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| **Citation** | Hawkyard, M., Laurel, B., Barr, Y., Hamre, K., & Langdon, C. (2015). Evaluation of liposomes for the enrichment of rotifers (Brachionus sp.) with taurine and their subsequent effects on the growth and development of northern rock sole (Lepidopsetta polyxystra) larvae. Aquaculture, 441, 118-125. doi:10.1016/j.aquaculture.2015.02.012 |
| **DOI** | 10.1016/j.aquaculture.2015.02.012 |
| **Publisher** | Elsevier |
| **Version** | Version of Record |
| **Terms of Use** | http://cdss.library.oregonstate.edu/sa-termsofuse |

Aquaculture 441 (2015) 118–125

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

# Evaluation of liposomes for the enrichment of rotifers (*Brachionus* sp.) with taurine and their subsequent effects on the growth and development of northern rock sole (*Lepidopsetta polyxystra*) larvae

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ARTICLE INFO

*Article history:*
Received 12 August 2014
Received in revised form 7 February 2015
Accepted 9 February 2015
Available online 14 February 2015

*Keywords:*
Taurine
Liposomes
Microparticles
Rotifers
Enrichment
Fish larvae

ABSTRACT

The naturally occurring taurine concentrations of rotifers (*Brachionus* sp.) may not be sufficient to meet the nutritional demands of several species of marine fish larvae. In this study, we evaluated the use of liposomes for taurine-enrichment of rotifers and compared them with standard methods in which taurine was dissolved in the rotifer enrichment water i.e., the "dissolved method". Each enrichment method was further evaluated by determining the growth performance of northern rock sole (*Lepidopsetta polyxystra*) larvae fed on the taurine-enriched rotifers. Results indicated that rotifers enriched with taurine-containing liposomes obtained approximately 1.2% taurine on a dry weight basis, similar to the upper concentrations reported in wild copepods and approximately 10-fold higher than in control rotifers. Northern rock sole larvae grew significantly larger, were more developed and had higher whole body taurine concentrations when fed rotifers enriched with taurine-containing liposomes, compared to larvae fed either unenriched rotifers or rotifers enriched with equivalent concentrations of taurine using the dissolved method. The dissolved method required 60× more taurine to achieve rotifer enrichment levels and larval growth performance observed using the liposome-enrichment method. Fluorescent markers indicated that rotifers were able to break down liposomes, liberating water-soluble nutrients into their guts and body cavity. Differences and similarities are discussed between the efficacy of liposomes and previously studied wax spray beads (WSB) for rotifer enrichment and subsequent nutritional effects on fish larvae.


## 1. Introduction

In commercial hatcheries, marine fish are often fed rotifers from first-feeding until they are weaned onto *Artemia* or particulate diets. With respect to the nutritional requirements of marine fish larvae, rotifers may be deficient in one or more water-soluble nutrients (Hamre et al., 2008; Mæhre et al., 2012; van der Meeren et al., 2008) notably among them the amino-sulfonic acid, taurine. Taurine is present in the free form in cells and body fluids and is an important compound in osmoregulation and bile salt production. It can be synthesized from methionine through cysteine (Espe et al., 2008), the rate-limiting enzyme being cysteine sulfinate decarboxylase (CSD; Griffith, 1987). Cysteine sulfinate decarboxylase deficiency may also be present in fish shown to benefit from taurine supplementation (Kim et al., 2008). Taurine has several important biological functions and deficiencies may result in lipid accumulation, mitochondrial damage and resulting oxidative stress, neurological anomalies and heart failure (Chesney et al., 1998; Espe et al., 2008, 2012; Jong et al., 2012; Militante and Lombardini, 2004).

The aquatic habitat makes delivery of water-soluble nutrients especially challenging due to loss of water-soluble substances from food particles to the surrounding seawater or "nutrient leaching". This problem is exacerbated with microparticulate diets because their surface area to volume ratios dramatically increase as particle sizes decrease (Langdon and Barrows, 2011). In living aquatic organisms, such as rotifers, cellular membranes serve to limit the losses of water-soluble substances. As such, living organisms may be used to bioencapsulate and deliver specific nutrients for fish larvae.

Enrichment of rotifers with water-soluble nutrients is most often accomplished by dissolving the nutrient in the rotifer's aqueous culture medium (Matsunari et al., 2005; Salze et al., 2012; Tonheim et al., 2000; Takahashi et al. 2005), hereafter referred to as the "dissolved method". However, there are several drawbacks to this approach in that: 1) only a small fraction of the aqueous water-soluble nutrient is taken up by rotifers during enrichment resulting in nutrient wastage, 2) water-soluble nutrients may interact with other solutes in the enrichment medium and are, therefore, prone to oxidation and/or other chemical reactions,

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http://dx.doi.org/10.1016/j.aquaculture.2015.02.012
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3) substances may be metabolized by the microbial community during enrichment processes and 4) high quantities of dissolved water-soluble nutrients may increase the likelihood of bacterial infection of cultured organisms. To ameliorate these issues, water-soluble substances may be encapsulated within microparticles, such as liposomes, which are fed to rotifers resulting in nutrient enrichment. Liposomes have been shown to elevate the concentrations of several water-soluble substances in rotifers (Barr and Helland, 2007; Monroig et al., 2003, 2007b; Pinto et al., 2013). However, the impact of liposome-enriched rotifers on marine fish larvae has not been thoroughly investigated. In one of the only documented studies in this area, taurine-liposome enriched rotifers were fed to Gilthead seabream but had little effect, presumably due to the low taurine requirements of seabream (Pinto et al., 2013). To our knowledge, there are no studies that demonstrate positive effects of rotifers enriched with taurine-containing liposomes on marine fish larvae.

