These results indicate that rotifers contain too low levels of taurine, in comparison to the requirements of the larvae. Srivastava *et al* (2006) showed that different forms of enrichment products can to some degree increase the concentration of taurine in rotifers. However, the achieved taurine concentrations presented in their study are still not close to the assumed optimum, found in zooplankton. Thus, unenriched rotifers, and rotifers enriched with commercial products seem to contain too low levels of taurine to cover dietary needs of Ballan wrasse larvae. Therefore, elevated taurine levels in rotifers fed to the Ballan wrasse larvae would most likely yield positive results, as shown in Karlsen *et al* (2015) for Atlantic cod, both from an economic standpoint and with regards to fish welfare.

Table 4.2.1: *Overview of taurine concentrations in different live feed presented in selected papers. Values are presented in estimated dietary taurine (unless otherwise specified), mg g^{-1} DW. "Enriched rotifers" were enriched with commercially available enrichment products. Not detected = nd. Not tested in the given study = -. Not presented = np.*

Author	Zooplankton	*Artemia*	Unenriched rotifers	Enriched rotifers	Taurine-enriched rotifers
Karlsen *et al* (2015)	6.26 – 7.88	3.63 – 5.01	-	0.09 – 0.10	-
Mæhre *et al* (2013)	7.7	-	nd	nd	-
van der Meeren *et al* (2008)	10.55 – 17.02	7.23 – 8.20	-	0.36	-
Øie *et al* (2017)	np	np	nd	nd	-
Rotman *et al* (2017)	-	6	nd	-	11.0*
Srivastava *et al* (2006)	-	-	-	≤ 0.56	-
Chen *et al* (2005)	-	-	-	-	2.052 – 4.535
Hawkyard *et al* (2015)	-	-	-	-	~ 6
Hawkyard *et al* (2016)	-	-	0.16 ± 0.01	-	23.5$^{\varphi}$
Present study	-	-	0.187***	0.157 ± 0.050	0.553**

**: Rotman* et al *(2017) enriched with 4 g pulverized taurine per liter enrichment water.*

**: *As the larvae were co-fed rotifers enriched with MultiGain®, the concentration of taurine delivered to the larvae were assumed to be half of the concentration found in the Tau treatment rotifers plus half the concentration found in the MultiGain treatment rotifers.*

***: *The unenriched rotifers in the present study had been fed* Chlorella *sp.*

$^{\varphi}$*: Dietary taurine in a group fed taurine-enriched rotifers (29.8 ± 1.6 mg g^{-1} DW taurine) and taurine-enriched* Artemia *metanauplii. (17.2 ± 1.1 mg g^{-1} DW taurine).*

Table 4.2.2: *Effects of increased supplementary taurine found in selected studies. Liposomes refers to the method described by Barr and Helland (2007), dissolved taurine refers to the method of adding taurine directly to the enrichment water. NRS= Northern rock sole (*Lepidopsetta polyxystra*), PC = Pacific cod (*Gadus macrocephalus*), JF = Japanese flounder (*Paralichthys olivaceus*), RSB = red sea bream (Pagrus major), CYT = California yellowtail (*Seriola lalandi*) SS = Senegalese sole (*Solea senegalensis*), PBT = Pacific bluefin tuna (*Thunnus orientalis*), YT = yellowfin tuna (*Thunnus albacares*)*

Species	Effect of increased supplementary taurine	Author	Taurine delivery method
NRS	Increased growth rate	Hawkyard *et al* (2015)	Liposomes
	Increased development rate, earlier onset of metamorphosis	Hawkyard *et al* (2014)	Microparticulate and dissolved taurine (4000 mg L^{-1})
PC	Increased growth rate	Matsunari *et al* (2005)	Dissolved taurine (up to 1200 mg L^{-1})
JF	Increased growth, improved development	Chen *et al* (2005)	Dissolved taurine (400 and 800 mg L^{-1})
	Increased feeding-rate, improved settlement behavior	Takeuchi *et al* (2001)	Mysids
RSB	Increased concentration of taurine in whole-body	Takeuchi *et al* (2001)	Microparticulate diet with 11.6 mg g^{-1} (assumed to be DW) taurine
SS	Increased protein retention in larval period, increased metamorphosis rate	Pinto *et al* (2010)	Microcapsules
PBT & YT	Improved growth, shortened development period	Katagiri *et al* (2017)	Dissolved taurine (800 mg L^{-1})
CYT	Increased growth rate	Hawkyard *et al* (2016)	Liposomes

4.3 Liposome method viability

Taurine is a water-soluble compound, and large amounts of the substance are needed for conventional rotifer enrichment (Karlsen *et al*, 2015), this is demonstrated by Rotman *et al* (2017), who used 4 g of pulverized taurine per liter enrichment water to achieve taurine concentrations of rotifers comparable to those found in copepods. One suggested solution to elevating taurine levels in rotifers has been the use of taurine-filled liposomes, as demonstrated by Hawkyard *et al* (2015), using the method developed by Barr and Helland (2007). In comparison to Rotman *et al* (2017, the present study obtained taurine levels in rotifers of up to 1.07 mg g^{-1} DW, while the taurine usage was considerably lower, up to 8.12 mg L^{-1} DW taurine, or 0.203 % of what was used in Rotman *et al* (2017). Similarly; Hawkyard *et al* (2015) enriched rotifers with liposomes (250 mg liposome phospholipid L^{-1}) rehydrated in 10 % (w v^{-1}) taurine solution; in addition, they enriched another group of rotifers with 15 g L^{-1} dissolved taurine. The resultant rotifers in these two groups had similar concentrations of taurine, even though the liposome-enriched group utilized considerably less taurine (Hawkyard *et al*, 2015).

The somewhat successful enrichment of taurine described in this paper, as well as Hawkyard *et al* (2015), show that it is possible to change the taurine profile of an organism quite effortlessly, by packing them with desired substances. This is contrary to what Cahu and Zambonino Infante (2001) suggests, for amino acid profiles. The method based on Barr and Helland (2007) made it possible to increase the taurine content of the rotifers to a level six times higher than the rotifers fed MultiGain®. However, when comparing the concentrations obtained in the present study with those of Hawkyard *et al* (2015) and Hawkyard *et al* (2016), the enrichment success in the present study was not at the expected level. These papers obtained taurine concentrations in rotifers of "approximately" 12 mg g^{-1} DW (Hawkyard *et al*, 2015) and 29.8 ± 1.6 mg g^{-1} DW (Hawkyard *et al*, 2016), while up to 1.07 mg g^{-1} DW was obtained in the present study. The reasons for the large discrepancy are not known, however there were some differences between the studies. Hawkyard *et al* (2015) and Hawkyard *et al* (2016) had FDEL rehydrated in 10 % (w v^{-1}) taurine solution, while 6 % (w v^{-1}) taurine solution was used in the present study. Additionally, cones were used in the mentioned studies, whereas buckets were used in the present study, to contain rotifers during enrichment. Hawkyard *et al* (2016) also enriched the rotifers with a liposome concentration of 1000 mg L^{-1}, while the present study used concentrations of 250 mg L^{-1}, as did Hawkyard *et al* (2015). Differences in liposome concentration during enrichment were presumably the factor

resulting in the differences between the two studies mentioned (Hawkyard *et al*, 2016). However, it is not likely that the different taurine concentrations between the present study and Hawkyard *et al* (2015) were due to a difference in design, but rather an unidentified production error in the present study when preparing liposomes, or during enrichment.

