AN ABSTRACT OF THE THESIS OF

Donald Matthew Hawkyard for the degree of Master of Science in Fisheries Science presented on March 12, 2010.
Title: The use of wax spray beads (WSB) for iodine enrichment of *Artemia* sp. for use as a live food for zebrafish (*Danio rerio*) larvae.

Abstract approved:

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Dietary iodine may play an important role in the nutritional health of freshwater fish larvae. *Artemia*, commonly used for the culture of larval zebrafish (*Danio rerio*), contain low concentrations of iodine when compared with wild zooplankton. Water-soluble micronutrients, such as iodine, are difficult to deliver to *Artemia* due to rapid diffusion from microparticles. Several methods have been developed to deliver water-soluble nutrients to live prey and are reviewed in this thesis. Wax spray beads (WSB) have been shown to retain greater than 50% of water-soluble micronutrients after 1h suspension in seawater. In addition, WSB have been used to bioencapsulate oxytetracycline (OTC; a water-soluble antibiotic) within *Artemia* but have not been previously used for the enrichment of micronutrients. It is currently unknown whether iodine, in the form of potassium iodide (KI), encapsulated within WSB is available to fish larvae and, if so, whether increased dietary iodine has a nutritional effect on larval zebrafish. Inert markers can been used to estimate ingestion and retention rates of diets by target organisms or to estimate feed preferences of organisms fed on multiple diets. In our study, yttrium (III) oxide (Y_2O_3) was used as an inert marker of WSB to provide detailed information about enrichment

processes. The objectives of this study were to: 1) evaluate the use of wax spray beads containing potassium iodide (KI WSB) and wax spray beads containing KI and Y_2O_3 (KI+Y WSB) for enrichment of *Artemia* with iodine; 2) evaluate the use of Y_2O_3 as an inert marker in feeding experiments with *Artemia* fed on WSB; 3) determine if zebrafish larvae were able to uptake iodine from KI WSB-enriched *Artemia*; 4) investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish; 5) determine if *Artemia* were a potential source of exogenous thyroid hormones (TH) for larval fish; and 6) determine if KI WSB had an effect on bacterial concentrations associated with *Artemia*.

We found that *Artemia* enriched with KI+Y WSB had higher levels of iodine than *Artemia* enriched with potassium iodide (KI) delivered in aqueous solution. WSB concentrations and the time of enrichment had significant effects on iodine and yttrium concentrations in *Artemia.* Enrichment with KI+Y WSB resulted in iodine absorption into *Artemia* tissues suggesting that a portion of the enriched iodine would be available to predators. Our results indicate that Y_2O_3 was an effective inert marker of WSB and was highly useful when interpreting data from enrichment trials. Zebrafish fed *Artemia* enriched with KI WSB showed a ten-fold increase in total iodine levels and increased survival when compared with larvae fed unenriched *Artemia*. Thirty-eight day post fertilization zebrafish larvae fed iodine-enriched *Artemia* had lower epithelium to colloid (v:v) ratios when compared to those fed unenriched *Artemia*. *Artemia* metanauplii were found to contain significant levels of deiodinase and thyroid hormones. KI WSB had no effect on the levels of marine bacteria associated with *Artemia*. The results of this study indicated that iodine

contained in KI WSB enriched *Artemia* was available to larval fish. It is also apparent that early stage zebrafish benefitted from increased levels of dietary iodine. In addition, *Artemia* may provide larval fish with significant levels of exogenous thyroid hormones and deiodinase.

The use of wax spray beads (WSB) for iodine enrichment of *Artemia* sp. for use as a live food for zebrafish (*Danio rerio*) larvae.

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I understand that my thesis will become part of the permanent collection of Oregon State University Libraries. My signature below authorizes the release my thesis to any reader upon request.

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Donald Matthew Hawkyard, Author

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CHAPTER 1 – GENERAL INTRODUCTION

Overview

Commercial hatcheries, public aquariums and biological research institutions often rely on live prey organisms, such as brine shrimp (*Artemia sp.*) and rotifers (*Brachionus sp.*), for larval fish food. Fish larvae fed *Artemia* have shown poor survival and reduced metamorphic success when compared to those fed wild zooplankton, primarily copepods (Shields et al., 1999; Hamre et al., 2005). Recent studies comparing the micronutrient profiles of *Artemia* and copepods indicate that copepods have much higher levels of several micronutrients than *Artemia* (Table 1.1) (Shields et al., 1999; Mæland et al., 2000; Hamre, 2006; van der Meeren et al., 2007). Current research efforts have been directed at elevating the micronutrient levels of *Artemia* to those found in copepods to determine if elevated levels improve growth and survival of larval fish.

Many methods have been developed for micronutrient enrichment of *Artemia* and will be addressed later in this chapter. While several methods have been shown to enrich lipid-soluble micronutrients in *Artemia*, few have the ability to enrich water-soluble micronutrients in a controlled and efficient manner. Microparticulate diets may have the potential for controlled enrichment of water-soluble micronutrients in *Artemia*; however, these diets are prone to rapid losses of water-soluble nutrients when suspended in seawater due to their high surface area to volume ratios (Langdon, 2003; Kvåle et al., 2006). Wax spray beads (WSB) are a type of microparticle in which water-soluble substances are embedded within a wax matrix. WSB show improved

retention of water-soluble nutrients and have been used to deliver water-soluble substances to *Artemia* (Langdon et al., 2008). However, the use of WSB for the micronutrient enrichment of *Artemia* has not been thoroughly evaluated.

In the following studies, we have chosen to focus on the effects of iodine enrichment on the nutritional value of *Artemia sp.* as a food for larval zebrafish. We chose iodine because: 1) iodine levels are often several orders of magnitude higher in copepods than in *Artemia* (Moren et al., 2006); 2) it is a highly water-soluble micronutrient and may be representative of a larger class of water-soluble micronutrients; and 3) iodine deficiencies have been shown to influence the metamorphic success and survival of several species of fish larvae (Hamre et al., 2002; Moren et al., 2006; Hamre et al., 2008; Ribeiro et al., 2009). If WSB are to be used for enrichment of iodine in *Artemia*, several parameters must be assessed including the effects of WSB concentration, duration of enrichment, as well as the impacts of starvation and gut purging on the iodine concentrations of *Artemia*. After enriching and purging *Artemia*, it can be difficult to determine if a substance is simply present in the gut of *Artemia* or if it has been absorbed into tissues. In the following studies we evaluated the use of yttrium oxide (Y_2O_3) as an inert marker for measuring rates of nutrient ingestion, retention and absorption during enrichment experiments.

Iodine is needed for the production of thyroid hormones (TH), which have an important role in the metamorphosis of fish (Power et al., 2008). In the following studies, we investigated the effect of increased dietary iodine on the survival, growth and thyroid status of larval zebrafish (*Danio rerio*). In addition, we investigated

whether *Artemia* are also a potential source of exogenous thyroid hormones and deiodinases for fish larvae (Chapter 3).

Micronutrients

Micronutrients (vitamins and trace minerals) are substances essential for growth, development, maintenance and reproduction of living organisms but are only required in small (ppm or μg g^{-1} diet) quantities (Webster and Lim, 2002). Vitamins are organic compounds that must be synthesized by living organisms. Fish are unable to synthesize most vitamins and must obtain these substances in their diet (Webster and Lim, 2002). Vitamins may be further categorized by their solubility in water or lipid; vitamins C (ascorbic acid), B_2 (riboflavin), and B_1 (thiamin) are examples of water-soluble vitamins, whereas vitamins A (retinoids), E (tocopherols) and D (calciferol) are examples of lipid-soluble vitamins. Trace minerals are elements that are required for the proper health of organisms and are required in very small (ppm or μg g^{-1} diet) quantities. In fish, trace minerals may be obtained in the diet or in some cases actively transported across the gills (Watanabe et al., 1997). Examples of trace minerals are selenium, copper, zinc and iodine. Micronutrient requirements for fish larvae are difficult to determine due to technical challenges associated with feeding larval fish (Waagbø, 2009). Delivery of water-soluble micronutrients, such as vitamin C or iodine, can be challenging since these substances tend to rapidly leach from microparticulate diets (Langdon, 2003; Kvåle et al., 2006) reducing their ability to enrich live-prey organisms. Due to the challenges of feeding fish larvae, micronutrient requirements for fish are often determined during the juvenile and adult stages (NRC,

1993). However, larvae may have significantly different nutritional needs compared to juveniles and adults (Dabrowski, 1984). Methods for the delivery of water-soluble substances to fish larvae are needed to address specific micronutrient requirements.

Micronutrient enrichment of *Artemia*

Various methods have been used to increase the vitamin and trace mineral content of *Artemia*. These methods include: a) algae, b) emulsions, c) aqueous solutions d) liposomes and e) wax spray beads (WSB). While additional enrichment methods may be possible, including various microparticle types, the following is a summary of methods that have been published in peer-reviewed literature.

Algae

Algae have been successfully used to elevate the micronutrient concentrations of *Artemia*. For example, Olsen et al. (2000) demonstrated that 3-day old *Artemia* enriched with *Isocrisis galbana* for 72 hours reached ascorbic acid (AA) levels of 1000-1200 μg g^{-1} DW that were similar to those measured directly in the algae. However, in the same study they measured a decrease in vitamin B_6 concentrations, which they suggest were associated with the low B_6 concentrations of *I. galbana* combined with the increased growth of the *Artemia* during the enrichment process. Vismara et al. (2003) demonstrated that several species of algae could be used to increase the vitamin E content of *Artemia* but the degree of enrichment depended on the species of algae used. Ritar et al. (2004) showed an increase in both vitamins C and E when *Artemia* were enriched with *Chaetoceros muelleri*, for 36 hours. These

studies suggest that live algae may be used to enrich both lipid and water-soluble micronutrient concentrations in *Artemia*.

There are three primary disadvantages to using algae for the enrichment of live-prey organisms for aquaculture. First, live cultures of algae are highly susceptible to contamination by bacteria (Duerr et al., 1998). Second, algae culture is an expensive process with an estimated cost of 50 USD kg^{-1} (Duerr et al., 1998). Third, specific micronutrient concentrations are not easily manipulated within algae, which may limit their use when complex micronutrient mixtures are desired.

Emulsions

Oil-in-water emulsions are one of the most common enrichment methods for delivering lipids and lipophilic substances to *Artemia*. Vitamins E and A are naturally lipid-soluble and are thought to be deficient in *Artemia*. Several studies have shown that oil-emulsions could be used to increase vitamin E concentrations of *Artemia* (Kolkovski et al., 2000; Ritar et al., 2004). In addition, several water-soluble micronutrients have been modified to create lipid-soluble forms, which improve their retention in lipid-emulsions. For instance, ascorbic acid, a highly water-soluble form of vitamin C, can be esterified with palmitic acid to produce ascorbyl palmitate, which is lipid-soluble. Ascorbyl palmitate, when included in a lipid-emulsion, resulted in a four-fold increase in ascorbic acid concentration when fed to *Artemia* (Merchie et al., 1997). Water-soluble nutrients are not effectively delivered by lipid-emulsions as they tend quickly dissolved into the aqueous phase during the enrichment process. For example, enriching *Artemia* with ascorbic phosphate mixed within an oil-in-water

emulsion had no effect on the vitamin C levels of *Artemia* after 18 h of enrichment (Ritar et al., 2004).

Aqueous solutions

Enrichment by dissolving nutrients in the culture water is a simple method of enriching *Artemia* with water-soluble substances. In this method of enrichment, *Artemia* acquire substances through drinking or adsorption onto the exoskeleton. Tonheim et *al.* (Tonheim et al., 2000) demonstrated a 20-30 fold increase in methionine levels of *Artemia* when enriched for 16 h with methionine dissolved in the culture water at 5.3 mM. This method of enrichment has the disadvantage of requiring large quantities of dissolved micronutrients, which could be expensive and be harmful to the environment when discharged from large-scale systems.

Liposomes

Liposomes are microparticles composed of an aqueous core surrounded by single or multiple phospholipid walls (lamellae). Liposomes can be manipulated to contain various lipids or lipophilic substances such as polyunsaturated fatty acids (Monroig et al 2003) or vitamin A (Monroig et al. 2007). In addition, water-soluble substances may be encapsulated within the aqueous core of liposomes. The effectiveness of liposomes to deliver water-soluble substances is dependent on the phospholipid composition and the lamallar structure of the liposomes.

Artemia have been shown to ingest and physically breakdown liposomes (Hontoria et al. 1994). Monroig et al. (2007) used liposomes to elevate the vitamin A

levels of *Artemia*. They showed an association between the retinyl palmitate delivered in liposomes with retinol levels measured in enriched *Artemia* and showed that these levels were much higher than those achieved by retinyl palmitate delivered by a lipid emulsion. High leakage rates were suspected when single-lamellar liposomes were no more effective at delivering vitamin C (sodium ascorbate; solubiltity 62g/100ml @ 25 ^{o}C) to *Artemia* than sodium ascorbate delivered in aqueous solution (Monroig et al. 2007). In contrast, multiple-lamellar liposomes effectively delivered methionine (25g/100ml @ 25 ^{o}C) to *Artemia* (Monroig et al., 2007).

In general, liposomes have shown promise for the enrichment of *Artemia* with lipophillic substances. Multiple-lamellar liposomes show improved delivery of the water-soluble substances to *Artemia* and may be used in future micronutrient enrichment applications. The drawback to liposomes is that the production process is complex and requires highly purified, expensive phospholipids, which may limit their use for large-scale enrichments (Tonheim et al., 2000; Langdon et al., 2008).

Wax spray beads (WSB)

WSB are microparticles in which an aqueous or particulate core is bound within a wax matrix. The hydrophobic wax matrix helps protect hydrophilic core materials, such as vitamins, amino acids, or antibiotics, from leaching into the surrounding water during feeding and enrichment. The advantages of using waxes rather than triglycerides are that waxes are less likely to react with core materials and are less likely to contribute to the nutrition of the target organism. WSB may be used

to enrich live-prey organisms or may be included in complex particles and fed directly to fish larvae.

WSB have recently been proposed for enrichment of water-soluble micronutrients in live-prey organisms (Langdon et al., 2008). They can be produced in the appropriate size range for rotifers and *Artemia* and are readily ingested by both of these live-prey organisms. *Artemia* fed WSB containing the water-soluble antibiotic oxytetracycline (OTC) have been shown to absorb and retain significant quantities of OTC (Langdon et al. 2008). Water-soluble substances, such as potassium iodide (KI), have not previously been delivered to larval fish using WSB-enriched *Artemia* nor has the availability of encapsulated nutrients been demonstrated for fish larvae. In chapter 2, we evaluate the use of WSB containing potassium iodide (KI) for the iodine enrichment of *Artemia* (objective 1).

Inert markers

We use the term "inert marker" to mean a non-nutritional substance added to a feed that can be used as a reference for the utilization of other dietary ingredients and carrier substances. Inert markers can been used to estimate ingestion and retention rates of diets by target organisms or to estimate feed preferences of organisms fed on multiple diets (Teshima et al., 2000; Otterå et al., 2003). Markers may also be used to determine digestibility or absorption of nutrients from feeds. Digestibility is often determined by comparing the marker to nutrient ratio measured in the feed with the ratio measured in fecal materials and is quantified as apparent digestion (or absorption efficiency) of the respective nutrient (Aksnes and Opstvedt, 1998; Austreng et al.,

2000; Hansen et al., 2009). Austreng (2000) suggests that an inert marker should: 1) be homogeneously incorporated into the feed and easily and accurately analyzed, 2) not be digested or metabolized by the animal, 3) pass through the gastro-intestinal tract at the same rate as the dietary nutrients and 4) be harmless to people and the environment. It has also been suggested that markers should not bias ingestion of diets by target organisms (Otterå et al., 2003).

Inert markers and live-prey

Cook et al. (2008) demonstrated that yttrium and lanthanide oxides could be used as inert markers for labeling live-prey organisms. They delivered inert markers to rotifers and *Artemia* by adding markers directly to the enrichment water. They demonstrated that yttrium oxide (Y_2O_3) could be used to enumerate live prey organisms ingested by larval Atlantic cod (*Gadus morhua*). In chapter 2, we evaluate the use of Y_2O_3 as an inert marker of WSB. We use Y_2O_3 to estimate ingestion and retention rates of WSB in response to various experimental conditions and to determine if water-soluble nutrients (iodine) are absorbed into the tissues of *Artemia* metanauplii (objective 2).

Fluorescent microspheres

Fluorescent microspheres have been shown to be effective markers of artificial diets used to feed larval fish by Hansen et al. (2009). They measured the number of microspheres in feed and in the fecal strands of fish larvae. The number of microspheres from each of these sources was compared to the respective nitrogen

levels and changes in the ratios of microspheres to nitrogen were used to determine apparent digestion (Hansen et al., 2009). The advantage of using fluorescent microspheres is that they can be easily visualized and counted with fluorescent microscopy, eliminating the need for expensive techniques and long sample analysis times. In chapter 2, we use fluorescent microspheres to check results obtained from yttrium measurements. In addition, we use fluorescent microspheres to obtain real-time data about particle ingestion and retention during enrichment and purging of *Artemia*.

Dietary iodine for larval fish

Uptake

Fish larvae are able to obtain iodide (I^-) via active transport across gill membranes from the surrounding water as well as from food (Hunn and Fromm, 1966; Hamre et al., 2005; Moren et al., 2006). The rate at which iodide is transported across gill membranes is likely to be species-specific and is dependent on the iodine/iodide concentrations of the surrounding water. In Atlantic halibut (*Hippoglossus hippoglossus*) larvae the uptake of iodide from the surrounding seawater is directly proportional to iodide concentration in the water column and uptake rates do not slow even at the highest concentrations measured (Moren et al., 2008). We may infer from these findings that iodide uptake is reduced when iodide levels are low in the surrounding water. Iodine levels are generally much higher in seawater than in freshwater and iodine deficiencies are much more prevalent in freshwater systems (Watanabe et al., 1997). Dietary iodine may be an important source for both marine

and freshwater fish. In chapter 3, we investigate the uptake of iodine from enriched *Artemia* by larval zebrafish (objective 3).

Thyroid hormones (TH)

Iodine is one of the main components of THs, which are related to cellular oxidation, neuro-muscular control, nutrient metabolism and growth (Watanabe et al., 1997). The transition from larval to juvenile forms, often termed metamorphosis, is an endocrine driven process in which THs play a central role (Power et al., 2008). In halibut (*Hippoglossus hippoglossus* L.), which undergo a radical transition from vertical swimming larvae to horizontally swimming juveniles, metamorphosis appears to be strongly related to TH production (Solbakken et al., 2002; Sæle et al., 2003; Hamre et al., 2007). Even in zebrafish, which undergo a subtle metamorphosis, termed direct development, the exposure of larvae to TH inhibitors results in reduced fin and ray development (Brown, 1997).

Thyroid hormones, triiodothyronine (T3) and thyroxine (T4), are made up of a tyrosine molecule bound with three (T3) or four (T4) iodine atoms. Outer-ring deiodinases (type I and II) convert T4 into the more bioactive T3 by cleaving an iodine molecule from T4 (Power et al., 2001). Therefore, the T3:T4 ratio is important when interpreting the thyroid status of fish. Teleost fishes do not have thyroid glands but rather produce thyroid hormones in thyroid follicles, which are generally located in the pharynx region, around the ventral aorta (Power et al., 2008). Thyroid follicles consist of an outer layer of epithelium cells, which surrounds an inner colloid region composed of precursors to thyroid hormone (Power et al., 2001).