Taurine concentrations are very low (not detected—0.05% DW) in rotifers when compared to those measured in copepods (0.5–1.9% DW), the natural prey of marine fish larvae (Mæhre et al., 2012; van der Meeren et al., 2008). Several species of marine fish have been shown to benefit from taurine concentrations well above those measured in rotifers (Chen et al., 2005; Matsunari et al., 2005; Omura and Inagaki, 2000; Salze et al., 2011, 2012). Specifically, northern rock sole larvae have been shown to grow and develop faster when fed rotifers enriched with taurine-containing lipid-spray beads, compared to those fed rotifers that were not enriched with taurine (Hawkyard et al., 2014). In this study, we investigate the use of taurine-liposomes to improve the nutritional value of rotifers for larval northern rock sole, a species that is known to benefit from elevated dietary taurine during the larval phase (Hawkyard et al., 2014). Improvements in the nutritional quality of live prey through enrichment with water-soluble nutrients provide opportunities for higher productivities and cost-savings for commercial hatcheries.

## 2. Material and methods

### 2.1. Production of liposomes

Liposomes were produced based on the methods described by Barr and Helland (2007). Liposomes were produced from Phospholipon 90H (294-KG-1, Lipoid, Newark, NJ, USA), which is a hydrogenated phosphatidylcholine (PC) with 16:0 and 18:0 as the predominant (>99%) fatty acids (product technical data). Hydrogenated PC was used because liposomes produced from saturated lipids have been shown to better retain amino acids when suspended in seawater compared to those containing polyunsaturated fatty acids (PUFA; Monroig et al., 2003). In addition, PUFA-containing liposomes are more susceptible to lipid-peroxidation (Monroig et al., 2007a) and could subject fish larvae to oxidative stress during feeding trials. Thirty-seven and a half grams of Phospholipon 90H were dissolved in 750 ml chloroform. An equal volume of distilled water was added to the organic solution and mixed until a stable emulsion was obtained. The emulsion was placed in a rotary evaporator (Labconco, Kansas City, MO, USA; water bath 60 °C) and rotated at 90–120 rpm. Vacuum pressure was maintained between 700 and 900 mbar with a KNF Laboport vacuum pump system (KNF Neuberger Inc., Trenton, NJ, USA). After 1.5 h of rotational evaporation, the resultant suspension was poured into plastic 250 ml beakers, frozen (−20 °C) and lyophilized in a Labconco Freezone (Labconco, Kansas City, MO, USA) freeze dryer resulting in freeze-dried, empty liposomes (FDEL).

Taurine (T-0625; Sigma-Aldrich, St. Louis, MO, USA) was ground for 1 h using a jar mill (U.S. Stoneware, NJ, USA) to obtain particle sizes smaller than 0.5 μm. The small particle size facilitated the dissolution of the taurine-crystals and reduced the need for heating during the preparation of taurine solutions. Liposomes containing taurine (taurine liposomes) were prepared by hydrating 100 mg FDEL $ml^{-1}$ with a preheated (60 °C) 10% (w/v) taurine and 0.86% (w/v) NaCl solution whereas "saline-liposomes" were prepared by hydrating 100 mg FDEL $ml^{-1}$ with a preheated 2.6% NaCl solution. Sodium chloride was added to both particle types to obtain isosmotic concentrations in the core with respect to the rotifer enrichment water. The resultant suspensions were stirred for 15 min and then extruded for 20 min (for every 100 ml suspension prepared) at a rate of 14.1 ml $min^{-1}$ through a 22 gauge smooth-flow tapered tip (Nordson EDF, Westlake, OH, USA) by means of a peristaltic pump (Heidolph pumpdrive 5201; SP Quick pumphead, Heidolph Instruments Schwabach, Germany). Liposomes were refrigerated (2–4 ° C) in suspension until use.

Fluorescent liposomes were prepared to qualitatively examine ingestion and digestion of liposomes by rotifers. These were made as described above with the following modifications: 5 mg DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; D282; Molecular Probes, Oregon, USA) were dissolved in the organic phase during rotary evaporation. DiI remained in the phospholipid-water suspension and was not observed in the chloroform distillate. A solution of sodium fluorescein (5% w/v) was prepared and encapsulated within DiI-stained liposomes, as described above for taurine. DiI was used to stain the outer-lipid lamellae of liposomes whereas sodium fluorescein was used to stain the aqueous core and, therefore, acts as a proxy for water-soluble nutrients. Using this methodology we could determine if water-soluble substances were released from ingested liposomes.

### 2.2. Taurine retention and particle size analyses

Liposomes were centrifuged at 3500 rpm for 15 min to obtain a liposome pellet, the supernatant was discarded and this process was repeated 3 times. The pellet was not disturbed between centrifugations thereby minimizing the leaching of particles during washing. For leaching trials, 25 mg (wet weight) liposomes were removed from the washed pellet and added to a 50 ml polypropylene centrifuge tube. Twenty-five ml of 1-μm filtered seawater (20 °C) was added to each tube at the beginning of the trial. Tubes were capped and rotated at 20 rpm on a culture-rotator. One and a half ml aliquots were removed at 5, 15, 30 and 60 min, placed in a 1.6 ml microcentrifuge tube and centrifuged for 1 min at 20,000 rcf to removed liposomes from suspension (sample leachate). The liposome-free supernatant (verified microscopically in preliminary trials) was then filtered though a 0.2 μm syringe filter to further ensure that any remaining liposomes were removed and the filtrate was collected in a clean microcentrifuge tube. An additional aliquot was removed to determine the total quantity of taurine present in the liposome suspension. Total taurine concentrations were determined after the liposomes were fractured to release taurine, using the freeze-fracture method described by Niesman et al. (1992). After-freeze fracturing (i.e. 100% loss), the samples were centrifuged and taurine concentrations in the supernatant were determined as described above and in Section 2.6. Taurine retention was calculated as follows:

$$\textit{Retention}\ (\%) = 100 - (\textit{taurine conc. measured in sample leachate} / \textit{total taurine conc. in sample} \times 100)$$

where, "*taurine conc. measured in sample leachate*" represents the taurine measured in the supernatant of a given sample after removal of the liposomes by centrifugation and "*total taurine conc. in sample*" represents the total taurine concentration measured in a given sample after taurine was liberated by the freeze fracture method.

