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A new method to increase and maintain the concentration of selenium in rotifers (*Brachionus* spp.)

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ABSTRACT

Rotifers are used as the first feeding diet for the larvae of many commercially produced marine fish species. However, the nutritional requirements of marine fish larvae appear to be better fulfilled by their wild feed, copepods. Consequently, rotifers are fed diets that alter their body composition to better imitate copepod composition and this results in greater success when rearing rotifer fed marine fish larvae. Despite this, copepod fed fish larvae still have higher growth rates and survival than rotifer fed larvae. This may occur because of the less investigated mineral differences that exist between rotifers and copepods. The concentration of selenium (Se) in rotifers *Brachionus* sp. (0.08–0.09 mg Se kg^{-1} dry weight) can be over 30 fold lower than the concentration found in copepods (3–5 mg Se kg^{-1} dry weight). In this study, the enrichment and retention of Se in rotifers fed Se enriched yeast (Se-yeast) were investigated. Rotifer Se concentration increased linearly with increasing levels of Se-yeast, with rotifers reaching a maximum of 138 mg Se kg^{-1} dry weight. The use of Se-yeast was highly effective, with only 1% of the batch culture or short term enrichment diet needed to be replaced with Se-yeast to achieve copepod Se levels. At this feeding level there were no negative effects on rotifer egg ratio or population growth. Uptake of Se from Se-yeast was over 40 fold higher than obtained from using sodium selenite in short term enrichments (3 h). Se-yeast enriched rotifers had a high retention (100–85%) of Se for up to 10 h storage in clear water at cold (10 °C) or warm (20 °C) temperatures, while storage in green water (with algae) resulted in a slightly lower Se retention (65%) in a similar time period (8 h). Overall, rotifers enrichment with Se-yeast resulted in reproducible concentrations of Se that were then retained for extended periods of time. This will allow Se-yeast enriched rotifers to be used as a Se delivery method for fish larvae nutritional requirement or toxicological studies.

1. Introduction

Rotifers are widely used as the start feeding diet of marine fish larvae, but in the wild marine fish larvae feed mainly on copepods. Past experience has shown that feeding rotifers diets that change the rotifer nutritional composition to more closely imitate that of copepods can yield substantial increases in fish larvae quality. For example, rotifer diets that alter their lipid composition to better imitate copepod lipid composition (van der Meeren et al., 2008) have increased larval rearing success for numerous marine fish species (Rainuzzo et al., 1997; Rodriguez et al., 1997; Park et al., 2006). However, these lipid altered rotifer fed larvae still fall short of obtaining the growth and survival rates obtained by copepod fed larvae (Payne et al., 2001; Rajkumar and Kumaraguru Vasagam, 2006; Schipp, 2006; Wilcox et al., 2006; Olivotto et al., 2008; Koedijk, 2009; Busch et al., in press). This may be because a large amount of research has gone into addressing some nutrient differences between rotifers and copepods, such as lipids, but other potential nutrient deficiencies in rotifers have been largely overlooked. The nutritional difference between rotifers and copepods occurs across all areas including lipids, free and total amino acids (Drillet et al., 2006; van der Meeren et al., 2008) as well as in vitamins and minerals (Hamre et al., 2008a). Recent findings by Hamre et al. (2008a) demonstrate that one of the largest unaddressed nutritional differences between copepods and rotifers is in their mineral composition. While copepods have ample concentrations of essential minerals, often much higher than the national research councils' recommendations for juvenile fish (NRC, 1993), rotifers appear to contain many minerals at low and possibly deficient concentrations. The mineral found with the largest difference and thus potentially the most deficient in rotifers was selenium (Se). Selenium levels of rotifers fed a range of commonly used culture diets (0.08–0.09 mg kg^{-1} dry weight (DW)) were over 30 fold lower than copepod levels (3–5 mg kg^{-1} DW; Hamre et al., 2008a) and 3–8 fold lower than the Se requirements for juvenile fish studied so far;

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channel catfish *Ictalurus punctatus* 0.25 mg kg^{-1}, rainbow trout *Oncorhynchus mykiss* 0.3 mg kg^{-1} and grouper *Epinephelus malabaricus* 0.7 mg kg^{-1}, of dry feed (Hilton et al., 1980; Gatlin and Wilson, 1984; Lin and Shiau, 2005). Furthermore, several recent studies have demonstrated that rotifers provide insufficient Se to meet the Se requirement, as measured by Se-dependent enzyme mRNA expression and/or activity, of Atlantic cod *Gadus morhua* (Penglase et al., 2010) and Senegalese sole *Solea senegalensis* (Ribeiro et al., submitted for publication) larvae.

Selenium is an essential nutrient for vertebrates (Johansson et al., 2005) but can also be toxic. The speciation of Se affects both its bioavailability and its potential toxicity. Selenium occurs in foods in numerous forms but generally the supplemental inorganic selenate (Se^{6+}) and selenite (Se^{4+}), and the naturally present Se containing amino acids, selenomethionine (Se-Met) and selenocysteine (Se-Cys) are the most prevalent (Combs and Combs, 1986). Both Se-Met and Se-Cys differ from methionine and cysteine respectively, by containing a Se atom in place of the sulphur atom. While Se-Cys differs greatly in chemistry to cysteine (Johansson et al., 2005), Se-Met shares similar chemistry to methionine (Kohrle et al., 2005). Transfer-RNA does not distinguish between methionine and Se-Met, and so both are readily incorporated into proteins at methionine positions (Waschulewski and Sunde, 1988; Bell and Cowey, 1989; Kohrle et al., 2005). This may explain why Se-Met has a higher retention (Lorentzen et al., 1994; Jaramillo et al., 2009; Rider et al., 2009a,b) and is more bioavailable (Wang and Lovell, 1997) than selenite in fish.

The window between Se requirement and toxicity is the smallest of any element (Chassaigne et al., 2002; Polatajko et al., 2006). For example, rainbow trout has a Se requirement of 0.3 mg kg^{-1} and a suggested chronic toxicity level of 3 mg kg^{-1} in dry feed (Hilton et al., 1980), a level only 10 fold higher. Selenium toxicity occurs through two main mechanisms. The first, is that Se can disrupt proteins via substituting as sulphur in sulphur bonds, resulting in incorrect protein shape and dysfunctional enzymes. The second mechanism is through oxidative stress caused by excess unbound Se (Lemly, 2002). Both mechanisms rely on Se in free forms, which explains why Se-Met which contains inert Se until catabolised (Ip, 1998), is regarded as less toxic than selenite and selenate. For example, while Hilton et al. (1980) suggested that 3 Se mg kg^{-1} supplied as Na-Se was chronically toxic for rainbow trout, Rider et al. (2009b) found no toxic effects of feeding 8 mg Se kg^{-1} supplied mainly as Se-Met via selenoyeast.

Interestingly, nutrient interactions between Se and other minerals can affect their retention. High dietary or waterborne Se concentrations have been shown to decrease the concentration of copper (Lorentzen et al., 1998; Lin and Shiau, 2007) and mercury (Belzile et al., 2006; Deng et al., 2008) in aquatic organisms. Therefore analysing other minerals alongside Se is necessary to identify any secondary effects of increasing rotifer Se concentration.

Selenoyeast (Se-yeast) is the common name for commercial bakers yeast *Saccharomyces cerevisiae* products which have been enriched with Se as a nutritional supplement. When cultured in the presence of high concentrations of inorganic Se, the yeast converts and then stores Se in organic forms (Polatajko et al., 2006). The exact speciation of Se within Se-yeast is unknown (Suhajda et al., 2000), and there is large variations in speciation between commercial products (Encinar et al., 2003). In general, around 70% of the Se in Se-yeast is in the form of Se-Met (Polatajko et al., 2006) and the remaining Se are inorganic forms such as selenite and selenate (Suhajda et al., 2000), or low molecular weight organic Se compounds other than Se-Met (Ip, 1998; Chassaigne et al., 2002). As rotifers can ingest and digest yeast (Rodriguez et al., 1996), feeding rotifers Se-yeast appears a logical method of increasing rotifer Se concentration.

Nutrients that are desirable for fish larvae can be increased in rotifers through their feed in both the culture phase, called long term (LT) enriching or for a short period before feeding to fish larvae, called short term (ST) enriching (Olsen et al., 1993). The culture diet has two purposes; to maintain healthy and high rotifer population growth rates, and to build up concentrations of nutrients in rotifers that are beneficial for fish larvae. Short term (ST) enrichment occurs for less than 24 h (Olsen et al., 1993), and aims to rapidly increase the concentration of desirable nutrients in the rotifer just prior to feeding to fish larvae. When combined, LT and ST enrichment results in higher levels of nutrients in rotifers and higher delivery of these nutrients to fish larvae than either enrichment technique alone.

Once enriched, rotifers are either fed directly to fish larvae, or stored in clean water and fed to fish larvae up to 24 h post enrichment. Rotifers are often stored after enrichment due to logistical constraints, where hatcheries may be limited in the number of batches of rotifers that can be enriched per day. In addition to storage, rotifers may remain uneaten in fish larvae tanks for extended periods of time, and fish larvae tanks may contain algae (Reitan et al., 1997). Rotifers metabolise and excrete ingested nutrients over time, which can result in large changes in rotifer nutrient composition after enrichment. For example rotifers have been shown to lose essential fatty acids (Rodriguez et al., 1996; Naz, 2008) and zinc (Matsumoto et al., 2009) after enrichment. Algae added to fish larvae tanks (green water technique) have also been shown to affect rotifer composition (Reitan et al., 1997; Yamamoto et al., 2009). Long term enrichment results in a greater uptake, assimilation and stabilisation of nutrients in the rotifer body than ST enrichment (reviewed by Dhert et al., 2001). Thus ST enriched nutrients are more prone to be lost quickly from rotifers, and hence are less likely to be passed on to fish larvae if the rotifers are not consumed within a short space of time (Olsen et al., 1993). The decrease of desirable nutrients in rotifers as they return to homeostasis is problematic for both marine fish larvae production and research. This loss of desirable nutrients lowers the nutritional value of rotifers to fish larvae, and also makes conclusions difficult on fish larvae nutrient intake and requirement studies.