Simple goiter occurs when inadequate iodine is available for the production of thyroid hormones (Watanabe et al., 1997). Circulating TH inhibits the production of thyroid stimulating hormone (TSH) from the pituitary. When insufficient iodine is available for TH production, elevated TSH levels promote the growth of thyroid epithelium and reduced colloid of thyroid follicles in order to mobilize TH. In severe cases of goiter, thyroid follicles will appear as a mass of swollen epithelium cells with no apparent colloid region (Ribeiro et al., 2009). Goiter has been documented in several species and is most common in freshwater and carnivorous fish species (Watanabe et al., 1997). In chapter 3, we investigate the effects of iodine-enriched *Artemia* on TH production and deiodinase activities of larval zebrafish (objective 4). In addition, we analyze thyroid follicles to determine if epithelium or colloid volumes changed in response to increased dietary iodine.

Exogenous hormones and enzymes

Artemia, rotifers and copepods produce hormones and enzymes related to their own physiological processes. Live prey organisms have been shown to contribute digestive enzymes to larval fish (Cahu and Zambonino-Infante, 1995; Kurokawa et al., 1998). However, the impact of these enzymes on larval fish digestion is under debate (Cahu and Zambonino-Infante, 2001). Though there is evidence that some invertebrates may produce iodine-regulatory hormones and enzymes (Heyland et al., 2006), THs and deiodinase have not been previously identified in *Artemia*. If *Artemia* produce THs or deiodinase, they may supply these substances to larval fish, which may impact the thyroid status of larvae. In the chapter 3, we measure the TH and

deiodinase activities of *Artemia* to determine if they are a potential source of these substances to larval fish (objective 5).

Antibacterial effects of iodine

Iodine is commonly used as a bacterial disinfectant. It is possible that the enrichment of *Artemia* with KI could have an effect on bacterial concentrations associated with metanauplii. If KI has an effect on bacterial concentrations, and if larvae fed iodine-enriched *Artemia* perform better than those fed unenriched *Artemia*, then the effects could be due to nutritional or microbiological factors. It has been shown that molecular iodine (I_2) has little impact on the bacterial levels of *Artemia* (Gomezgil-RS et al., 1994); However, in chapter 3, we investigate the effect of enrichment with KI WSB on the bacterial concentrations of *Artemia* (objective 6).

Summary

Artemia have significantly lower levels of several micronutrients, especially iodine, when compared with levels measured in wild zooplankton. The enrichment of water-soluble micronutrients in *Artemia* is challenging. While algae may be efficient at delivery of certain micronutrients, the specific levels of micronutrients are not easily manipulated. Dissolving substances directly in the enrichment water may lead to uptake by live prey but this approach is highly inefficient. Particulate diets are prone to high leakage rates due to high surface area to volume ratios. WSB have improved retention of water-soluble nutrients and have been used to deliver water-soluble substances to *Artemia*. Likewise, WSB may be used to increase the iodine content of

Artemia. The extent to which iodine is absorbed by the tissues of *Artemia* may affect its availability for fish larvae. Yttrium oxide may be used as an inert marker of WSB to determine the extent to which iodine is absorbed by *Artemia.* In addition, yttrium may be used to estimate ingestion and retention rates of WSB. Iodine is an essential trace element for fish and larvae may benefit from iodine levels that are significantly higher than those recommended for adult fish. Iodine is expected to affect the thyroid-endocrine system of fish since iodine plays a crucial role in the production of thyroid hormones. Live prey organisms have been shown to contribute digestive enzymes to larval fish. If *Artemia* produce THs or deiodinases, they may be a potential source of these substances to larval fish.

Primary objectives

1. Evaluate the use of wax spray beads containing KI and Y_2O_3 (KI+Y WSB) for enrichment of *Artemia* with iodine.
2. Evaluate the use of Y_2O_3 as an inert marker in feeding experiments with *Artemia* fed on WSB.
3. Determine if zebrafish larvae were able to uptake iodine from KI WSB-enriched *Artemia*.
4. Investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish.
5. Determine if *Artemia* were a potential source of exogenous THs for larval fish.
6. Determine if KI WSB had an effect on the bacterial concentrations of enriched *Artemia.*

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Table 1.1 Micronutrient profiles of *Artemia*, rotifers and copepods (Mæland et al., 2000; Hamre, 2006; van der Meeren et al., 2007) as well as NRC recommendations (NRC, 1993) for adult fish (mg kg^{-1} DW unless otherwise noted). N/A = not available, R = required

	Nutrient concentrations in live-prey			NRC recommendations for adult fish		
	Artemia[1]	Rotifers[1]	Copepods[1]	Rainbow trout[2]	Common carp[2]	Pacific salmon[2]
Vitamins						
Riboflavin	30-60	23-43	14-35	4	7	7
Thiamine	6-21	2-125	13-46	1	0.5	R
Vit C	361-690	181-576	38-1106	50	R	50
Vit E	100-500	85-889	23-209	50	100	50
Carotenoids	650-750	4-16	321-1422	N/A	N/A	N/A
Vitamin A	0	0.5-5	0.03-0.18	2500	4000(IU)	2500
Minerals						
Iodine	0.5-4.6	3-8	50-350	1.1	N/A	0.6-1.1
Manganese	4-30	4-5	8-25	13	13	R
Copper	7-40	3-8	12-38	3	3	N/A
Zinc	120-310	62-64	340-570	30	15-30	R
Selenium	2.2	0.08-0.09	2-5	0.3	N/A	R
Iron	63-130	57-114	85-371	13	150	N/A

CHAPTER 2 – THE USE OF WAX SPRAY BEADS (WSB) FOR ENRICHMENT OF *ARTEMIA* WITH IODINE AND THE USE OF YTTRIUM OXIDE AS AN INERT MARKER DURING ENRICHMENT TRIALS

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Abstract

Iodine concentrations in *Artemia* have been reported to be 50-700 times lower than in wild-caught zooplankton. Water-soluble micronutrients are difficult to deliver to *Artemia* due to their rapid loss from microparticles. Wax spray beads (WSB) have been shown to retain greater than 50% of water-soluble micronutrients, including potassium iodide (KI), after 1h suspension in seawater (SW). In addition, WSB were used to bioencapsulate oxytetracycline (OTC; a water-soluble antibiotic) within *Artemia* but have not been previously used to for micronutrient enrichment of *Artemia*. In addition, yttrium (III) oxide (Y_2O_3) may be used as an inert marker of WSB to provide detailed information about enrichment processes. The objectives of this study were to evaluate: 1) the use of WSB containing KI and Y_2O_3 (KI+Y WSB) for enrichment of *Artemia* with iodine; and 2) the use of Y_2O_3 as an inert marker in feeding experiments with *Artemia* fed on WSB. Leakage trials were performed to determine the retention of iodine and yttrium by WSB when suspended in SW. Enrichment trials were conducted to test the effects of: WSB concentrations, duration of enrichment, starvation, and purging with algae on the total iodine and yttrium

concentrations in *Artemia*. We found that *Artemia* enriched with KI+Y WSB had higher levels of iodine than *Artemia* enriched with the same amount of KI delivered in aqueous solution. Increasing the particle concentrations of WSB in the enrichment water resulted in significant increases in iodine and yttrium concentrations in *Artemia.* In a timed enrichment trial, both iodine and yttrium concentrations reached their maximum values within the first fifteen minutes. When transferred to clean SW, enriched *Artemia* did not lose significant amounts of yttrium and only small amounts of iodine after 4 hours in clean SW. Yttrium oxide was: 1) highly retained by WSB, 2) was not leached from WSB suspended in SW and 3) was not absorbed from WSB by *Artemia.* The results of this study suggest that KI+Y WSB can be used to elevate the iodine levels of *Artemia*. Enrichment with KI+Y WSB resulted in iodine absorption into *Artemia* tissues suggesting that a portion of the enriched iodine would likely be available to predators. Our results indicate that Y_2O_3 was an effective inert marker of WSB and was highly useful when interpreting data from enrichment trials.

Introduction

The concentrations of several micronutrients in *Artemia* have been reported to be significantly lower than in wild-caught zooplankton (Ronnestad et al., 1998; Mæland et al., 2000; Hamre et al., 2002; Solbakken et al., 2002; Moren et al., 2006). Specifically, iodine concentrations were 50-700 times lower in *Artemia* that in wild-caught zooplankton (Moren et al., 2006). It is often impractical to enrich *Artemia* with water-soluble nutrients, such as iodine, by dissolving them directly in the culture water because this approach requires large quantities of nutrients and results in low delivery

efficiencies. Oil-in-water emulsions have been used to enrich lipid-soluble nutrients in *Artemia* but have not been effective in delivering water-soluble components (Gapasin et al., 1998; Kolkovski et al., 2000; Ritar et al., 2004; Moren et al., 2006; Noshirvani et al., 2006). Algae and yeasts have been shown to deliver water-soluble vitamins to *Artemia* (Olsen et al., 2000; Vismara et al., 2003; Ritar et al., 2004; Nguyen et al., 2008). However, algae and yeasts are limited in their ability to deliver complex micronutrient mixtures since nutrients concentrations are not easily manipulated within these organisms. The use of microparticulate diets for the delivery of water-soluble substances to marine suspension feeders, such as *Artemia*, is problematic due to rapid leaching rates during aqueous suspension (Langdon, 2003). Single-lamellar liposomes were shown to have limited success elevating the vitamin C content of *Artemia* due to high nutrient loss during suspension (Monroig et al., 2007). Multiple-lamellar liposomes may have increased retention of water-soluble substances and were shown to elevate the methionine levels of *Artemia* (Monroig et al., 2007). However, liposome production is costly due to the need for highly purified phospholipids and low production volumes making them uneconomical at larger scales of production (Tonheim et al., 2000; Langdon et al., 2008).

Wax spray beads (WSB) have the potential to be used for the delivery of water-soluble substances to marine suspension-feeders. Beeswax WSB retained greater than 50% of several water-soluble micronutrients, including potassium iodide (KI), after 1h of suspension in seawater (SW) (Langdon et al., 2008). In addition, WSB are inexpensive and the production process is easy to scale-up to large

production volumes. Previous research has shown that WSB can be used to encapsulate water-soluble substances (oxytetracycline; OTC) within *Artemia* (Langdon et al., 2008). However, specific methodologies such as duration of enrichment and concentration of WSB in the enrichment water have not been optimized. In addition, the ability of target organisms to absorb and retain released materials from ingested WSB has not been thoroughly evaluated. The purpose of this study was to evaluate the use of WSB containing KI and yttrium oxide (KI+Y WSB) for the iodine enrichment of *Artemia*.

One of the difficulties in enrichment studies is quantifying material retained in the gut versus material absorbed and retained in the tissues of the target organism. Inert markers can been used to estimate ingestion and retention rates of diets by target organisms (Teshima et al., 2000; Otterå et al., 2003). Cook et al. (2008) demonstrated that yttrium oxide (Y_2O_3) and lanthanide oxides could be used as inert markers for labeling live prey organisms. They delivered inert markers to rotifers (*Brachionus sp*.) and *Artemia* by adding markers directly to the enrichment water. In this study, Y_2O_3 was included within the wax-matrix of WSB in order to measure WSB ingestion and retention by *Artemia* and to determine if iodine was absorbed into the tissues of *Artemia*.

For Y_2O_3 to be used as an inert marker of WSB it was necessary that it: 1) was not lost from WSB during suspension in SW, 2) was not digested or absorbed by *Artemia,* 3) did not inhibit the uptake of WSB by target organisms and 4) did not have a negative effect on the health of target organisms. Y_2O_3 has been a common marker

in nutritional studies with no adverse effects on target organisms (Austreng et al., 2000; Jagger et al., 2007; Cook et al., 2008) and therefore we assumed that Y_2O_3 would not impact the health of *Artemia*.

The objectives of this study on enrichment of *Artemia* nauplii with KI+Y WSB were to evaluate: 1) the use of KI+Y WSB for enrichment of *Artemia* with iodine; and 2) the use of Y_2O_3 as an inert marker in experiments with *Artemia* fed on WSB.

Methods

Production of WSB

WSB were produced using methods described by Langdon et al. (2008). All WSB formulations included beeswax (refined; Sigma-Aldrich, St. Louis, MO, USA) and 5% (w/w of lipid fraction) sorbitan tristearate (Sigma-Aldrich, St. Louis, MO, USA), which was added as an emulsifying agent and to improve bead dispersion in SW. An aqueous core was included in both KI+Y WSB and WSB containing Y_2O_3 only (Y WSB) to obtain a 1:4 water to lipid ratio. In KI+Y WSB and Y WSB formulations, 2% (w/w of total bead formulation) yttrium (III) oxide (Sigma-Aldrich, St. Louis, MO, USA) was added to the lipid mixture to act as an inert marker. Y_2O_3 was chosen because it was: 1) insoluble in water and would therefore be highly retained by WSB during leakage and enrichment trials and 2) ingested Y_2O_3 was assumed to be nutritionally inert and unlikely to be absorbed by *Artemia*. For KI+Y WSB, 10% (w/w of total bead formulation or 47% w/v of the aqueous core) KI (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the aqueous core prior to emulsification.

Fluorescent WSB ("DiI WSB") were produced as previously described with 95% (w/w) beeswax and 5% sorbitan tristearate (w/w). The wax fraction of WSB was labeled with a lipophilic fluorescent tracer (1,1′- dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiI; Molecular Probes, Eugene, OR, USA; 0.1 mg DiI g^{-1} lipid).

Particle size

WSB were dispersed in 0.5% sodium n-dodecyl sulphate (SDS; Sigma-Aldrich, Oslo, Norway) filtered (0.4 μm Durapore® membrane filter; Millipore, Oslo, Norway) SW. One drop of WSB suspension was added to a glass slide and with cover slip. Digital images were taken with an Olympus BX51 microscope (Tokyo, Japan) fitted with a Olympus DP50 digital camera (Tokyo, Japan). Particle diameters were measured and analyzed using Image-J software (National Institute of Mental Health, Bethesda, MD, USA) by measuring all in-focus particles (>20 particles $image^{-1}$) in a given image. Image measurements were calibrated using a 0.1 mm calibration slide (Olympus, Tokyo, Japan).

Encapsulation (EE), Inclusion (IE) and Retention Efficiencies (RE) of iodine and yttrium in WSB

Encapsulation efficiencies (EE) were expressed as the percentage (% w/w) of core material to total bead weight after preparation, modified from Buchal and Langdon (1998).

EE (%) = (weight of core material)/(total WSB weight) x 100

Where “weight of core material” is the concentration (w/w) of yttrium or iodine with respect to the “total WSB weight”.

Inclusion efficiencies (IE) were expressed as the percentage of core material originally present in the lipid mixture that was successfully incorporated (Önal and Langdon, 2004) and was calculated as:

IE (%) = (measured concentration at time 0)/(expected concentration) x 100

Where “measured concentration” is the wet weight concentration (w/w) of yttrium or iodine in unleached WSB and “expected concentration” is the concentration of iodine or yttrium expected in WSB based on initial production formula (based on total formula weight).

Retention efficiencies (RE) were expressed as the percentage (% w/w) of initial core material after suspension in SW (Önal and Langdon, 2004) and were determined as follows: Approximately 20 mg of WSB were weighed into 50 ml polypropylene centrifuge tubes. Fifteen milliliters of 4° C, SDS filtered (0.4 μm) SW solution was added to each tube. WSB were dispersed using low-powered sonication (Vibracell™; Sonics & Materials Inc., CT, USA) and placed on a Cel-Gro tissue culture rotator (Barnstead International, Dubuque, IA, USA) to keep WSB in

suspension. At the end of each suspension period (5, 30, 60, 360 and 720 min), WSB were collected on a 0.65 μm Durapore® membrane filter (Millipore, Oslo, Norway) and returned to the original 50 ml centrifuge tube for temporary storage. In addition, membrane filters were added to unleached WSB samples and blanks to control for background effects of the filter during extraction and analyses. WSB samples were stored at -20° C until extraction. Retention efficiencies were calculated using the formula:

$$RE\ (\%) = (\text{concentration time X})/(\text{concentration time 0}) \times 100$$

Where "concentration time X" is the wet weight concentration (w/w) of iodine or yttrium at a given time period and "concentration time 0" is the measured concentration on iodine or yttrium in unleached WSB.

Artemia culture

Great Salt Lake *Artemia* cysts (1.4 g l^{-1}; INVE Tech., Dendermonde, Belgium) were added to 1 μm filtered SW (20-24 ppt, 26° C) in 15 L polycarbonate hatching cones (Aquatic Habitats, Apopka, FL, USA). Aeration was provided by an air pump fitted with a rigid air tube and was set to provide ~0.1-0.2 l min^{-1}. After 24 hours of incubation, the aeration was removed and bottom lighting was utilized to attract metanauplii to the bottom of the cone while cysts floated to the surface. *Artemia* metanauplii were then drained from the bottom of the cone onto a 200 μm sieve, rinsed with clean SW and transferred to a clean polycarbonate hatching cone

containing 1 µm filtered SW. Unfed, 24 hour post-hatch (hph) *Artemia* metanaulpii were used for all enrichment trials.

Yttrium and iodine uptake by Artemia at increasing particle concentrations of WSB

The purpose of this trial was to determine if the concentration of WSB in the enrichment water could be altered to manipulate the concentrations of iodine and yttrium in enriched *Artemia*. Metanauplii (24hph; approx 150 metanauplii ml^{-1}) were enriched in 26º C SW for 12 h with three concentrations of KI+Y WSB; 100, 200 and 400 mg WSB l^{-1}. A control of unenriched *Artemia* was also included. All treatments were reproduced each day over a three-day period. After 12h of enrichment, *Artemia* were drained from the bottom of the enrichment cone and collected on a 200 µm sieve, repeatedly rinsed in sequential baths of clean SW to remove adsorbed beads and then washed with distilled water into 100 ml plastic beakers with lids. *Artemia* samples were frozen at -20° C, freeze-dried (Freezone® freeze-dry system, Labconco® Corp., Kansas city, MO, USA) and stored at -20° C for yttrium analysis.

Yttrium and iodine uptake by Artemia over time

Uptake of yttrium and iodine by *Artemia* from KI+Y WSB was determined throughout a 12h enrichment period. Twenty-four hph metanauplii (approx 150 metanauplii ml^{-1}) were enriched at one level of KI+Y WSB (200 mg WSB l^{-1}) in 2 L cultures. Independent cultures were sampled after 0, 15, and 30 min and 1, 4, 8 and 12 h of enrichment. All time periods were replicated each day of a three-day period.

Artemia samples were taken and stored for yttrium and iodine analyses as previously described.

Retention of yttrium and iodine by Artemia following transfer to clean SW

The following trial was conducted to determine if *Artemia* retained significant quantities of iodine after transfer to clean SW and to determine if "cold storage" had an effect on the retention of iodine. Yttrium concetrations were used to estimate the uptake and retention of WSB by *Artemia* during enrichment. Twenty-four hph *Artemia* (approx. 150 ind. ml^{-1}) were enriched for 12 h with 200mg KI+Y WSB l^{-1} as previously described. At the end of the enrichment period, *Artemia* metanauplii were collected on a 200 µm sieve, rinsed in three sequential baths of clean SW and transferred into 2 l of clean SW at either 26° or 4-10° C and maintained at these temperatures until sampling. *Artemia* were collected after the initial rinsing (T0) and at 1, 4, and 8 h after transfer. Each temperature and time point was produced in an individual cone to maintain sample independence and all treatments were reproduced each day of a three-day period. *Artemia* were sampled and stored as previously described.

