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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-caught 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) evaluate the use of KI WSB for enrichment of *Artemia* with iodine; 2) determine if zebrafish larvae were able to obtain iodine from KI WSB-enriched *Artemia*; 3) investigate the effects of KI WSB-enriched *Artemia* on the growth, survival and thyroid status of larval zebrafish; 4) determine if *Artemia* were a potential source of exogenous thyroid hormones (TH) for larval fish; and 5) 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 outer-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.

1. 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) have been shown to effectively deliver water-soluble antibiotics (oxytetracycline; OTC) to *Artemia* (Langdon et al. 2008) suggesting that WSB may be used to enrich live prey with water-soluble micronutrients. In this study, WSB were produced containing potassium iodide (KI WSB) and were evaluated for iodine enrichment of *Artemia*.

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 $\mu g\,l^{-1}$ but is much more variable in freshwater and seldom exceeds 15 $\mu g\,l^{-1}$ (Fuge, 1996). Therefore, 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 is 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

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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, both eye migration and bone ossification are 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 canceled 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* 1) evaluate the use of KI WSB for enrichment of *Artemia* with iodine; 2) determine if zebrafish larvae were able to obtain iodine from KI WSB-enriched *Artemia*; 3) investigate the effects of KI WSB-enriched Artemia on the growth, survival and thyroid status of larval zebrafish; 4) determine if *Artemia* were a potential source of exogenous thyroid hormones (TH) for larval fish; and 5) determine if KI WSB had an effect on bacterial concentrations associated with *Artemia*.

2. Methods

2.1. 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. Briefly: The wax fraction was melted at ~90 ° C, emulsified with the aqueous core by sonication (Vibracell™; Sonics & Materials Inc., Danbury, CT, USA). The emulsification was sprayed with dry nitrogen gas (20 PSI) through an air-atomizing nozzle (1/4 JBCJ) fitted with a 35100-SS fluid nozzle and 120-SS air cup. (Spray Systems co., Wheaton, Illinois, USA). The emulsified droplets (WSB) hardened when in contact with a stainless steel cone which has been pre-cooled to −80 °C with liquid nitrogen vapor.

2.2. 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) seawater (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 an 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).

2.3. Inclusion (IE) and retention efficiencies (RE) of iodine for WSB

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:

$$\text{IE (\%)} = (\text{measured concentration at time 0}) / (\text{expected concentration}) \times 100.$$

Where "measured concentration" is the wet weight concentration (w/w) of iodine in unleached WSB and "expected concentration" is the concentration of iodine expected in WSB based on the initial production formula (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 solution (prepared as described in Section 2.2) was added to each tube. WSB were dispersed using low-powered sonication (Vibracell™; Sonics & Materials Inc., Danbury, 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:

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

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

2.4. 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, pH 7.3–7.9, 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 h 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* metanauplii were used for all enrichment trials.

2.5. Iodine enrichment of Artemia over time

Artemia metanauplii (24 hph; approx. 150 ind. ml^{-1}) were enriched for 12 h 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 12 h 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 12 h to be consistent with recommended protocols for commercial enrichment products. After the 12 h 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 debris and undigested WSB 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.

2.6. Zebrafish culture systems

Zebrafish embryos were obtained from the Department of Biology, 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 days post fertilization (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 (37 larvae tank^{-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 growth and histological measurements.

2.7. Zebrafish feeding trial and sampling protocol

Larvae were fed Schwarz larval diet (Aquarien-Bau Schwarz, Göttingen, Germany) from first feeding (5–6 dpf) 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 (37 larvae tank^{-1}) and fed *Artemia* and Schwartz diet three times a day. A small fraction of zebrafish larvae were found to feed on *Artemia* as early as 10 dpf in preliminary trials. However, in this feeding trial, larvae were fed *Artemia* beginning on 14 dpf because this was found to be the age at which the majority of larvae would ingest *Artemia*. Zebrafish were co-fed with Schwartz diet until 18 dpf to facilitate weaning onto *Artemia*. *Artemia* were 1) enriched for 12–16 h with either KI WSB (100 mg l^{-1}) or 2) kept in SW with no additional 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 transferred to 3 l tanks at 21 dpf to avoid crowding as the larvae grew. Larvae were starved for 24 h 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 with an Olympus SZX microscope fitted with a DP50 digital camera (except for 38 dpf larvae which were measured with a ruler due to size limitations). Due to the need for a large numbers of larvae in hormone and enzyme analyses, only one larva per tank could be spared from each tank for growth estimates at each sampling date. However, given the high number of tanks used per treatment (12) and since larvae were taken from separate (statistically independent) tanks, this sampling protocol could be used to accurately estimate treatment effects. Total lengths (TL) were measured from digital images ($n = 12$) using Image-J software (NIMH, Bethesda, MD, USA). Larvae were then prepared for histological sectioning as described in Section 2.6. 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 and were obtained by counting all remaining larvae at 28 dpf. No additional mortality occurred during the 10-day grow-out period (ending 38 dpf).