There were two aims of this study. The first aim was to investigate the effect of LT and ST enrichment of rotifers with increasing concentrations of Se-yeast on rotifer Se concentration. As a sub factor of the first aim, rotifer egg ratio, population growth and mineral composition other than Se were also measured to determine the suitability for application of feeding Se-yeast to rotifers in commercial hatcheries. The second aim was to determine the rate of Se retention in rotifers enriched to copepod levels with Se-yeast, after storage in clear or green water.

2. Material and methods

2.1. Yeast specification

A commercially available Se-yeast (Sel-Plex® 2000, 2000 mg Se kg^{-1}, Alltech, Lexington, KY) was used in the experiments. Speciation of Se in Sel-Plex consists of 63–66% Se-Met, 34–36% low molecular weight Se compounds and <0.5% inorganic Se (S. Elliott, personal communication). The total Se level of the Se-yeast was analysed as per Section 2.3.

To determine yeast diameter, individual Se-yeast ($n = 359$) were measured using an Olympus BX51 binocular microscope fitted with an Olympus DP50 3.0 Camera and the program cell^B V.2.6 (Olympus, Germany). A drop of yeast sample suspended in distilled water (ddH_2O) was placed on a microscope slide before viewing at 1000× magnification.

2.1.1. Selenium solubility

The Se-yeast were crushed to destroy cell wall and membrane integrity and enable the extraction of the entire water soluble Se fraction in seawater or in fresh water adjusted to pH 2. The Se that remained associated with the Se-yeast was the non soluble fraction and its percentage of total Se may affect uptake of Se from Se-yeast by aquatic organisms.

Se-yeast were weighed into two ball mills (≈5 g total), and then milled for 1 min intervals for a total of 5 min at 30 shakes second^{-1} (Retsch MM301 ball mill, Haan, Germany). Between 1 min intervals the ball mills were stored at −20 °C for 10 min, to prevent heat build up. Yeast were checked under a microscope (Section 2.1) for evidence of cell destruction. Approximately 0.1 g (accurately weighed) of the crushed yeast was placed into each 15 ml plastic tube. For treatment one, 15 ml of 0.45 μm filtered, 22‰ salinity, pH 7.8 seawater, along with 50 μl protease inhibitor (Cat. no. P8215, Sigma, Germany) was added ($n=4$). For treatment two, 15 ml of pH 2 water (0.017 M HCl, Merck, Germany) and 50 μl protease inhibitor were added ($n=4$). Tubes were then placed in an ultrasonic bath (Bandelin Sonorex RK 100, Sigma-Aldrich, Germany) for 90 min, using ice to keep the temperature below 31 °C. Tubes were then placed on a rotating wheel at 5 rotations per minute for 180 min. The tubes were then centrifuged (Eppendorf Centrifuge 5810 R; 3220×g for 15 min at 20 °C) and the supernatant decanted off. The pellet was re-suspended in 15 ml of seawater or pH 2 water (same as starting solution), centrifuged and the supernatant decanted off, two more times. The remaining pellet was then rinsed from the tube using 5% nitric acid and wet digested as per Section 2.3, except samples were rinsed out of the Teflon bombs after digestion with 50 ml ddH_2O water instead of 25 ml. Samples were diluted 200× before analyses with ICP-MS.

2.1.2. Selenium leaching from whole yeast

Whole Se-yeast was mixed in seawater for increasing periods to determine the percentage of water soluble Se (Se solubility, Section 2.1.1) that was leachable. The percentage of leached Se from uncrushed Se-yeast may affect the subsequent availability and uptake of Se from Se-yeast fed to aquatic organisms.

Approximately 0.1 g (accurately weighed) of Se-yeast was placed into 15 ml plastic tubes to which 15 ml of seawater (as per Section 2.1.1), was added. The yeast was then suspended into solution with a vortex. Tubes were either mixed by hand for 1 min from the time after vortexing, or by a rotating wheel at 5 rotations min^{-1}, for 30, 90 or 180 min ($n=4$ tubes for each time). The contents of the tubes were then filtered through a 0.65 μm filter and the remaining yeast in the tube were rinsed onto the filter using ddH_2O. The yeast were then scraped off the filter into a wet digestion bomb and a small amount of (<10 ml) 5% nitric acid (Merck, Germany) was used to rinse the remaining yeast off the filter and spatula and into the bomb. Samples were then wet digested and prepared for ICP-MS analyses (Section 2.3) as in Section 2.1.1.

2.2. Rotifer trials

Two different strains of rotifers (Stock culture 1 and 2) were used in these experiments. All (Sections 2.2.2–2.2.4.2) but one of the experiments were performed at the NIFES facility, Bergen, Norway with stock culture one (Section 2.2.1). Rotifers from stock culture 2 (Section 2.2.5) were used to determine the retention of Se in rotifers stored in green water (Section 2.2.6).

2.2.1. Rotifer stock culture one

A strain of *Brachionus plicatilis* (adult lorica length 202 ± 10 μm, width 173 ± 11 μm; mean ± SD) was obtained from the Sagafjord Cod Hatchery, Stord, Norway. Rotifers were cultured in two 200 l tanks at 25 °C, 18–22‰ salinity, 1 μm filtered seawater (from now on referred to as warm seawater), with gentle aeration and at an average concentration of 500 rotifers ml^{-1}. They were fed 3–4 times daily with 2.5 ml (338 mg DW) of live *Chlorella* sp. (Docosa, SV12, Japan) and 0.021 ml of synthetic fish oil (Rich S.A., Rich, Greece; from now on referred to as 'fish oil') million^{-1} rotifers day^{-1}. *Chlorella* was measured in a volumetric flask then poured directly into the rotifer tanks. Fish oil was emulsified in tap water as per directions, and the emulsion was pipetted into tanks. Rotifers were washed every 3–4 days in a commercial rotifer cleaner (mesh size 62 μm) for 30 min with warm seawater. Rotifers were cultured with the above method for at least 10 days before use in trials.

2.2.2. Long term rotifer enrichment with Se-yeast

Rotifers were batch cultured for 6 days (2 culture cycles of 3 days) with treatments consisting of increasing concentrations of Se-yeast as a dry weight percentage of the total daily diet (Table 1). Rotifers were taken from the stock culture and washed as described (Section 2.2.1). Rotifers were then placed in 18 l (15 l volume) tanks (*Artemia* hatchers, Aquatic Habitats, USA) and cultured in warm seawater at initial concentrations of 250–350 rotifers ml^{-1}. Air was bubbled into the base of each culture at a rate of ≈0.8 l min^{-1}.

The diets were divided into four time weighted portions and fed at 7:00, 12:00, 18:00 and 22:00 daily. *Chlorella* was pipetted directly into tanks. A Se-yeast solution was made by mixing 1.00 g Se-yeast with 100 ml tap water in a volumetric flask. This solution was added to a beaker and mixed with a magnetic stirrer while pipetting to tanks. Fish oil was prepared as in Section 2.2.1. Both the fish oil and Se-yeast were prepared at each feeding time. Rotifer concentration and rotifers with eggs were counted at experiment start, then at the same time daily for each tank. Rotifer concentrations were used to calculate the feeding rate for the following 24 h. Changes in rotifer concentration are referred to as population growth. The egg ratio (ER) of rotifers was calculated as a basis to quantify rotifer health, using the following equation.

$$ER = \text{rotifers with eggs} / \text{total rotifers}$$

Rotifer samples for mineral analyses were taken at 72 h (end of first culture cycle; day 3) and again at 144 h (end of second culture cycle; day 6). At 72 h the rotifer concentration was counted and the total rotifer population calculated in each tank. A portion of rotifers were then drained from the base of each tank, leaving approximately 4.5 million rotifers per tank to continue on the diets for the second culture cycle. The sampled rotifers were collected on a 62 μm mesh and washed for 5–10 min with warm seawater. The rotifers were then patted dry from underneath the mesh with paper towel and transferred to 50 ml sampling containers on ice. The samples were stored at −20 °C before and after lyophilising and then analysed for minerals (Section 2.3).

After the 72 h sampling, the rotifers remaining in tanks were washed on 62 μm mesh, placed back in cleaned tanks with 15 l of new warm seawater, and the rotifer concentration counted. They were cultured for a further 3 days on the diets (second culture cycle, days 3–6). On day 6, sampling was the same as day 3 with the exception that all rotifers were sampled and the experiment terminated. The experiment was repeated in triplicate.

Table 1
Rotifer batch culture feeding rates, showing increasing levels of Se-yeast in diets were offset by equivalent reductions in *Chlorella*. The resulting total feeding rates were 359 mg DW feed million rotifers^{-1} day^{-1} for all treatments.*

Feed as fed per million rotifers per day			
Se-yeast (mg DW)	Se-yeast % of total diet (DW)	*Chlorella* (mg DW)	Se concentration in diet (mg Se kg^{-1} DW)
0	0	338	0.01
1.7	0.5	336.3	8.17
3.4	1	334.6	16.3
6.8	2	331.2	32.7
13.5	4	324.5	64.9
33.8	10	304.2	162
67.5	20	270.5	324

*Includes 21 μl of fish oil included as a gravimetric measurement.