For particle size analysis, one drop of the liposome suspension was placed on a glass slide with cover slip. The samples were diluted as necessary to obtain 20–50 particles within the field of view and to minimize clumping. Digital images were taken with a Leica DM 1000 (Leica Microsystems, Wetzlar, Germany) microscope fitted with a Leica DFC 400 camera (Leica Microsystems, Wetzlar, Germany). Particle diameters were measured using Image-J (National Institute of Health, Bethesda, MD, USA) software. Only individual liposome diameters were measured. If two particles were in contact, each liposome was

measured independently; however, clumping of liposomes was rarely observed.

### 2.3. Rotifer culture and enrichments

Rotifers were cultured on a diet of RotiGrow algae paste (Reed Mariculture, Campbell, CA, USA) using methods described in Hawkyard et al. (2014). Liposome enrichments were carried out in 15 l polycarbonate enrichment cones (Aquatic Habitats, Apopka, FL, USA) in 2.5 l of filtered seawater (32 ppt, 26 °C) at a density of 500-rotifers $ml^{-1}$. Rotifers were enriched for 1 h with: 1) saline-liposomes (250 mg liposome phospholipid $l^{-1}$; "Control"); 2) 250 mg dissolved taurine $l^{-1}$ in addition to saline-liposomes (250 mg liposome phospholipid $l^{-1}$; "Dissolved Low"); 3) saline-liposomes (250 mg liposome phospholipid $l^{-1}$) and 15 g dissolved taurine $l^{-1}$ ("Dissolved High"); or 4) taurine-liposomes (250 mg liposome phospholipid $l^{-1}$; "Liposomes"). The concentration of dissolved taurine in the "Dissolved Low" treatment was chosen so that rotifers were exposed to the same concentration of taurine as in the "Liposome" treatment during the enrichment process. The concentration of taurine in the "Dissolved High" treatment was chosen to enrich rotifers with the same concentration of taurine as obtained with the "Liposome" treatment, based on the results of preliminary experiments. Saline-liposomes were provided to rotifers in the Dissolved Low, Dissolved High and Control treatments to ensure that rotifers had equivalent energy and lipid contents in all treatments. In the Dissolved Low and Dissolved High treatments, taurine (T-0625; Sigma-Aldrich, St. Louis, MO, USA) was ground into a powder for 1 h using a jar mill (U.S. Stoneware, NJ, USA) to facilitate the dissolution in filtered seawater prior to adding to the rotifer enrichment water. Rotifers were collected, washed and sampled using the methods described in Hawkyard et al.'s (2014) study.

Rotifers were enriched with a commercial enrichment product, Algamac 3050 (Bio-Marine, CA, USA), using the methods described by Hawkyard et al. (2014). Briefly, rotifers were enriched twice daily for 6–16 h using 0.3 g Algamac 3050 per million rotifers. Algamac-enriched rotifers were collected on an 80 μm sieve and reconstituted in cold (8 °C) seawater.

In a separate trial, rotifers were fed fluorescently-stained liposomes for 1 h (500 rotifers $ml^{-1}$; 250 mg liposomes [by lipid dry weight] $l^{-1}$; 32 ppt, 26 °C). Rotifers were collected on an 80-μm sieve, repeatedly rinsed with clean seawater and observed under an epifluorescent microscope (Leica DM 1000 microscope with DFC 400 camera; Leica Microsystems, Wetzlar, Germany). Fluorescence of DiI (excitation max 549 nm, emission max 565 nm) and fluorescein (excitation max 460 nm and emission max 515 nm) were visually isolated using an Endow GFP Bandpass Emission and TRITC filter sets (Chroma Inc., VT, USA), respectively (Fig. 1).

### 2.4. Northern rock sole husbandry

Northern rock sole larvae were hatched and reared using the methods described by Hawkyard et al. (2014). Larvae were transferred to 20, 100 l black-walled fiberglass tanks (2000 larvae $tank^{-1}$) in a flow-through system maintained at 9 °C. The larval tanks were set up in a flow-through system supplied with sand-filtered seawater from Yaquina Bay, Oregon, and temperatures were maintained at 9 °C (32–35 ppt). Temperature was adjusted by controlling the flow rates of ambient and chilled seawater inputs. Tanks were configured to provide upwelled seawater from the base of the tank (flow rates ~0.6 l $min^{-1}$) and the outflow of each tank was fitted with a "banjo" covered with a 250 μm Nitex® screen. Gentle aeration was provided to each tank by an airstone placed at the bottom of each tank. Due to the high turnover rates of water in the larval tanks (~9 times $day^{-1}$) nitrogenous compounds were not monitored. Larvae were fed Algamac-enriched rotifers twice daily at a feeding density of five rotifers per ml until feeding was observed in >95% of larvae (determined using digital microscopy) which marked the beginning of the feeding trial (Section 2.5). In addition, 5 ml of RotiGrow algae paste (Reed Mariculture, Campbell, CA, USA) was added to each larval tank prior to feeding to obtain "greenwater".