However, as the larvae group fed supplemental dietary taurine did in fact have a significantly higher concentration of taurine than the other groups, the experiment did succeed in elevating the concentrations of taurine in the larvae. Furthermore, the Tau rotifer group from the larval experiment contained approximately six times more taurine than the MultiGain rotifers. This finding suggests that the method is effective for the enrichment of rotifers with taurine, which is in accordance with findings from Hawkyard *et al* (2015) and Hawkyard *et al* (2016). These findings highlight the efficiency of the liposome method developed by Barr and Helland (2007) in regard to the delivery of water-soluble nutrients to rotifers. As mass production of copepods is challenging to obtain, relative to rotifers and *Artemia* (Støttrup, 2002), these results are promising in regard to an increase of dietary taurine in larval marine fish feed, and the method used in the present study seems be a viable option for live-feed enrichment for industrial production of marine larvae.

The Tau larvae group was fed more taurine than the other groups in the larval experiment, but also less marine fatty acids than the MultiGain group. This fact may have had an effect on the larval growth and development, however; such an effect was not observed among the larvae groups. Tau rotifers were not enriched with anything other than the taurine-filled liposomes, and the larvae were therefore co-fed conventionally enriched (MultiGain®) rotifers, to cover their dietary needs for marine fatty acids, among other nutrients. Likewise, the Empty group was co-fed normally enriched rotifers (MultiGain®). This meant that the dietary taurine fed to the larvae were not the same as the concentrations found in these treatment groups, the dietary taurine concentrations are presented in table 3.3.2. The Empty rotifer group had a lower concentration of taurine than the MultiGain rotifer group. The concentrations were found to be 0.157 ± 0.046 and 0.107 ± 0.010 mg g^{-1} DW for MultiGain and Empty rotifer groups, respectively. These findings show that the MultiGain® enrichment may to some degree contribute taurine to the rotifers, there were not however, found a significant difference between the MultiGain and Empty rotifer groups regarding taurine concentration. The hypothesis in the present study that taurine is an essential nutrient for Ballan wrasse larvae is strengthened by the fact that larvae fed taurine-enriched rotifers had a higher concentration of taurine (whole body, WW), compared to the other groups.

4.4 Multivariate enrichment experiment

Even though rotifers have low concentrations of taurine, it is apparent that rotifers are highly suitable for providing beneficial nutrients and substances to the larvae. The rotifers can be enriched with desired substances through normal enrichment products and protocols, but also water-soluble substances such as taurine by the use of liposomes. Furthermore, Srivastava *et al* (2012) showed that rotifers have low retention rates of iodine, which is almost absent 2 hours after enrichment. Rotifers were proven to have high a retention rate of taurine, when stored in 4 °C, as shown in figure 3.2.1, with no significant reduction in taurine concentration 21 hours after enrichment with liposomes.

The multivariate experiment revealed that enrichment success (in terms of taurine concentration) depended mainly on the concentration of taurine in the liposomes ($p< 0.001$). Duration of enrichment and amount of liposomes in the enrichment water was not shown to have a significant effect on enrichment success, which was surprising; as Park *et al* (2006) showed that increased duration of enrichment increased enrichment success. Park *et al* (2006) found that 24 hours enrichment improved enrichment success, in comparison to 8 and 16 hours, whereas in the present study; 0.5, 1 and 1.5 hours were tested. Thus, enrichment success may have been improved with longer enrichment duration. On the other hand; Baer *et al* (2008) showed that maximum gut filling were achieved after 35 minutes of enrichment, which indicates that approximately 1 hours of enrichment should be sufficient for elevating the concentrations of wanted substances (Mæhre *et al*, 2013). Liposome concentration was also expected to have a significant effect on taurine concentration in rotifers, as the improved enrichment success of taurine in Hawkyard *et al* (2016) compared to that of Hawkyard *et al* (2015) were most likely due to differences in liposome concentration (Hawkyard *et al*, 2016). No interaction terms were found to be significant on enrichment success.

With the significant variable identified, an “optimal” practice based on the variable values used in the multivariate regression experiment was obtained. As only one variable were significant, the regression equation consisted only of this variable. This constituted in using a concentration of 9 % (w v^{-1}) taurine in the liposomes. However, by increasing the taurine concentration in the liposomes, the amount of liposomes in the enrichment water or enrichment duration; the “optimal” protocol may change. In this study, rotifers enriched using this concentration obtained a taurine concentration of up to 1.07 ± 0.001 mg g^{-1} DW. It is therefore recommended for eventual later studies using this method to use 9 % (w v^{-1}) taurine

when preparing liposomes. Furthermore, taurine may start to crystallize when stored at 4 °C with concentrations exceeding 6 % in liposomes (Matthew Hawkyard, personal communication), and thereby destroying the liposomes. It may therefore be beneficial to perform tests with larvae in conjunction with rotifers enriched with liposomes rehydrated in taurine solution with a concentration of 9 % (w v^{-1}) to investigate if the increased concentration has an effect on the larval uptake of taurine. In the present study, the enrichment with 9 % (w v^{-1}) taurine liposomes was successful, which suggests that taurine is contained in the liposomes at this concentration, and thereby also be available for the fish larvae. However, this may have been different if the liposomes were stored for a longer period prior to enrichment, as the retention-rate of taurine in liposomes may be affected by taurine-concentration of the rehydration medium. In the present study, liposomes were prepared the day prior to the multivariate experiment, and therefore had a storage duration of 1 day. It is also the candidates' recommendation for potential further research on the topic of liposome enrichment of rotifers to spread out the liposome production throughout an eventual larval experiment, as this would probably also allow for a higher concentration of taurine in the liposomes, as storage duration of liposomes decreases. Based on results from the liposome analysis, the liposomes seem to leech taurine relatively fast when stored in 4 °C, see table 3.3.2. Even though the rotifer experiment yielded reproducible results and identified the most impactful variable (taurine concentration in liposomes), more research on the subject is recommended.

4.5 Growth of *L. bergylta* larvae

Larvae from all treatments in the present study presented a similar growth-rate, there was found significant differences in SL on specific sampling dates, see chapter 3.3.1, however no significant difference were found between the treatments at 23 dph. It was expected that the Tau larvae would have the highest growth rate, based on findings on other species. The mean standard length of the larvae in the present study was found to be 2.96 ± 0.45 mm at day 1 (n=34). Other papers examining the standard length of *L. bergylta* has reported similar lengths. Skiftevik and Bjelland (2003) found that the mean length of the species' newly hatched larvae was 3.6 mm, while Fives (1976) determined the length to 3 mm. It is however unclear whether these publications have measured the standard length of the larvae or the total length. Dunaevskaya (2010) found that the newly hatched *L. bergylta* larvae examined had an SL of 3.64 ± 0.05 mm, see table 4.2.3. The Tau larvae in the present study exhibited a higher SL-value at 23 dph than the Dunaevskaya (2010) larvae at 25 dph. However, there were other

differences than the treatment between the two groups which may have impacted the results. Tank size was equal, however temperature was generally higher in the present study. Other abiotic factors may have been different, as well as population density and genetic factors, differences in study design such as these makes conclusions difficult, or even impossible to produce.