2.2.3. Short term rotifer enrichment with Se-yeast

Rotifers were ST enriched for 3 h at 25 °C with fish oil and increasing concentrations of Se-yeast or sodium selenite pentahydrate (Table 2). The sodium selenite pentahydrate ($Na_2Se0_3.5H_2O$, Merck, Germany, from now on referred to as Na-Se) concentrations were calculated to make available the same concentration of Se per rotifer as ST enrichments with 2, 10 or 20 mg Se-yeast. Se-yeast and fish oil were prepared as in Sections 2.2.2 and 2.2.1, respectively. A selenite solution was prepared by diluting 0.100 g Na-Se with 100 ml tap water in a volumetric flask. Rotifers were taken from the stock culture (Section 2.2.1) and prepared in 18 l tanks as per Section 2.2.2, but with densities between 900 and 1100 rotifers ml^{-1}. Rotifer concentration was counted at the start of the trial for each tank. Rotifers were sampled and analysed as per Section 2.2.2. The experiment was repeated in triplicate.

2.2.4. Selenium retention in Se-yeast fed rotifers stored in clear water without feed

Rotifers that had been LT or ST enriched with Se-yeast to copepod levels were stored without food for up to 10 h in clean seawater to determine the level of Se retention. Trials were conducted at both room temperature (≈20 °C; warm treatment) and in a cold room (≈10 °C; cold treatment).

2.2.4.1. Selenium retention of rotifers stored in clear water after LT enrichment with Se-yeast. Rotifers were batch cultured in a 300 l tank as per Section 2.2.1, but with the 1.7 mg Se-yeast LT enrichment feeding rate (Table 1) for ≥6 days. This Se-yeast LT enrichment rate was predetermined to give 3–5 mg Se kg^{-1} DW in rotifers from the LT enrichment trials (Section 2.2.2). A portion of these rotifers were washed with warm seawater, and enriched for 3 h at ≈1000 rotifers ml^{-1}, with 0.2 ml fish oil $million^{-1}$ rotifers, as per ST enriched control rotifers (Section 2.2.3), in 100 l warm seawater. Rotifers were then cleaned for 10 min in warm seawater to remove residual fish oil, then seawater at 18 °C for 15 min to decrease the temperature of the rotifers from 25 °C to 18 °C. The rotifers were then transferred in the 18 °C water to a cold room (≈9 °C) and diluted to 600–800 rotifers ml^{-1} with 9 °C seawater. Rotifers were then placed in 15 l buckets (10 l volume) with aeration to create a gentle turbulence throughout the experiment. Buckets ($n=3$) at times 1, 2, 5 and 10 h after rotifer transfer into the cold room, were then randomly sampled by collecting rotifers on 62 μm mesh and washing with 9 °C seawater for 5 min. The rotifers were then patted dry with paper towel from underneath and transferred to 50 ml containers. The samples were stored at −20 °C before and after lyophilising prior to analyses of minerals (Section 2.3).

Table 2
Rotifer ST enrichment feeding rates, showing increasing Se-yeast levels were offset by equivalent reductions in fish oil. The resulting feeding rates were 200 mg DW feed million $rotifers^{-1}$ for all treatments.*

Enrichment diet as fed per million rotifers for 3 h				
Treatment (mg DW)		Se-yeast or the equivalent Na-Se, % of total enrichment (DW)**	Fish oil (μl)	Se concentration in enrichment diet (mg Se kg^{-1} DW)
Se-yeast	0	Control	200	0
	2	1	198	17.2
	4	2	196	34.4
	8	4	192	68.9
	16	8	184	138
	32	16	168	276
Na-Se	0	Control	200	0
	0.012	1	200	0
	0.057	5	200	0
	0.115	10	200	0

*Fish oil volume was included as a gravimetric measurement.
**Na-Se (sodium selenite) rate was based on the equivalent level of Se in Se-yeast, and was added directly to the water.

The temperature of the rotifers at time 0 h was 14 °C and gradually fell to 9 °C within 10 h.

The above experiment was repeated at room temperature (≈20 °C) for the storage period. The experiment followed the same procedure as the above cold temperature treatment, except rotifers were washed with only warm seawater after the fish oil ST enrichment and held at room temperature (≈20 °C) for the 10 h storage period. Rotifer temperature decreased from 25 °C at time 0 h to 21.5–20 °C within 10 h.

2.2.4.2. Selenium retention of rotifers stored in clear water after ST enrichment with Se-yeast. Rotifers from the stock culture (Section 2.2.1) were ST enriched with Se-yeast as per Section 2.2.3, except the enrichment level was 3.2 mg Se-yeast (treatment level between 2 and 4 mg, Table 2, predetermined to give rotifers with 3–5 mg Se kg^{-1} DW). The amount of rotifers enriched and the procedure were identical to Section 2.2.4.1, from the stage of cleaning after the 3 h fish oil enrichment.

2.2.5. Rotifer stock culture two

A separate rotifer trial using a different rotifer culture to the previous sections was performed at the Institute of Marine Research, Austevoll, Norway. Rotifers (*Brachionus* 'Cayman', adult lorica length 184 ± 10 μm, width 134 ± 11 μm; mean ± SD) were batch cultured in three tanks (550 l max capacity), with seawater (24–26 °C, 1 μm filtered, ≈27‰ salinity; from now on referred to as 'salt water'). Gentle aeration and oxygen were provided from the base, keeping oxygen saturation above 100%. The cultures were fed continuously with a 50:50 mix (w/w) of bakers yeast (Idun, Norway) and Rotimac (Biomarine Aquafauna Inc., California, USA), at a rate of 180–300 g feed $tank^{-1}$ day^{-1}. The cultures were cleaned once weekly for 30 min, using a rotifer cleaner with 62 μm mesh, salt water and aeration.

2.2.6. Selenium retention of rotifers stored in green water after ST enrichment with Se-yeast

A portion of rotifers were siphoned from stock culture two (Section 2.2.5), and washed for 10 min (62 μm mesh rotifer cleaner, salt water, aeration). Rotifers were then ST enriched with either a control diet or a treatment diet. The control enrichment was 150 mg Multigain (Dana Feed, DK-8700 Horsens, Denmark) $million^{-1}$ rotifers, plus 200 mg sodium iodide (VMR, Belgium art. no. 27915.297) l^{-1} of enrichment volume. The treatment enrichment was 1.1 mg Se-yeast and 148.9 mg Multigain (equalling the same amount of dry feed $million^{-1}$ rotifers for both the control and treatment), and sodium iodide as per the control. Up to 30 g of Multigain was mixed with a blender for 3 min in ≈500 ml of lukewarm tap water. Se-yeast and sodium iodide were prepared separately by hand mixing with a spoon in cold tap water. Enrichment time was 3 h at rotifer densities between 1000 and 2000 ml^{-1}, in salt water with continual aeration and oxygenation (oxygen saturation was above 80%). After enrichment, rotifer treatments were washed separately as per before enrichment. The rotifers were then concentrated to 2000–4500 rotifers ml^{-1}, and transferred to storage tanks with clean salt water and aeration, and rapidly cooled (<10 min) to 8.5 °C. After 2.5 h a portion of rotifers were transferred to tanks (algae tanks). Algae tanks were filled with a mixture of filtered seawater (temperature ≈12 °C, ≈34.5‰ salinity, 20 μm sand/lamella filtered, degassed, from 160 m depth), algae paste (2–3 ml *Nannochloropsis* spp. l^{-1}; Nanno 3600, Reed Mariculture Inc, USA), and either control or treatment rotifers, to a volume of 60 l. The rotifer concentrations in the algae tanks ranged between 200 and 700 rotifers ml^{-1}. The algae tanks received gentle aeration from the base and were held at room temperature (≈12.5 °C). Samples of rotifers were taken directly after the enrichment (0 h; $n=6$), just prior to transfer to the algae tanks (2.5 h; $n=4$), and then after 5.5 and 15.5 h ($n=4$) in the algae tanks (8 and 18.5 h after time 0 h, respectively). As a comparison for Se retention in rotifers held in clean water, a sample

of rotifers remaining in the original holding tank was taken 18.5 h after time 0 h. Samples were collected on 62 μm mesh, washed for 5 min with 12 °C seawater (same specification as in algae tanks), placed in 25 ml containers and stored at −20 °C before and after lyophilising, and then analysed for minerals (Section 2.3).

2.3. Mineral analysis

Mineral concentrations were determined by wet digesting samples with nitric acid (65% HNO_3 Suprapur®, Merck, Germany) and hydrogen peroxide (30% H_2O_2, Merck, Germany), in a microwave (Ethos 1600, Milestone, USA) as described by Julshamn et al. (2004). Samples were then analysed with ICP-MS (Agilent 7500 series, USA). A standard curve was made using a multi element standard (Merck, art. no. 1.11355, Germany) plus a mercury standard (Spectrascan, Teknolab, Norway) diluted in 5% nitric acid. Standards contained either 0, 1, 2, 5, 10, 20 or 50 μg l^{-1} of Se, Zn and As, and one order of magnitude lower concentrations of Mn, Co, Cu, Mo, Cd, Sn, Hg and Pb. Samples were analysed using rhodium (0.5 μg l^{-1}) as a general internal standard, gold (5 μg l^{-1}) to amalgamate and stabilise mercury, and yttrium (0.5 μg l^{-1}) as an internal standard for Se. Certified reference materials (Oyster tissue, CRM 1566, NIST; TORT-2, NRC) were digested and analysed with ICP-MS concurrently as a measure of analysis accuracy. Blank samples were also digested and analysed concurrently to account for background mineral contamination.