### 2.5. Feeding trial

A six-week feeding trial was conducted to compare the growth and survival response of northern rock sole larva fed rotifers enriched with either taurine-liposomes or taurine dissolved in the enrichment water. Five replicate tanks of larvae were assigned to one of the four dietary treatments described in Section 2.3: 1) Control, 2) Dissolved Low, 3) Dissolved High and 4) Liposomes. It has been well established that marine fish larvae benefit from live prey enriched with polyunsaturated fatty acids (see reviews by Izquierdo and Koven, 2011; Izquierdo et al., 2000; Rainuzzo et al., 1997; Sargent et al., 1999). However, the liposomes in this study were produced from saturated-PC and did provide rotifers with PUFA. Likewise, fish larvae were fed Algamac-enriched rotifers for half of their daily ration. This was done to ensure that the fish larvae obtained essential fatty acids and lipids not provided by liposome-enriched rotifers. We chose to avoid simultaneous enrichment of rotifers with both Algamac and liposomes because rotifers are highly selective of particles based on size (Baer et al., 2008). Likewise, day-to-day differences in Algamac preparations (i.e. blending) would have resulted in elevated variability in the liposome uptake, and therefore, taurine concentrations of rotifers. Since

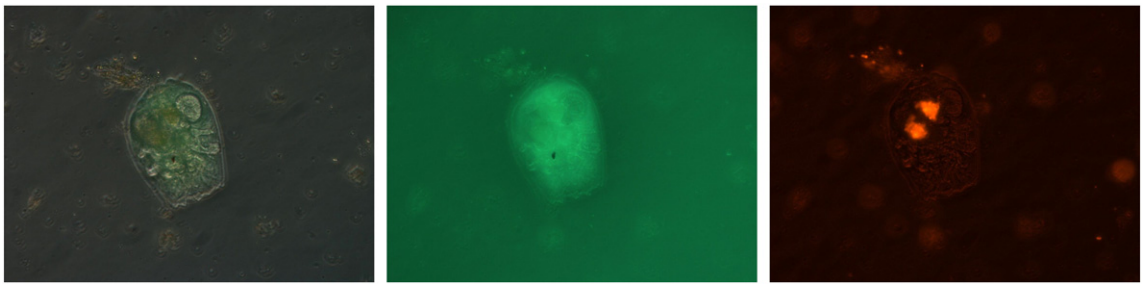

**Fig. 1.** Digital images of a rotifer fed fluorescent liposomes for 1 h taken with a 40× objective. A 5% solution of sodium fluorescein (w/v; green fluorescence) was encapsulated in liposomes as a proxy for taurine. The lipid fraction of liposomes was stained with DiI (0.5 mg $g^{-1}$ w/w orange fluoresence). Liposome-fed rotifers were viewed under an epifluorescent microscope using filters to optimize visualization of the fluorescence of these two markers. Left: rotifer viewed under non-fluorescent conditions. Center: same rotifer viewed under conditions to show fluorescence of fluorescein. Right: same rotifer viewed under conditions to show DiI fluorescence.

Algamac-enriched rotifers were not enriched with taurine, the provision of Algamac-enriched rotifers had a dilution effect on the dietary taurine concentrations experienced by the larvae during the feeding trial. We accounted for this dilution by reporting the "estimated dietary taurine" (est. dietary TAU), which was calculated as follows:

$$Est.\ dietary\ TAU = (taurine\ conc.\ of\ Algamac\ rotifers + taurine\ conc.\ of\ experimental\ rotifers)/2$$

where, "taurine conc. of Algamac rotifers" were the average taurine concentrations (% DW) measured in rotifers enriched with Algamac over the study period and "taurine conc. of experimental rotifers" were the average taurine concentrations (% DW) measured in rotifers enriched with either 1) no added taurine (Control), 2) taurine dissolved in the rotifer enrichment water (Dissolved Low, Dissolved High) or 3) taurine-liposomes. These calculations were based on the assumption that both Algamac- and liposome-enriched rotifers were consumed at equal rates by the fish larvae. Rotifers were added to larval tanks two times per day to obtain a prey density of 5 rotifers $ml^{-1}$, greenwater was also provided as previously described.

Ten larvae from each tank were sampled on day one of the trial and at the end of every week over the six-week period. Larvae were euthanized with MS-222 and individually photographed with a Leica dissecting scope (Wetzlar, Germany) fitted with a Spot insight QE camera (Sterling Heights, MI, USA). Digital images of larvae were analyzed using Image J (NIH, Bethesda, MD, USA) for standard length (SL) and body depth. Developmental stages were determined from digital images based on the criteria described by Hawkyard et al. (2014). Stage 2 was identified by the appearance of a node on an unbent notochord. Stage 3 was identified by a bent notochord that protruded past the caudal peduncle. Stage 4 was identified by a bent notochord that was enveloped by the caudal peduncle. Larval dry weights (DW) were taken by rinsing ten larvae from each tank with 0.5 M ammonium formate to remove salts and then placing them on a pre-weighed aluminum weighing dish. The samples were dried in an oven (65 °C) for 72 h. Once dried, the samples were cooled for 5 min at room temperature (~20 °C) and then weighed to the nearest microgram using a Mettler Toledo MT5 microbalance (Columbus, OH, USA).

Twenty larvae from each tank were pooled and sampled on week six for amino acid analysis. Twenty-four hours prior to sampling, larvae in all treatments were fed Algamac-enriched rotifers to purge them of experimentally enriched rotifers. This method of purging was found to sufficiently purge experimentally enriched rotifers from the larval gut in previous trials (Hawkyard et al., 2014). Larvae were frozen (−20 °C) and then freeze dried (Labconco Freezone freeze dryer) for 72 h. Dried samples were ground with a mortar and pestle and stored under an atmosphere of nitrogen at −20 °C for amino acid analysis.

### 2.6. Amino acid and taurine analyses

Total amino acids were extracted and analyzed by the UC Davis Amino Acid laboratory (Davis, CA, USA) as described in Hawkyard et al.'s (2014) study. The amino acids were measured with a Biochrom 30 ion exchange column with post-ninhydrin derivatization (Biochrom, Holliston, MA, USA). L-Norleucine (General Biochemicals Inc.) was used as an internal standard.