Yttrium and iodine absorption by Artemia

The following experiments were conducted to determine if yttrium and iodide were absorbed from WSB into *Artemia* tissues:

Particle ingestion and retention: The purpose of this experiment was to determine if YG microspheres (Fluoresbrite® YG, Polysciences, , USA; *ex.* 441 nm,

em. 486 nm) could be used to indicate WSB ingestion and retention and could therefore be used to quickly determine if *Artemia* guts had been purged in subsequent trials. For YG microspheres to be used as a proxy of WSB ingestion and retention it was necessary that WSB and YG microsperes were treated similarly in the gut (i.e. similar gut passage and no sorting of beads). Two-liter cultures of *Artemia* were co-enriched with 200 mg DiI WSB l^{-1} (*ex.* 549 nm, *em.* 565 nm) and 0.25 ml YG microsphere suspension l^{-1} (approx. 5 mg YG microspheres l^{-1}). Metanauplii containing DiI WSB and YG microspheres were digitally photographed with a Leica DM1000 microscope (Leica, Bannockburn, IL, USA) fitted with a Spot insight QE camera (Diagnostic Instruments, Sterling Heights, MI, USA) using GFP (*ex.* 450-490, *em.* 500-545) and TRITC (*ex.* 510-560, *em.* 573-647) filter sets, respectively.

Iodine absorption in *Artemia*: *Artemia* (~150 metanauplii ml^{-1}) were enriched with 200 mg KI+Y WSB l^{-1} or 200 mg Y WSB l^{-1} along with 20 mg aqueous KI l^{-1} for 1h. Following enrichment, *Artemia* were collected on a 180 um sieve, transferred to clean SW and fed Ori-green (1.0 g l^{-1}) for 20 minutes. Since *Artemia* were not fully emptied of WSB by a single purging, this process was repeated 7 times over a 2.5h period. *Artemia* samples were taken immediately after the 1h enrichment period, after 2.5 h (7 washes), and after an additional 2.5 h of purging (no additional washes). Individual enrichment cones were used for each time and treatment combination to preserve sample independence and were reproduced in triplicate. In order to observe gut purging of *Artemia* during the experimental period, three-micron fluorescent polystyrene microspheres (Fluoresbrite® YG, Polysciences Inc, Warrington, PA,

USA) were added to the enrichment medium. The number of fluoresecent microspheres was estimated in the guts of *Artemia* metanauplii from digital images taken with an epifluorescent microscope fitted with a GFP filter set (*ex.* 450-490 nm, *em.* 500-545 nm). Images were taken after 1h of enrichment and after 2.5h and 5 h of purging. *Artemia* were sampled as follows: aeration was removed and bottom lighting was used to attract swimming *Artemia* towards the bottom of the cone. *Artemia* were collected and stored as described for the 12h enrichment trials.

Yttrium concentrations were used to estimate the amount of WSB that remained in the guts of *Artemia* after purging. It was assumed that the pattern of iodine leakage from WSB during enrichment of *Artemia* was consistent with the results from leakage trials. Iodine contained within WSB was estimated based on residual (post-purging) yttrium concentrations and predetermined leakage rates of iodine from WSB. The quantities of estimated iodine were subtracted from measured iodine concentrations in *Artemia* to yield the "absorbed" iodine levels (i.e. iodine measured in *Artemia* not associated with ingested WSB).

$$\text{"Absorbed" iodine} = (\text{I conc.}) - [(\text{Y conc.}) \times (\text{I:Y ratio in WSB})]$$

Where "I conc." was the concentration of iodine measured in *Artemia* after purging with algae, "Y conc." was the concentration of yttrium measured in *Artemia* after purging with algae and "I:Y ratio in WSB" was the concentration of iodine with respect to the concentration of yttrium measured in WSB after 6h suspension in SW.

Analysis of yttrium

Yttrium (Y) was extracted using an acidic digestion technique utilizing nitric acid (Suprapur®; Merck, Darmstadt, Germany) and hydrogen peroxide (Merck, Darmstadt, Germany) and was measured using ICP-MS (Agilent 7500; Agilent Technologies Inc., Santa Clara, CA, USA) as described by Otterå (2003). Rhodium (Teknolab a/s, Oslo, Norway) was used as an internal standard and fish fecal material (NIFES, Bergen, Norway) containing yttrium was used as standard reference material.

Analysis of iodine

Total iodine was extracted from samples with tetramethylammonium hydroxide (TMAH) (Tama Chemicals Co., Tempapure-AA, Kawasaki city, Japan) and concentrations determined by ICP-MS (Julshamn et al., 2001). Extracted samples were diluted as necessary to fall within the quantifiable range (1-5 $\mu g\ l^{-1}$) of this method. Standard curves were produced with an iodine standard (prod # 8034; Teknolab as, Kolbotn, Norway) for each sample type and dilution to account for matrix effects caused by the interference and interactions of co-occurring elements (all R^2 values ≥ 0.97). Milk powder no. 150 (Community Bureau of Reference, Brussels, Belgium) was used as standard reference material and tellurium (prod. # 8062; Teknolab as, Kolbotn, Norway) was used as an internal standard for ICP-MS.

Statistics

Statistical tests were performed with JMP® version 8.0 (SAS Institute Inc, Cary, NC, USA) and StatPlus®:mac version 2009 (AnalystSoft, Vancouver, BC,

Canada). Particle sizes and REs were analyzed using one-way ANOVAs. Homogeneity of variance was checked with Levene's test and normality was checked graphically. If necessary, data were *ln* or arc-sin transformed to better meet the assumptions of the ANOVA. When transformation was insufficient to meet the assumption of equal variance, Wilcoxon's rank-sum test was used. Iodine and yttrium concentrations from *Artemia* enrichment trials were analyzed using a mixed model (referred to as REML). Fixed effects were estimated from the Standard Least Squares and random effects were calculated using the Restricted Maximum Likelihood (REML) method. Homogeneity of variance and data normality were checked graphically. When variance was not homogenous among groups, *ln* or arc-sin transformations were employed. Multiple pair-wise comparisons were performed with Tukey's Honest Significant Difference (Tukey's HSD) test at a significance level of 0.05. In the event that a *t*-test was used, normality was checked graphically and homogeneity of variances was verified with an *F*-test for equal variances. If needed, data were *ln* or arc-sin transformed to better meet the assumptions of the *t*-test.

Results

Particle size

Mean particle diameters (±1 SD) were 8.0±2.0 µm and were not significantly different between WSB formulations (ANOVA, $p = 0.609$).

Encapsulation (EE), Inclusion (IE) and Retention efficiencies (RE) of iodine and yttrium in WSB

The mean EE (% of total WSB weight) of iodine in KI+Y WSB was 6.8±1.0%. The mean EE for Y WSB and KI+Y WSB were not significantly different (*t*-test, $p = 0.241$) and had a grand mean of 1.3±0.2%. IE for iodine and yttrium were 89±13.5% and 84.9±17.4%, respectively. RE of iodine in KI+Y WSB significantly declined over time during the leakage trial (Wilcoxon's rank-sum, $p < 0.001$). On average, 63% of the iodine contained in WSB was lost within the first 5 minutes when suspended in SW (Figure 2.1). RE of iodine remained constant, at approximately 13% of initial concentration, after 30 minutes suspension (Tukey's HSD, threshold $p = 0.05$). RE of yttrium was 102±15.7% overall and was not significantly different among time periods (Figure 2.1; ANOVA, $p = 0.842$).

Yttrium and iodine uptake by Artemia at increasing particle concentrations of WSB

Artemia yttrium concentrations increased with increasing particle concentrations (Figure 2.2; REML, $p = 0.004$). *Artemia* enriched with KI+Y WSB had significantly higher levels of iodine than *Artemia* enriched with KI in aqueous solutions (REML, $p < 0.001$). Specific pair-wise comparisons are given in Figure 2.2. Within KI+Y WSB enriched *Artemia*, there was a strong correlation between yttrium and iodine concentrations (Linear regression, $R^2 = 0.94$, $p > 0.001$).

Iodine and yttrium uptake by Artemia over time

Artemia enriched with KI+Y WSB at 200 mg l^{-1} had a mean iodine concentration of 1220 μg I g^{-1} after 15 minutes of enrichment (Figure 2.3). The mean iodine concentration then decreased to 630 μg I g^{-1} DW after one hour and 350 μg I g^{-1}

DW after 12 hours. *Artemia* had a mean yttrium concentration of 600 µg Y g^{-1} DW at 15 minutes, which underwent an insignificant decline to 430 µg Y g^{-1} DW after 12 hours of enrichment (Tukeys HSD, threshold $p = 0.05$). Iodine concentrations varied significantly over time, (REML, $p < 0.001$) whereas yttrium concentrations did not change after the first 15 min of enrichment (Tukey's HSD, threshold $p = 0.05$).

Retention of iodine and yttrium by Artemia following enrichment

Iodine concentrations in KI+Y WSB enriched *Artemia* significantly declined from 220 µg I g^{-1} prior to starvation to 130 µg I g^{-1} after 8 h of starvation (REML, $p < 0.001$) but were not effected by SW temperature (REML, $p = 0.427$; Figure 2.4). Mean yttrium concentrations in *Artemia* varied with the duration of starvation and SW temperature (REML, $p = 0.022$ and 0.015, respectively). However, only *Artemia* starved for 8 h in cold SW had significantly lower yttrium concentrations when compared to fully enriched *Artemia* (Tukey's HSD, threshold $p = 0.05$; Figure 2.4).

Iodine and yttrium retention by Artemia after purging

Particle ingestion and retention: DiI WSB and YG microspheres were easily identifiable in the guts of *Artemia* and were visually isolated from one another with GFP (*ex.* 450-490, *em.* 500-545) and TRITC (*ex.* 510-560, *em.* 573-647) filter sets. *Artemia* guts appeared to be full of both particle types after 1h of enrichment (Figure 2.5). The numbers of YG microspheres were consistent amoung metanauplii with a mean of ~100 microshperes nauplii^{-1}. Sorting of WSB and microspheres did not

appear to occur in the guts of metanauplii and both particle types appeared to pass through the gut at similar rates (Figure 2.5).

Iodine absorption in *Artemia*: After purging *Artemia* with Ori-green, 2.5 and 0.9% of yttrium remained in *Artemia* after 2.5 and 5 h, respectively (Figure 2.6). These levels were highly correlated with the number of YG microspheres remaining in *Artemia* after purging (Figure 2.7; Linear regression, $R^2 = 0.95$, $p = 0.001$) suggesting that *Artemia* were not fully purged and that remaining yttrium concentrations were likely associated with retained WSB in the gut rather than absorbed yttrium. "Absorbed" iodine levels (iodine in *Artemia* that was not associated with ingested WSB) were significantly higher in KI+Y WSB enriched *Artemia* than in *Artemia* enriched with aqueous KI (Figure 2.8; REML, $p = 0.006$). After purging, *Artemia* enriched with KI+Y WSB had a mean total iodine concentration of 15.2 μg g^{-1} DW and a mean "absorbed" concentration of 10.6 μg g^{-1} DW. *Artemia* enriched with aqueous KI had a mean total iodine concentration of 4.6 μg g^{-1} DW.

Discussion

Artemia enriched with KI+Y WSB had higher levels of iodine than *Artemia* enriched with similar concentrations of KI in aqueous solution indicating that WSB can be used to elevate the concentrations of iodine in *Artemia*. Changing the particle density of WSB in the enrichment water significantly affected concentrations of both iodine and yttrium in *Artemia* (Figure 2.2). These results are consistent with previous research indicating that increasing particle density can result in a higher proportion of *Artemia* with full guts (Gelabert, 2003). Manipulation of WSB rations could be used

to adjust concentrations of encapsulated materials within *Artemia* and, consequently, their nutritional value for aquaculture.

Enrichment trials showed that KI+Y WSB rapidly increased the total iodine concentrations in *Artemia* (Figure 2.3). Iodine concentrations reached their maximum values within the first fifteen minutes of addition of WSB, at which point, *Artemia* began to lose iodine as the enrichment period progressed. Yttrium levels indicated that *Artemia* filled their guts with WSB within the first fifteen minutes of enrichment. After fifteen minutes, yttrium concentrations remained constant, representing equilibrium between ingestion and defecation of WSB. The decrease in iodine levels of *Artemia* after the first fifteen minutes of enrichment was likely due to the ingestion of WSB containing less iodine due to leakage from the suspended beads. It is also possible that *Artemia* down-regulated tissue iodine concentrations during the enrichment period. Recent investigations suggest that *Artemia* have measurable quantities of thyroid hormones and deiodinase (Chapter 3) suggesting that *Artemia* are capable of iodine self-regulation.

When transferred to clean SW, regardless of water temperature, enriched *Artemia* retained more than 50% of their fully enriched iodine concentrations (Figure 2.4). Yttrium concentrations were relatively constant throughout the starvation period, only fully enriched *Artemia* and *Artemia* starved in cold SW for 8 h showed a significant difference (Figure 2.4). These results suggest that *Artemia* retain ingested particles for at least 4 h following starvation. Similarly, Smith et al. (2002) reported that *Artemia* would not evacuate their gut contents unless purged by feeding on

additional food particles. This response could be useful for both research and commercial applications when retention of enrichment materials is desired. This is in contrast to enriched rotifers that show a rapid loss of stomach contents if not rapidly transferred to chilled SW (Baer et al., 2008).

YG microspheres were readily ingested and did not appear to be sorted in the guts of *Artemia* (Figure 2.5). In addition, it is highly unlikely that YG microspheres were digested or absorbed by metanauplii. We were, therefore, able to use YG microspheres to validate our findings with yttrium as well as use YG microspheres to obtain "real time" data about the enrichment and purging processes.

We found that Y_2O_3 was an effective marker of WSB during the enrichment of *Artemia*. Yttrium was highly retained within WSB suspended in SW (Figure 2.1) and yttrium levels remained constant in WSB prior to ingestion by *Artemia*. Metanauplii readily consumed WSB containing Y_2O_3, suggesting that inclusion of yttrium did not inhibit WSB ingestion by *Artemia*. Furthermore, our data suggests that *Artemia* did not absorb yttrium from WSB. Two and a half percent and 1% of the enriched yttrium levels remained after 2.5 h and 5 h of purging, after 7 transfers to clean SW and fresh suspensions of Ori-green (Figure 2.6). The percent of yttrium remaining after purging corresponded with retention estimates of YG microspheres, suggesting that residual yttrium was likely contained within unpurged WSB retained in the gut. Given these properties, we were able to use yttrium concentrations to determine the quantities of WSB that were ingested and retained by *Artemia*, providing a more detailed

explanation of the enrichment process than would have been known in the absence of an inert marker.

Based on our data, WSB were composed of 1.6% yttrium (WW) and *Artemia* enriched with the highest concentrations of WSB had yttrium concentrations of 750 µg Y g^{-1} DW. If *Artemia* nauplii are approximately 85% water (Goldan et al., 1998), the wet weight concentration of yttrium in *Artemia* would have been 112.5 µg Y g^{-1} and an *Artemia* would have 7030 µg WSB g^{-1} WW. Based on these calculations, we estimate that the weight of WSB in the gut of a given *Artemia* was approximately 1% of its total body weight. If we assume that *Artemia* and WSB have similar densities (~1 g ml^{-1}), then the volume percent and weight percent would be equivalent. If we also assume that the volume of WSB in the gut was representative of the total gut volume, then we estimate that the gut of an *Artemia* represents approximately 1% of its total volume. Though this estimate is approximate, these data suggest that the gut of an *Artemia* represents a very small fraction of its total body volume.

In the purging trial, we found that enrichment with KI+Y WSB resulted in iodine absorption into *Artemia* tissues. *Artemia* enriched with KI+Y WSB and purged with Ori-green had significantly higher iodine levels than those enriched with aqueous KI even after accounting for iodine associated with residual WSB in the digestive tracks of the metanauplii (Figure 2.8). This suggests that *Artemia* absorbed iodide directly from ingested WSB. With respect to fully enriched *Artemia*, metanauplii retained only a small fraction of iodine after purging; however, it should be noted that purged *Artemia* were suspended for at least 2.5h in clean SW before being preserved

for analysis. It is possible that *Artemia* down-regulated iodine concentrations within their tissues or lost iodine via excretion.

This study illustrated the difficulty in "purging" the gut contents of *Artemia*. When transferred to clean SW, metanauplii retained gut contents for at least eight hours (Figure 2.4). A second food type was required to replace ("purge") the initial food type contained in the guts of *Artemia* (Figure 2.6). The difficulty in purging WSB from the guts of *Artemia* may be attributed to re-ingestion of defecated beads by *Artemia* or it may be due to preferential sorting of particles in the gut. In this study, to remove 98% of ingested WSB, it was necessary to transfer *Artemia* to clean SW seven times, at 20-minute intervals, with new Ori-green added each time. These findings emphasize the importance of using inert markers in order to estimate retained material in the guts of *Artemia*.

Though conflicting data exist, 100% beeswax WSB do not appear to be physically broken down by 36 hph *Artemia*. 'Softening' WSB may allow them to be broken-down or manipulated by peristalsis, which would improve the availability of encapsulated nutrients. Though this may improve breakdown, particle breakdown may not be required for live prey enrichment. In this study we have shown that *Artemia* absorb iodine from WSB even without significant particle breakdown. Short-term enrichments may be utilized to take advantage of high rates of nutrient loss by microparticles. For instance, *Artemia* will fill their guts in about fifteen minutes and therefore can ingest particles that are still rapidly leaching their nutrients. This mode of enrichment may reduce the need to develop particles that can be physically broken

down by target organisms. In addition, microparticles may be formulated to control the rate of losses of target substances and therefore maximize the uptake of diffused substances by live prey.

WSB had an average bead diameter of 8.0±2.0 µm regardless of WSB formulation. Approximately 61% of the number of particles and 12% of the total volume of WSB used were in the optimal size range reported for consumption by *Artemia* metanauplii (Makridis and Vadstein, 1999). Decreasing the variability in bead diameters would likely have increased the ingestion rates by *Artemia*. However, reducing the variation in particle size, specifically by eliminating the larger WSB, may have reduced the RE of water-soluble substances due to increased surface area to volume ratios associated with smaller particles. Beeswax WSB were homogenously dispersed by low-powered sonication in 1% gum arabic. Once added to the 15 l poly-carbonate enrichment cones containing SW (without *Artemia*), observable quantities of WSB remained in suspension for greater than 12h but no WSB remained after 24 h (unpublished data). WSB appeared to be lost from suspension due to particles flocculating and floating to the surface where they collected on the edge of the cones. If long-term enrichments (>12h) are desired it may be necessary to add WSB in "pulses" throughout the enrichment period.

The fatty acid composition of beeswax is primarily made up of saturated fatty acids (Hepburn et al., 1991), which are not desirable for larval fish in levels exceeding those found in *Artemia* (Sargent et al., 1999; Sorgeloos et al., 2001). Based on yttrium concentrations and WSB formulas, we estimate that the wax fraction of WSB ingested

by metanauplii comprise approximately 4% of the DW of *Artemia*. Total lipid levels of *Artemia* (on a DW basis) have been reported to be between 12 and 20% (Lèger et al., 1987; Sargent et al., 1999). Based on these values, we estimate that the wax in WSB represented between a 20-33% increase in total lipids and that wax represented 17-25% of the total lipids in WSB-enriched *Artemia*. It is currently unknown if *Artemia* or larval fish are able to digest or absorb waxes. If *Artemia* and fish larvae are unable to digest beeswax, then the increased quantities of lipids, in the form of wax, may not have a nutritional impact on larval fish. Further research is needed to address the digestibility and nutritional impacts of waxes on larval fish.