2.4. Data analyses

Statistica software (Statsoft Inc., 2008, Tulsa, USA, Ver.8) was used for statistical analysis. Population growth and ER of rotifers were tested with repeated measures ANOVA. The mineral concentrations in LT and ST enriched rotifers were tested with multiple regression analysis. Data from the Se retention in rotifers with storage, and data for yeast specification were analysed using one-way ANOVA. Data were checked for homogeneity of variances using Levene's test before ANOVA. Differences among means were considered significant at $p<0.05$.

3. Results

3.1. Yeast specification

The mean diameter of the Se-yeast was 5.0 ± 1.2 μm (mean ± SD). The total Se concentration determined in the Se-yeast was 1722 mg Se kg^{-1} (Table 3). There was no statistical difference between total water solubility of Se in crushed Se-yeast in either seawater or at pH 2 ($p=0.14$) (Table 3). Approximately 26% of the Se in the crushed Se-yeast was water soluble. Approximately 25% of the Se in whole Se-yeast was leached within 1 min of dispersion in seawater while there was no significant leakage of Se in the following 179 min ($p=0.08$, Table 3). There was no significant difference between leakage of Se from whole Se-yeast within 1 min and the total water soluble Se content of the yeast ($p=0.95$, Table 3).

Table 3
Total Se concentration in unadulterated Se-yeast, and after leaching 1 min in seawater or removal of the total water soluble fraction in either seawater or at pH 2. Data are mean ± SD and except for total Se, were analysed with one-way ANOVA, $p<0.05$.

Treatment	Se concentration (mg kg^{-1} DW)
Total Se	1722±107 ($n=2$)
Total insoluble Se; seawater	1237 ± 97^{ab} ($n=4$)
Total insoluble Se; pH 2	1322 ± 45^{a} ($n=4$)
Total unleached Se; 1 min	1283 ± 73^{ab} ($n=4$)
Total unleached Se; 30 min	1272 ± 62^{ab} ($n=4$)
Total unleached Se; 90 min	1221 ± 49^{b} ($n=4$)
Total unleached Se; 180 min	1198 ± 70^{b} ($n=4$)

3.2. Rotifer LT enrichment with Se-yeast

3.2.1. Population concentration, egg ratios and morphological effects of rotifers LT enriched with Se-yeast

Se-yeast in the rotifer diet had no effect on the population concentration of rotifers over 6 days (2×3 day batch cultures), except at the highest feeding level (67.5 mg Se-yeast, $p=0.04$). The 67.5 mg feeding level resulted in lower rotifer population concentrations than controls by the end of the first batch culture on day 3 (≈550 versus 750 rotifers ml^{-1} for 67.5 mg and control treatments respectively, $p=0.01$, Fig. 1a). These same rotifers had stagnant growth from days 3 to 6, resulting in lower rotifer concentrations by the end of the second 3 day batch culture at day 6 (≈250 versus 550 rotifers ml^{-1} for 67.5 mg and control treatments respectively, $p<0.01$). Additionally, observation of the rotifer populations revealed that the 67.5 mg treatment had a near total absence of juveniles (small rotifers without eggs) during the second culture cycle (day 3–6).

The egg ratios were not significantly affected by different Se-yeast feeding levels (Fig. 1b). High variation between replicates may have masked effects, as a visual trend of lower egg ratios at the 67.5 mg Se-yeast feeding level (0.17 ± 0.11 (mean ± SD), $p=0.21$) and higher egg ratios for the 13.5 mg feeding level (0.26 ± 0.10 (mean ± SD), $p=0.10$), than the controls (0.21 ± 0.08, mean ± SD) was apparent (Fig. 1b). There was a significant ($p<0.01$) decrease in the egg ratio for all rotifer groups after washing at the end of the first batch culture on

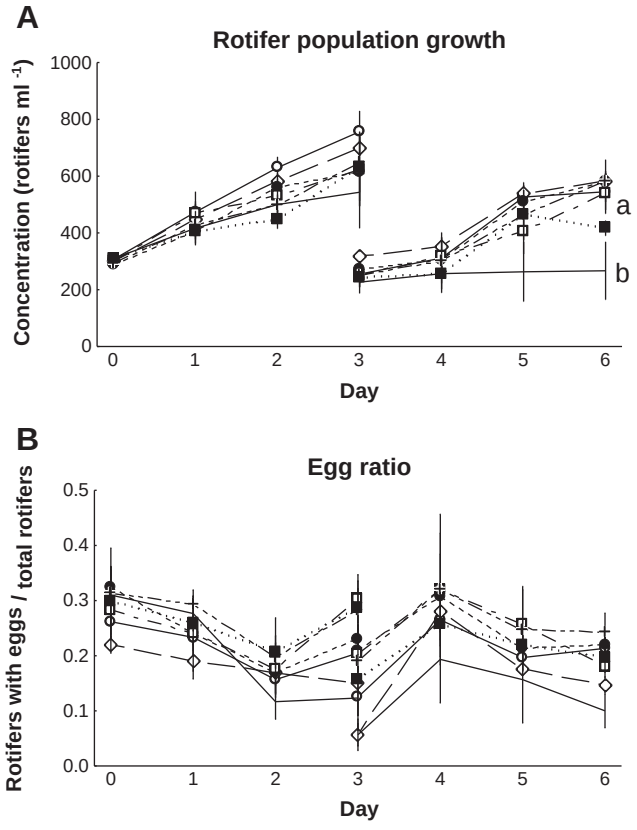

Fig. 1. The effect of Se-yeast fed as increasing percentages of batch culture diets for 6 days on rotifer population growth (rotifers ml^{-1}, Graph A) and egg ratio (rotifers with an egg/total rotifers, Graph B). Day 3 includes data from before and after sampling. Letters denote significantly different groups (repeated measures ANOVA; $p<0.05$). Points represent mean ± SEM ($n=3$), except the control which are mean ± SEM ($n=6$). Legend; (○) control, (◊) 1.7 mg, (●) 3.4 mg, (□) 6.8 mg, (+) 13.5 mg, (■) 33.8 mg, (−) 67.5 mg of Se-yeast fed million rot^{-1} day^{-1}.

day 3 (before washing 0.22 ± 0.08; after 0.14 ± 0.09; mean ± SD). The general trend of egg ratios for all rotifers groups during the 6 day trial was to fluctuate daily, within a range of 0.19 to 0.28, with an average egg ratio of 0.22 ± 0.10 (mean ± SD).

Rotifers from the 67.5 mg Se-yeast group were significantly ($p<0.01$) larger than controls after 6 days of LT enrichment. Controls had an average adult lorica length of 202 ± 10 μm and width of 173 ± 11 μm (mean ± SD, $n=85$) while the 67.5 mg group had an average adult lorica length of 227 ± 22 μm and width of 199 ± 23 μm (mean ± SD, $n=85$). Thus the 67.5 mg rotifer group grew approximately 12% longer and 15% wider than controls. Substantial malformations of the rotifer lorica resulting in loss of symmetry were seen in many of these individuals. These rotifers were also observed to have a slower swimming speed and a slower or absent response to shock (withdrawal of soft body mouth parts into the protective lorica) than the control rotifers.

3.2.2. Selenium levels of rotifers LT enriched with Se-yeast

Rotifer Se concentration increased linearly with increasing levels of Se-yeast in LT enrichment diets ($p<0.01$, Fig. 2). The levels of Se in rotifers were not significantly different at the end of the first (day 3) and second (day 6) batch culture cycle ($p=0.40$, Fig. 2). Rotifer Se concentrations, averaged for day 3 and 6, ranged from 0.43 ± 0.11 in controls up to 131.67 ± 24.47 mg kg^{-1} DW (mean ± SD) at the highest Se-yeast feeding level (67.5 mg group). There was no difference in the concentration of Mn, Co, Cu, Zn, Sn, Pb, or Hg, between the rotifer control and 1.7 mg Se-yeast (copepod level Se) groups after 6 days of LT enrichment. Differences were observed in arsenic (control; 0.315 ± 0.021 mg As kg^{-1} DW, treatment; 0.281 ± 0.016 mg As kg^{-1} DW; mean ± SD) and cadmium (control; 0.070 ± 0.004 mg Cd kg^{-1} DW, treatment; 0.058 ± 0.004 mg Cd kg^{-1} DW; mean ± SD) concentrations which were 11 and 17% lower respectively ($p<0.05$), in the 1.7 mg rotifer group than controls.

Rotifers batch cultured with the 1.7 mg Se-yeast feeding rate for 14 days contained Se levels of ≈4 mg kg^{-1} DW by the end of the first culture cycle (day 3) and maintained a level of ≈4.5 mg Se kg^{-1} DW from day 3 to 14 (Fig. 3).

3.3. Rotifer ST enrichment with Se-yeast or sodium selenite

Rotifer Se concentration increased linearly with increasing ST enrichment levels of Se-yeast or Na-Se ($p<0.01$, Fig. 4). The level of Se in rotifers fed Se-yeast was significantly ($p<0.01$) higher than rotifers enriched with equivalent levels of Se added directly to the enrichment water as Na-Se.

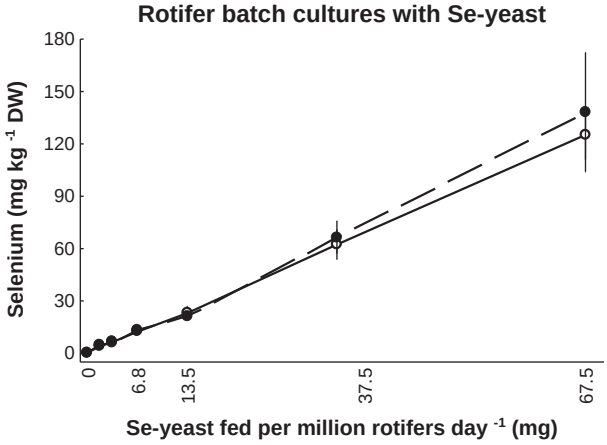

Fig. 2. The effect of Se-yeast fed at increasing levels of rotifer culture diets for 3 days (○) or 6 days (●) on the Se concentration (mg kg^{-1} DW) in rotifers. Data are mean ± SD ($n=3$), except for the control which are mean ± SD ($n=6$). Regression line for the combined data from days 3 and 6 yields; $y=-0.38+1.94x$, $R^2=0.96$, $p<0.01$.