### 2.7. Statistics

Statistics were performed using JMP software, version 10.0.0 (SAS Institute Inc., Cary, NC, USA). Leakage data was analyzed with an ANOVA and post-hoc comparisons were conducted with Tukey's HSD. Homogeneity of variances was checked with Levene's test and normality was assessed graphically. Repeated-measures models, using restricted maximum likelihood (REML) methods, were used to analyze standard lengths (SL), body depths (BD) and larval dry weights (DW) among treatments. The repeated-measures models were used because larvae were repeatedly sampled from the same tanks on a weekly basis. In all cases, the resultant p-values were based on the F-statistic derived from the fixed effects within the REML model. For growth data, "tank" was the statistical unit and therefore the tank means (i.e. average size of larvae per tank) were used to estimate model parameters. As necessary, growth data were normalized with natural-log transformations to ensure data met the assumptions of the model. Larval survival and stage data were analyzed at the end of the trial using ANOVA. Larval survival data were arcsin square root transformed to meet the assumption of equal variance. Post-hoc comparisons were performed with Tukey's HSD with a threshold of $p < 0.05$. The amino acids in fish and rotifers were analyzed with ANOVAs and multiple comparisons were corrected for using the Bonferroni method.

## 3. Results

### 3.1. Liposome particle size and leakage

Liposomes were found to have an average diameter of 4.0 ±0.3 μm (1 S.D.; maximum diameter = 8.9 μm; minimum diameter = 1.3 μm). All liposomes measured were within the range reported to be appropriate for consumption by rotifers (Baer et al., 2008; Hotos, 2003). Taurine concentrations of liposomes suspended in seawater declined to approximately 85 ± 5% of the initial concentration but did not decline further during the remaining 55 min of the test period (Fig. 2).

### 3.2. Rotifer enrichments

After a 1 h feeding period with fluorescently-stained liposomes, the lipid-soluble dye (DiI) was observed in the digestive tract and fecal material of rotifers. DiI stained liposomes appeared both spherical and as fragments in both the gut and fecal material. The water-soluble fluorescent dye sodium fluorescein was also observed in the gut and fecal material of rotifers. However, unlike DiI, sodium fluorescein was also visible in the body region (beyond the gut) of rotifers. Images of a rotifer fed fluorescent-stained liposomes are shown in Fig. 1.

Taurine concentrations in rotifers (% DW; Fig. 3) were higher in Dissolved High and Liposome treatments when compared to the Dissolved Low and Control treatments (Tukey's HSD, significance level $p = 0.05$); however, taurine concentrations of rotifers in the Dissolved High treatment were not significantly different than those measured in Liposome treatment (Tukey's HSD, significance level $p = 0.05$; Fig. 3). Taurine concentrations in the Dissolved Low treatment were not significantly different from those of the Control (Tukey's HSD,

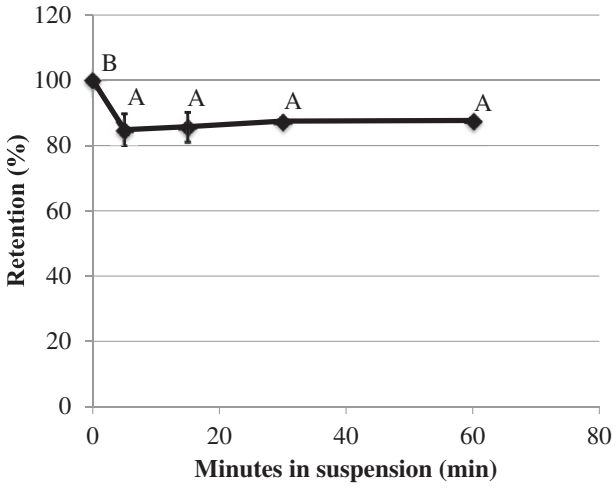


**Fig. 2.** Retention of taurine in liposomes suspended in seawater (%; ±1 SD). Different letters denote significant differences in % retention among time points (Tukey's HSD, significance level $p = 0.05$).

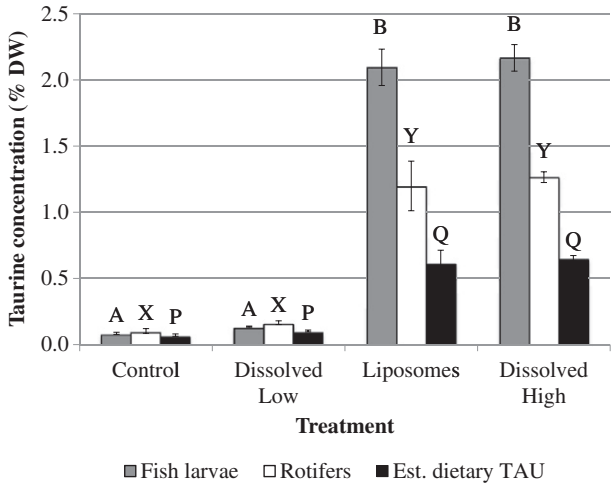


**Fig. 3.** Taurine concentrations (% dry weight; %DW; ±1 SD) measured in taurine-enriched (Dissolved Low, Liposome and Dissolved High) and non-taurine supplemented (control) rotifers, as well as the whole body tissue concentrations of northern rock sole larvae fed each of the experimental diets at the end of the trial. Estimated dietary taurine concentrations are corrected for 50% dilution of the taurine-enriched rotifers with Algamac-enriched rotifers (see Section 2.5 for calculations). Letters denote significant differences among treatments (Tukey's HSD, significance level $p = 0.05$).

significance level $p = 0.05$). With the exception of taurine, no statistical differences were observed in the amino acid concentrations (% DW) of rotifers in the different treatments (ANOVA, Bonferroni correction, p-values above $p = 0.003$ were not significant).