2.8. Analysis of iodine

Total iodine was extracted from pooled samples of larval zebrafish (>16 larvae sample^{-1}) and freeze-dried *Artemia* samples using 1% tetramethylammonium hydroxide (TMAH) to dissolve tissues (Tama Chemicals Co., Tempapure-AA, Kawasaki city, Japan). Total iodine concentrations were 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 be 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”.

2.9. Stereohistology of zebrafish thyroid follicles

Larval zebrafish were fixed in 4% paraformaldahyde for 24 h 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 and stained with buffered methylene blue (1% Mallinckrodt chem., Phillipsburg, NJ, USA). 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 a 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.

2.10. Determination of protein

Protein was determined from pooled samples of larvae (5 larvae - sample^{-1}) using a BCA protein assay kit (Thermo scientific, Waltham MA, USA) following the manufacturer's instructions.

2.11. Determination of type I and II iodothyronine deiodinase activity

Enzyme activities in pooled samples of zebrafish larvae (5 larvae - sample^{-1}) and freeze-dried *Artemia* were determined as follows: 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, as described in Section 2.8.

2.12. Determination of thyroid hormones in zebrafish and Artemia

Thyroid hormones, T3 and T4, were extracted from pooled samples of frozen (−80 °C) zebrafish larvae (>35 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 24 h for extraction and then centrifuged at 3000 rpm, 4 °C, for 30 min. 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° 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 et al. (2006) as modified by Hamre et al. (2008).

2.13. 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 12 h 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).

2.14. 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 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, natural logarithmic (*ln*) or arc-sin transformations were employed. Zebrafish growth parameters (total length, TL; wet weight, Wt; and condition factor, 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 natural logarithmic (*ln*) or arc-sin transformed to better meet the assumptions of the *t*-test. Mann-Whitney *U*-tests for non-parametric data were used if transformation was not sufficient, such as for larval survival, colloid volumes and epithelium to colloid (v:v) ratios in 38 dpf larvae.

3. Results

3.1. Particle size

Mean particle diameter (±1 SD) was 8.0 ± 2.0 μm and was not significantly different among batches (n = 3) of WSB (p = 0.81).

3.2. Inclusion (IE) and retention efficiencies (RE) of iodine for WSB

Iodine represented 68 ± 10 mg g^{-1} (w/w) of the total weight of KI + Y WSB. IE for iodine was 89 ± 13.5%. RE of iodine for KI WSB significantly declined over time during the leakage trial ($p<0.001$). On average, 63% of the iodine contained in WSB was lost within the first 5 min when suspended in SW. RE of iodine remained constant, at approximately 13% of initial concentration, after 3 min suspension ($p<0.05$).

3.3. Iodine enrichment of Artemia over time

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

3.4. 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* (Fig. 2; $p = 0.001$). 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*.

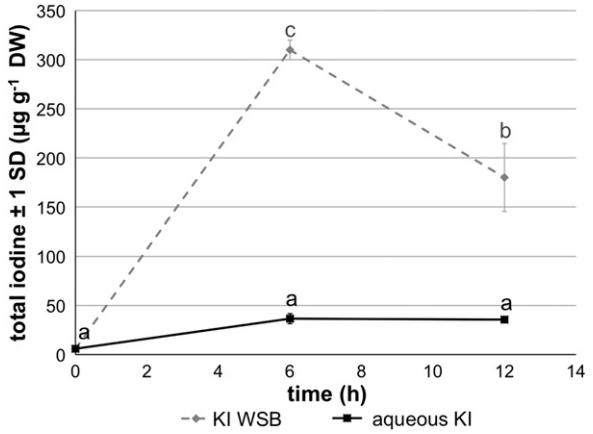

Fig. 1. Total iodine concentrations in *Artemia* enriched with either 100 mg l^{-1} KI WSB ("KI WSB") or 10 mg l^{-1} KI added in aqueous solution ("aqueous KI") for 12 h. The amount of KI delivered in the aqueous KI treatment was equivalent to the total amount of KI delivered in the KI WSB treatment.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

3.5. Survival and growth of zebrafish larvae

Zebrafish larvae fed KI WSB-enriched *Artemia* had higher rates of survival (61.5% ± 8.8%) when compared larvae fed unenriched Artemia (49.8% ± 8.7%; $p = 0.004$) as well as marginally significant increases in mean total lengths (TL) and wet weights (Wt) when compared with those of larvae fed unenriched *Artemia* ($p = 0.053$ and 0.048, respectively). However, neither TL nor Wt were significantly different between treatments at any specific sampling time ($p < 0.05$; Fig. 3). Iodine enrichment of *Artemia* had no effect on the condition factor (K; $p > 0.967$; Fig. 3).