Table 4.2.3: *Comparison between reported SL values (mean ± SD, mm) for Ballan wrasse in selected papers. The present study SL are from Tau group larvae (1dph: n=34, 9 dph: n=82, 14 dph: n=82, 23 dph: n= 101).*

Dph ->	0-1	2-5	7	9	13	14	23	25
Present study (Tau)	2.96 ± 0.45	-	-	4.08 ± 0.37	-	4.71 ± 0.48	5.82 ± 0.91	-
Dunaevskaya (2010)	3.64 ± 0.05	4.18 ± 0.06	4.40 ± 0.15	4.28 ± 0.11	4.78 ± 0.19	-	-	5.35 ± 0.30
Hansen (2012)	-	3.97 ± 0.25	-	-	-	-	-	-

Hansen (2012) showed that the growth rate of Ballan wrasse was relatively slow initially, until "around 35 dph", where an exponential pattern in growth rate was observed. At this date, the larvae had been weaned onto a formulated diet (Gemma Micro 150 µm, Skretting, Stavanger, Norway). The formulated diet consisted of higher levels of proteins, oils and fats than the enriched rotifers at the time, which may contribute to the change of growth rate. However, as suggested by Hansen (2012); the maturation and increased digestion efficiency of the digestive system may play a role in the increased growth rate, as the pancreas in Ballan wrasse has been shown to increase markedly in size around 17 dph (Dunaevskaya, 2010). Therefore, the improved digestive functions, as well as a food-source promoting higher growth-rates than rotifers are probable causes for the observed pattern (Hansen, 2012). Sudden bursts in growth-rate such as this have also been observed for other species of fish at the larval stage (Atlantic cod, *G. morhua*, Sæle *et al*, 2010; Bay snook, *Petenia splendida,* Uscanga-Martínez *et al*, 2011). The larvae observed in the present study did not show any bursts of exponential growth, the observed growth rate was relatively linear, it is likely

however, that this would be observed if the experiment had lasted until weaning, which would be in accordance to the findings of Hansen (2012) with the species. Hansen (2012) also showed that SL was dependent of the age of the larvae, which were also the case for the present study (ANOVA, $p < 0.001$). Because of the delayed increase in growth rate, and the early termination of the larval experiment, potential persistent effects of supplemental dietary taurine could not be investigated. It is likely that taurine would have a positive effect on growth rate on Ballan wrasse larvae.

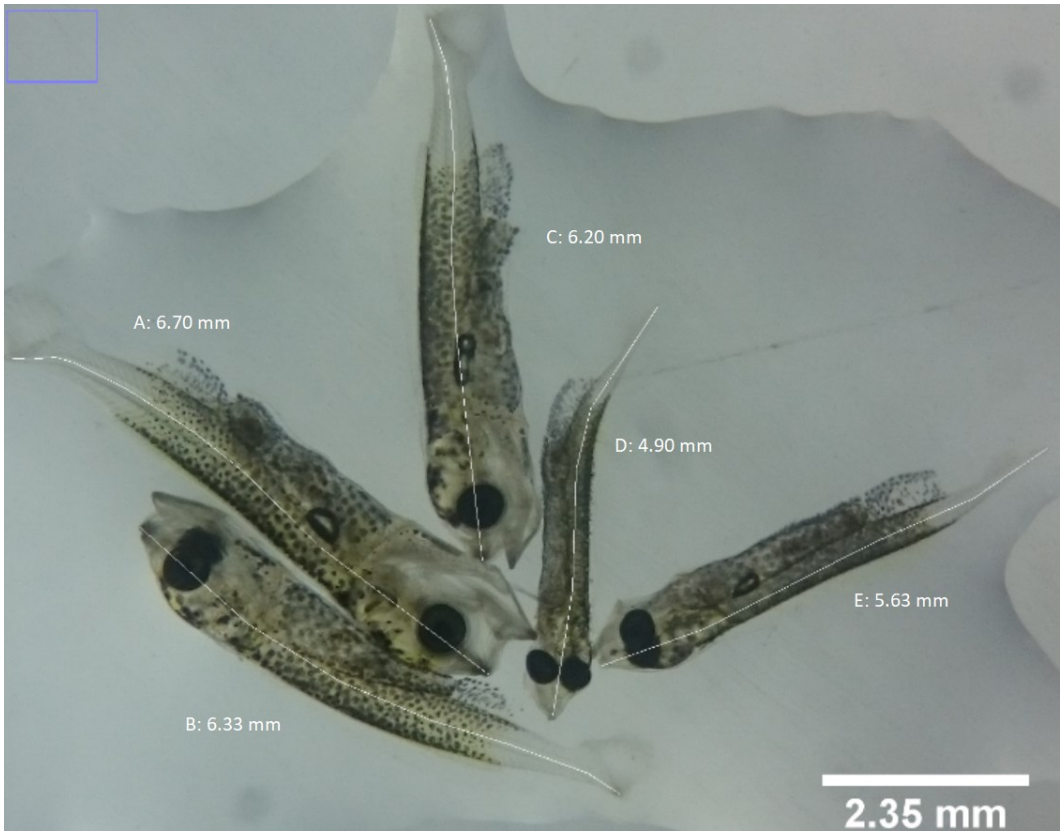

Figure 4.4.1: *Example of difference in SL. Larvae from tank 3 at 23 dph, Tau treatment. Examples such as this was found in all tanks. The differences in SL are quite clear, however the term "loserfish" has not been given a quantified value in SL and are based on subjective observations. For this particular case the individuals D and E are considered loserfish, as they are shorter, in terms of SL, and seem to be thinner and less developed.*

Between the SL measurements at 19 dph and 23 dph, the larvae in the tanks started to differentiate into two groups, based on SL observations. One group grew fast, while the other stopped growing, and was termed "loser-fish", figure 4.4.1. This phenomenon was present in all tanks, and there was no apparent difference in the prevalence of "loserfish" between tanks or treatments, based on SL. There may have been a difference between treatments if other

factors were examined. Measuring mean DW and establishing a threshold and definition of a "loserfish" in regard to DW could potentially yield results, another factor to measure regarding "loserfish" would be myotome height. The emergence of loserfish may be explained by the burst in growth-rate demonstrated by Hansen (2012) in Ballan wrasse. Some individuals may not be successful enough in the exogenous feeding, perhaps due to deformations in the jaw (Espen Grøtan, personal communication), or experience digestion-related issues, and therefore may not experience a burst in growth. Individuals with repressed stress tolerance may also end up in this group, for example if the water quality is sub-optimal, which may act as a persistent stressor. It may be of interest to investigate eventual correlations between dietary taurine levels and the prevalence of "loserfish".

4.6 Effect of taurine on marine fish larvae

Taurine has a wide range of effects on marine fish species, with several physiological roles. Among these are functions connected to cell volume regulation, taurine also functions as an antioxidant, is involved with bile salt synthesis and has osmoregulatory functions (Salze and Davis, 2015; Pinto *et al*, 2010; Huxtable, 1992).