### 3.3. Feeding trial

Sudden and high larval mortality was observed in 3 larval tanks (two Control, one Dissolved Low) during the feeding trial. The cause of these high-mortality events is unknown but appeared to be associated with a fungal-like growth in the affected tanks. Tanks that did not contain larvae at week 6 were excluded from statistical analyses (except estimates of survival). Northern rock sole larvae in the Liposome and Dissolved High treatment had higher whole body taurine concentrations (statistics performed with arc-sin square-root transformed proportional dry weight concentrations; raw data shown in Fig. 3) when compared to the Control and Dissolved Low treatments (ANOVA, significance level, $p < 0.001$). With the exception of taurine, there was no difference in the whole body amino acid concentrations measured in fish larvae (% DW) among treatments (Bonferroni estimate, significance level $p = 0.003$). When data from all weeks were analyzed, larval standard lengths (SL), body depths, and ln-transformed dry weights (raw data shown in Figs. 4 and 5) were significantly different among dietary treatments (repeated-measures REML, fixed-effect p-values < 0.001; Figs. 4 and 5). All growth measurements were significantly higher in the Dissolved High and taurine-liposome treatments when compared to the Dissolved Low and the Control treatments (Tukey's HSD, significance level $p = 0.05$). Larval survival (statistics performed on arcsine square root transformed survival; reported as % survival) varied among treatments (ANOVA, $p = 0.012$) with the highest survival in the Liposome treatment; specific comparisons are shown in Fig. 6. Larval flexion stage, measured on week 6, varied among treatments (ANOVA, $p = 0.0032$, 0.05 for stages 2 and 3, respectively; specific pairwise comparisons shown in Fig. 7); however larvae in flexion stage 4 were only observed in the taurine-liposome and Dissolved High treatments and were not significantly different between these two treatments (T-test, $p = 0.10$; Fig. 7).

## 4. Discussion

### 4.1. Retention of taurine by liposomes

In this study, 85% of the taurine encapsulated within liposomes remained after 1 h of suspension in filtered seawater, with most of the loss occurring in the first 5 min of suspension. Barr and Helland (2007) reported 90.9% retention of free amino acids by liposomes prepared with soybean phosphaidylcholine (Epikuron® 200 SH, Degussa Texturant Systems GmbH, Germany) following 120 min of suspension in seawater, with the greatest losses occurring in the first 5 min. In the same study, taurine was lost in proportion to its inclusion rate in the free amino acid solution, suggesting that approximately 90% of encapsulated taurine was retained after 120 min. Liposomes show higher retention of taurine than wax spray beads (WSB) when suspended in seawater. Langdon et al. (2008) reported 55% and 23% retentions of taurine following 4 min in aqueous suspension for WSB produced from 100% beeswax and 95% beeswax/5% marine phospholipids, respectively. Hawkyard et al. (2014) reported 40% retention of taurine in WSB (99% beeswax, 1% sorbitan tristearate) following 1 h of suspension. Structurally, WSB are comprised of emulsified aqueous droplets or crystals within a lipid phase to form a particle matrix. The interconnected nature of the aqueous droplets and/or crystals in the matrix will likely facilitate loss of encapsulated substances during suspension, especially if the core material is not fully encapsulated. In contrast, liposomes are

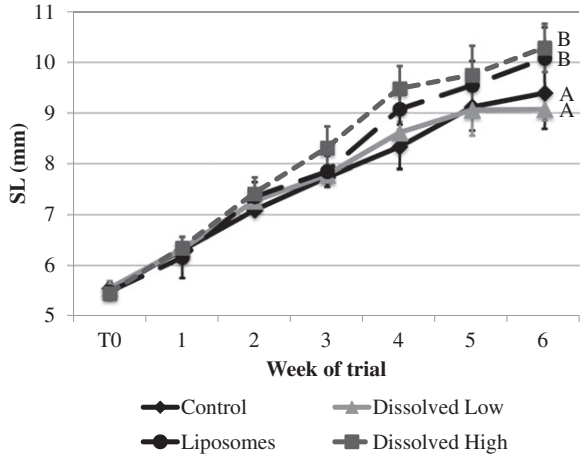


**Fig. 4.** Standard lengths (SL; ±1 SD) of northern rock sole fed experimentally enriched rotifers. Different letters denote significant differences among treatments over all weeks (Tukey's HSD, significance level $p = 0.05$).

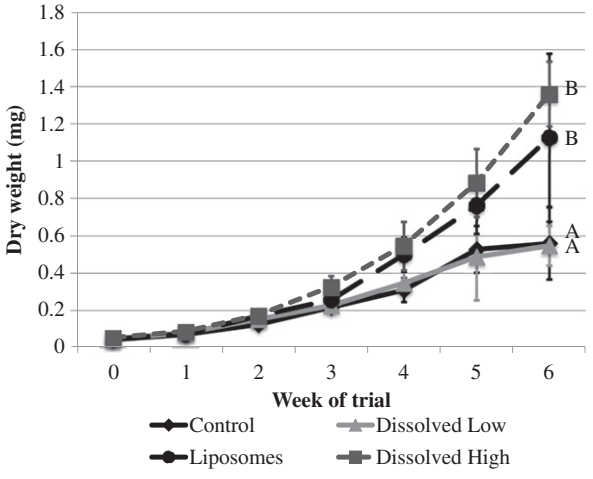


**Fig. 5.** Individual larval dry weight (mg) of northern rock sole fed experimentally enriched rotifers. Different letters denote significant differences among treatments over all weeks (Tukey's HSD, significance level $p = 0.05$).