3.6. Stereohistology of zebrafish thyroid follicles

Epithelium to colloid ratios (v:v) were not significantly different between treatments at 28 dpf ($p = 0.825$). 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* (Fig. 4; $p = 0.029$).

3.7. 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* ($p = 0.19$; Table 1). Unenriched *Artemia* were found to have ORD activities significantly higher than measured in sample blanks and similar to those measured in zebrafish larvae (Table 1).

3.8. 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* ($p = 0.08$ and 0.859, respectively). T3:T4 ratios were also not significantly different between treatments for zebrafish larvae ($p = 0.506$). 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* ($p = 0.023$ and 0.006, respectively). There was no difference in the levels of T4 in *Artemia* between treatments ($p = 0.373$; Table 1).

3.9. 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 colony forming units (CFU) per *Artemia* between treatments ($p = 0.719$), *e.g.* 10.9 (± 5.6) $\times 10^3$ and 9.6 (± 4.3) $\times 10^3$ CFU *Artemia*$^{-1}$ in the KI WSB enriched and unenriched treatments, respectively. *Vibrio* sp. were not detected at significant levels with TCBS agar plates for either treatment.

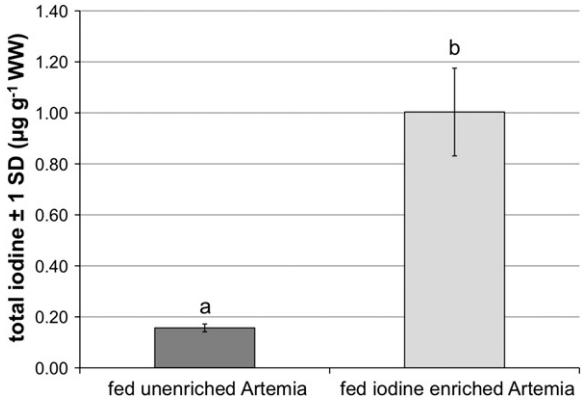

Fig. 2. Total iodine concentrations (µg g^{-1} wet weight) of 28 dpf zebrafish larvae fed either unenriched *Artemia* or *Artemia* enriched with 100 mg l^{-1} KI WSB for two weeks. In addition to *Artemia*, both treatments were fed a particulate diet (Schwartz larval diet; 4.2 ug I g^{-1} DW) for the first 10 days of feeding. Data are given as mean ± 1 SD (n = 3). Different letters denote significant differences ($p = 0.001$).