As the larval experiment were affected by what is assumed to be bacterial problems, and low survival-rates in all tanks were experienced, samples for all analytic tests planned were not possible to be taken. For example, analysis of the cellular redox couple 2GSH/GSSG (reduced/ oxidized glutathione) were planned. The redox system is important to overall health in fish and is affected by the nutrients ingested by the larvae (Penglase *et al*, 2015). 2GSH/GSSG is an important redox couple for maintaining cellular redox homeostasis, and such an analysis could be used to assess the general redox-environment. Penglase *et al* (2015) investigated 100 genes related to redox regulation during development of cod. The paper aimed at examining the differences in regulation of these genes between two groups of Atlantic cod (*G. morhua*), one group were fed zooplankton, mainly copepods, and another were fed according to commercially normal protocols, with rotifers and *Artemia*. Of the investigated genes, 46 % were differentially regulated in the rotifer-fed larval group. Deficiency of taurine was identified as one probable cause for the differences in redox regulation.

Several studies have highlighted the positive effects of feeding marine fish with zooplankton in the larval stages of the lifecycle (Sørøy, 2012; Penglase *et al*, 2015; Øie *et al*, 2017; Karlsen *et al*, 2015). Karlsen *et al* (2015) and Øie *et al* (2017) identified the higher concentrations of taurine in zooplankton as one of the most probable causes for the improved effects seen in marine fish larvae, in comparison to larvae fed other live-feed. Sørøy (2012) found that Ballan wrasse larvae fed copepods presented a lower frequency of skeletal anomalies, than those fed rotifers. Furthermore, Sørøy (2012) showed that copepod-fed larvae had better stress-tolerance, growth and feeding success, which may be caused by the elevated levels of taurine in copepods compared with those found in rotifers.

By increasing the concentration of taurine in rotifers, the nutrient profile of the rotifers becomes more similar to that of the copepods. Especially when combined with rotifers enriched with marine fatty acids (DHA and EPA). As the nutrient composition found in copepods can be viewed as the "optimum" (van der Meeren *et al*, 2008), as Penglase *et al* (2015) hypothesized for redox-regulation, the increased taurine levels could cascade positive effects in the larvae, such as the growth rate, which is observed in a range of species, as well as other positive effects (table 4.2.2, Salze and Davis (2015)). When using the same enrichment method as the present study, Hawkyard *et al* (2015) presented an increased growth-rate in northern rock sole (*L. polyxystra*), furthermore, the northern rock sole fed taurine was the most developed at any given point in time, in terms of flexion development. These findings were also in accordance to those of Hawkyard *et al* (2014), when a different method of taurine-enrichment was used (microparticulate). It was expected that the present study would yield similar results as those discussed in this segment. However, since the experiment was affected by what is assumed to be bacterial problems, this could not be proven for Ballan wrasse.

In a study examining the effect of taurine on Senegalese sole (*S. senegalensis*) larvae, Pinto *et al* (2010) found that the benefits of early dietary taurine supplements became visible only after the larvae had started settling on the bottom and were fed *Artemia*. The positive effects were in other words "hidden" from the researchers until a later life-stage of the fish. Koedijk *et al* (2010) reported a persisting positive growth effect in Atlantic cod (*Gadus morhua*) larvae fed zooplankton, compared to rotifers. Furthermore, they reported that there was a "lack of a diet-induced growth difference until 29 dph". With these findings in mind, it may seem like the potential effects of the elevated taurine levels would present themselves at a later stage in the larvae, which would also be in accordance to the findings of Hansen

(2012). Salze *et al* (2012) found that taurine supplementation increases the specific activity of digestive enzymes in larval cobia (*R. canadum*). However, there was no significant difference between the treatments in the present study regarding SL, and the elevated taurine levels of the Tau group may have caused other effects than those investigated in this paper. There is also a possibility that the assumed bacterial issues may have depressed these potential effects. As the experiment was ended before the start of the *Artemia* phase, and the successful elevation of taurine levels in the larvae, positive effects of taurine may have become apparent later in their life.

4.7 Taurine requirement for Ballan wrasse larvae

Species of marine fish vary in their ability to convert cysteine to taurine (Goto *et al*, 2003) due to differences in the activity of the CSAD enzyme, taurine is generally considered an essential nutrient for larval marine fish species (Matsunari *et al* 2005). The requirement of dietary taurine for Ballan wrasse at the larval stage is not known (Hamre *et al*, 2013a). Salze and Davis (2015) write in their review that "A nutrient is required in the diet if endogenous production from precursors is absent or insufficient to meet physiological needs". Li *et al* (2009) describes taurine as a conditionally essential amino acid; referring to the suggestion that taurine "Conditionally essential AA (amino acids) must be provided under conditions where rates of utilization are greater than rates of synthesis". Several authors also report or suggest that taurine as an essential or conditionally essential nutrient for several marine fish species, among these; larval cobia (*Rachycentron canadum*), yellowtail (*Seriola quinqueradiata*), red sea bream (*Pagrus major*) and juvenile Japanese flounder (*P. olivaceus*) (Salze *et al*, 2012; Takagi *et al*, 2008; Takeuchi *et al*, 2001 and Hano *et al*, 2017, Kim *et al*, 2008 and Takeuchi *et al*, 2001). Furthermore, it is suggested that a high level of free amino acids is required for marine fish larvae (Rønnestad *et al*, 1999; Rønnestad *et al*, 2003).

There is increasing amount of evidence that taurine supplements improve several aspects of production, health and welfare of marine fish species. In the case of Ballan wrasse, larval weaning success has been shown to depend on the presence of specific raw materials in the weaning diets (Kousoulaki *et al*, 2015). Weaning success of Ballan wrasse larvae is suggested to be dependent on raw materials related to crustaceans (Kousoulaki *et al*, 2015), which generally have high levels of taurine. Also, free amino acids appeared to have positive effects on weaning success (Kousoulaki *et al*, 2015). Thus, supplemented dietary taurine may increase the weaning success of Ballan wrasse, which is identified as a bottleneck in intensive

production of the species. There is evidence presented in the present study that suggests that taurine is an essential nutrient for Ballan wrasse larvae. In accordance with findings of Karlsen *et al* (2015) with Atlantic cod larvae (*G. morhua*), Ballan wrasse larvae fed elevated levels of taurine contained significantly higher concentrations of taurine than the other groups, it seems likely that taurine is not produced in sufficient amounts endogenously in the larvae. Therefore, it is suggested that inclusion of taurine in the enrichment is required, when fed rotifers, as taurine is likely to be essential for Ballan wrasse larvae. This would also make sense when considering the natural feed of the species at the larval stage, which is zooplankton, and has a much higher taurine content than the rotifers (table 4.2.1; Karlsen *et al*, 2015). Furthermore, Fiogbé and Kestemont (1995) found that the requirements for essential amino acids in goldfish (*Carassius auratus*) are higher for larvae than for juveniles, this may also be true for marine fish species. This would in that case be in accordance to the findings of Huxtable (1992), who pointed out that the concentration of taurine in the developing brain of several organisms are higher in neonates than in adults. Takeuchi *et al* (2001) complements this finding, as they found that the taurine content of red sea bream (*P. major*) larvae decreased immediately after hatching. In the present study, all treatment groups of larvae exhibited a decrease in whole body taurine concentration from 1 dph to 23 dph. However, the larvae fed increased levels of taurine contained significantly more taurine than the other groups, which is also in accordance to the findings of Takeuchi *et al* (2001) in red sea bream.