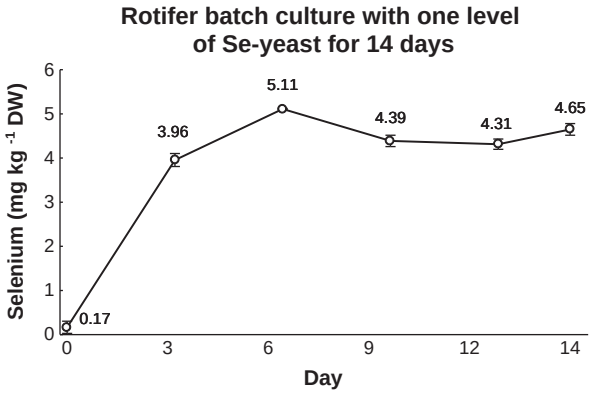

Fig. 3. The effect of culturing rotifers with 1.7 mg Se-yeast fed daily as part of the culture diet on the concentration of Se (mg kg^{-1} DW; concentration given at each point) in rotifers over a 14 day period. Rotifers were cultured in a 300 l tank, and harvested at day 6 and day 14 for storage experiments. Data are mean and the range of analytical parallels.

The Se-yeast enriched rotifers had Se levels ranging from 0.52 ± 0.04 for controls up to 36.22 ± 4.99 mg kg^{-1} DW (mean ± SD) for the highest Se-yeast enrichment level (32 mg per million rotifers). The Na-Se enriched rotifers had Se levels ranging from 0.35 ± 0.06 for controls to 0.54 ± 0.02 mg kg^{-1} DW (mean ± SD) for the highest Na-Se enrichment level (20 mg Se-yeast equivalent group). The uptake efficiency of Se by rotifers (percentage of Se fed in Se-yeast or Na-Se that was retained in rotifers) was 16.0 ± 1.2% and 0.41 ± 0.21% (mean ± SD) from Se-yeast or Na-Se, respectively. Thus, uptake of Se from Se-yeast was over 40 fold higher than uptake from Na-Se after 3 h of enrichment.

3.4. Selenium retention of rotifers stored in clear water after LT or ST enriching with Se-yeast

Rotifers retained a high percentage of Se up to 10 h after LT or ST enrichment with Se-yeast (Fig. 5). Between 99 and 95% of the Se was retained after 10 h by rotifers LT enriched with Se-yeast, and rotifers ST enriched with Se-yeast and stored at cold (≈10 °C) temperatures. Selenium retention was significantly lower ($p<0.01$) after 10 h in ST enriched rotifers stored at warm temperatures (86% Se retention, Fig. 5).

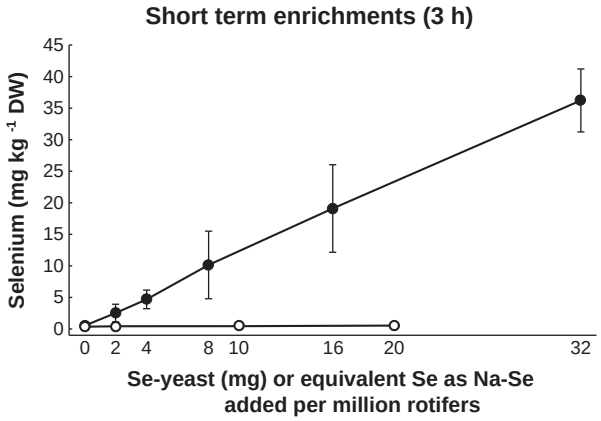

Fig. 4. Concentration of Se (mg kg^{-1} DW) in rotifers in response to increasing percentages of either Se-yeast (mg) included in short term enrichments with fish oil (●), or sodium selenite ($Na_2SeO_3 \cdot 5H_2O$) added as equivalent levels of Se as in Se-yeast (mg) short term enrichment diets with fish oil (○). Data are mean ± SD ($n=3$). Regression line for Se-yeast enriched rotifers yields; $y=0.59+1.12x$, $R^2=0.93$, $p<0.01$.

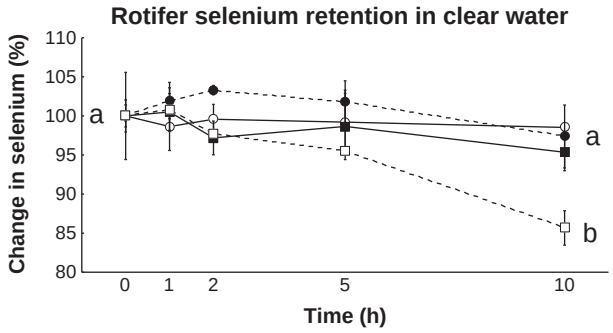

Fig. 5. The effect of storage time on the retention of Se (% of starting Se) in rotifers cultured with Se-yeast then stored cold (○) or warm (■), or short term enriched with Se-yeast then stored cold (●) or warm (□). Long term enriched rotifers were batch cultured with 1.7 mg Se-yeast as part of their diet for ≥6 days, then short term enriched with fish oil before washing and storage. The short term enriched rotifers were enriched for 3 h with 3.2 mg of Se-yeast and fish oil, before washing and storage. Rotifers were stored at either warm (20 °C) or cold temperatures (10 °C). Letters denote statistically significant difference in Se % between 0 and 10 h (one-way ANOVA, $p<0.05$). Data are mean ± SD ($n=3$), except at data for 0 h which are the mean±SD of analytical parallels.

3.5. Selenium retention of rotifers stored in green water after ST enriching with Se-yeast

After ST enrichment (time 0 h), rotifers ST enriched with Se-yeast contained 2.95 ± 0.48 mg Se kg^{-1} DW while control rotifers contained 0.66 ± 0.02 mg Se kg^{-1} DW (mean ± SD) (Fig. 6). Rotifers that were placed in algae tanks were observed to have a green digestive tract after 30 min in the presence of algae, indicating algae ingestion at the storage temperature of 12.5 °C. Both the control rotifers that were held in storage without food for 18.5 h, and those held in the presence of algae fell slightly, but significantly ($p<0.05$) in Se concentration from time 0 h (Fig. 6). Rotifers ST enriched with Se-yeast decreased to 1.73 ± 0.23 mg Se kg^{-1} DW (mean ± SD) after 18.5 h when algae was present ($p<0.01$), but did not change significantly in the same period when algae was absent (2.71 ±0.34 mg Se kg^{-1} DW; mean±SD, $p=0.98$). Rotifers ST enriched with Se-yeast lost 35% of their Se by 8 h (5.5 h in the presence of algae) and 41% after 18.5 h (16 h in the presence of algae) (Fig. 6). There was no difference in the concentration of Mn, Co, Cu, Zn, Sn, Pb, Cd or As in rotifers ST enriched with Se-yeast or controls at time 0. However, mercury levels were 23% lower in rotifers ST enriched with

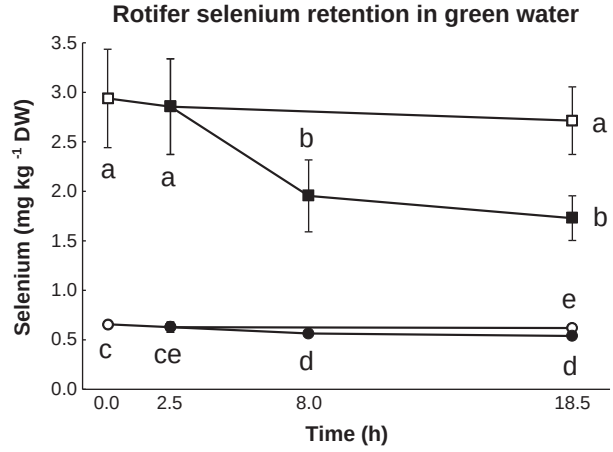

Fig. 6. The effect of storage time on the Se concentration (mg kg^{-1} DW) in rotifers fed 1.1 mg of Se-yeast per million rotifers as part of a short term enrichment (3 h) diet then stored with (■) or without algae (□), or control rotifers that were short term enriched without Se-yeast and then stored with (●) or without algae (○). All rotifer groups were stored without food for the first 2.5 h. Data are mean ± SD ($n=4$), except data for 0 h which are mean ± SD ($n=6$). Letters denote statistical significant differences in Se concentration (one-way ANOVA, $p<0.05$).

Se-yeast (0.20 ± 0.02 mg Hg kg^{-1} DW; mean ± SD) compared to controls (0.26 ± 0.02 mg Hg kg^{-1} DW; mean ± SD) at time 0 h ($p<0.01$). The Se concentration of the *Nannochloropsis* spp. of algae used for the green water study, along with the Se concentrations of other feed ingredients used in this paper are given in Table 4.

4. Discussion

4.1. Se-yeast specification

The percentage and speciation of insoluble Se are important considerations when Se-yeast is fed to aquatic organisms, where soluble Se may be quickly lost to the environment. The present study found that approximately 74% of the Se in the Se-yeast is in a non water soluble form and remains associated with the crushed Se-yeast fragments after dispersal in either seawater or water at pH 2. This result is within range of the typical 75–85% Se remaining after aqueous extraction of various Se-yeast products found by other researchers (Chassaigne et al., 2002; Encinar et al., 2003; Polatajko et al., 2006).