It is currently unknown to what extent WSB can be used for the delivery of lipid-soluble nutrients. It is possible that lipid soluble nutrients could be included in the wax matrix of WSB, however, doing so would likely compromise the RE of water-soluble substances. A better approach may be to co-enrich *Artemia* with both WSB and an enrichment product aimed at delivering lipid-soluble nutrients. This could be accomplished by varying the ratio of WSB to lipid-enrichment product in the same culture (unpublished data) or by enriching two separate cultures with the two different methods and then combining the cultures prior to feeding larval fish. Further research is needed to develop methods capable of delivering both water and lipid-soluble nutrients.

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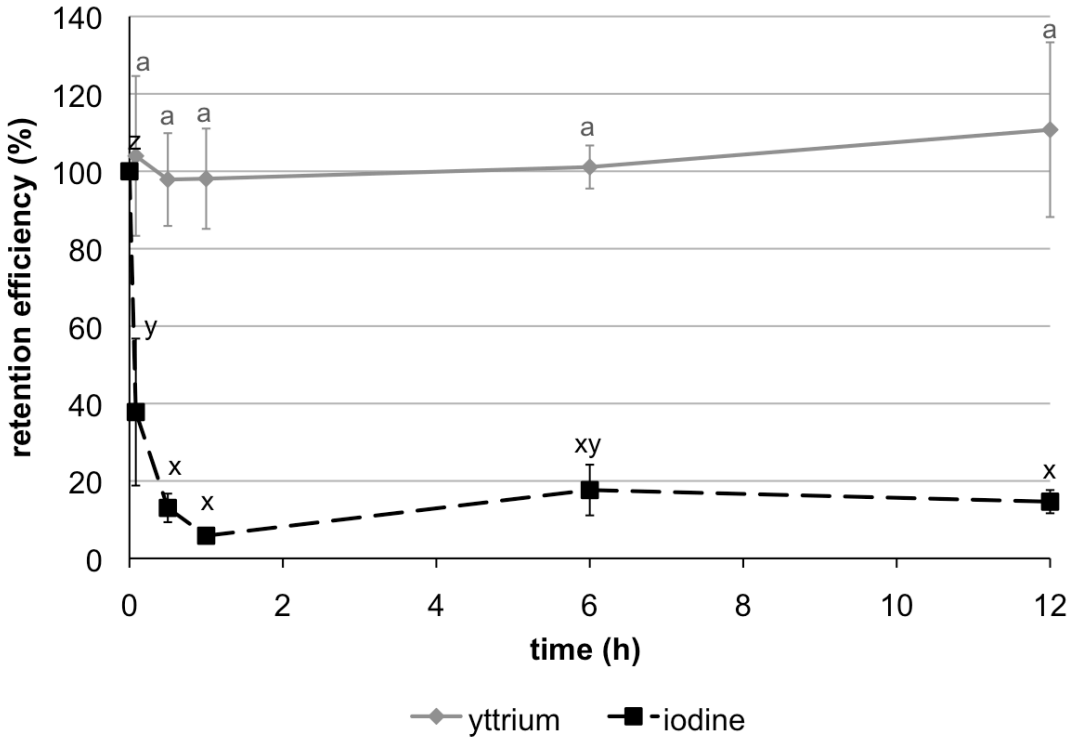

Figure 2.1 Retention of iodine and yttrium by KI+Y WSB and Y WSB after suspension in 0.5% SDS in SW solution. Independent samples were taken at 0, 5 and 30 minutes as well as at 1, 6 and 12 hours. Different letters denote significant differences (Tukey's HSD, $p < 0.05$). Iodine levels and yttrium levels were not compared.

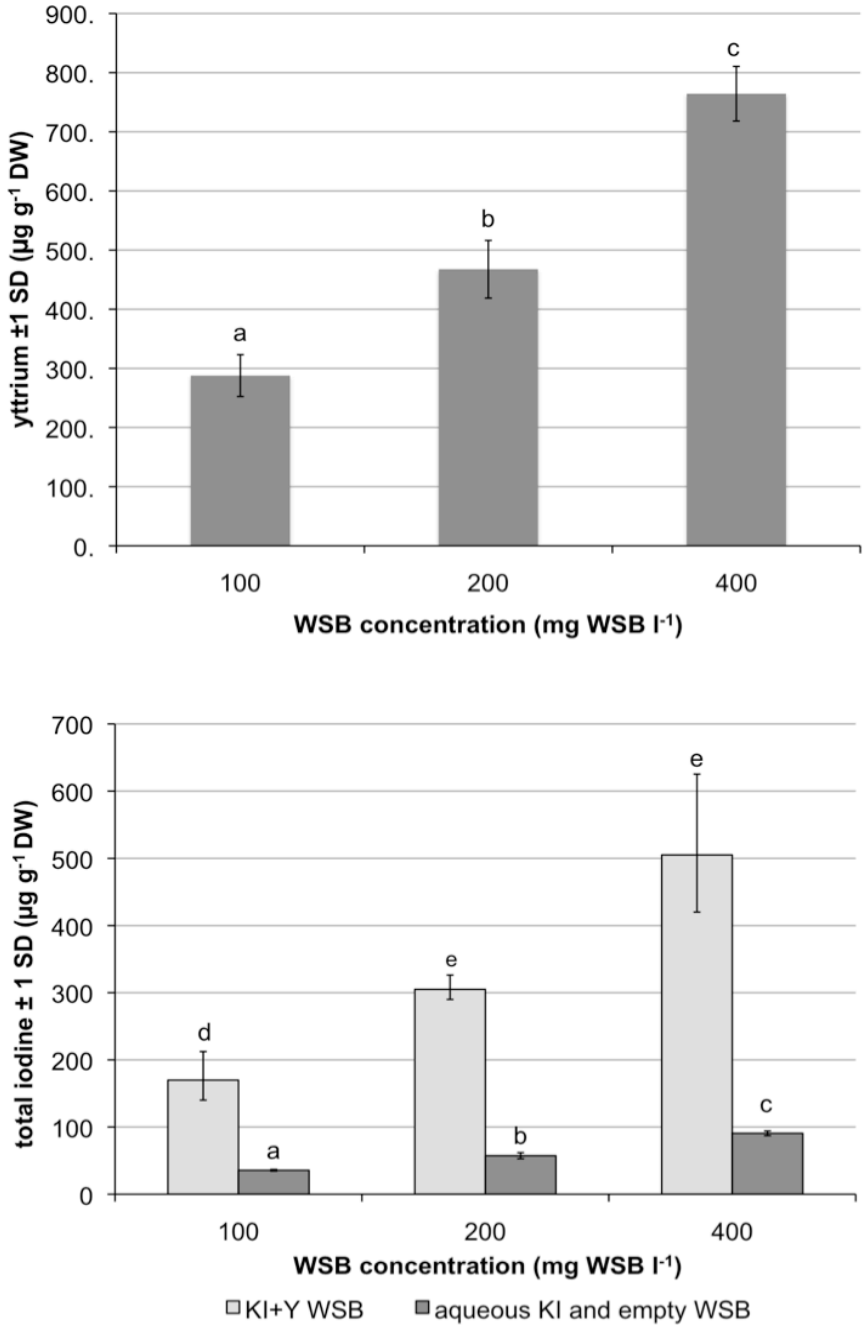

Figure 2.2 Yttrium (above) iodine (below) concentrations measured in *Artemia* after a 12h enrichment period with KI+Y WSB at three concentrations of WSB (100, 200 and 400 mg WSB l^{-1}). Different letters denote significant differences (Tukey's HSD, $p < 0.05$).

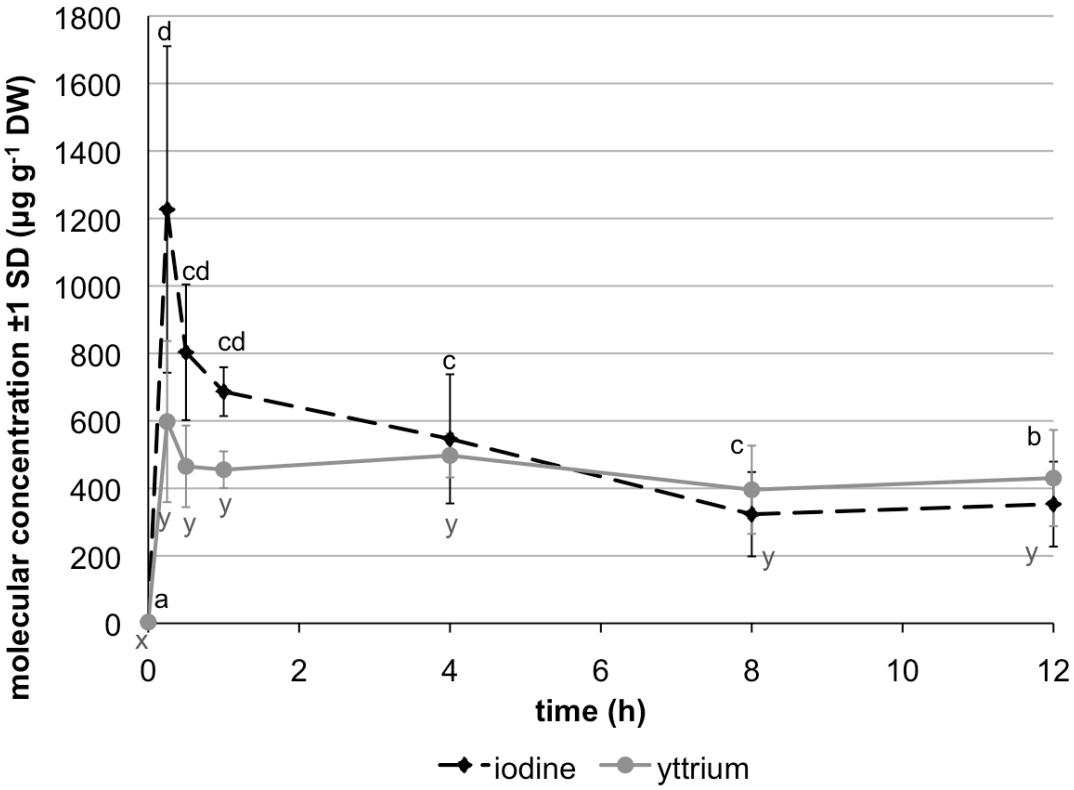

Figure 2.3 Total iodine and yttrium concentrations of *Artemia* enriched with 200 mg KI+Y WSB l^{-1} over a 12 h enrichment period. Samples were taken at 0, 15 and 30 minutes as well as at 4, 8 and 12 h. Different letters denote significant differences (Tukey's HSD, $p < 0.05$). Comparisons were not made between iodine (a-d) and yttrium (x-y) concentrations.

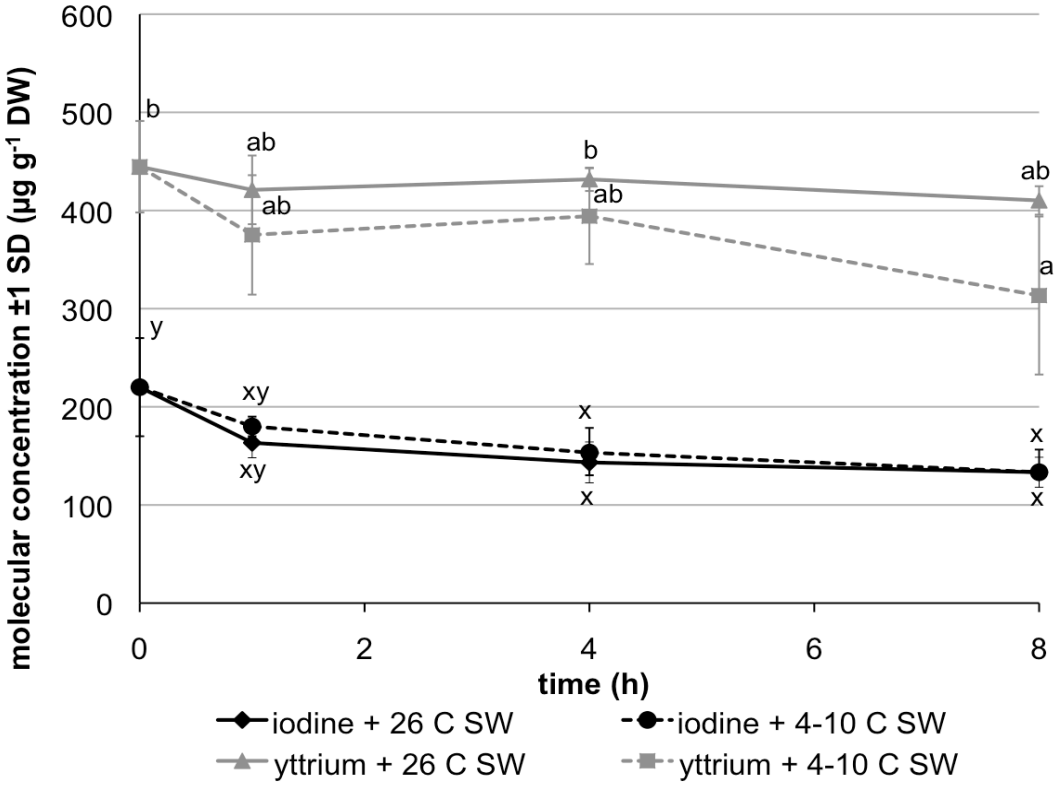

Figure 2.4 Total iodine and yttrium concentrations in *Artemia* starved in clean SW following a 12 h enrichment with 200 mg KI+Y WSB. *Artemia* were starved in either warm (26° C) or cold (4-10° C) SW. Different letters denote significant differences (Tukey's HSD, $p < 0.05$). Comparisons were not made between iodine (a-b) and yttrium (x-y) concentrations.

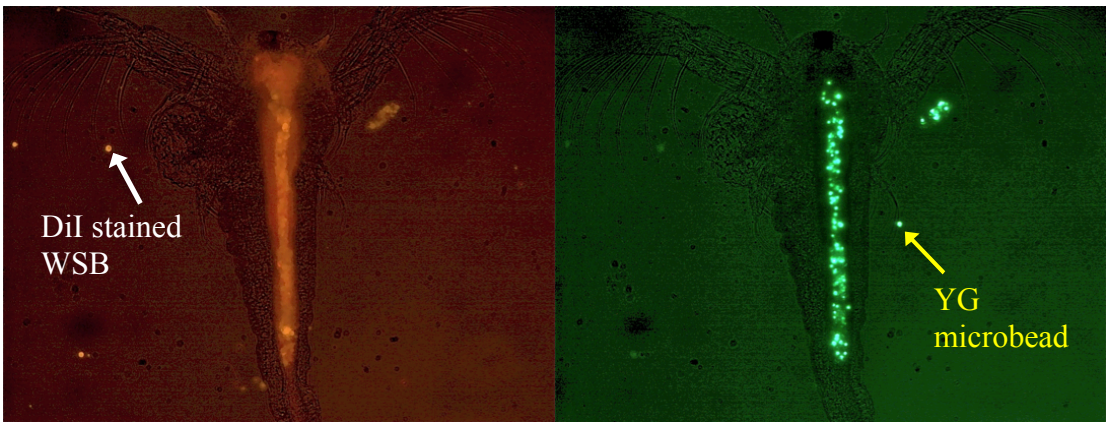

Figure 2.5 *Artemia* co-fed with DiI stained WSB (200 mg l^{-1}) combined with 3 µm YG microbeads (5 mg l^{-1}) for 1h. (Left) Image taken with TRITC bandpass filter (*em.* 573-647 nm) to visualize DiI stained WSB. (Right) Image taken with GFP bandpass filter (*em.* 500-545nm) to visualize YG microbeads.

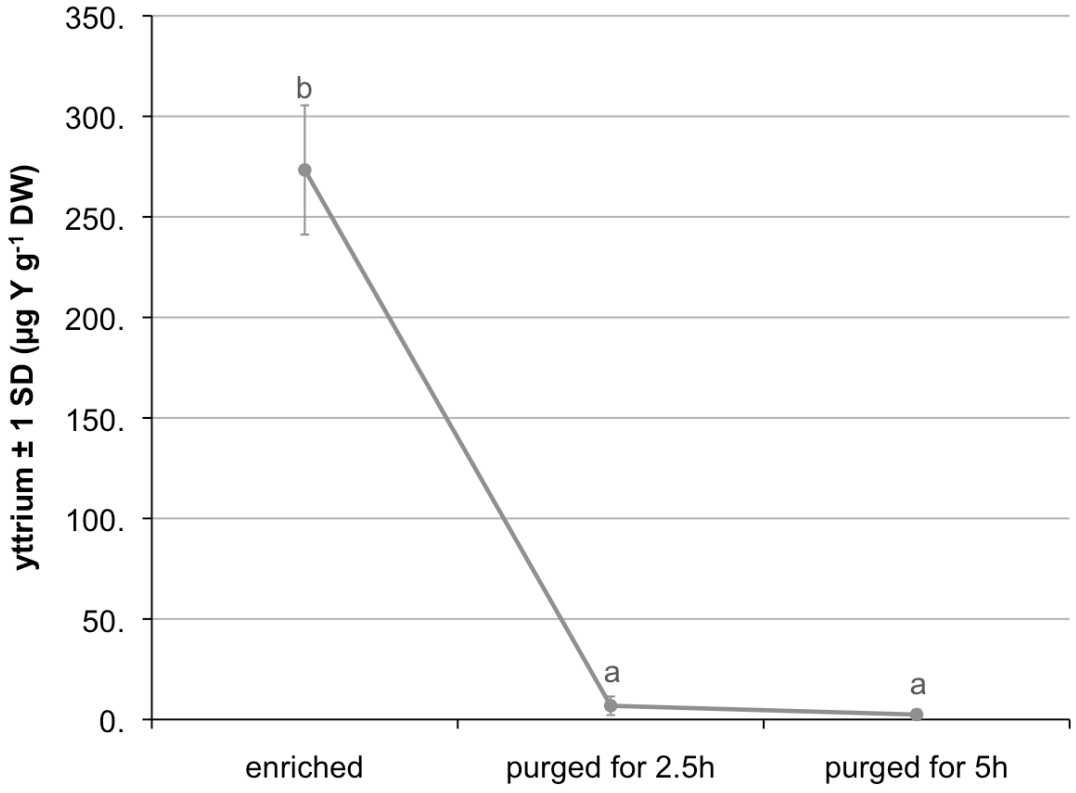

Figure 2.6 Yttrium concentrations in *Artemia* after 1h enrichment with Y WSB (200 mg l^{-1}; "enriched") and after *Artemia* were transferred to clean SW and fed Ori-green to purge their guts of WSB (repeated seven times in 20 minute intervals). *Artemia* were sampled after 2.5h and again after an additional 2.5h period with no additional transfers. Different letters denote significant differences (Tukey's HSD, $p < 0.05$).