There are some degree of variation between species, as well as during different stages of ontogenesis, regarding the ability to synthesize taurine (Goto *et al*, 2001; Goto *et al*, 2003, Kim *et al*, 2008). For example, Kim *et al* (2008) found that the Japanese flounder (*P. olivaceus*) have a poor ability for taurine biosynthesis when compared to common carp (*Cyprinus carpio*) at the juvenile stage. This is an indication that taurine may be an essential nutrient for Japanese flounder at the juvenile stage, and is in accordance with the findings of Chen *et al* (2005), but not for the common carp, at the same stage. Furthermore, Pinto *et al* (2013) found that dietary taurine supplementation is not likely to be essential for gilthead seabream (*Sparus aurata*) at its larval stage, as this did not cause a benefit for larval growth. However, the experiment on gilthead seabream did indicate that methionine became more available for the larvae, when fed taurine supplements, which in turn indicate that taurine supplements in the diet may lead to an increase of larval growth, by saving methionine for protein synthesis (Pinto *et al*, 2013). It is on the other hand, unwise to conclude that taurine is

not essential for larval gilthead seabream based on one study. It is apparent that taurine requirements are differing between species, as the activity of the CSAD varies between species (Goto *et al*, 2001; Goto *et al*, 2003). No assessment of the CSAD activity has been conducted on Ballan wrasse, as per the candidates' knowledge. Considering that the Ballan wrasse larvae in the present study that were fed Tau rotifers had a significantly higher concentration of taurine than the other groups, it is probable that Ballan wrasse has a low activity of CSAD.

Marine fish larvae experience massive morphological and physiological changes during metamorphosis, as well as exhibiting rapid growth and feeding continuously (Hamre *et al*, 2013b). Therefore, marine fish larvae demand a high amount of nutrients. A review by El-Sayed (2014) presents results and recommended required taurine levels in feed for a range of marine species. For all investigated species' the recommended taurine concentration of the feed was reported to be between 2 and 15 mg g^{-1} DW supplementation. Salze and Davis (2015) also reports similar recommended concentrations in their review. In Japanese flounder (*P. olivaceus*), Chen *et al* (2005) demonstrated an increase in growth when taurine enrichment of rotifers was elevated from 0.533 mg g^{-1} to 2.05 mg g^{-1} DW of rotifers. However, increasing the taurine concentration further, to 4.54 mg g^{-1} DW in rotifers, did not yield an additional increase in growth. The requirement for a nutrient is often defined as "requirement for maximal growth and/or survival" (Hamre *et al*, 2013b), thus, the requirement for taurine in Japanese flounder is probably between 0.533 and 2.052 mg g^{-1} DW in rotifers. The concentrations of taurine in the Tau-rotifers (0.948 mg g^{-1} DW) in the present study would therefore be approximately half that of the assumed required level for Japanese flounder. However, as the Tau-group larvae were co-fed rotifers enriched with MultiGain®, the actual contribution of taurine from rotifers in the Tau-group larvae were 0.552 mg g^{-1} DW. A similar approach to determining the requirement for Ballan wrasse has, to the best of the candidates' knowledge, not been conducted. Al-Feky *et al* (2016) found that a concentration of 9.7 mg g^{-1} dietary taurine is required for optimal growth rate, feed efficiency and survival for Nile tilapia (*Oreochromis niloticus*) larvae fed plant based diets.

Karlsen *et al* (2015) demonstrated that a concentration of 1.45 ± 0.035 mg g^{-1} WW taurine (values modified from Karlsen *et al* (2015)) were achieved in cod (*G. morhua*) larvae at 20 dph when fed exclusively zooplankton (6.26 ± 0.751 mg g^{-1} DW taurine). Taurine content in the cod larvae presented by Karlsen *et al* (2015) exceeds that of the Ballan wrasse larvae in the present study, which were 0.292 ± 0.019 mg g^{-1} WW at 23 dph, after being fed taurine- and MultiGain® enriched rotifers (in total 0.533 mg g^{-1} DW taurine). This finding may indicate that the taurine concentrations obtained in rotifers in the present study was too low to cover dietary needs of Ballan wrasse, as Atlantic cod fed the assumed optimal feed contained higher concentrations of taurine. To determine the exact requirement of taurine for Ballan wrasse, a similar study as Chen *et al* (2005) performed with Japanese flounder would need to be done. Furthermore, it seems probable that the requirement for taurine in Ballan wrasse is higher than that of gilthead seabream (*S. aurata*) and zebrafish (*Danio rerio*), which has high activity of the enzyme CSAD (Pinto *et al*, 2013; Guimarães *et al*, 2018). Requirement of taurine for Ballan wrasse larvae are probably closer to the level found in zooplankton.

Guimarães *et al* (2018) showed that the whole-body taurine content increases with increased dietary taurine, until a threshold is reached, in mature zebrafish (*Danio rerio*). However, juvenile zebrafish did not reach such a threshold, and whole body taurine content had a linear relationship with dietary taurine concentration. It may therefore be a good first approximation to compare the dietary and larval taurine profiles when assessing requirement for nutrients. This could increase knowledge about how dietary taurine affect the Ballan wrasse larvae, and the requirement for taurine could be better understood. In the present study, such a comparison is attempted to be made, such as to determine the requirement of taurine for Ballan wrasse larvae. However, even though the enrichment method with liposomes succeed in elevating the taurine concentration of the rotifers, enrichment success of taurine was not severe enough to obtain taurine concentrations in the rotifers at the assumed optimal level, found in zooplankton (approximately 7 mg g^{-1} DW). Therefore it is recommended for future studies to test the effect of different levels of dietary taurine on Ballan wrasse larvae, and thereby a dietary taurine requirement can be obtained for Ballan wrasse larvae.

4.8 Conclusions

Because taurine-enrichment elevated the concentration of taurine in the larvae, it is suggested that the Ballan wrasse may not have an efficient system for biosynthesis of taurine at the larval stage. It is therefore likely that Ballan wrasse has a low activity of the CSAD enzyme, which indicate that the taurine is an essential nutrient for Ballan wrasse larvae. Furthermore, the low concentrations taurine found in rotifers are not covering the requirements of taurine for Ballan wrasse larvae, and probably causes a nutritional deficiency of taurine in the larvae. The current practices of rotifer enrichment would likely be improved by elevating the amount of available taurine in the feeds. A dietary taurine concentration like that found in zooplankton are probably covering the taurine requirements of larval Ballan wrasse. These conclusions are also in accordance with other papers' findings, for a range of marine species. The enrichment method used in the present study contributes to elevating the concentration of taurine in rotifers. However, the method did not provide enough taurine in the rotifers to cover the assumed dietary requirements for Ballan wrasse.