While the current study did not determine the Se species in the Se-yeast sample, a large amount of research has been conducted in this area. Chassaigne et al. (2002) determined that the water soluble Se fraction of one Se-yeast product consisted of proteins, selenite, selenate, free seleno-amino acids (Se-Met, Se-Cys) and possibly selenoglutathione. Encinar et al. (2003) found that 30% of the water soluble Se fraction in Se-yeast was protein bound. Assuming that different Se-yeast samples have similar speciation within the water soluble Se fraction, then 70% of the water soluble Se, or close to 25% of the total Se in the current studies Se-yeast samples was small organic Se species such as free Se-Met and ionic Se such as selenite. This is important when considering the potential uptake of Se from Se-yeast by rotifers. For example, the current study determined that uptake of sodium selenite (Na-Se) during 3 h enrichments was extremely low by rotifers (≈0.4%). Thus, the water soluble Se fraction leaches rapidly from Se-yeast and probably becomes largely unavailable for rotifer uptake. Consequently, Se uptake by rotifers would be mainly dependent on the ingestion of the ≈1250 mg Se kg^{-1} DW of the Se-yeast present in an insoluble form. Previous investigations have shown that the insoluble Se in Se-yeast is approximately evenly distributed between protein associated Se and polysaccharides associated Se (Chassaigne et al., 2002; Encinar et al., 2003). While the Se contained in proteins is predominately contained in Se-Met residues (Polatajko et al., 2006; Encinar et al., 2003; Chassaigne et al., 2002) the exact speciation of Se within the insoluble polysaccharide fraction is not known. Therefore, rotifers fed Se-yeast would probably ingest mainly insoluble Se, with at least half in the form of Se-Met associated with the insoluble protein fraction.

4.2. The effect of Se-yeast on rotifer health, Se uptake and retention

4.2.1. The effects of Se-yeast on rotifer health

Although uptake and retention of certain nutrients by rotifers have been studied extensively (reviewed by Dhert et al., 2001) these data are the first to show uptake, and the retention over time, of Se from an organic source by rotifers. Furthermore, this study is the first to show

Table 4
The Se concentrations in feed ingredients used in the rotifer feeding trials.

Rotifer food	Se (mg kg^{-1} DW)
Chlorella sp.*	0.01
Nannochloropsis spp.	0.35±0.05
Multigain	0.37±0.02
Rotimac	1.05±0.29
Bakers yeast	0.04±0.00

Chlorella sp. data obtained from Hamre et al. (2008a).
Data are mean±SD of analytical parallels.

the effects of dietary Se-yeast on rotifer population growth and egg ratio. Population growth and egg ratio were not affected by Se-yeast inclusion in the batch culture diets up to and including 13.5 mg Se-yeast per million rotifers per day. At this level, rotifers contained ≈22 mg Se kg^{-1} DW, which is 4–7 fold higher than copepod Se levels (3–5 mg Se kg^{-1} DW, Hamre et al., 2008a). Therefore, using Se-yeast to increase the level of Se in rotifers to copepod levels would have no negative effects on rotifer cultures in commercial fish hatcheries.

The highest (67.5 mg Se-yeast) LT feeding rate had a statistically significant negative effect (population growth) or showed a visual negative trend (egg ratio) on rotifers. At the highest Se-yeast feeding rate, the yeast made up approximately 20% of the total rotifer diet. At this level, the rotifer diet containing 324 mg Se kg^{-1} DW, and resulted in rotifers with ≈130 mg Se kg^{-1} DW (over 40 fold higher than copepod Se levels). The biochemical basis of Se toxicity occurs from distortion of protein shape with erroneous Se substitution in sulphur bonds and oxidative damage (reviewed by Lemly, 1999, 2002). The rotifer lorica consists of proteins that are probably disulfide cross-linked keratin (Bender and Kleinow, 1988). Excessive Se may have resulted in substitution of Se in the disulfide cross-links, and resulted in protein distortion, and subsequently the lorica malformations and/or increased lorica size observed in the 67.5 mg group.

While there were no statistically significant differences in the overall rotifer population concentration or egg ratios at the second highest feeding rate (33.8 mg Se-yeast, 10% of diet), there was a trend of lower rotifer concentrations towards the end of the second culture cycle (Fig. 1a). The 33.8 mg rotifer group had whole body Se concentrations of ≈64 mg kg^{-1} DW, and the subtle negative effects indicate this to be a chronically toxic whole body Se level for rotifers. This study indicates that non toxic whole body levels of Se in rotifers are between 22 and 64 mg kg^{-1} DW. This Se level is substantially higher than the whole body Se toxicity threshold average for freshwater fish of 4 mg Se kg^{-1} (reviewed by Hamilton, 2003), but similar to the whole body toxic threshold of 15–32 mg Se kg^{-1} DW for the water flea *Daphnia magna* (Ingersoll et al., 1990). The results demonstrate that up to 13.5 mg of Se-yeast per million rotifers per day can be safely included in rotifer culture diets. In addition, this study indicates that the negative effects of the highest feeding rate, 67.5 mg Se-yeast per day, begin to develop after 24 h. Thus levels up to and including 67.5 mg Se-yeast are recommended as a safe ST (≤24 h) enrichment rate for rotifers. This gives the possibilities of future studies using Se-yeast fed rotifers as a dietary Se delivery medium for Se toxicity trials with fish larvae and other small predatory aquatic organisms.

4.2.2. Rotifer selenium uptake

Rotifer Se uptake increased linearly with increasing amounts of Se-yeast fed in LT and ST enrichments. To obtain rotifers with Se concentrations in the lower range of copepods (3 mg Se kg^{-1} DW, Hamre et al., 2008a) approximately 1.7 mg of Se-yeast per day in rotifer batch cultures or 2.1 mg of Se-yeast in 3 h enrichments, per million rotifers was required (Figs. 2 and 4). This represented a replacement of approximately 0.5% of the total rotifer batch culture diet, or 1.1% of the total ST enrichment diet. The amount of Se-yeast required in ST enrichments to reach copepod levels was affected by the other enrichment ingredients (Multigain versus fish oil), and a regression of uptake should be established when using Se-yeast with different enrichment feeds. The low replacement of other rotifer feed ingredients is an important requirement for fish larvae production. Rotifers fed solely on yeast are unsuitable for feeding to marine fish larvae as they lack sufficient quantities of DHA, EPA and AA (Rodriguez et al., 1996, 1997) which are essential for marine fish larvae growth and survival (reviewed by Rainuzzo et al., 1997). The small quantities of Se-yeast required to increase rotifer Se to copepod levels are unlikely to significantly alter lipid levels in rotifers.

Minimal effects of feeding Se-yeast were seen in the analysed essential mineral composition of rotifers after either ST or LT enrichment. However, effects were found in the toxic mineral composition. Rotifers enriched to copepod Se levels with Se-yeast had lower mercury levels when ST enriched, and lower arsenic and cadmium levels when LT enriched versus controls. The decrease in these toxic metals may have been due to direct binding of the metals to Se that decreased their absorption or facilitating their excretion. It has been demonstrated that Se binds to mercury and arsenic in vivo (Gailer, 2007) and cadmium in vitro (Sasakura and Suzuki, 1998), and the formation of Se and mercury complexes has been a suggested reason for increased Se reducing body accumulation of mercury (Belzile et al., 2006; Deng et al., 2008). It is unknown why enriching rotifers ST or LT with Se-yeast affected different minerals. It may be that LT enriching rotifers with Se-yeast did decrease mercury levels but the effects were not seen as mercury levels in both the controls and Se-yeast enriched rotifers were below the limit of quantification of the analysis method. If true, it may be that LT enrichment allows more complex interactions between Se and other minerals to occur, and insufficient time may be why no differences in arsenic or cadmium were observed in the rotifers ST enriched with Se-yeast.

Rotifer uptake of Se during ST enrichment was 40 fold greater from Se-yeast than from Na-Se (≈16% for Se-yeast versus ≈0.4% for Na-Se). The 0.41 ± 0.21% absorption of Se from Na-Se is in the range of that found by Hamre et al. (2008b) who used 7 mg l^{-1} Na-Se (2.1 mg Se l^{-1}) to enrich rotifers to 4.8 ± 0.5 mg kg^{-1} Se DW. Assuming these rotifers were similar in size to the current studies, then their uptake of Se was ≈0.3%. The slightly higher average uptake found in the current study could have been due to the longer enrichment period of 3 h as opposed to the 1.5 h used by Hamre et al. (2008b). Other minerals in inorganic/soluble forms have also been observed to have low uptakes by rotifers. Rotifers took up less than 0.1% of the iodine added as sodium iodide during a 1.5 h enrichment by Hamre et al. (2008b). Matsumoto et al. (2009) found that rotifers had an extremely low uptake of ionic zinc, and that by first enriching algae (*Chlorella* sp.) with zinc and feeding the algae to rotifers, zinc uptake was greatly increased. Thus, evidence is accumulating which strongly suggests that rotifer enrichment with many minerals may be most effective when rotifers can physically ingest the mineral as part of a food particle, such as insoluble Se associated with Se-yeast or zinc enriched algae. Therefore, future studies involving mineral enrichment of rotifers may obtain greater success by focusing on using food items to deliver minerals into rotifers, rather than adding ionic forms of the minerals directly to the water.

Long term enriched rotifers had approximately the same Se concentrations after the 1st (day 3) and 2nd (day 6) batch culture cycle. This indicates that Se concentration is mediated by the rotifers, where ingestion of Se equals excretion, even at the highest Se-yeast feeding rates. The mechanism for this cannot be determined from the available data, but Se mediation was apparent even after extended periods of Se-yeast feeding. For example, rotifers maintained a level of approximately 4.5 mg Se kg^{-1} DW over a 14 day batch culture period with Se-yeast.