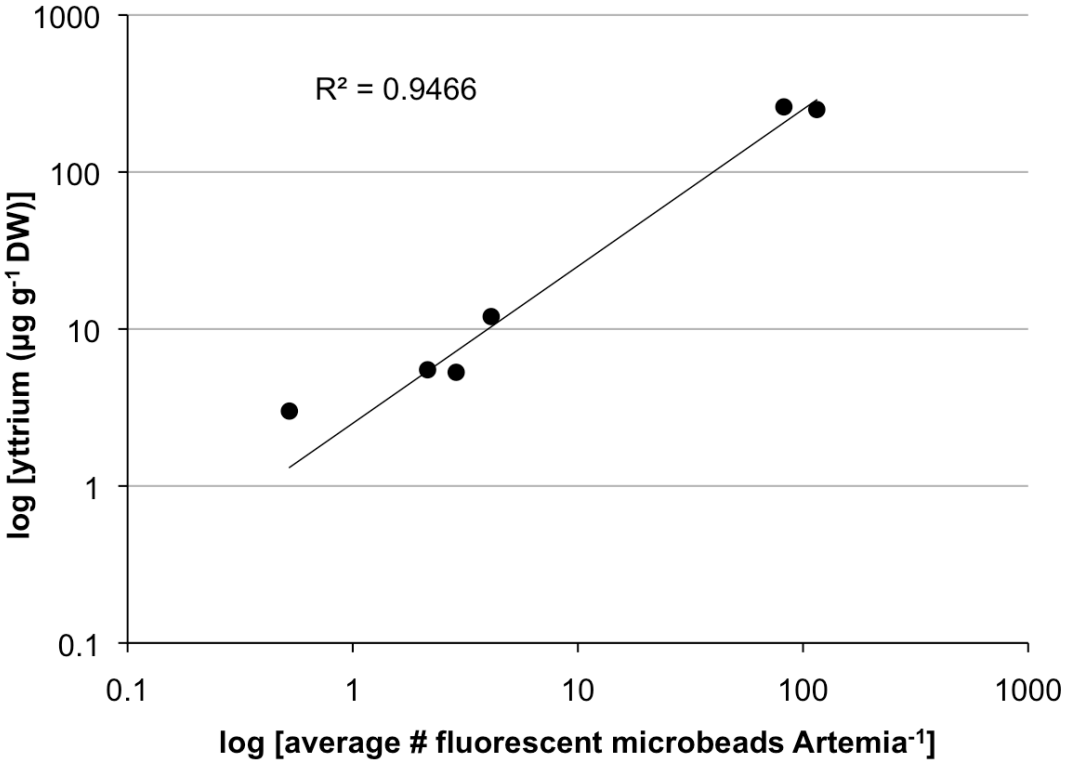

Figure 2.7 Correlation between yttrium concentrations and the average number of fluorescent YG microbeads measured per *Artemia* (> 10 *Artemia* measured at each point shown) after a 1h enrichment period and after 2.5 and 5h of purging with Ori-green. Yttrium concentrations were highly correlated ($R^2 = 0.95$, $p = 0.001$) with the average number of microbeads measure in *Artemia* suggesting that both particle types were ingested and retained similarly by *Artemia*.

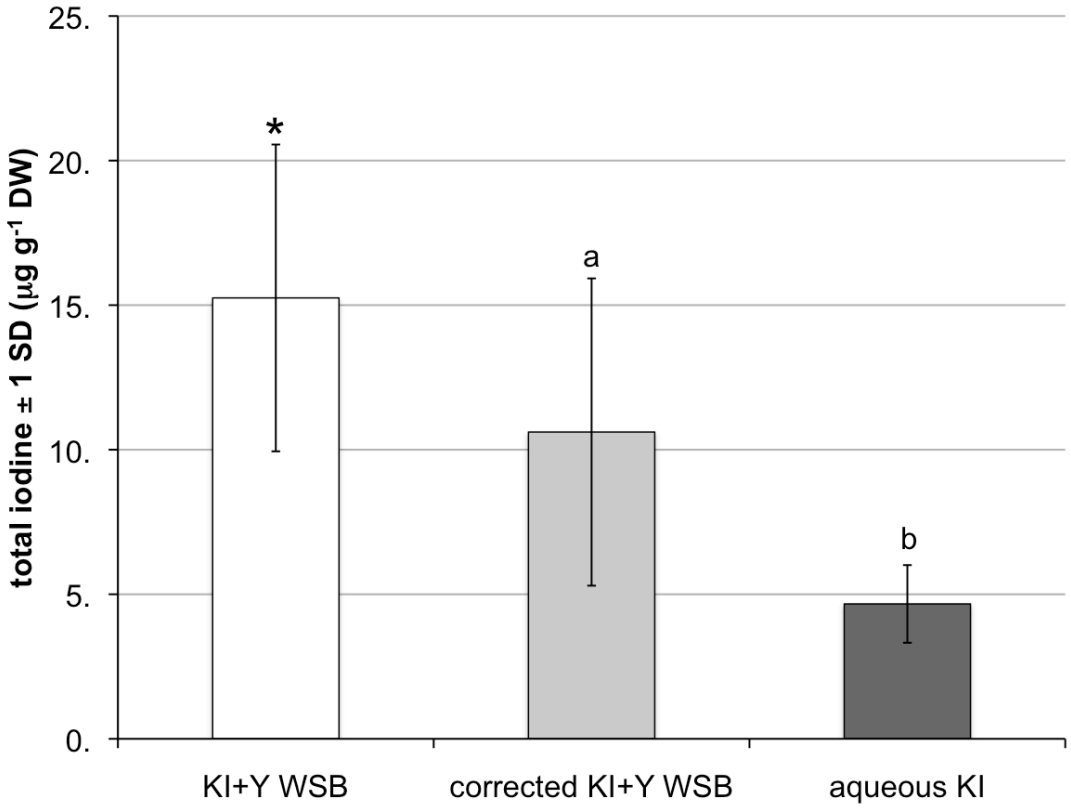

Figure 2.8 Total iodine concentrations of *Artemia* enriched for 1h with either a) KI+Y WSB or b) KI in aqueous solution (aqueous KI) at levels equivalent to the WSB treatment. *Artemia* were purged for 2.5h or 5h and transferred to clean Ori-green seven times to remove ingested WSB. There was no difference in iodine levels between *Artemia* purged for 2.5h and *Artemia* purged for 5h (REML, $p = 0.46$), therefore the combined data are shown. "Corrected KI +Y WSB" levels were calculated by subtracting KI contained in WSB (based on leakage rates) that was contained in the guts of *Artemia* after purging as determined from yttrium levels. Error bars represent untransformed data values. Uncorrected values (*) are shown for qualitative comparison only. Different letters denote significant differences based on *ln*-transformed data (REML, $p = 0.006$).

CHAPTER 3 –EFFECT OF IODINE ENRICHMENT OF *ARTEMIA* SP. ON THEIR NUTRITIONAL VALUE FOR LARVAL ZEBRAFISH (*DANIO RERIO*).

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Abstract

Dietary iodine may play an important role in the nutritional health of freshwater fish larvae. *Artemia*, commonly used for the culture of larval zebrafish (*Danio rerio*), contain low concentrations of iodine when compared with wild zooplankton. Iodine concentrations of *Artemia* can be increased using wax spray beads (WSB) containing potassium iodide (KI; KI WSB); however, the availability of iodine in enriched *Artemia* for fish larvae is currently unknown. The objectives of this study were to: 1) determine if zebrafish larvae were able to obtain iodine from KI WSB-enriched *Artemia*; 2) investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish; 3) determine if *Artemia* were a potential source of exogenous THs for larval fish; and 4) determine if KI WSB had an effect on bacterial concentrations associated with *Artemia*. A 24-day feeding trial was conducted to compare the effects of iodine-enriched *Artemia* with unenriched *Artemia* on the survival and growth of larval zebrafish. Zebrafish fed *Artemia* enriched with KI

WSB showed a ten-fold increase in total iodine levels and increased survival when compared with larvae fed unenriched *Artemia*. Thirty-eight days-post-fertilization (dpf) zebrafish larvae fed iodine-enriched *Artemia* had lower epithelium to colloid (v:v) ratios when compared to those fed unenriched *Artemia*. *Artemia* were found to contain significant levels of out-ring deiodinase and THs. KI WSB had no effect on the levels of marine bacteria associated with *Artemia*. The results of this study indicate that iodine contained in KI WSB enriched *Artemia* is available to larval fish. There was also evidence to suggest that early-stage zebrafish benefit from increased levels of dietary iodine. In addition, *Artemia* may provide larval fish with significant levels of exogenous THs and deiodinase.

Introduction

Recent studies have shown that *Artemia sp.* have significantly lower concentrations of iodine when compared with wild-caught zooplankton (Hamre et al., 2002; Solbakken et al., 2002; Moren et al., 2006) which may explain higher metamorphic success of marine fish larvae fed wild-caught zooplankton compared with larvae fed *Artemia*. *Artemia* are often used for the culture of freshwater fishes, such as zebrafish (*Danio rerio*) (Lawrence, 2007). However, it is currently unknown whether freshwater species benefit from levels of dietary iodine exceeding those normally measured in *Artemia*. Commonly, *Artemia* and other prey species used as food for cultured marine larvae are enriched by adding water-soluble micronutrients, such as potassium iodide (KI), directly to the enrichment water to facilitate uptake via drinking or adsorption (Moren et al., 2006; Hamre et al., 2008; Ribeiro et al., 2009).

This enrichment approach typically results in wastage of large amounts of nutrients due to low uptake efficiencies by prey organisms. Beeswax wax spray beads (WSB) containing potassium iodide (KI WSB) have been shown to significantly elevate the iodine levels of *Artemia* and were shown to deliver iodine to *Artemia* more efficiently than when KI was dissolved directly into the seawater (Chapter 2). It was shown that iodine was absorbed from WSB into the tissues of *Artemia* suggesting that iodine contained in WSB is available to *Artemia* and is likely available to larval fish.

Freshwater is generally much lower in iodine than seawater and iodine deficiencies are believed to be more common in freshwater systems (Watanabe et al., 1997). Iodine in seawater averages approximately 58 μg l^{-1} but is much more variable in freshwater and seldom exceeds 15 μg l^{-1} (Fuge, 1996). Therefore, the intake of dietary iodine may be essential for freshwater species. We have chosen to investigate the role of dietary iodine in the larval stages of a commonly cultured freshwater species, zebrafish (*Danio rerio*). The natural prey of zebrafish in composed of both aquatic and terrestrial invertebrates (McClure et al., 2006); however, the iodine content of these prey organisms has not been reported. Like many marine fish species, captive reared zebrafish are typically fed *Artemia* as a major component of their diet (Lawrence, 2007) and may therefore be subjected to low levels of dietary iodine. Zebrafish are often used as a model organism in biomedical and genetic research and it is necessary to ensure that experimental animals are not stressed due to nutritional deficiencies.

Fish require iodine for the production of thyroid hormones (THs), thyroxine (T4) triiodothyronine (T3), which are composed of 65 and 58% iodine by molecular weight, respectively (Power et al., 2008). THs play a central role in the development and ontogeny of teleost fish (Power et al., 2001). For instance, in Atlantic halibut (*Hippoglossus hippoglossus* L.), which undergo a dramatic metamorphosis, eye migration and bone ossification is strongly influenced by TH levels (Sæle et al., 2003). While zebrafish undergo a much more subtle metamorphosis, THs have been shown to be strongly linked to early development. Brown (1997) found that the inhibition of THs in larval zebrafish reduced fin development, scale formation and pigmentation but that these effects could be cancelled with the simultaneous addition of exogenous T4. Although exogenous THs may have an effect on larval fish development, little attention has been given to the possible introduction of THs via live-prey organisms, mainly because there has been insufficient evidence to suggest that invertebrates contain significant quantities of THs and related enzymes.

Iodine (I_2), sometimes in combination with KI, is commonly used as a bacterial disinfectant. It is possible that enriching *Artemia* with elevated levels of iodine or iodide could reduce the bacterial levels of enriched metanauplii. If so, changes in the growth or survival of fish larvae fed iodine-enriched *Artemia* could be due to pathogenic rather than nutritional effects. It has been shown that molecular iodine (I_2) has little impact on the bacterial levels of *Artemia* (Gomezgil-RS et al., 1994). Likewise, we hypothesized that enrichment with KI WSB would not have a significant effect on bacterial concentrations associated with *Artemia*. If KI WSB did not affect

bacterial concentrations, then changes in the growth and survival of larval zebrafish could be attributed to nutritional effects.

The primary objectives of this study were: 1) determine if zebrafish larvae were able to take up iodine from KI WSB-enriched *Artemia*; 2) investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish; 3) determine if *Artemia* were a potential source of exogenous THs for larval fish; and 4) determine if KI WSB had an effect on bacterial concentrations associated with *Artemia*.

Methods

Production of WSB

Wax spray beads were produced using methods described by Langdon et al. (2008). WSB were made with beeswax (refined; Sigma-Aldrich, St. Louis, MO, USA) and 5% (w/w of lipid) sorbitan tristearate (Sigma-Aldrich, St. Louis, MO, USA) that was added as an emulsifying agent and to improve bead dispersion in seawater. WSB had an aqueous core to lipid ratio of 1:4. In KI WSB, 10% (w/w of total bead formulation or 47% w/v of the aqueous core) potassium iodide (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the aqueous core prior to emulsification.

Artemia culture

Great Salt Lake *Artemia* cysts (1.4 g l^{-1}; INVE Tech., Dendermonde, Belgium) were added to 1-µm filtered seawater (20-24 ppt, 26° C) in 15 L polycarbonate hatching cones (Aquatic Habitats, Apopka, FL, USA). Aeration was provided by an

air pump fitted with a rigid air tube and was set to provide ~120-150 bubbles min^{-1}. After 24 hours of incubation, the aeration was removed and bottom lighting was utilized to attract metanauplii to the bottom of the cone while cysts floated to the surface. *Artemia* metanauplii were then drained from the bottom of the cone onto a 200 µm sieve, rinsed with clean seawater and transferred to a clean polycarbonate hatching cone containing 1 µm filtered seawater. Unfed, 24 hours-post-hatch (hph) *Artemia* metanaulpii were used for all enrichment trials.

Iodine enrichment of Artemia over time

Artemia metanauplii (24 hph; approx. 150 ind. ml^{-1}) were enriched for 12h with 100 mg l^{-1} KI WSB in 2 L cultures (~0.67 µg $metanauplii^{-1}$). In addition, metanauplii were enriched with WSB that did not contain KI together with 10 mg l^{-1} aqueous KI dissolved in the culture water, which was an equivalent KI concentration to that delivered by the KI WSB. Unenriched *Artemia* were sampled at the beginning of the enrichment period and both treatments were sampled at 6 and 12h after initiation of feeding. Each treatment and time point combination was set up in triplicate in separate enrichment cones to maintain sample independence. *Artemia* were enriched for 12h to be consistent with recommended protocols for commercial enrichment products. After the 12h enrichment period, aeration was removed and bottom lighting was used to attract swimming metanauplii towards the bottom of the cone. *Artemia* were then drained from the bottom and collected on a 200 µm sieve, repeatedly rinsed in sequential baths of clean seawater to remove adsorbed beads and then washed with distilled water into 100 ml plastic beakers with lids. *Artemia*

samples were frozen at -20° C, freeze-dried (Freezone® freeze-dry system, Labconco® Corp., Kansas city, MO, USA)) and stored at -20° C until extraction.

Zebrafish culture systems

Zebrafish embryos were obtained from the department of biology at University of Bergen. Embryos were hatched in 100 ml beakers (approx. 50 embryos beaker^{-1}) containing water from the zebrafish culture system with methylene blue (approx. 50 µl l^{-1} culture water) added to reduce bacterial and fungal growth. At 4 dpf, larvae were transferred to 250 ml beakers where they were held until 10 dpf. Temperature was maintained at 28° C by partial submersion of beakers in a temperature-controlled bath. Larvae were then pooled and transferred to 24, 1.5 l tanks (25 larvae l^{-1}) and placed in a multi-rack, self-regulated freshwater recirculation system (Aquatic Habitats, FL, USA). At 21 dpf, larvae were transferred to 3 l tanks where they were held until 28 dpf when the majority of larvae were sampled. Remaining larvae were retained for an additional 10-day grow-out period and sampled at 38 dpf for LT, K and histological measurements.

Zebrafish feeding trial and sampling protocol

Larvae were fed Schwarz larval diet (Aquarien-Bau Schwarz, Göttingen, Germany) from first feeding until 18 dpf. Schwarz larval diet was used because it had been shown in preliminary studies to be low in iodine (approx. 4.2 µg I g^{-1} DW).

At 14 dpf, zebrafish were split into two groups of 12, 1.5 l tanks and fed either unenriched *Artemia* or *Artemia* enriched with 100 mg l^{-1} KI WSB three times a day.

Larvae were transferred to 3 l tanks at 21 dpf to avoid crowding as the larvae grew. Zebrafish were co-fed with Schwartz diet until 18 dpf to facilitate weaning onto *Artemia*. *Artemia* were enriched for 12-16h with either KI WSB (100 mg l^{-1}) or with no enrichment, then collected on a 180 µm sieve, rinsed and re-suspended in freshwater before being fed to zebrafish larvae at a density of 1 nauplii ml^{-1}. Larvae were co-fed Schwartz particulate diet (4.2 ± 0.7 µg I g^{-1} DW) until 18 dpf at which point they were fed on *Artemia* only. Larvae were starved for 24h prior to sampling in order to ensure that their guts were empty of metanauplii (verified visually). At 21, 28 and 38 dpf, 12 larvae from each treatment (1 larvae from each tank) were euthanized in MS-222 (13 ppm), were individually weighed and digitally photographed (except for 38 dpf larvae which were measured with a ruler due to size limitations). Larvae were fixed in 4% paraformaldehyde for 24 hours and stored in 70% ethanol for histological analysis. At day 28, all but 12 larvae from each treatment were euthanized in MS-222, weighed, counted and stored at -80° C for iodine analysis. Survival rates were expressed as the percentage of larvae per tank that survived from 14 dpf (beginning of dietary treatments) to 28 dpf; however, no additional mortality occurred during the 10-day grow-out period (ending 38 dpf). For 21 and 28 dpf larvae, total lengths (TL) were measured from digital images (n = 12) using Image-J software (NIMH, Bethesda, MD, USA). Thirty-eight dpf larvae were too large to be captured in a single image and so TL was measured with a ruler.

Analysis of Iodine

Total iodine was extracted from samples with tetramethylammonium hydroxide (TMAH) (Tama Chemicals Co., Tempapure-AA, Kawasaki city, Japan) and concentrations determined by ICP-MS (Agilent 7500; Agilent Technologies Inc., Santa Clara, CA, USA) as described by Julshamn et al. (2001). Extracted samples were diluted as necessary to fall within the quantifiable range (1-5 μg l^{-1}) of this method. Standard curves were produced with an iodine standard (product # 8034; Teknolab as, Kolbotn, Norway) for each sample type and dilutions carried out to account for possible matrix effects resulting from interference and interactions of co-occurring elements (all standard curve R^2 values ≥ 0.97). Milk powder (product # 150; Community Bureau of Reference, Brussels, Belgium) was used as a standard reference material and tellurium (product # 8062; Teknolab as, Kolbotn, Norway) was used as an internal standard for ICP-MS. This method determines total iodine levels and does not discriminate between species (*i.e.* iodine, iodide etc.), thus all results from this analysis are described as "iodine" or "total iodine".

Stereohistology of zebrafish thyroid follicles

Larval zebrafish were fixed in 4% paraformaldahyde for 24h and then transferred to 70% ethanol for storage. Larvae were rehydrated in 50% ethanol and decalcified in 0.5 M autoclaved, buffered EDTA (pH 6.5) for 10 days with solution renewal every 2-3 days. Larvae were dehydrated and fixed in Technovit 7100 epoxy-resin (Haraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Histological sectioning was conducted with a Leica RM 2165 motorized rotary microtome to obtain 5 μm vertical sections; every second section was retained

for analysis. Stereological measurements of thyroid follicles were performed on a compound microscope (Axioscop, Zeiss, Germany) and Olympus DP72 digital camera (Olympus, Center Valley PA, USA). Surface area estimates were performed using a Cavaleiri point estimate grid with full coverage meander sampling (Visphram A/S software, Denmark). Colloid and epithelium volumes were estimated from surface area measurements by multiplying by the sum of the section thicknesses including discarded regions.