5 Methodological considerations

5.1 Liposome production and rehydration

The production of saline liposomes, to be used in the Empty treatment, were delayed one week, due to a mistake when rehydrating the FDEL (freeze-dried empty liposomes). Originally, half the FDEL produced were supposed to be rehydrated in taurine-solution, while the other was meant to be rehydrated in a saline solution. All the FDEL were rehydrated in taurine-solution, and FDEL to be used in the Empty treatment had to be produced once again. Because of the duration of the freeze-drying process (at least 72 hours) and the availability of the freeze-dryer, this was delayed. The liposomes were prepared at 18. September 2017, eight days after the larvae were placed in the experimental tanks. This meant that for the first five days of feeding, the larvae in the Empty treatment were fed unenriched rotifers, in place of Empty rotifers. It is unlikely that this has influenced the larval development in a significant manner, as there were no significant differences between the different treatments in larval standard length. However, it is possible that this has had an undiscovered effect on the results. Also, the only difference between the unenriched rotifers and the Empty rotifers (disregarding natural variation) is the liposomes themselves. Whether the fatty acids that constitute the liposomes have had a positive or negative effect is not known. However, it is possible that these fatty acids would have a negative effect on digestion (Øystein Sæle, personal

communication). Thus, the effect of liposomes on the larvae is not known, however, there was not found a significant difference in SL at 23 dph, or survival-rate among the groups. Therefore, it seems probable that the liposomes do not cause a negative effect on the larvae.

5.2 General sampling

Since it became apparent that not all tanks had enough individuals to conduct proper statistical analyzes, samples for dry-weight were prioritized over the HAA (hydrolyzed amino acid, for taurine analysis) samples. This meant that the paper has less data on taurine concentration from the experiment, since some tanks either hasn't been analyzed, or have been analyzed with few parallels. Because of this, some results may not be as statistically strong as they potentially could have been. Furthermore, the dry-weight samples were not properly analyzed based on a misunderstanding between the candidate and the laboratory technicians at Nifes. This means that the size-related results in this paper is the standard-length samples, which does not provide perfect estimations for the larvae's size and mass, as larvae occupy three dimensions. Non-larvae samples were not taken during the last day of sampling, day 23, as rotifers were not enriched at this day. Taurine concentration in Empty liposomes were not analyzed, since no amino-acids were used in preparation of these liposomes, the results from the rotifers amino-acid tests also show that this group contained the lowest concentration of taurine. Samples for all wanted tests were not taken due to the high mortality in the tanks. For example, samples for 2GSH/GSSG levels in the larvae were planned but not conducted. 2GSH/GSSG is identified as the most important cellular redox couple and is commonly used as an assessment of the redox environment in an organism (Penglase *et al*, 2015). Such an analysis could have provided depth in the understanding of the effect of dietary taurine.

5.3 Liposome production

The concentration of taurine in the taurine liposomes were shown to be halved (see table 3.3.2) during the experiment. Samples taken day 4 had almost exactly double the concentration of taurine than samples taken day 19. This may have had an impact on the amounts of taurine the larvae were fed, because the taurine concentration in the rotifers could have been reduced. However, the rotifers were not shown to have a dramatic decrease in taurine concentration as the liposomes presented, see table 3.3.2. It would make sense to assume that, as the liposomes concentration of taurine is reduced, so should the concentrations in the rotifers. It is unknown why this not the case in the present study.

For the larval experiment, taurine-filled liposomes had a concentration of 6 % (w v^{-1}) taurine. The reasoning for choosing 6 % was that at higher concentrations, taurine could start to crystallize when stored in 4 °C or less (Matthew Hawkyard, personal communication). Furthermore, preliminary tests were performed using both 6 and 10 % (w v^{-1}) taurine, out of which only the 6 % group yielded an acceptable result. However, the trials with 10 % (w v^{-1}) taurine were unsuccessful, as no taurine were found in these rotifers. The leeching of taurine from liposomes with higher concentration of taurine was not tested, as the rotifers were enriched with newly produced liposomes. In the multivariate experiment, the leeching effect may have been more significant in rotifers enriched with liposomes rehydrated in 9 than 6 % (w v^{-1}) taurine solution, however retention rate was examined for the center-point group (6 % (w v^{-1}) taurine), and retention-rate of taurine was found to be high in rotifers.

5.4 The larval tank environment

Temperature in the experimental tanks mimicked the temperature in the facility's own production tanks, as the water in the systems had the same origin. The tanks had continuous lighting from above the tanks, in accordance to the facility's protocols. The rotifers had equal origin and the rotifer enrichment water for the enrichment of the Tau and Empty treatments had the same origin as the water for the enrichment of the MultiGain® treatment rotifers. Oxygen saturation levels were satisfactory, throughout the water column. Clay was taken from the same clay storage as the clay used in the facility's own production. Apart from the liposomes used in the enrichment of the Tau and Empty treatments, no outside substance (substance not used in the facility's production) were added to the tanks. As the production cycle at the facility at the time of the experiment performed badly (<2 % survival), it seems likely that the high mortalities displayed in the experimental tanks originated from the facility, and not the treatments used in the experiment. There are some factors that may have affected the experimental tanks. The tanks themselves are smaller than the ones normally used at the facility (0.1 m^3 in experimental tanks, 9 m^3 in larval rearing tanks), and even though the concentration of larvae were similar (100 larvae per liter) stressors such as momentary changes in abiotic factors may have had a higher impact on the environment in the experimental tanks, than in the larval rearing tanks.

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Appendices

Appendix 1: Detailed description of amino acid analysis

The samples were weighted in 100 mL Florence flasks (Pyrex) and added 300 µl 0.1 M Dithiothreitol (DTT, Sigma), a spatula tip of crystallic Phenol (Sigma) and 9.7 ml internal standard. HCl with intern standard were produced by weighing exactly 0.3661 g DL-Norvalin ($C_5H_{11}NO_2$, Fluka) and adding 6M hydrochloric acid (HCl, Sigma), the mixture was then transferred to a 1000 ml volumetric flask and diluted to the 1000 ml mark with 6 M HCl. The Florence flasks were marked and stirred before a coat of nitrogen gas (N_2) filled the flasks. The flasks were then placed in a metal box with a lid and hydrolyzed for 22 hours in 110 °C. Cysteine and tryptophan are destroyed during acid hydrolysis, DTT are added as an antioxidant do prevent methionine from oxidizing. After hydrolyzation, 500µl hydrolysate were pipetted from the Florence flasks to 10 ml centrifugation tubes and centrifuged in a vacuum centrifuge (CentriVap Benchtop Vacuum Concentrator) connected to a cold trap system (CentriVap Cold Trap System, Labconco), until all HCl were removed from the samples. This process lasted for approximately 2.5 hours. After centrifugation, 5 ml filtered water were pipetted to each tube and mixed on a whirling mixer (Vortex Genie 2, Scientific Industries) for 40 seconds. Following this, the samples were filtrated using a syringe and a filter (0.45 µm mesh size), 1 ml of each sample were filtrated and transferred to Eppendorf tubes. After each filtration, the syringe and filter were discarded.

Following the filtration, the samples were derivatized using Accq.Tag™ Ultra Derivatization Kit, which consisted of Borat buffer, reagent powder and reagent dilution. A heating block were preheated to 55 °C, and glass-vials (Waters) were prepared for blank sample, 2 standard samples and the real samples. Each vial was added 70 µl Borat buffer, except for the blank vial which was added 80 µl. Then 10 µl sample were pipetted into the sample-vials, and 10 µl external standard* into the standard vials. Then, 20 µl derivatization reagent** were added to all vials, and the vial caps were screwed on immediately after the last addition. All vials were then mixed on whirling mixer for 10 seconds and were not disturbed for 1 minute after mixing. Then the vials were put into the preheated heating block for exactly 10 minutes, after which the samples were ready for analysis.