4.2.3. Rotifer selenium retention

The retention of Se in rotifers that were LT or ST enriched with Se-yeast was >95% after 10 h of storage in clear seawater, except for ST enriched rotifers stored at warm temperatures (≈20 °C; STW). After 10 h of storage STW rotifers retained 86% of their enrichment Se level. The reason for a lower Se retention in STW rotifers than other rotifer groups is probably twofold. Firstly, rotifers would have higher excretion rates at the warmer temperature of the treatment, as opposed to the cold treatment (≈10 °C). For example, Baer et al. (2008) found that rotifers excreted approximately 100%, 65% or 30% of ingested latex beads within 3 h at 26 °C, 10 °C or 4 °C, respectively. Thus rotifers would have excreted Se that was in or was cycled into the digestive tract, at a faster rate at the higher temperature. Secondly, rotifers enriched with Se-yeast for shorter periods appeared to retain more Se in or associated closely to the digestive tract than those batch

cultured with Se-yeast. Demonstrating this, rotifers LT enriched with Se-yeast and stored at warm temperatures (LTW) retained 95% of their Se, versus the 86% for the STW rotifers. In this example the excretion rate of gut contents should be the same between the two rotifer groups, which indicates the excretion of STW rotifers contained more Se. As discussed, it is likely that the majority of Se ingested by rotifers fed Se-yeast comes from the non soluble Se fraction, of which at least half is Se-Met (Chassaigne et al., 2002; Encinar et al., 2003). Selenomethionine is actively incorporated into proteins in place of methionine (Waschulewski and Sunde, 1988) and methionine is present in rotifers (Makridis and Olsen, 1999; Aragão et al., 2004; Srivastava et al., 2006; van der Meeren et al., 2008). Hence it is probable that at least a portion of the Se present in rotifers fed the Se-yeast was incorporated in rotifer proteins as Se-Met. Greater protein incorporation of Se-Met in LT rather than ST Se-yeast enriched rotifers may provide one explanation for the higher retention of Se observed in the LTW rather than STW rotifers.

The rotifer storage experiment in clear seawater without food (Section 3.4) was conducted to simulate enriched rotifers stored in a hatchery, before distribution to fish larvae tanks. In commercial hatcheries algae are often added to fish larvae tanks (Reitan et al., 1997; Rosenlund and Halldórsson, 2007; Yamamoto et al., 2009), and hence rotifers not eaten immediately by larva can be exposed to algae after enrichment. This may increase rotifer Se loss by increasing excretion of Se present in the rotifer digestive tract. The experiment with ST Se-yeast enriched rotifers stored in green water (water with algae; Section 3.5), confirms this. Rotifers retained 59% of their Se after 18.5 h (2.5 h stored without food followed by 16 h in green water), while rotifers stored in the absence of food for 18.5 h retained 92% of their Se. Thus, this indicates that rotifer excretion increases in the presence of food particles.

Interestingly, there was no statistically significant difference in rotifer Se concentration after 5.5 h (65% Se retention) or 16 h in the presence of algae. The rotifers clearly had entirely green digestive tracts indicating large amounts of algae were ingested within 30 min. Aoki and Hino (1995, cited in Hino et al., 1997) found that only 20 min was required for food to pass through the rotifer digestive tract. Thus, it is reasonable to assume that food items, including Se-yeast remaining in the gut after ST enrichment, would have been excreted within 5.5 h in the presence of algae. Therefore, the remaining 65% Se in the rotifers probably represents Se that had been digested and taken up into the rotifer body. This is lower than the 88% Se retention found when rotifers were LT enriched with Se-yeast to copepod levels and then had their digestive tract purged by 3 h of ST enrichment with fish oil (data not shown). Thus, the higher Se retention in LT rather than ST enriched rotifers after digestive tract purging further demonstrates a higher uptake of Se into the rotifer body when Se-yeast is fed for longer periods. In addition, the data indicate that 3 h is sufficient time to digest and absorb Se from Se-yeast.

Overall, the rotifer Se retention data demonstrate that storing LT or ST Se-yeast enriched rotifers results in minimal and controllable Se loss. Nutrient loss from enriched rotifers has proved problematic for the industry. For example, Naz (2008) found that enriched rotifers lost approximately 25% of their EPA and AA, and the omega 3 to 6 ratio decreased by 62% after 16 h storage at 4 °C in filtered seawater. Matsumoto et al. (2009) found that rotifers fed zinc enriched algae (*Chlorella* sp.) lost approximately 50% of their zinc after 24 h in storage in clean seawater. The higher retention of Se from Se-yeast under similar conditions indicates a higher uptake in the rotifer digestive tract, a high incorporation into body composition and/or a slower turnover time than the essential fatty acids and zinc examples given.

5. Conclusion

This study concludes that Se-yeast is a suitable medium to increase Se levels in rotifers. Generally, less than 1% of the rotifer culture diet or ST enrichment must be replaced with Se-yeast to obtain copepod Se levels in rotifers. At this feeding rate, Se-yeast had no negative effect on rotifer egg ratio or population growth. Se-yeast enriched rotifers retained a high percentage of Se after extended periods of storage in both the presence and absence of food. The results show rotifers had 40 fold higher uptake of Se from Se-yeast than from Na-Se. This study builds on evidence suggesting that uptake of many minerals by rotifers may benefit from being associated with an ingestible food particle, rather than being added to enrichments in soluble forms.

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References

Aoki, S., Hino, A., 1995. Measurements of the nitrogen budget in the rotifer *Brachionus plicatilis* by using ^{15}N. Fisheries Science 61, 406–410.
Aragão, C., Conceição, L.E.C., Dinis, M.T., Fyhn, H.J., 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquaculture 234 (1–4), 429–445.
Baer, A., Langdon, C., Mills, S., Schulz, C., Hamre, K., 2008. Particle size preference, gut filling and evacuation rates of the rotifer *Brachionus* "Cayman" using polystyrene latex beads. Aquaculture 282 (1–4), 75–82.
Bell, J.G., Cowey, C.B., 1989. Digestibility and bioavailability of dietary selenium from fishmeal, selenite, selenomethionine and selenocystine in Atlantic salmon (*Salmo salar*). Aquaculture 81, 61–68.
Belzile, N., Chen, Y.W., Gunn, J.M., Tong, J., Alarie, Y., Delonchamp, T., Lang, C.Y., 2006. The effect of selenium on mercury assimilation by freshwater organisms. Canadian Journal of Fisheries and Aquatic Sciences 63, 1–10.
Bender, K., Kleinow, W., 1988. Chemical properties of the lorica and related parts from the integument of *Brachionus plicatilis*. Comparative Biochemistry and Physiology. Part B: Biochemistry & Molecular Biology 89 (3), 483–487.
Busch, K., Peruzzi, S., Tonning, F., Falk-Petersen, I.B., in press. Effect of prey type and size on growth, survival and pigmentation of cod (*Gadus morhua*) larvae fed rotifers (*Brachionus* 'Cayman') or natural zooplankton. Aquaculture Research.
Chassaigne, H., Chéry, C.C., Bordin, G., Rodriguez, A.R., 2002. Development of new analytical methods for selenium speciation in selenium-enriched yeast material. Journal of Chromatography A 976 (1–2), 409–422.
Combs, G.F., Combs, S.B., 1986. The biological availability of selenium in foods and feeds. The Role of Selenium in Nutrition. Academic Press Inc, New York, pp. 127–177.
Deng, D.F., Teh, F.C., Teh, S.J., 2008. Effect of dietary methylmercury and selenomethionine on Sacramento splittail larvae. Science of The Total Environment 407 (1), 197–203.
Dhert, P., Rombaut, G., Suantika, G., Sorgeloos, P., 2001. Advancement of rotifer culture and manipulation techniques in Europe. Aquaculture 200 (1–2), 129–146.
Drillet, G., Jørgensen, N., Sørensen, T., Ramløv, H., Hansen, B., 2006. Biochemical and technical observations supporting the use of copepods as live feed organisms in marine larviculture. Aquaculture Research 37 (8), 756–772.
Encinar, J.R., Sliwka-Kaszynska, M., Polatajko, A., Vacchina, V., Szpunar, J., 2003. Methodological advances for selenium speciation analysis in yeast. Analytica Chimica Acta 500 (1–2), 171–183.
Gailer, J., 2007. Arsenic–Selenium and mercury–selenium bonds in biology. Coordination Chemistry Reviews 251, 234–254.
Gatlin III, D.M., Wilson, R.P., 1984. Dietary selenium requirement of fingerling channel catfish. The Journal of Nutrition 114 (3), 627–633.
Hamilton, S.J., 2003. Review of residue-based selenium toxicity thresholds for freshwater fish. Ecotoxicology and Environmental Safety 56 (2), 201–210.
Hamre, K., Mollan, T.A., Sæle, Ø., Erstad, B., 2008a. Rotifers enriched with iodine and selenium increase survival in Atlantic cod (*Gadus morhua*) larvae. Aquaculture 284 (1–4), 190–195.
Hamre, K., Srivastava, A., Rønnestad, I., Mangor-Jensen, A., Stoss, J., 2008b. Several micronutrients in the rotifer *Brachionus* sp. may not fulfil the nutritional requirements of marine fish larvae. Aquaculture Nutrition 14, 51–60.
Hilton, J.W., Hodson, P.V., Slinger, S.J., 1980. The requirement and toxicity of selenium in rainbow trout (*Salmo Gairdneri*). The Journal of Nutrition 110, 2527–2535.
Hino, A., Aoki, S., Ushiro, M., 1997. Nitrogen-flow in the rotifer *Brachionus rotundiformis* and its significance in mass cultures. Hydrobiologia 358 (1), 77–82.
Ingersoll, C.G., Dwyer, F.J., May, T.W., 1990. Toxicity of inorganic and organic selenium to *Daphnia-Magna* (*Cladocera*) and *Chironomus-Riparius* (*Diptera*). Environmental Toxicology and Chemistry 9 (9), 1171–1181.
Ip, C., 1998. Lessons from basic research in selenium and cancer prevention. The Journal of Nutrition 128 (11), 1845–1854.
Jaramillo, F., Peng, L., Gatlin, D., 2009. Selenium nutrition of hybrid striped bass (*Morone chrysops*×*M. saxatilis*) bioavailability, toxicity and interaction with vitamin E. Aquaculture Nutrition 15 (2), 160–165.