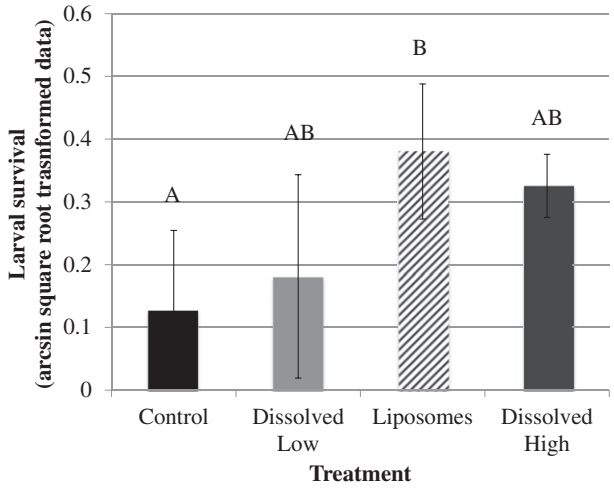


**Fig. 6.** Survival rates (%; ±1 SD) of northern rock sole larvae at the end of the trial. Different letters denote significant differences (Tukey's HSD, significance level, p = 0.05).

composed of layers of phospholipid-based lamellae that fully encapsulate the aqueous core materials. Therefore, losses from liposomes are likely due to diffusion of substances across the membrane as well as their physical rupture. The higher retention of taurine by liposomes when compared to WSB partially explains their higher efficacy during the enrichment of rotifers with taurine.

### 4.2. Rotifer enrichments

Taurine-liposome enriched rotifers contained approximately 1.2% taurine on a dry weight basis and were within the range of concentrations previously reported in copepods (~0.5–1.9% DW; Mæhre et al., 2012; van der Meeren et al., 2008). The average taurine concentration achieved in taurine-liposome enriched rotifers was greater than that obtained with equivalent concentrations (250 mg taurine $l^{-1}$) of taurine dissolved in the rotifer enrichment water (Dissolved Low; 0.17% taurine by rotifer DW). Furthermore, 60-times more taurine had to be dissolved in the enrichment water to obtain taurine concentrations (1.3% taurine by rotifer dry weight) equal to those obtained by enrichment with taurine-liposomes. It should be noted that rotifers in the present study were enriched for 1 h and that longer enrichment

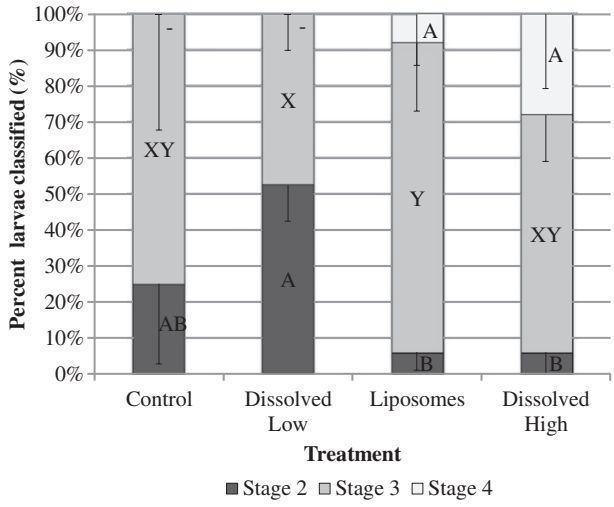


**Fig. 7.** Percent (%; ±1 SD) of larvae classified in each of three developmental (flexion) stages at the end of the trial (see text for criteria; Section 2.5). Different letters denote significant differences (Tukey's HSD, p = 0.05); (−) indicates groups excluded from statistical comparisons due to lack of data.

durations would likely impact the efficiencies for both methods. We chose 1 h enrichment periods because it allowed for direct comparisons with a parallel trial that focused on the use of taurine-wax spray beads (taurine-WSB) for the enrichment of rotifers (Hawkyard et al., 2014). These results are similar to those reported for taurine-WSB, whereby, enrichment of rotifers with taurine-WSB beads for 1 h required 80-fold less taurine than dissolved methods to obtain similar concentrations (0.35% by rotifers DW) in rotifers (Hawkyard et al., 2014). The taurine concentrations of taurine-liposome enriched rotifers in this study were 4-fold higher in taurine in terms of percent indispensible amino acids (%IAA; data not shown) than those previously reported for taurine-liposomes by Pinto et al. (2013). This difference was likely a result of differences in the formulations of liposomes as well as differences in enrichment methods. In the current study, enrichment of rotifers with taurine-liposomes resulted in 3.5-fold higher dry weight concentrations of taurine in rotifers when compared to a previous study using taurine-WSB (~0.34 % DW; Hawkyard et al., 2014). The higher concentrations of taurine achieved in liposomes may have been attributed to a variety of factors including: 1) higher leaching rates of taurine from wax spray beads, 2) differences in ingestion rates by rotifers associated with particle size selectivity and/or 3) high losses of particles during the enrichment process due to floating and clumping of wax spray beads.

There are physical constraints as to the maximum particle size that a rotifer can ingest which varies by rotifer size and strain. For instance, *Brachionus* "Cayman" do not ingest inert particles over 12 μm in diameter (Baer et al., 2008). Hotos (2003) found that L-type *Brachionus plicatilis*, similar to those used in this study, could not ingest particles over 22 μm. Differences in particle size distributions may have partially explained the differences in efficacies between WSB and liposomes. Hawkyard et al. (2014) estimated that only 71% of the WSB particles (by number) and 1.5% of the particles by volume were in the size range appropriate for consumption by L-type rotifers. In contrast, all of the liposomes measured (100% by number and volume) in the present study were under 10-μm and were therefore ingestible by rotifers.