*: External standard was produced by pipetting 200 µl amino acid standard (Amino Acid standard H, Pierce), 100 µl 5.0 mM Norvalin (Sigma-Aldrich), 100 µl 5.0 mM hydroxyproline and taurine (Hydroproxyproline; Sigma-Aldrich, Taurine; Sigma-Aldrich) and 600 µl filtered water into a 1.8 ml cryotubes. The cryotubes were then thoroughly shaken on a whirling mixer.

**: Derivatization reagent was produced by pipetting 1 ml reagent dilution, marked 2B, to the vial with the reagent powder, marked 2A. Then the vial was shaken on a whirling mixer for 10 seconds before being placed in the heating block (55 °C) for 12 minutes.

The vials were then placed in a HPLC instrument (Waters Acquity UPLC with Photodiode Array (PDA) Detector) and analyzed. The column temperature of the instrument was set to 55 °C, the flow to 0.7 ml min^{-1} and the eluents to 99.9 and 0.1 for A1 (5 % AccQ.Tag™ Ultra Eluent A) and B1 (AccQ.Tag™ Ultra Eluent B) respectively. After the delta presented in Empower (the program that controls the instrument) showed a steady value beneath 15, the instrument was ready start the analysis. Then sample names and weights of the samples were written into Empower, and the analysis were started. The instrument detects the samples using UV-absorption at 260 nm. The results are then quantified using intern and extern standard-curves as calibration.

The output from the instrument was presented in Empower. In Empower, the output was shown in a diagram, as one continuous curve with peaks. These peaks represent the concentration of certain amino acids in a given sample. Using a previously set of identification tools, which consisted of lines marking the peaks with the corresponding amino acid, a calibration was conducted based on the standard samples. This calibration was then added to the samples of interest. Empower automatically locates peaks, however, if the peaks have certain traits, this isn't always accurate. One such trait is that the concentration of one or more amino acids were too low to make Empower automatically identify it as a peak. These were added manually. Using the area of the peak, the program then calculates the concentration of the amino acid, given in milligrams per gram sample (mg g^{-1}).

The HPLC system is based on the difference in migration speed of different amino acids. Due to molecular structure and composition, different amino acids have varying degrees of interaction with the stationary phase, causing them to move through it at different rates, and components in the sample with higher degrees of interaction with the stationary

phase moves more slowly. As the components in the sample move through the stationary phase at unequal rates, the components become separated, and concentrations may then be analyzed separately. The mobile phase consists of a constant slow of solvent, in which the sample is injected and led to the column, in the beginning of the analysis, a weaker solvent is used (Eluent A1) and over time higher concentrations of a stronger solute is added (Eluent B1). After the components have exited the column, they are quantified by a detector. The quantification of amino acids is performed by UV absorbance detection at 254 nm, after HPLC separation (RP18 column, internal diameters: 2.1 x 100 mm), the higher concentration of a component, a higher peak is presented in the HPLC software (Empower). In the system used in the present study, the chromatography is reverse phase. This means that the stationary phase is non-polar, and the mobile phase is polar.

Appendix 2: Multivariate experiment

The data from the rotifer experiment were set up in Microsoft Excel and transferred to Statistica 13. Multivariate Regression was then used to analyze the data. As dependent variable, concentration of taurine was selected, as this were the variable that expressed the enrichment success of each trial. As independent variables, the percent of taurine in the liposomes (1), the amount of liposomes used to enrich the rotifers (2) and the enrichment time of the rotifers (3) were selected, as well as the interactions between these variables (1x2, 1x3, 2x3 and 1x2x3).

	1	2	3	1x2	1x3	2x3	1x2x3	tau DW (mg/g)
1	3	200	0,5	600	1,5	100	300	0,315087761
2	3	300	0,5	900	1,5	150	450	0,471835821
3	3	200	1,5	600	4,5	300	900	0,296516976
4	3	300	1,5	900	4,5	450	1350	0,38519315
5	9	200	0,5	1800	4,5	100	900	0,866723933
6	9	300	0,5	2700	4,5	150	1350	0,797288442
7	9	200	1,5	1800	13,5	300	2700	0,707818356
8	9	300	1,5	2700	13,5	450	4050	1,07259675
9	6	250	1	1500	6	250	1500	0,552169389
10	6	250	1	1500	6	250	1500	0,570923516
11	6	250	1	1500	6	250	1500	0,53942973

Figure A.2.1: *Variables used in multiple regression analysis.*

The output from the analysis is seen in figure A.2.2. Variables highlighted in red are identified as significant. These variables or interactions between variables have a significant effect of the dependent variable. The "adjusted R^2" value explains to what grade the chosen variables explain the dependent variable, the closer to 1 this value is, the more of the dependent variable is explained by the chosen variables.

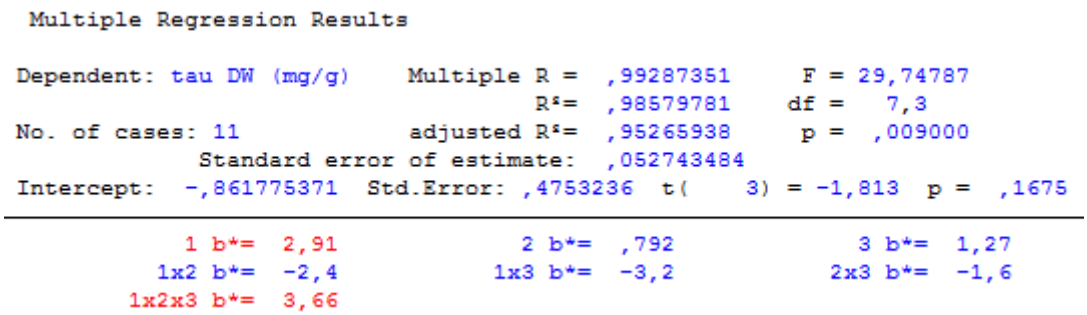

Figure A.2.2: *Output from first multiple regression. Significant variables are highlighted in red ($p < 0.05$).*

As variables 1 and the interaction term 1x2x3 are identified as significant, another multivariate regression analysis was conducted by selecting these variables, results shown in figure A.2.3, while the dependent variable stays the same.

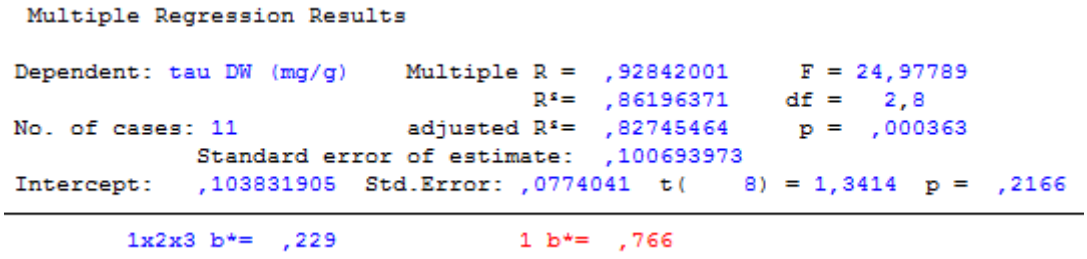

Figure A.2.3: *Output from second multiple regression. Significant variables are highlighted in red ($p < 0.05$).*

The second multiple regression analysis identified only the concentration of taurine in liposomes as a significant variable, therefore a third analysis were conducted, using only this variable.

```
 Multiple Regression Results

Dependent: tau DW (mg/g)     Multiple R =  ,91126519      F = 44,06737
                                      R²=  ,83040425     df =   1,9
No. of cases: 11             adjusted R²=  ,81156028      p =  ,000095
             Standard error of estimate:  ,105229633
Intercept:    ,103831905  Std.Error: ,0808907  t(    9) = 1,2836  p =  ,2313

             1 b*=  ,911
```

Figure A.2.4: *Output from third multiple regression. Red color indicates that a variable is significant ($p < 0.05$).*

Regression summary and analysis of variance is provided in figure A.2.5.