Johansson, L., Gafvelin, G., Arnér, E.S.J., 2005. Selenocysteine in proteins—properties and biotechnological use. Biochimica et Biophysica Acta (BBA) - General Subjects 1726 (1), 1–13.
Julshamn, K., Lundebye, A.K., Heggstad, K., Berntssen, M.H.G., Boe, B., 2004. Norwegian monitoring programme on the inorganic and organic contaminants in fish caught in the Barents Sea, Norwegian Sea and North Sea, 1994-2001. Food Additives & Contaminants: Part A 21 (4), 365–376.
Koedijk, R., 2009. "Phenotypic plasticity influenced by diet during early development—a comparative study on Atlantic cod." Doctoral Thesis, University of Bergen, Norway. ISBN 978-82-308-0829-0.
Kohrle, J., Jakob, F., Contempre, B., Dumont, J.E., 2005. Selenium, the thyroid, and the endocrine system. Endocrine Reviews 26 (7), 944–984.
Lemly, A.D., 1999. Selenium impacts on fish: an insidious time bomb. Human & Ecological Risk Assessment 5 (6), 1139–1151.
Lemly, A.D., 2002. Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. Aquatic Toxicology 57 (1–2), 39–49.
Lin, Y.H., Shiau, S.Y., 2005. Dietary selenium requirements of juvenile grouper, *Epinephelus malabaricus*. Aquaculture 250 (1–2), 356–363.
Lin, Y.H., Shiau, S.Y., 2007. The effects of dietary selenium on the oxidative stress of grouper, *Epinephelus malabaricus*, fed high copper. Aquaculture 267, 38–43.
Lorentzen, M., Maage, A., Julshamn, K., 1994. Effects of dietary selenite or selenomethionine on tissue selenium levels of Atlantic salmon (*Salmo salar*). Aquaculture 121 (4), 359–367.
Lorentzen, M., Maage, A., Julshamn, K., 1998. Supplementing copper to a fish meal based diet fed to Atlantic salmon parr affects liver copper and selenium concentrations. Aquaculture Nutrition 4, 67–72.
Makridis, P., Olsen, Y., 1999. Protein depletion of the rotifer *Brachionus plicatilis* during starvation. Aquaculture 174 (3–4), 343–353.
Matsumoto, S., Satoh, S., Kotani, T., Fushimi, H., 2009. Examination of a practical method for zinc enrichment of euryhaline rotifers (*Brachionus plicatilis*). Aquaculture 286 (1–2), 113–120.
Naz, M., 2008. The changes in the biochemical compositions and enzymatic activities of rotifer (*Brachionus plicatilis*, Müller) and *Artemia* during the enrichment and starvation periods. Fish Physiology and Biochemistry 34 (4), 391–404.
NRC, 1993. Nutrient requirements of fish. National Research Council, National Academy Press, Washington, DC.
Olivotto, I., Buttino, I., Borroni, M., Piccinetti, C.C., Malzone, M.G., Carnevali, O., 2008. The use of the Mediterranean calanoid copepod *Centropages typicus* in yellowtail clownfish (*Amphiprion clarkii*) larviculture. Aquaculture 284 (1–4), 211–216.
Olsen, Y., Inge Reitan, K., Vadstein, O., 1993. Dependence of temperature on loss rates of rotifers, lipids, and ω3 fatty acids in starved *Brachionus plicatilis* cultures. Hydrobiologia 255–256 (1), 13–20.
Park, H.G., Puvanendran, V., Kellett, A., Parrish, C.C., Brown, J.A., 2006. Effect of enriched rotifers on growth, survival, and composition of larval Atlantic cod (*Gadus morhua*). Journal Of Marine Science 63 (2), 285–295.
Payne, M.F., Rippingale, R.J., Cleary, J.J., 2001. Cultured copepods as food for West Australian dhufish (*Glaucosoma hebraicum*) and pink snapper (*Pagrus auratus*) larvae. Aquaculture 194 (1–2), 137–150.
Penglase, S., Nordgreen, A., van der Meeren, T., Olsvik, P.A., Sæle, Ø., Sweetman, J.W., Baeverfjord, G., Helland, S., Hamre, K., 2010. Increasing the level of selenium in rotifers (*Brachionus plicatilis* 'Cayman') enhances the mRNA expression and activity of glutathione peroxidase in cod (*Gadus morhua* L.) larvae. Aquaculture 306, 259–269.
Polatajko, A., Jakubowski, N., Szpunar, J., 2006. State of the art report of selenium speciation in biological samples. Journal of Analytical Atomic Spectrometry 21, 639–654.
Rainuzzo, J.R., Reitan, K.I., Olsen, Y., 1997. The significance of lipids at early stages of marine fish: a review. Aquaculture 155, 103–115.
Rajkumar, M., Kumaraguru Vasagam, K.P., 2006. Suitability of the copepod, *Acartia clausi* as a live feed for seabass larvae (*Lates calcarifer* Bloch): compared to traditional live-food organisms with special emphasis on the nutritional value. Aquaculture 261 (2), 649–658.
Reitan, K.I., Rainuzzo, J.R., Øie, G., Olsen, Y., 1997. A review of the nutritional effects of algae in marine fish larvae. Aquaculture 155 (1–4), 207–221.
Ribeiro, A.R.A., Ribeiro, L., Sæle, O., Hamre, K., Dinis, M.T., Moren, M., submitted for publication. "Selenium supplementation enhances glutathione peroxidase activity and thyroid hormone production in Senegalese sole (*Solea senegalensis*) larvae." Aquaculture.
Rider, S.A., Davies, S.J., Jha, A.N., Clough, R., Sweetman, J.W., 2009a. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (*Oncorhynchus mykiss*) diet. Journal of Animal Physiology and Animal Nutrition (http://dx.doi.org/10.1111/j.1439-0396.2008.00888.x).
Rider, S.A., Davies, S.J., Jha, A.N., Fisher, A.A., Knight, J., Sweetman, J.W., 2009b. Supra-nutritional dietary intake of selenite and selenium yeast in normal and stressed rainbow trout (*Oncorhynchus mykiss*): implications on selenium status and health responses. Aquaculture 295 (3–4), 282–291.
Rodriguez, C., Perez, J.A., Izquierdo, M.S., Cejas, J.R., Bolanos, A., Lorenzo, A., 1996. Improvement of the nutritional value of rotifers by varying the type and concentration of oil and the enrichment period. Aquaculture 147 (1–2), 93–105.
Rodriguez, C., Perez, J.A., Diaz, M., Izquierdo, M.S., Fernandez Palacios, H., Lorenzo, A., 1997. Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. Aquaculture 150 (1–2), 77–89.
Rosenlund, G., Halldórsson, Ó., 2007. Cod juvenile production: research and commercial developments. Aquaculture 268 (1–4), 188–194.
Sasakura, C.T., Suzuki, K., 1998. Biological interaction between transition metals (Ag, Cd and Hg), selenide/sulfide and selenoprotein P. Journal of Inorganic Biochemistry 71, 159–162.
Schipp, G., 2006. The use of calanoid copepods in semi-intensive, tropical marine fish larviculture. Avances en Nutrición Acuícola VIII. 8th Int. Symp. Aquatic Nutrition, University Autón. Nuevo León, Monterrey.
Srivastava, A., Hamre, K., Stoss, J., Chakrabarti, R., Tonheim, S.K., 2006. Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): with emphasis on the water soluble fraction. Aquaculture 254 (1–4), 534–543.
Suhajda, Á., Hegóczki, J., Janzsó, B., Pais, I., Vereczkey, G., 2000. Preparation of selenium yeasts I. Preparation of selenium-enriched *Saccharomyces cerevisiae*. Journal of Trace Elements in Medicine and Biology 14 (1), 43–47.
van der Meeren, T., Olsen, R.E., Hamre, K., Fyhn, H.J., 2008. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture 274 (2–4), 375–397.
Wang, C., Lovell, R.T., 1997. Organic selenium sources, selenomethionine and selenoyeast, have higher bioavailability than an inorganic selenium source, sodium selenite, in diets for channel catfish (*Ictalurus punctatus*). Aquaculture 152 (1–4), 223–234.
Waschulewski, I.H., Sunde, R.A., 1988. Effect of dietary methionine on utilization of tissue selenium from dietary selenomethionine for glutathione peroxidase in the rat. Jounal of Nutrition 118 (3), 367–374.
Wilcox, J., Tracy, P., Marcus, N., 2006. Improving live feeds: effect of a mixed diet of copepod nauplii (*Acartia tonsa*) and rotifers on the survival and growth of first-feeding larvae of the southern flounder, *Paralichthys lethostigma*. Journal of the World Aquaculture Society 37 (1), 113–120.
Yamamoto, T., Teruya, K., Hara, T., Hokazono, H., Kai, I., Hashimoto, H., Furuita, H., Matsunari, H., Mushiake, K., 2009. Nutritional evaluation of rotifers in rearing tanks without water exchange during seed production of amberjack *Seriola dumerili*. Fisheries Science 75, 697–705.