Determination of protein

Protein was determined using a BCA protein assay kit (Thermo scientific, Waltham MA, USA) following the manufacturer's instructions.

Determiniation of type I and II iodothyronine deiodinase activity

Outer-ring deiodinase (ORD) activities were determined by measuring the amount of radio-labeled iodine that was liberated from [I^{125}] rT3 (Perkin Elmer, USA) by type I and II deiodinases as described by Klaren et al. (2005) and modified by Hamre et al. (2008). Liberated radio-labeled iodine was isolated from [I^{125}] rT3 by chromatographic separation on a sephadex column. Ultima Gold™ liquid scintillation cocktail (Perkin-Elmer, Waltham MA, USA) was added to all samples and γ-radiation was measured on a Tri-Carb 1900 TR Liquid Scintillation counter (Packard, MN, USA). ORD activities were standardized by protein concentration, determined as previously described.

Determination of thyroid hormones in zebrafish and Artemia

Thyroid hormones, T3 and T4, were extracted from frozen (-80º C) zebrafish larvae and freeze-dried *Artemia* samples by homogenizing the sample in ten times the volume of ice-cold methanol. Each sample was stored at 4°C for 24h for extraction and then centrifuged at 3000 rpm, 4°C, for 30 minutes. The supernatant was removed and the pellet was re-homogenized in clean ice-cold methanol. This process was repeated two additional times. To dispose of high levels of lipid, extracts were mixed with barbital buffer (0.1 M pH 8.6) and chloroform (buffer:methanol:chloroform 1:1:2). The aqueous phase containing T_4 and T_3 was transferred to a fresh tube, evaporated with nitrogen and stored at -20^{o} C until use. Pellets were re-suspended in 400 µl barbital buffer (0.1 M pH 8.6) before analysis. T3 and T4 were analyzed using competitive RIA methods described by Einarsdóttir (2006) as modified by Hamre et al. (2008).

Effects of KI WSB on the bacterial levels of Artemia

Artemia culture and enrichment protocols were conducted similarly to those used in the zebrafish feeding trial. *Artemia* (approx 150 metanauplii ml^{-1}) were cultured in unsterilized seawater and were incubated for 12h with either 200 mg l^{-1} KI WSB or were not enriched. Each treatment was tested in quadruplicate cultures. Sterile conditions were maintained for all post-enrichment methods. At the end of the enrichment period, 20 ml of suspended *Artemia* were removed with a pipette, screened using a 100 µm sieve and washed into a 100 ml graduated cylinder with 100 ml autoclaved seawater. One milliliter of *Artemia* (30-40 metanauplii) was drawn into a

sterile serological pipette and nauplii were counted under a dissecting scope. *Artemia* were then transferred to a sterile 15 ml centrifuge tube and sonicated six times for one-second to disrupt tissues as well as dislodge bacteria from external surfaces of the metanauplii. *Artemia* extracts were serially diluted with sterile seawater. Triplicate 100 µl samples of each diluted extract were cultured on Difco™ marine agar (BD & co., Sparks MD, USA) and Difco™ TCBS agar (BD & co., Sparks MD, USA). After 24 h incubation, plates with between 20 and 200 colony-forming units (CFU) were visually counted using a Quebec® colony counter (American Optical corp., Buffalo NY, USA).

Statistics

Statistical tests were performed with JMP© version 8.0 (SAS Institute Inc, Cary, NC, USA) and StatPlus®:mac version 2009 (AnalystSoft, Vancouver, BC, Canada). Iodine and yttrium concentrations from the *Artemia* enrichment trial were analyzed using one-way ANOVA. Homogeneity of variance was checked using Levene's test and verified graphically, normality was checked graphically. When variance was not homogenous among groups, *ln* or arc-sin transformations were employed. Zebrafish growth parameters (TL, Wt and K) were analyzed using a Repeated-measures model and random effects were calculated using the Restricted Maximum Likelihood (REML) method. Multiple pair-wise comparisons were performed with Tukey's Honest Significant Difference (Tukey's HSD) test at a significance level of 0.05. TH concentrations, epithelium and colloid volumes from 28 dpf larvae, epithelium volumes of 38 dpf larvae, ORD activities and bacterial

concentrations of *Artemia* were analyzed with *t*-tests. Normality was checked graphically and homogeneity of variances was verified with an *F*-test for equal variances. If needed, data were *ln* or arc-sin transformed to better meet the assumptions of the *t*-test. In the event that transformation was not sufficient, such as for larval survival, colloid volumes and epithelium to colloid (v:v) ratios in 38 dpf larvae, Mann-Whitney *U*-tests for non-parametric data were used.

Results

Iodine enrichment of Artemia over time

Iodine levels underwent significant change with time of enrichment (ANOVA, $p < 0.001$) and were higher in *Artemia* enriched with 100 mg KI WSB l^{-1} than in *Artemia* enriched by immersion in a solution of 10 mg (aqueous) KI l^{-1} (ANOVA, $p < 0.001$). *Artemia* enriched with KI WSB showed significantly higher iodine levels than *Artemia* enriched with aqueous KI after 6h and 12h of enrichment (Tukey's HSD, threshold $p = 0.05$). Specific values and pair-wise comparisons are shown in Figure 3.1.

Iodine levels in zebrafish larvae

Iodine levels were significantly higher in 28 dpf zebrafish larvae fed KI WSB enriched *Artemia* compared with larvae fed unenriched *Artemia* (Figure 3.2; *t*-test, $p = 0.001$). The mean iodine concentration for larvae fed iodine WSB enriched *Artemia* was 1.0 µg g^{-1} DW and was 0.16 µg g^{-1} DW for larvae fed unenriched *Artemia*.

Survival and growth of zebrafish larvae

Mean survival was significantly higher in larvae fed KI WSB enriched *Artemia* when compared with larvae fed unenriched *Artemia* (Table 3.1; *t*-test, $p = 0.004$). Zebrafish larvae fed KI WSB-enriched *Artemia* had higher rates of survival (*t*-test, $p = 0.004$) and weakly significant increases in TL and Wt when compared with larvae fed unenriched *Artemia* (Repeated-measures REML, $p = 0.053$ and 0.048, respectively). However, neither TL nor Wt were significantly different between treatments at any specific sampling time (Tukey's HSD, threshold $p = 0.05$; Table 3.1). Iodine enrichment of *Artemia* had no effect on the condition factor (K; $K = \text{weight}/TL^3$; Repeated-measures REML, $p > 0.967$; Table 3.1).

Stereohistology of zebrafish thyroid follicles

Epithelium to colloid ratios (v:v) were not significantly different between treatments at 28 dpf (*t*-test, $p = 0.825$). However, after an additional grow-out period, 38 dpf zebrafish larvae fed KI WSB enriched *Artemia* had lower epithelium to colloid ratios than larvae fed unenriched *Artemia* (Figure 3.3; Mann-Whitney *U*-test, $p = 0.029$).

Outer-ring deiodinase (ORD) activity in zebrafish and Artemia

ORD activities were not significantly different between zebrafish larvae fed iodine-enriched *Artemia* and larvae fed unenriched *Artemia* (*t*-test, $p = 0.19$; Table 3.2). Unenriched *Artemia* were found to have ORD activities significantly higher than

measured in sample blanks and similar to those measured in zebrafish larvae (Table 3.2).

Thyroid hormones in zebrafish and Artemia

T3 and T4 levels were not significantly different in 28 dpf zebrafish larvae fed KI WSB-enriched *Artemia* when compared to those fed unenriched *Artemia* (*t*-test, $p = 0.08$ and 0.859, respectively). T3:T4 ratios were also not significantly different between treatments for zebrafish larvae (*t*-test, $p = 0.506$). Both T3 and T4 were detected in *Artemia*. Levels of T3 as well as the ratio of T3:T4 were significantly higher in *Artemia* enriched with KI WSB that in unenriched *Artemia* (*t*-tests, $p = 0.023$ and 0.006, respectively). There was no difference in the levels of T4 in *Artemia* between treatments (*t*-test, $p = 0.373$; Table 3.2).

Effects of KI WSB on the bacterial concentrations associated with Artemia

With a marine agar substrate, there was no significant difference in the number of CFU per *Artemia* between treatments (*t*-test $p = 0.719$), *e.g.* 10.9 (± 5.6) x 10^3 and 9.6 (± 4.3) x 10^3 CFU *Artemia*$^{-1}$ in the KI WSB enriched and unenriched treatments, respectively. *Vibrio* sp. were not detected at significant levels with TCBS agar for either treatment.

Discussion

Zebrafish larvae fed *Artemia* enriched with KI WSB showed a ten-fold increase in total iodine levels when compared with larvae fed unenriched *Artemia*

(Figure 3.2). We can be confident that the iodine measured was in the larval tissues because larvae were starved for the last 24h before sampling and examined visually to ensure that *Artemia* metanaupllii were not present in the gut at the time of sampling. We found that larvae fed KI WSB-enriched *Artemia* had higher rates of survival and slight overall improvements in TL and Wt compared with larvae fed unenriched *Artemia* though TL and Wt. Neither TL nor Wt were significantly different between treatments at any specific sampling time (Table 3.1). Though iodine requirements may be considerably different between freshwater and marine fish species, it is noteworthy that our findings are consistent with those of previous studies involving marine fish larvae. Hamre et al. (2008) found that, in Atlantic cod (*Gadus morhua*), larval survival was significantly higher when larvae were fed rotifers enriched with both selenium and aqueous sodium iodide (200 mg NaI l^{-1}). Moren et al. (2006) found similar increases in total body iodine levels when feeding halibut larvae *Artemia* enriched with lipiodol (iodinated poppy-seed oil). However, their study did not show improvements in the growth or survival of halibut larvae, which they suggested was due to the limited bioavailability of the lipid-bound iodine. Ribeiro et al. (2009) found that Senegalese sole (*Solea senaegalensis*) larvae fed *Artemia* enriched with sodium iodide (NaI) had 5-10 times higher tissue iodine concentrations than those fed unenriched *Artemia*. They found that larvae fed iodine-enriched *Artemia* had greater total lengths after 31 dph and greater dry weights after 15 dph than larvae fed unenriched *Artemia*. However, they did not observe a change in larval survival associated with iodine enrichment.

In our study, 38 dpf zebrafish fed iodine-enriched *Artemia* had lower epithelium to colloid ratios when compared to those fed unenriched *Artemia* (Figure 3.3) suggesting that increased dietary iodine had an effect on the thyroid status of late-stage larvae. Fish fed unenriched *Artemia* may have been showing the onset of goiter and therefore had reduced colloid and increased epithelium volumes as is seen in goiterous zebrafish larvae (Brown, 1997). It may also be that larvae fed iodine-enriched *Artemia* were producing more colloid in order to store additional iodine or iodinated compounds. The colloid region of the thyroid follicle contains thyroglobulin, an iodinated protein, which is a precursor to thyroid hormones (Power et al., 2008).

There was a small, but statistically insignificant, increase in the ORD activity of 28 dpf zebrafish fed KI WSB enriched *Artemia* when compared to larvae fed unenriched *Artemia* (Table 3.1) but no evidence in the thyroid measurements in 28 dpf larval fish to suggest that TH levels were significantly affected by iodine enrichment. It may be that differences in thyroid hormone levels did not occur until after 28 dpf (THs in 38 dpf were not measured in this study). It should be noted that THs measured in zebrafish showed a high degree of variation and are prone to type II error.

It is possible that the levels of iodine used in this experiment were "beyond deficiency requirements" (Waagbo 2009) and that increased dietary iodine has added health benefits beyond avoiding goiter. Venturi and Venturi (1999) have suggested that iodine and TH have an important functions as antioxidants. In human blood serum it has been shown that 15 μM sodium iodide (NaI) has an equivalent physiological

effect as 50 μM ascorbic acid (Winkler et al., 2000). Further research is needed to address the role of iodine as an antioxidant in larval fish.

A surprising outcome from this study is that *Artemia* contained significant levels of both ORD and THs (Table 3.2) and the high ORD activity suggests that THs are regulated and thus biologically important. Furthermore, T3 production in *Artemia* increased as a result of iodine enrichment. These findings suggest that *Artemia* may be providing fish larvae with significant quantities of exogenous THs and that these levels may be affected by iodine-enrichment. While we did not find a difference in zebrafish THs, both treatments consisted of *Artemia* as the primary food source, meaning that larvae in both treatments would have been exposed to significant levels of exogenous THs. Exogenous THs provided by *Artemia* may have compensated for reduced endogenous thyroid hormone production by fish larvae and thus ameliorated the effects of reduced dietary iodine in this study. This hypothesis is supported by the finding that the addition of exogenous T4 was able to reverse the effects goiter in larval zebrafish exposed to goitrogens (Brown, 1997). It is currently unknown to what extent ingested THs or deoidinases can be utilized by fish larvae since these substances may be denatured in the gut or may not be absorbed. Future studies investigating dietary-iodine for fish larvae that utilize *Artemia* as a live-prey should consider the potential impacts of exogenous THs and deiodinases provided by *Artemia*. It is currently unknown whether rotifers or copepods have similar iodine-related hormones. The analytical methods used in this study for TH and ORD were highly specific. RIA of T3 and T4 had less than 0.01% cross-reactivity for each other

or for related molecules including diiodo-L-thyronine (T2), diiodo-L-tyrosine (DIT), monoiodo-L-tyrosine, reverse T3 (rT3), D-thyroxine and triiodo-D-thyronine (Einarsdóttir et al., 2006). Even so, we should interpret TH and ORD measurements in *Artemia* with caution since the methods employed had been optimized for fish larvae.

The results of the bacteriological assay suggest that KI WSB had no effect on the levels of marine bacteria associated with *Artemia*. Therefore, it is highly unlikely that the increased survival of larval zebrafish associated with KI WSB-enriched *Artemia* was due to the antibiotic qualities of potassium iodide. Our results are consistent with those of Gomezgil-RS et al. (1994) who found that iodine (I_2) does little to reduce the levels of bacteria in iodine-treated *Artemia.*

Increased tissue-iodine levels in zebrafish larvae indicate that some portion of iodine from KI WSB enriched *Artemia* was taken-up by zebrafish larvae. Our results suggest that larval zebrafish benefit from dietary iodine levels exceeding those found in unenriched *Artemia*. The exact amount of iodine that was available from KI WSB-enriched *Artemia* to larval zebrafish in this experiment is unknown. Assuming that 100% of the iodine that *Artemia* absorbed by drinking was available to fish larvae then, at a minimum, zebrafish larvae would have been exposed to iodine levels near those measured in aqueous KI-enriched *Artemia* (*approx*. 36 ug g^{-1} DW; Figure 3.1). If 100% of iodine contained within KI WSB enriched *Artemia* was available to fish larvae then, at maximum, larvae would have been exposed to levels equivalent with those measured in KI WSB enriched *Artemia* (*approx*. 180 ug g^{-1} DW; Figure 3.1). It is likely that the amount of iodine available to fish larvae was somewhere in between

these two values. This is supported by the finding that iodine contained in WSB was absorbed by *Artemia* beyond that taken-up from aqueous solution (Chapter 2). However, more research is needed to determine the specific availability of substances delivered via beeswax WSB as well as recommendations for levels of dietary iodine for larval zebrafish. To our knowledge, this study has been the first to demonstrate significant levels of THs and ORD activity in *Artemia* as well as the impact of iodine enrichment on these levels. Future research should be aimed at better describing the thyroid-endocrine systems of *Artemia*. When conducting enrichment studies, researchers should consider the effects of enrichment on the endocrine systems of live prey organisms.

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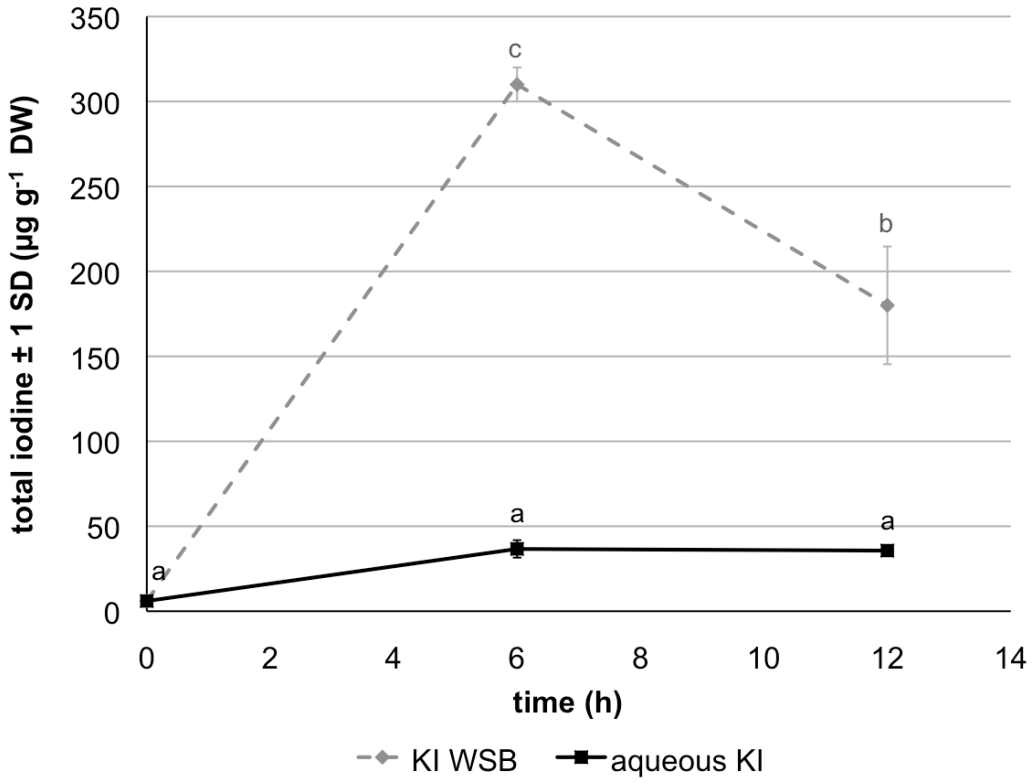


Figure 3.1 Total iodine concentrations in *Artemia* enriched with either 100 mg l^{-1} KI WSB ("KI WSB") or 10 mg l^{-1} KI added in aqueous solution ("aqueous KI") for 12 hours. The amount of KI delivered in the aqueous KI treatment was equivalent to the total amount of KI delivered in the KI WSB treatment. Data are given as mean ± 1 SD (n = 3). Different letters denote significant differences (Tukey's HSD, threshold $p = 0.05$).