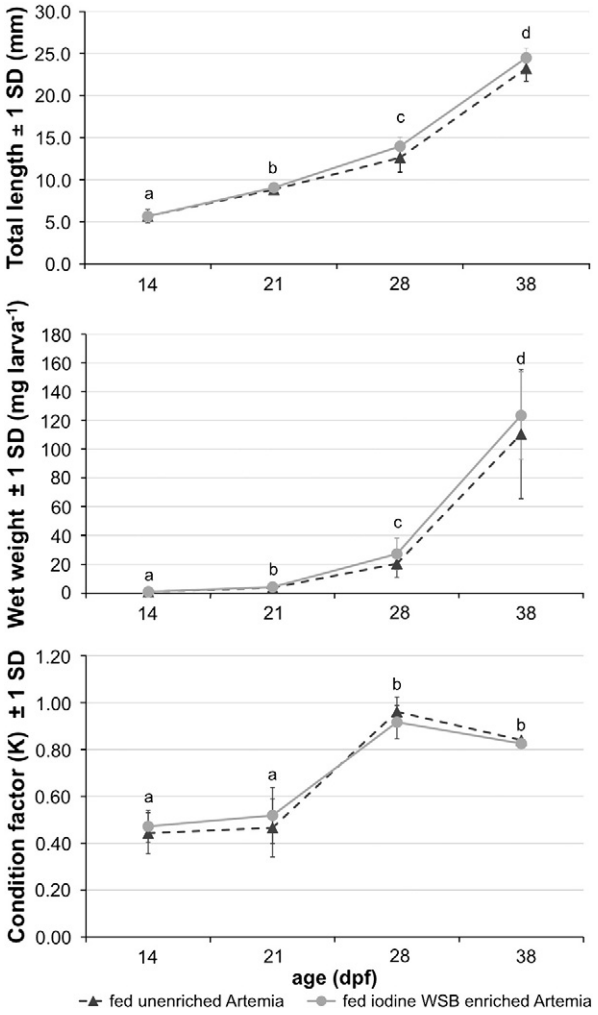

Fig. 3. Total length (TL; top), wet weight (Wt; middle), condition factor (K; bottom) of zebrafish during a feeding trial from 14 to 38 dph. Zebrafish larvae were fed either "iodine WSB enriched *Artemia*" or unenriched *Artemia*. Condition factor was calculated: K = weight/TL^3. Data are given as means ± 1 SD (n = 12). Different letters denote significant differences ($p < 0.05$) between ages (dpf). There was no difference between treatments at any single age.

4. 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* (Fig. 2). We can be confident that the iodine measured was in the larval tissues because larvae were starved for the last 24 h 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 growth parameters compared with larvae fed unenriched *Artemia*. However, neither total lengths, weights nor condition factors were significantly different between treatments at any specific sampling time (Fig. 3). Although 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

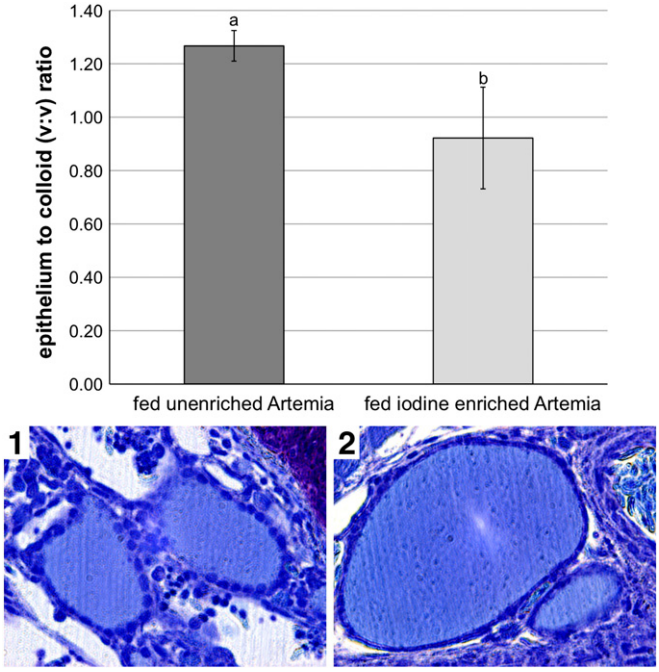

Fig. 4. (Above) Epithelium to colloid volume ratios in 38 dpf zebrafish larvae fed KI WSB enriched *Artemia* and unenriched *Artemia* from 14 dpf. Zebrafish thyroid follicle epithelium and colloid volumes were measured from histological sections at 400× magnification using a Cavaleiri point estimate. 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 WSB enriched *Artemia*. Digital photographs were taken from histological sections at 400× magnification.

halibut larvae *Artemia* enriched with lipiodol (iodinated poppy-seed oil). However, their studies 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*. They did not observe a change in larval survival associated with iodine enrichment but found that larvae that did not receive iodine supplementation showed signs of goiter.

In our study, 38 dpf zebrafish fed iodine-enriched *Artemia* had lower epithelium to colloid ratios when compared to those fed unenriched *Artemia* (Fig. 4) 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 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, which may have masked any real difference between treatments.

It is possible that the levels of iodine used in this experiment were "beyond deficiency requirements" (Waagbø, 2009) and that increased dietary iodine has added health benefits beyond avoiding goiter. Venturi and Venturi (1999) have suggested that iodine and TH have 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 1) 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 are utilized by fish larvae since these substances may be degraded 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 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. More research is needed to determine the specific availability of substances delivered via beeswax WSB as well as recommendations for levels of

Table 1
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 12 h with 100 mg l^{-1} KI WSB (KI WSB). Zebrafish larvae were fed either KI WSB enriched *Artemia* or unenriched *Artemia* (control) from 14 to 28 dpf.

	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

Data given as mean ± 1 SD (n = 3). N/A = data not available.
* Denotes treatments that were significantly different from the respective control ($p < 0.05$).

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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