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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

Matt Hawkyard [a,b,*], Ben Laurel [c], Yoav Barr, Kristin Hamre [d], Chris Langdon [a,b]

[a] Coastal Oregon Marine Experiment Station, Oregon State University, Newport, OR 97365, USA
[b] Department of Fisheries and Wildlife, Oregon State University, Corvallis, OR 97331, USA
[c] Fisheries Behavioral Ecology Program, Alaska Fisheries Science Center, NOAA, Hatfield Marine Science Center, Newport, OR 97365, USA
[d] National Institute of Nutrition and Seafood Research (NIFES), PO. Box 2029 Nordnes, N-5006 Bergen, Norway

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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.
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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,

* Corresponding author. Tel.: +1 5418670254; fax: +1 5418670345.
E-mail address: Hawkyard@onid.orst.edu (M. Hawkyard).

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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

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