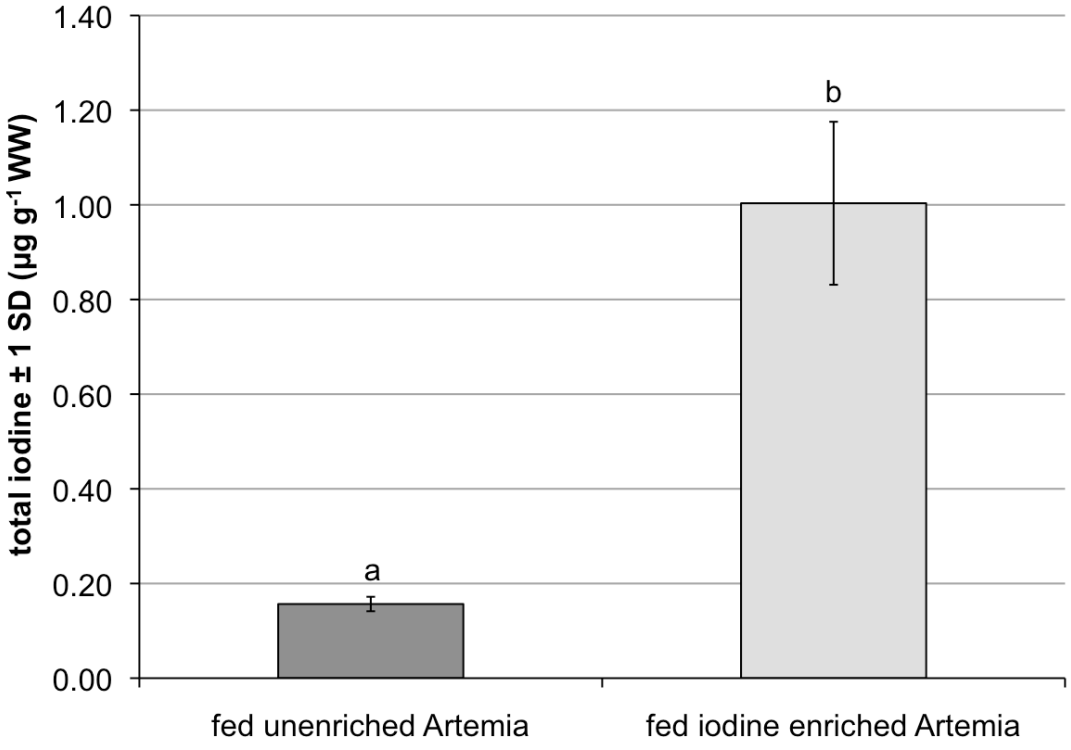
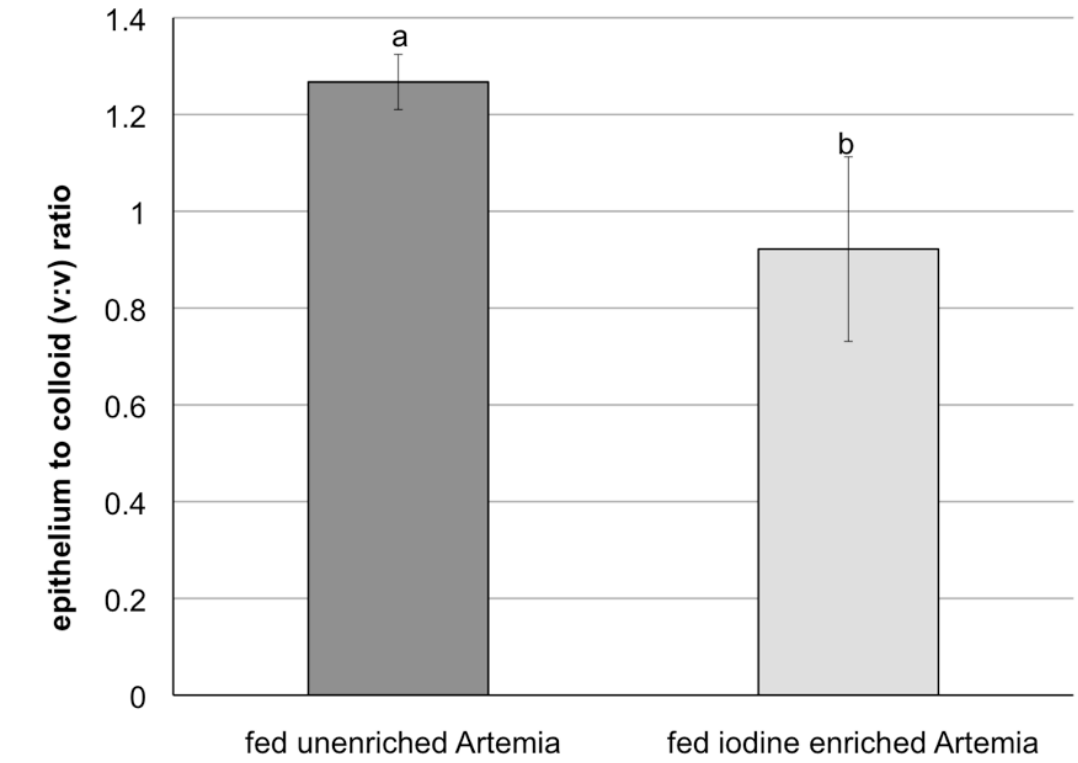


Figure 3.2 Total iodine concentrations (µg g^{-1} wet weight) of 28 dpf zebrafish larvae fed either unenriched *Artemia* or *Artemia* enriched with 100 mg l^{-1} KI WSB for two weeks. In addition to *Artemia*, both treatments were fed a particulate diet (Schwartz larval diet; 4.2 ug I g^{-1} DW) for the first 10 days of feeding. Data are given as mean ± 1 SD (n = 3). Different letters denote significant differences (*t*-test, $p = 0.001$).



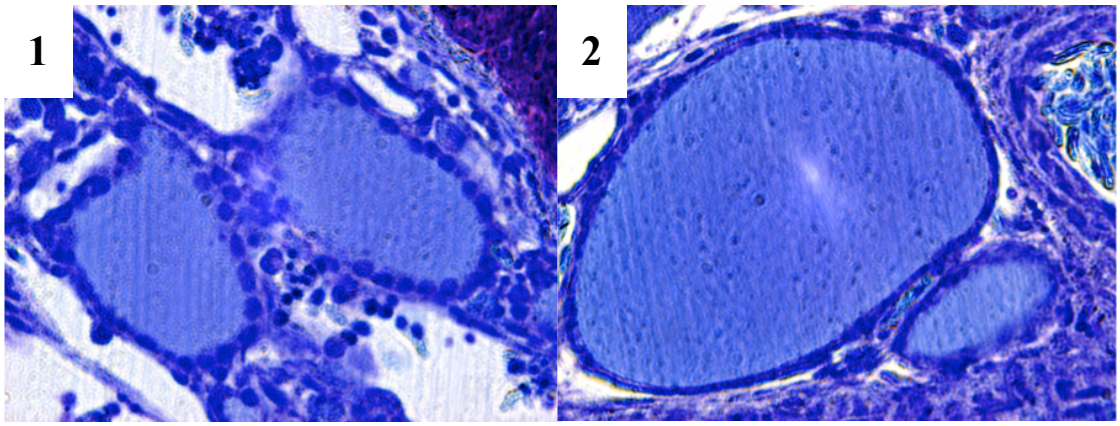


Figure 3.3 (Above) Epithelium to colloid volume ratios in 38 dpf zebrafish larvae fed KI WSB enriched *Artemia* and unenriched *Artemia* from 14 dpf. Zebrafish thyroid follicle epithelium and colloid volumes were measured from histological sections at 400X magnification using a Cavaleiri point estimate. Data are given in mean ±1 SD, (n = 4). (Below) Thyroid follicles of 38 dpf zebrafish larvae fed either (1) unenriched *Artemia* or (2) KI+Y WSB enriched *Artemia*. Digital photographs were taken from histological sections at 400X magnification.

Table 3.1 Total length (TL), wet weight (Wt; mg), condition factor (K) and survival (%) of zebrafish during a feeding trial from 14 to 38 dph. Zebrafish larvae were fed either KI WSB enriched *Artemia* ("KI+Y WSB") or unenriched *Artemia* ("control"). Data are given as mean ± 1 SD (n = 12). Differences between treatments ($p < 0.05$) are denoted with an asterisk (*). N/A = data not available

	21 dpf		28 dpf		38 dpf	
	KI+Y WSB	**control**	**KI+Y WSB**	**control**	**KI+Y WSB**	**control**
TL (mm)	9.0±1.4	8.9±1.4	14.5±1.7	12.7±2.6	24.5±2.4	23.3±3.6
Wt (mg)	4.2±2.3	3.7±2.2	29.0±9.8	20.8±10	122±32	113±46
K	0.52±0.07	0.47±0.09	0.92±0.1*	0.96±0.13	0.83±0.07	0.82±0.06
survival (%)	N/A	N/A	*61.5±8.8	49.8±8.7	N/A	N/A

Table 3.2 Triiodothyronine (T3; ng T3 mg^{-1} DW), thyroxine (T4; ng T4 mg^{-1} DW), T3:T4 ratios and deiodinase activities (ORD; fmol min^{-1} µg $protein^{-1}$) measured in *Artemia* and zebrafish larvae. *Artemia* were either unenriched (control) or enriched for 12h with 100 mg l^{-1} KI WSB (KI WSB). Zebrafish larvae were fed either KI WSB enriched *Artemia* or unenriched *Artemia* (control) diets from 14 to 28 dpf. Data given as mean ± 1 SD (n = 3). (*) denotes treatments that were significantly different from the respective control (*t*-test, $p < 0.05$). N/A = data not available.

	Artemia		**Zebrafish**	
	KI WSB	**control**	**KI WSB**	**control**
T3	30±11	11±0.58	3.0±1.0	7.0±4.0
T4	143±48*	107±41	67±65	81±115
T3:T4	0.21±0.02*	0.11±0.02	0.08±0.05	0.32±0.28
ORD	N/A	0.99±0.10	0.88±0.11	0.73±0.11

CHAPTER 4 –GENERAL DISCUSSION

Overview

The aim of this study was to evaluate the use of wax spray beads (WSB) for the enrichment of a water-soluble micronutrient (iodine) in *Artemia* and to determine if iodine enrichment had a biological effect on zebrafish larvae (*Danio rerio*). The specific objectives of this study were to: 1) evaluate the use of WSB containing potassium iodide (KI; KI WSB or KI+Y WSB) for enrichment of *Artemia* with iodine; 2) evaluate the use of yttrium oxide (Y_2O_3) as an inert marker of WSB; 3) determine if zebrafish larvae were able to uptake iodine from KI WSB-enriched *Artemia*; 4) investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish; 5) determine if *Artemia* were a potential source of exogenous thyroid hormones (TH) for larval fish; and 6) determine if KI WSB had an effect on the bacterial concentrations of enriched *Artemia.* In general, we found that WSB containing KI could be used to increase the iodine concentrations of *Artemia*. Yttrium oxide, used as an inert marker of WSB, provided detailed information about enrichment processes, and was instrumental in the evaluation of WSB. Survival of larval zebrafish was higher when fed WSB enriched *Artemia* compared with those fed unenriched *Artemia*. *Artemia* had significant levels of THs, which increased as a result of iodine enrichment. Bacteria concentrations in *Artemia* were not affected by enrichment with KI WSB. The following is a discussion of our findings as they relate to the objectives of this research.

Objective 1- Evaluate the use of WSB containing KI and Y_2O_3 (KI+Y WSB) for enrichment of *Artemia* with iodine.

Our results indicate that KI+Y WSB can be used to increase the iodine concentrations of *Artemia* in a controlled and consistent manner. We found that enrichment with KI+Y WSB was more effective than KI delivered in aqueous solution at elevating the total concentrations of iodine in *Artemia*. Iodine concentrations in *Artemia* were elevated to levels equivalent with those measured in copepods (100-350 ug g^{-1} DW; (Hamre et al., 2002; Moren et al., 2006)) and were easily manipulated by changing the concentrations of WSB in the enrichment water (Chapter 2, Figure 2.2). *Artemia* enriched with KI+Y WSB higher concentrations of absorbed iodine when compared with *Artemia* that had been enriched with aqueous KI suggesting that iodide contained in WSB was available to *Artemia*.

Ribeiro et al. (2009) used aqueous sodium iodide (NaI; 100 to 200 mg I^- l^{-1}) to enrich *Artemia* (at *Artemia* densities similar to our study) for 6h, which resulted in 64.22 µg I g^{-1} *Artemia* (WW) (Std. Dev. = 8.11). For comparison, it is necessary to assume that the dry weight of *Artemia* is approximately 15% of the wet weight (Goldan et al., 1998); therefore we may estimate that the dry weight concentration of iodine in *Artemia* may have been approximately 430 µg I g^{-1}. In our study, the highest concentrations of WSB (400 mg WSB l^{-1}; 30.6 mg I^- l^{-1}) used to enrich *Artemia* resulted in 505 µg I g^{-1} *Artemia* (DW). Based on these estimates, enrichment with KI+Y WSB achieved 117% of the iodine concentrations measured in *Artemia* by Ribeiro et al. (op. cit.) with a 70% reduction in the amount of iodine used.

WSB may be used to enrich additional micronutrients within *Artemia.* Langdon et al. (2008) found that non-labile micronutrients were efficiently incorporated in WSB. However, labile micronutrients, specifically vitamin C and selenium, were lost during the production process. Further research is needed to improve production techniques and formulations so that labile micronutrients can be included in WSB. When WSB containing a complex micronutrient mix were suspended in water, retention efficiencies (RE) varied among micronutrients, which was likely related to the solubility and molecular weight of constituent micronutrients (Langdon et al., 2008). The RE of KI was low in the current study (~20% after 1 h) when compared to the RE of other micronutrients (>50% after 1h) measured by Langdon et al. (2008). Specifically, Langdon et al. measured ~50% retention of KI after 1h which was much higher than the 13% retention of KI in the current study. The low RE in our study may have been due to the addition of sorbitan tristearate (STS) to WSB, which may have decreased the hydrophobic nature of the wax matrix and increased the susceptibility of water-soluble nutrients to interact with the surrounding water. Sorbitan tristearate was added to stabilize the pre-spray emulsion during WSB preparation and to aid in particle dispersion in seawater. While emulsification agents may be necessary for the production and dispersal of WSB, future research should be directed at evaluating alternative emulsification agents, such as sorbitan monopalmitate. Finally, WSB formulations should be optimized to balance the retention of water-soluble nutrients with other desired traits, such as, particle dispersion and nutrient availability to target organisms.

Objective 2.- Evaluate the use of yttrium oxide as an inert marker in feeding experiments with *Artemia* fed on WSB.

Y_2O_3 was an effective marker of WSB during the enrichment of *Artemia*. Yttrium was not leached from WSB when suspended in seawater (Chapter 2, Figure 2.1) indicating that yttrium levels remained constant in WSB prior to ingestion by *Artemia*. Metanauplii readily consumed WSB containing Y_2O_3, suggesting that the inclusion of yttrium Y_2O_3 did not inhibit ingestion of WSB by *Artemia*. *Artemia* did not absorb yttrium from WSB meaning that yttrium concentrations measured in *Artemia* were associated with WSB contained in the gut. These findings suggest that Y_2O_3 met the requirements of an inert marker (Austreng et al., 2000; Otterå et al., 2003) and could be used in concurrent feeding studies.

Yttrium data provided detailed information about enrichment processes. It was possible to use yttrium concentrations to calculate rates of WSB ingestion and retention by *Artemia*. Based on yttrium concentrations, we were able to determine that: 1) *Artemia* filled their guts with WSB in approximately 15 min and then maintained a constant level of WSB for the duration of enrichment; 2) *Artemia* retained WSB for at least 4h after transfer to clean seawater; 3) *Artemia* ingested WSB at a rate proportional to WSB concentrations in the seawater; and 4) *Artemia* absorbed iodine from WSB. These findings suggest that yttrium concentrations may be used to optimize WSB enrichment protocols.

Previous studies have used inert markers to determine the digestibility of proteins from microparticulate diets (Aksnes and Opstvedt, 1998; Austreng et al.,

2000; Hansen et al., 2009). To our knowledge, our study is the first to utilize an inert marker to reference uptake and retention rates of water-soluble substances from a microparticulate diet. Cook et al. (2008) used Y_2O_3 to label live-prey and therefore determine prey ingestion rates by fish larvae. Future studies could use Y_2O_3-labeled WSB in a similar manner to estimate rates of ingestion and retention of WSB-enriched live prey by fish larvae.

Objective 3.- Determine if zebrafish larvae were able to take up iodine from KI WSB-enriched *Artemia*.

Prior to this study, water-soluble substances, such as KI, had not been delivered to larval fish using WSB-enriched *Artemia*. Zebrafish fed KI WSB-enriched *Artemia* showed a ten-fold increase in iodine levels when compared with larvae fed unenriched *Artemia* (Chapter 3, Figure 3.2). These findings suggest that a portion of KI contained in KI WSB-enriched *Artemia* was available to fish larvae.

Few studies have investigated the uptake of iodine by fish larvae fed iodine-enriched live-prey and none of these have been with freshwater species. Halibut larvae (*Hippoglossus hippoglossus*) fed *Artemia* enriched with iodinated poppy-seed oil (lipiodol) showed more than a 2-fold increase in whole-body iodine concentrations after a 51-day feeding trial (Moren et al., 2006). Atlantic cod (*Gadus morhua*) larvae fed rotifers (*Brachionus sp*.) enriched with NaI showed no increase in whole-body iodine levels when compared to larvae fed unenriched rotifers (Hamre et al., 2008). In all studies reviewed, increased levels of dietary iodine resulted in increased iodine concentrations in larval tissues. The extent to which iodine was absorbed by larvae in

these studies may vary because of differences in species-specific iodine requirements, the method used to enrich live prey as well as the type of live prey used (rotifers vs. *Artemia*).

Objective 4.-Investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish.

We found that KI WSB-enriched *Artemia* had an effect on the survival and thyroid status of larval zebrafish but had weak effects on growth parameters. Zebrafish fed KI WSB-enriched *Artemia* had better survival than those fed unenriched *Artemia* (Chapter 3, Table 3.1). Overall, mean total length (TL) and mean larval weight (Wt) of zebrafish were higher when larvae were fed iodine-enriched *Artemia* when compared to unenriched *Artemia* (Chapter 3, Results); however, significant differences were not found between treatments at any specific sampling point (Chapter 3, Table 3.1). At 38 dpf, zebrafish fed iodine-enriched *Artemia* had lower epithelium to colloid (v:v) ratios when compared to those fed unenriched *Artemia* (Chapter 3, Figure 3.3) suggesting that increased dietary iodine affected the thyroid status of late-stage larvae. Fish fed unenriched *Artemia* may have been showing the onset of goiter and, therefore, had reduced colloid and increased epithelium volumes as is seen in goiterous zebrafish larvae (Brown, 1997). A more likely explanation is that larvae fed iodine-enriched *Artemia* produced additional colloid in order to store iodine or iodinated compounds. There was no evidence in 28 dpf zebrafish larvae that TH or outer-ring deiodinase (ORD) activities were significantly affected by iodine enrichment.

Concerning species of freshwater fishes, there are no published studies that have investigated the effects of increased dietary iodine on the growth and thyroid status of larvae. However, the effects of dietary iodine have been examined for several marine fish species. Hamre et al. (2008) observed a 32% increase in survival when Atlantic cod larvae were fed iodine and selenium enriched rotifers but did not observe significant changes in TH or ORD activities. Ribeiro et al. (2009) fed rotifers and *Artemia* (in series) enriched with NaI (780 mg NaI g-1 emulsion) to Senegalese sole (*Solea senagalensis*) and found that increased dietary iodine resulted in increased larval weights and lengths when compared with larvae fed unenriched rotifers and *Artemia* (control). They measured lower colloid volumes and higher epithelium volumes in the control when compared to larvae fed iodine-enriched rotifers and *Artemia*. In several cases, larvae in the control had completely depleted colloid regions displaying typical symptoms of goiter. In the current study, we observed similar trends of colloid and epithelium volumes but did not observe complete colloid depletion as in the study by Ribeiro et al. (2009). Moren et al. (2006) found no difference in the growth or survival (measured as mortality) of Atlantic halibut larvae fed lipiodol-enriched *Artemia,* which may have been due to the limited bioavailability of lipid-bound iodine in lipiodol (Moren et al., 2006). Based on studies involving Senegalese sole, Atlantic cod larvae and now zebrafish larvae, there is accumulating evidence that rotifers and *Artemia* may be insufficient for the proper thyroid function and health or larval fish. More research is needed to determine optimal iodine levels that should be supplied to larval zebrafish. In addition, studies should be conducted to determine if

additional freshwater fish species, especially those normally reared on rotifers or *Artemia*, benefit from increased dietary iodine.