N=11	Regression Summary for Dependent Variable: tau DW (mg/g) (raw data stat R= ,91126519 R²= ,83040425 Adjusted R²= ,81156028 F(1,9)=44,067 p<,00010 Std.Error of estimate: ,10523					
	b*	Std.Err. of b*	b	Std.Err. of b	t(9)	p-value
Intercept			0,103832	0,080891	1,283608	0,231341
1	0,911265	0,137273	0,082325	0,012401	6,638326	0,000095

Effect	Analysis of Variance; DV: tau DW (mg/g) (raw data stat				
	Sums of Squares	df	Mean Squares	F	p-value
Regress.	0,487970	1	0,487970	44,06737	0,000095
Residual	0,099659	9	0,011073		
Total	0,587630				

Figure A.2.5: *Regression summary and analysis of variance.*

Cell No.	% tau lip	{1} ,36716	{2} ,55417	{3} ,86111
1	3		0,115838	0,000652
2	6	0,115838		0,013925
3	9	0,000652	0,013925	

Figure A.2.6: *Output Tukey HSD between low, medium and high taurine group.*

Appendix 3: Standard length and taurine concentrations from larval experiment

dph →	**1**		**9**	**14**	**19**	**23**	
Treatment ↓	Tau (mg g^{-1})	SL (mm)	SL (mm)	SL (mm)	SL (mm)	Tau (mg g^{-1})	SL (mm)
Tau (1)	0.6195	2.96	4.12	4.70	4.95	-	5.493 ± 0.850
Tau (2)	0.6195	2.96	3.87	4.49	4.75	0.3054*	5.651 ± 0.704
Tau (3)	0.6195	2.96	4.14	4.92	5.26	0.2784	6.358 ± 0.955
MultiGain (4)	0.6195	2.96	3.73	4.32	4.77	0.0495*	5.666 ± 0.899
MultiGain (5)	0.6195	2.96	3.91	4.25	4.74	-	5.759 ± 1.002
Empty (14)	0.6195	2.96	4.10	4.76	5.53	0.1715	6.896 ± 0.945
Empty (15)	0.6195	2.96	4.11	4.43	4.98	0.1410	5.481 ± 0.756
Empty (16)	0.6195	2.96	4.02	4.40	5.12	0.1508*	5.851 ± 1.010

Table A.3.1: *Overview of SL (standard length) and taurine concentration (Tau-group larvae) WW. * indicates 1 parallel were available, other samples have 2 parallels. Tank 6 is not presented in this table, as the tank was discontinued due to low survival rate. Brackets in the treatment column represents tank number.*

Appendix 4: Post hoc Unequal N HSD analysis for SL at 23 dph. Nested design ANOVA.

Unequal N HSD; variable Length (mm) (lengder larver)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = ,79321, df = 241,00

Cell No.	Tank	{1} 5,4934	{2} 5,6507	{3} 6,3577	{4} 5,6658	{5} 5,7588	{6} 6,8961	{7} 5,4806	{8} 5,8511
1	1		0,996189	0,002636	0,995884	0,949295	0,000032	1,000000	0,761846
2	2	0,996189		0,032295	1,000000	0,999801	0,000039	0,995341	0,987359
3	3	0,002636	0,032295		0,061656	0,170594	0,338848	0,002682	0,328089
4	4	0,995884	1,000000	0,061656		0,999928	0,000041	0,993586	0,993552
5	5	0,949295	0,999801	0,170594	0,999928		0,000100	0,935175	0,999932
6	14	0,000032	0,000039	0,338848	0,000041	0,000100		0,000032	0,000450
7	15	1,000000	0,995341	0,002682	0,993586	0,935175	0,000032		0,727230
8	16	0,761846	0,987359	0,328089	0,993552	0,999932	0,000450	0,727230	

Figure A.4.1: *Post hoc analysis for SL at 23 dph. Values in red represents significant differences ($p < 0.05$).*

Appendix 5: Post hoc Unequal N HSD analysis for SL from repeated measures ANOVA.

Unequal N HSD; variable DV_1 (lengder larver)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = ,41858, df = 603,93

Cell No.	Treatment	R1	{1} 4,0766	{2} 4,7635	{3} 5,0538	{4} 5,7789	{5} 3,8376	{6} 4,2687	{7} 4,7495	**{8} 5,6619**	{9} 4,0389	{10} 4,6011	{11} 5,2549	{12} 6,0285
1	Tau	SL 9 dph		0,000018	0,000018	0,000018	0,853566	0,965120	0,000082	0,000018	1,000000	0,001303	0,000018	0,000018
2	Tau	SL 14 dph	0,000018		0,368938	0,000018	0,000018	0,017430	1,000000	0,000018	0,000018	0,977208	0,003935	0,000018
3	Tau	SL 19 dph	0,000018	0,368938		0,000018	0,000018	0,000018	0,545428	0,000646	0,000018	0,013011	0,898221	0,000018
4	Tau	SL 23 dph	0,000018	0,000018	0,000018		0,000018	0,000018	0,000018	0,999505	0,000018	0,000018	0,001327	0,677777
5	MultiGain	SL 9 dph	0,853566	0,000018	0,000018	0,000018		0,051059	0,000018	0,000018	0,951341	0,000019	0,000018	0,000018
6	MultiGain	SL 14 dph	0,965120	0,017430	0,000018	0,000018	0,051059		0,014582	0,000018	0,883598	0,399161	0,000018	0,000018
7	MultiGain	SL 19 dph	0,000082	1,000000	0,545428	0,000018	0,000018	0,014582		0,000018	0,000033	0,995662	0,013260	0,000018
8	MultiGain	SL 23 dph	0,000018	0,000018	0,000646	0,999505	0,000018	0,000018	0,000018		0,000018	0,000018	0,123569	0,247567
9	Empty	SL 9 dph	1,000000	0,000018	0,000018	0,000018	0,951341	0,883598	0,000033	0,000018		0,000080	0,000018	0,000018
10	Empty	SL 14 dph	0,001303	0,977208	0,013011	0,000018	0,000019	0,399161	0,995662	0,000018	0,000080		0,000018	0,000018
11	Empty	SL 19 dph	0,000018	0,003935	0,898221	0,001327	0,000018	0,000018	0,013260	0,123569	0,000018	0,000018		0,000018
12	Empty	SL 23 dph	0,000018	0,000018	0,000018	0,677777	0,000018	0,000018	0,000018	0,247567	0,000018	0,000018	0,000018	

Figure A.5.1: *Post hoc analysis for SL from repeated measures ANOVA. Values in red represents significant differences ($p < 0.05$).*