### 4.3. Larval feeding trial

All metrics of growth indicated that the northern rock sole larva fed liposome-enriched rotifers grew significantly larger than larvae in the Control and Dissolved Low treatment. In addition, the most developed larvae, in terms of flexion, were observed in the Liposome and Dissolved High treatments (Fig. 7). These findings are consistent with a previous study that showed positive effects of taurine-enriched rotifers on the growth and development of northern rock sole larvae (Hawkyard et al., 2014). In contrast, Pinto et al. (2013) did not find differences in growth indices of gilthead seabream (*Sparus aurata*) after a feeding trial comparing taurine-liposome enriched rotifers and non-taurine enriched rotifers. Such differences may reflect species-specific taurine requirements or abilities to use taurine precursors (methionine) to meet taurine requirements. Alternatively, the differences observed between studies may reflect the taurine concentrations used in growth trials.

Rotifers in the present study were not purged of liposomes and therefore taurine concentrations measured in rotifers represented taurine contained in the rotifer gut and body tissues as well as taurine that was encapsulated within undigested liposomes in the gut (supported by fluorescent images; Fig. 1). Likewise, it was possible that taurine encapsulated within liposome-enriched rotifers could have been unavailable to the fish larvae. To address this issue, we included the "Dissolved High" treatment, whereby rotifers contained equal concentrations of taurine (by percent rotifer DW) when compared to the liposome-enriched rotifers, but taurine was not delivered in an encapsulated formulation. Therefore, reduced digestibility of microparticles (by rotifers or fish larvae) should have manifested as differences in larval growth rates or whole body taurine concentrations.

However, in this study larvae grew equally well when fed liposome-enriched rotifers when compared to those fed rotifers with equal concentrations of taurine (by rotifer DW) obtained by the dissolved method. In addition, whole body taurine concentrations measured in the larvae at the end of the trial were similar in the Dissolved High and Liposome treatments (Fig. 3). These findings suggest that a large fraction of the taurine provided in liposome-enriched rotifers was biologically available to the larvae. In contrast, rock sole larvae fed taurine-wax spray bead enriched rotifers had lower larval growth rates and lower whole body taurine concentrations when compared to the larvae fed rotifers with equal taurine concentrations (by rotifer DW) obtained by dissolved methods (Hawkyard et al., 2014). It is likely that these differences were related to differences in the digestibility of wax spray beads and liposomes. It has been well established that marine fish larvae have high capabilities to digest and metabolize phospholipids (see reviews by Izquierdo et al., 2000, 2011; Rainuzzo et al., 1997; Sargent et al., 1999), which may explain the high availability of liposome-encapsulated substances to northern rock sole larvae in the present study. Waxes are highly concentrated in copepods (Parrish, 2013) and therefore it seems to follow that fish larvae should have high capacities for wax digestion. However, as previously noted, larvae do not appear to digest beeswax to a great extent, which may have been attributed to the degree of saturation, and the respective hardness, of beeswax. Alternatively, the physical structure of wax spray beads (solid wax with aqueous core droplets) may be less digestible than the cell-like structure of liposomes, which may release their contents upon rupture. Fluorescent techniques reveal that rotifers appear to break down liposomes, liberating water-soluble nutrients into their body cavity, prior to ingestion by larval fish (Fig. 1). These findings suggest that rotifers may have aided in the digestion of liposomes and therefore increased the availability of encapsulated substances to the fish larvae.

In the present study it was necessary to co-feed larvae with Algamac- and liposome-enriched rotifers to provide both lipid- and water-soluble nutrients, respectively. However, this strategy resulted in lower dietary taurine concentrations for the larvae than would have been achieved using l00% liposome-enriched rotifers. We assumed equal ingestion rates of the differently enriched rotifers by the larvae and therefore estimated dietary taurine concentrations were 0.6 and 0.65% DW for Liposome and Dissolved High treatments, respectively (Fig. 3). However, ingestion rates of the liposome- and Algamac-enriched live prey by fish larvae were not explicitly examined in this study and therefore such estimates should be interpreted with caution. Furthermore, liposome-enriched rotifers likely acted to dilute the concentrations of dietary PUFAs delivered to fish larvae compared to larvae fed a 100% Algamac-enriched rotifers diet. Future work should be aimed at the simultaneous enrichment of the rotifers with both water- and lipid-soluble substances. This might be accomplished by adding PUFA to the liposome membranes or by developing suspensions of both liposomes and PUFA rich algae or oil emulsions. The addition of PUFA to the liposome membranes will likely reduce their retention of water-soluble substances by liposomes, whereas, algae or oil droplets may be preferentially ingested or may compete for space in the rotifer guts. In both cases, there is likely a trade-off between the enrichment of lipid- and water-soluble nutrients. These different enrichment methods should be tested for their ability to obtain the concentrations of taurine and PUFAs that meet the nutritional needs of marine fish larvae.

### 4.4. Summary

We have found that liposomes are highly effective for the taurine-enrichment of rotifers. Specifically, the enrichment with taurine liposomes elevated the taurine concentrations of rotifers to those previously reported in copepods. Furthermore, the taurine-liposome enriched rotifers promoted the rapid growth of northern rock sole larvae suggesting that enriched taurine was utilized and biologically available to the larvae. While similar taurine concentrations can be achieved with dissolved taurine, taurine liposomes required 60 times less taurine to achieve similar concentrations. It should be noted that liposomes also have the potential to deliver a suite of water-soluble substances to marine suspension feeders e.g., vitamins, minerals and antibiotics. The use of liposomes for the enrichment of rotifers has the potential to reduce the wastage of water-soluble substances and improve water-quality during live prey culture. Future work should be aimed at investigating the efficacy of liposomes in delivering additional water-soluble substances as well as co-delivery of lipids and lipid-soluble nutrients.

## Acknowledgments

This work was supported by the Agriculture and Food Research Initiative Competitive Grant no. 2012-67015-19454 from the USDA National Institute of Food and Agriculture. We are grateful for all of the help and assistance provided by researchers and technicians at the NOAA Alaska Fisheries Science Center and for the use of facilities and resources at the Hatfield Marine Science Center.

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