Objective 5.- Determine if *Artemia* are a potential source of exogenous THs for larval fish.

Artemia contained measurable quantities of both ORD and THs (Chapter 3, Table 3.2) and may be a significant source of these substances to fish larvae. Triiodothyronine (T3) production in *Artemia* increased as a result of iodine enrichment. Exogenous THs provided by *Artemia* may have compensated for reduced endogenous TH production by fish larvae and thus ameliorated the effects of reduced dietary iodine in this study. This hypothesis is supported by the finding that the addition of exogenous thyroxine (T4) was able to reverse the effects goiter in larval zebrafish exposed to goitrogens (Brown, 1997). To our knowledge, this study has been the first to demonstrate significant levels of THs and ORD activity in *Artemia*; however, since our methods were originally optimized for larval fish, further research should be conducted to validate these findings. In addition, future studies should consider the potential impacts of exogenous THs and deiodinase provided by *Artemia*.

It is currently unknown whether rotifers or copepods have similar iodine-related hormones. *Artemia* generally inhabit hyper-saline waters and may have evolved an iodine-regulatory mechanism to deal with high levels of iodine found in these environments. However, TH production in invertebrates is not limited to *Artemia*. There is evidence that several taxa of invertebrates may be capable of TH regulation (Heyland et al., 2006). Further evidence suggests the sea hare (*Aplysia*

californica) and the sea urchin (*Lytechinus variegates*) are both capable of endogenous TH synthesis (Heyland et al., 2006).The physiological role of THs in invertebrates is currently unknown and is beyond the scope of this study.

Objective 6.- Determine if KI WSB have an effect on the bacterial concentrations of enriched *Artemia.*

Due to the antibacterial qualities of iodine, it was necessary to determine if bacterial densities of *Artemia* were affected by enrichment with KI WSB. If enrichment with KI WSB had an effect on the bacterial concentrations of *Artemia*, then fish larvae fed KI WSB-enriched *Artemia* may have had higher survival and growth than control larvae due to bacteriological factors. We found that enrichment with KI WSB had no effect on the bacterial concentrations of *Artemia*. Our results are consistent with those of Gomezgil-RS et al. (1994) who found that iodine (I_2; 1 mg I_2 l^{-1}) had minimal effects on the bacterial concentrations of iodine-treated *Artemia.* It is possible that we did not observe an effect on the bacterial densities because the relatively low concentrations of KI (approx 10 mg KI l^{-1}) used in this study. The method of iodine enrichment (aqueous vs. WSB) may also affect the bacterial densities of *Artemia*. Additional research is needed to determine the effect of increasing iodine concentrations and delivery methods on the bacterial densities of *Artemia*. In addition, research should be conducted to determine if the composition of the bacteria species is affected by iodine enrichment.

Summary

KI+Y WSB were successfully used to enrich the iodine levels of *Artemia.* Iodide was absorbed by the tissues of *Artemia* was available to larval fish. Y_2O_3 was a good marker of WSB and was easily used to estimate ingestion and retention rates of WSB in response to various experimental conditions. Zebrafish larvae benefited from iodine levels that were significantly higher than those recommended for adult fish. *Artemia* produce THs and deiodinase and are a potential source of these substances to larval fish. Enrichment with KI+Y WSB did not alter the bacterial concentrations in *Artemia* and, therefore, improvements in larval survival were likely due to the nutritional benefits of increased dietary iodine.

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APPENDIX A – ADDITIONAL DATA

Table A.1 Total iodine in various products used (or were candidates for use) in *Artemia* and zebrafish trials. Iodine was extracted and obtained by ICP-MS as described in chapter 3. Ori-green is an algae-based enrichment product was used, in our study, to purge WSB from the guts of *Artemia*. Rich is an emulsion-type enrichment product commonly used to enrich *Artemia*. Schwartz and JBL larval diets are particulate feeds commonly used to feed fish larvae. Crystal sea marine salt was used to adjust the salinity of the water in the zebrafish rearing system.

Product	**Total iodine concentration (µg g $^{-1}$ product weight)**	
	Mean	**1 SD**
Ori-Green enrichment	1.60	0.26
RICH enrichment	1.30	0.00
Schwartz larval diet	4.20	0.69
JBL larval diet	13.33	0.58
Artemia cysts (GSL)	6.23	0.21
Crystal Sea marine salt	0.35	0.03

APPENDIX B – STATISTICS

Table B.1 Results from ANOVA and Tukey's HSD for mean particle diameters (μm) between different formulations of WSB (Treatment). Statically comparisons were based on the mean particle diameter per digital image. Different letters denote significant differences (threshold, $p = 0.05$).

Source	Sum of Squares	DF	Mean Square	*F*-ratio	*p*-value
Treatment	4.3461	2	2.1731	0.5248	0.609
Error	37.266	9	4.1407		
Total	41.412	11			

Treatment	Significance	Least Square Mean
KI WSB	A	8.7
Y WSB	A	8.2
KI+Y WSB	A	7.2

Table B.2 Results from *t*-test comparing yttrium concentrations (μg Y g^{-1} WSB) between KI+Y WSB and Y WSB (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	3	1.476	0.265

Treatment group	Mean	n	Std. Dev.
KI+Y WSB	11505	2	1987
Y WSB	14569	3	2690

Table B.3 Results from *t*-test comparing iodine concentrations (μg I g^{-1} WSB) between KI+Y WSB and KI WSB (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	2	0.790	0.5172

Treatment group	Mean	n	Std. Dev.
KI WSB	65333	3	6808
KI+Y WSB	71000	2	8485

Table B.4 Results from ANOVA, Wilcoxon's rank-sum test, and Tukey's HSD for arcsine-transformed Retention Efficiencies (RE) of iodine by WSB following suspension in SW. Wilcoxon's rank-sum test was performed due to unequal variances between groups identified by Levene's test (p-value < 0.001). Different letters denote significant differences (threshold, $p = 0.05$).

Source	Sum of Squares	DF	Mean Square	F-ratio	p-value
Time	5.1152	5	1.0230	68.098	<0.0001
Error	0.3455	23	0.0150		
Total	5.4607	28			

Chi-Square	DF	p-value
Total	28	0.0003

Treatment	Significance	Least Square Mean
0 min	Z	1.2605
5 min	Y	0.3941
30 min	X	0.1308
60 min	X	0.0582
360 min	XY	0.1778
720 min	X	0.1471

Table B.5 Results from ANOVA and Tukey's HSD for arcsine-transformed Retention Efficiencies (RE) of yttrium by WSB following suspension in SW. Different letters denote significant differences (threshold, $p = 0.05$).

Source	Sum of Squares	DF	Mean Square	*F*-ratio	*p*-value
Time	0.1254	5	0.0251	0.4021	0.8423
Error	1.4352	23	0.0624		
Total	1.5606	28			

Treatment	Significance	Least Square Mean
0 min	A	1.4440
5 min	A	1.3863
30 min	A	1.3825
60 min	A	1.2857
360 min	A	1.2767
720 min	A	1.2747

Table B.6 Results from REML and Tukey's HSD for *ln*-transformed iodine concentrations in *Artemia* enriched with increasing concentrations of KI (Level) delivered by KI+Y WSB or in aqueous solution (Treatment). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Treatment	1	7	564.9	<0.0001
Level	1	8.286	59.5	<0.0001
Level x Treatment	2	8.286	0.5	0.6278

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	-0.0697	-0.0011	0.003	-7.5
Residual		0.0171	0.009	107.5
Total		0.0159		100.0

Treatment	Significance	Least Square Mean
WSB, 40 mg I l^{-1}	E	6.21
WSB, 20 mg I l^{-1}	E	5.73
WSB, 10 mg I l^{-1}	D	5.11
Aqueous KI, 40 mg I l^{-1}	C	4.51
Aqueous KI, 20 mg I l^{-1}	B	4.05
Aqueous KI, 10 mg I l^{-1}	A	3.57

Table B.7 Results from REML and Tukey's HSD for yttrium concentrations measured in *Artemia* enriched with increasing concentrations of KI+Y WSB. Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Level	2	2	277.0	<0.0036

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	8	3333.3	4912.4	88.9
Residual		416.7	416.7	11.1
Total		3750		100.0

Treatment	Significance	Least Square Mean
400 mg WSB l^{-1}	C	750
200 mg WSB l^{-1}	B	450
100 mg WSB l^{-1}	A	275

Table B.8 Results from REML and Tukey's HSD for *ln*-transformed iodine concentrations measured in *Artemia* enriched with KI+Y WSB over time (Time). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Time	6	12	300.4	<0.0001

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	2.309	0.088	0.094	69.8
Residual		0.015	0.016	30.2
Total		0.126		100.0

Treatment	Significance	Least Square Mean
0	A	1.31
15	D	7.05
30	CD	6.67
60	CD	6.53
240	BC	6.26
480	B	5.72
720	B	5.82

Table B.9 Results from REML and Tukey's HSD for *ln*-transformed yttrium concentrations measured in *Artemia* enriched with KI+Y WSB over time (Time). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Time	6	12	265.1	<0.0001

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	1.214	0.046	0.052	54.8
Residual		0.038	0.016	45.2
Total		0.085		100.0

Treatment	Significance	Least Square Mean
0	X	1.27
15	Y	6.33
30	Y	6.12
60	Y	6.12
240	Y	6.20
480	Y	5.94
720	Y	6.02

Table B.10 Results from REML and Tukey's HSD for yttrium concentrations in enriched *Artemia* starved in clean SW for increasing durations (Time) at two different SW temperatures (Temperature). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Time	3	14	4.440	0.0216
Temperature	1	14	7.636	0.0152
Time x Temperature	3	14	1.494	0.2594

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	0.4466	713.14	915.9	30.9
Residual		1596.96	603.6	69.1
Total		2310.10		100.0

Treatment	Significance	Least Square Mean
0 h, 4-10º C (cold SW)	B	444.6
0 h, 26º C (warm SW)	B	444.6
1 h, 4-10º C (cold SW)	AB	375.1
1 h, 26º C (warm SW)	AB	421.0
4 h, 4-10º C (cold SW)	AB	394.2
4 h, 26º C (warm SW)	B	431.8
8 h, 4-10º C (cold SW)	A	313.4
8 h, 26º C (warm SW)	AB	410.2

Table B.11 Results from REML and Tukey's HSD for iodine concentrations in enriched *Artemia* starved in clean SW for increasing durations (Time) at two different SW temperatures (Temperature). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Time	3	14	21.624	<0.0001
Temperature	1	14	0.6697	0.4269
Time x Temperature	3	14	0.2511	0.8592

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	1.249	497.6	547.7	55.5
Residual		398.2	150.5	44.5
Total		895.8		100.0

Treatment	Significance	Least Square Mean
0 h, 4-10º C (cold SW)	Y	220.0
0 h, 26º C (warm SW)	Y	220.0
1 h, 4-10º C (cold SW)	XY	180.0
1 h, 26º C (warm SW)	XY	163.3
4 h, 4-10º C (cold SW)	X	153.3
4 h, 26º C (warm SW)	X	143.3
8 h, 4-10º C (cold SW)	X	133.3
8 h, 26º C (warm SW)	X	133.3

Table B.12 Results from REML for "corrected" iodine concentrations in enriched *Artemia* following gut purging with Ori-green. Iodine values were corrected by subtracting iodine concentrations associated with unpurged WSB (based on yttrium levels). Different letters denote significant differences (threshold, p = 0.05).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Treatment	1	8	5.753	0.043

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Day	0.951	9.427	11.97	48.7
Residual		9.916	4.958	51.3
Total		19.34		100.0

Table B.13 Results from two-way ANOVA and Tukey's HSD for iodine concentrations in *Artemia* enriched with either 100 mg l^{-1} KI WSB or 10 mg l^{-1} aqueous KI (Treatment) measured over time (Time). Different letters denote significant differences (threshold, p = 0.05).

Source	Sum of Squares	DF	Mean Square	*F*-ratio	*p*-value
Treatment	88480	1		420.2	<0.0001
Time	86704	2		205.9	<0.0001
Treatment x Time	57584	2		136.7	<0.0001
Error	2526.7	12	210.6		<0.0001
Total	235296	17		221.1	

Treatment	Significance	Least Square Mean
KI WSB, 0h	A	6.3
Aqueous KI 0h	A	6.0
KI WSB, 6h	C	310
Aqueous KI 6h	A	36
KI WSB, 12h	B	180
Aqueous KI 12h	A	36

Table B.14 Results from Mann-Whitney U-test for iodine concentrations measured in zebrafish fed either KI WSB enriched *Artemia* or unenriched *Artemia*.

Group	Rank-sum for unenriched *Artemia*	Rank-sum for KI WSB enriched *Artemia*	*Z*	*p*-value
Treatment	6.0	15.0	-1.98	0.049

Table B.15 Results from *t*-test comparing arcsine-transformed percent survival to 28 dpf between zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	22	3.223	0.0039

Treatment group	Mean	n	SD
KI WSB enriched *Artemia*	0.667	12	0.1179
Unenriched *Artemia*	0.524	12	0.1000

Table B.16 Results from Repeated-measures REML and Tukey's HSD for total length (TL) of zebrafish larvae fed either KI WSB enriched *Artemia* (KI WSB) or unenriched *Artemia* (Unenriched; Treatment) and sampled at three time points (21, 28 and 38 dpf). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Treatment	1	20	4.200	0.0538
Day	2	40	207.4	<0.0001
Treatment x Day	2	40	0.733	0.4867

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Treatment nested within Tank	-0.1200	-0.7437	0.6228	-13.6
Residual		6.1953	1.3853	113.6
Total		5.1547		100.0

Treatment	Significance	Least Square Mean
KI WSB, 21 dpf	A	9.0
Unenriched, 21 dpf	A	8.9
KI WSB, 28 dpf	B	14.5
Unenriched, 28 dpf	B	12.7
KI WSB, 38 dpf	C	24.5
Unenriched, 38 dpf	C	23.3

Table B.17 Results from Repeated-measures REML and Tukey's HSD for ln-weight (Wt) of zebrafish larvae fed either KI WSB enriched *Artemia* (KI WSB) or unenriched *Artemia* (Unenriched; Treatment) and sampled at three time points (21, 28 and 38 dpf). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Treatment	1	20	4.453	0.0476
Day	2	40	225.1	<0.0001
Treatment x Day	2	40	0.492	0.6150

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Treatment nested within Tank	-0.1181	-0.0351	0.0300	-13.4
Residual		0.2972	0.0665	113.4
Total		0.2621		100.0

Treatment	Significance	Least Square Mean
KI WSB, 21 dpf	A	0.0036
Unenriched, 21 dpf	A	0.0032
KI WSB, 28 dpf	B	0.0274
Unenriched, 28 dpf	B	0.0181
KI WSB, 38 dpf	C	0.1177
Unenriched, 38 dpf	C	0.1017

Table B.18 Results from Repeated-measures REML and Tukey's HSD for condition factor (K) of zebrafish larvae fed either KI WSB enriched *Artemia* (KI WSB) or unenriched *Artemia* (Unenriched; Treatment) and sampled at three time points (21, 28 and 38 dpf). Different letters denote significant differences (threshold, $p = 0.05$).

Fixed Effect (Source)	DF	DFDen	*F*-ratio	*p*-value
Treatment	1	20	0.0018	0.9665
Day	2	40	171.2	<0.0001
Treatment x Day	2	40	1.717	0.1927

Random Effect	Variance Ratio	Variance Component	Std Error	Percent of total
Treatment nested within Tank	0.2579	0.00176	0.00137	20.5
Residual		0.00683	0.00153	79.5
Total		0.00858		100.0

Treatment	Significance	Least Square Mean
KI WSB, 21 dpf	A	0.5188
Unenriched, 21 dpf	A	0.4660
KI WSB, 28 dpf	BC	0.9526
Unenriched, 28 dpf	C	0.9166
KI WSB, 38 dpf	B	0.8327
Unenriched, 38 dpf	B	0.8193

Table B.19 Results from *t*-test comparing thyroid follicle epithelium volumes between 28 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	1.1313	0.3212

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	12026750	3	5069130
Larvae fed Unenriched *Artemia*	7850110	3	3897870

Table B.20 Results from *t*-test comparing thyroid follicle colloid volumes between 28 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.6133	0.5728

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	10957500	3	7140980
Larvae fed Unenriched *Artemia*	7630450	3	6105630

Table B.21 Results from *t*-test comparing epithelium to colloid (v:v) ratios between 28 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.2353	0.8255

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	1.3266	3	0.5484
Larvae fed Unenriched *Artemia*	1.2353	3	0.3883

Table B.22 Results from *t*-test comparing thyroid follicle epithelium volumes between 38 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.2965	0.7768

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	45008870	3	3381370
Larvae fed Unenriched *Artemia*	45905640	3	5015350

Table B.23 Results from Mann-Whitney U-test comparing thyroid follicle colloid volumes between 38 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Group	Rank-sum for larvae fed unenriched *Artemia*	Rank-sum for larvae fed KI WSB enriched *Artemia*	*Z*	*p*-value
Treatment	23.0	13.0	1.299	0.1939

Table B.24 Results from Mann-Whitney U-test comparing epithelium to colloid (v:v) ratios between 38 dpf zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Group	Rank-sum for larvae fed unenriched *Artemia*	Rank-sum for larvae fed KI WSB enriched *Artemia*	*Z*	*p*-value
Treatment	26.0	10.0	2.309	0.0286

Table B.25 Results from *t*-test comparing outer-ring deiodinase activities between zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	-1.584	0.1884

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	0.8757	3	0.11
Larvae fed Unenriched *Artemia*	0.7332	3	0.11

Table B.26 Results from *t*-test comparing T3 concentrations between zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	1.713	0.1618

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	0.030	3	0.0118
Larvae fed Unenriched *Artemia*	0.072	3	0.0407

Table B.27 Results from *t*-test comparing T4 concentrations between zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.1894	0.859

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	0.674	3	0.649
Larvae fed Unenriched *Artemia*	0.811	3	1.070

Table B.28 Results from *t*-test comparing *ln*-transformed T3:T4 ratios between zebrafish larvae fed either KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.7290	0.5064

Treatment group	Mean	n	Std. Dev.
Larvae fed KI WSB enriched *Artemia*	-2.851	3	1.061
Larvae fed Unenriched *Artemia*	-1.899	3	1.994

Table B.29 Results from *t*-test comparing *ln*-transformed T3 concentrations between KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	3.589	0.023

Treatment group	Mean	n	Std. Dev.
KI WSB enriched *Artemia*	1.045	3	0.4129
Unenriched *Artemia*	0.085	3	0.2099

Table B.30 Results from *t*-test comparing T4 concentrations KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	1.002	0.3726

Treatment group	Mean	n	Std. Dev.
KI WSB enriched *Artemia*	14.32	3	4.802
Unenriched *Artemia*	10.67	3	4.072

Table B.31 Results from *t*-test comparing T3:T4 ratios between KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	5.407	0.0056

Treatment group	Mean	n	Std. Dev.
KI WSB enriched *Artemia*	0.2077	3	0.0205
Unenriched *Artemia*	0.1083	3	0.0243

Table B.32 Results from *t*-test comparing concentrations of marine bacteria between KI WSB enriched *Artemia* and unenriched *Artemia* (Treatment).

Variable	DF	*t-value*	*p*-value
Treatment	4	0.3775	0.7188

Treatment group	Mean	n	Std. Dev.
KI WSB enriched *Artemia*	10903	3	5595
Unenriched *Artemia*	9566.7	3	4337

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