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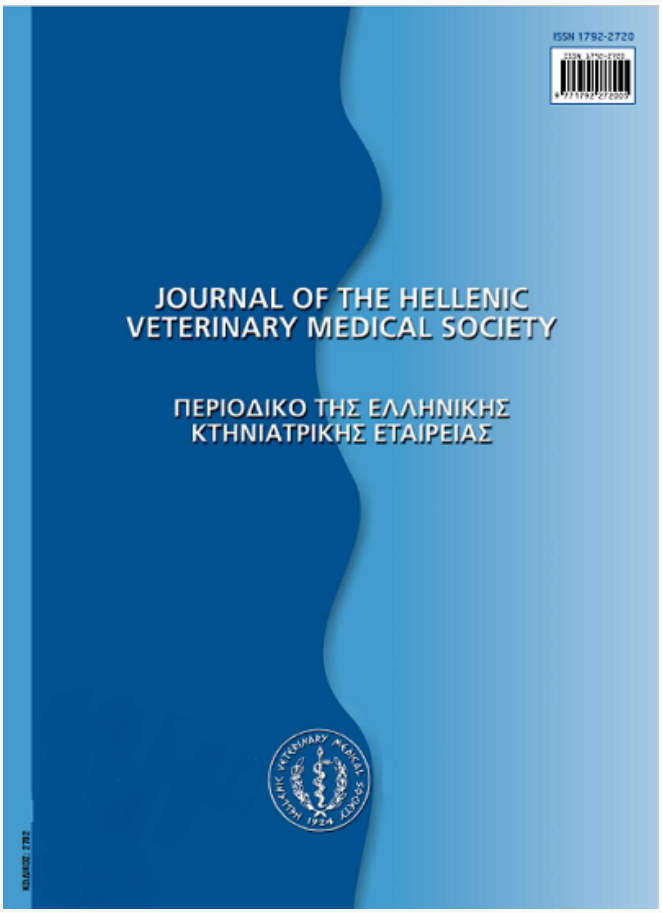

Total lipids content and fatty acids composition of the rotifer Brachionus plicatilis using artificial enrichments

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Research article
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Total lipids content and fatty acids composition of the rotifer *Brachionus plicatilis* using artificial enrichme nts.

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ABSTRACT. Secondary feeding with commercial lipids enrichments such as the microencapsulated diets Diet A (33.42±3.00%) and Diet B (55.88±3.5%) compared with the marine yeast type product Diet C(8.59±1.0%) as a method of increasing the total lipids and **ω**-3 PUFA content of rotifers, hence enhancing their nutritional value as live feed prey for fish larvae in hatcheries. The total lipids the rotifers was affected analogous to the levels of these components in the feeds showing its maximum percentage uptake within 4 hours from the enrichment (20.27±3.52, 26.64±3.91, 11.31±2.30 respectively). There was not any significant toxicity to the animals due to the diets during the 16 hours experiment. The DHA/EPA for Diet A(1.31), Diet B(0.42) and Diet C(absent) as well as the DHA/EPA/ARA ratios for Diet A(10.70 ± 1.60 / 8.18 ± 1.10 / 2.08 ± 0.20), Diet B(6.20 ± 2.30 / 14.60 ±1.00 / 1.12 ± 0.40) and Diet C (0 / 5.14 ± 3.40 / 1.30 ± 1.10) indicates that Diet A is closer to the suggested DHA/EPA/ARA optimal value 10/5/1 for marine fish larval growth.

Keywords: rotifers, *Brachionus plicatilis,* lipids, PUFA, enrichment diets, toxicity, microcapsules, marine yeast.

ΠΕΡΙΛΗΨΗ. Δευτερογενές τάϊσμα με εμπορικά εμπλουτιστικά λιπιδίων όπως οι μικροενκαψυλιωμένες τροφές Ενδιαίτημα Α (33.42±3.00%) και Ενδιαίτημα Β (55.88±3.5%) συγκρίθηκαν με το Ενδιαίτημα Γ (8.59±1.0%), το οποίο είναι τύπου θαλάσσια μαγιάς, ως μέθοδος αύξησης των ολικών λιπαρών και των ω-3 PUFA των τροχόζωων, έτσι ώστε να ενισχύσουν την διατροφική αξία της ζωντανών θηραμάτων των ιχθυονυμφών στους ιχθυογεννητικούς

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σταθμούς. Τα ολικά λιπίδια των τροχόζωων επιρεάζονται από τα επίπεδα των συστατικών αυτών στις τροφές έχοντας μια μέγιστη αναλογία πρόσληψης 4 ώρες μετά τον εμπλουτισμό (20.27±3.52, 26.64±3.91, 11.31±2.30 αντίστοιχα). Αυτό δεν έχει καμιά επίπτωση τοξικότητας στα τροχόζωα λόγω διατροφής κατά την διάρκεια των 16ωρου πειράματος. Η αναλογία DHA/EPA του Ενδιαιτήματος Α (1.31), Ενδιαιτήματος Β (0.42) and Ενδιαιτήματος Γ (απουσία) όπως επίσης και της αναλογίας DHA/EPA/ARA για το Ενδιαίτημα Α(10.70 ± 1.60 / 8.18 ± 1.10 / 2.08 ± 0.20), Ενδιαίτημα Β(6.20 ± 2.30 / 14.60 ±1.00 / 1.12 ± 0.40) και Ενδιαίτημα Γ (0 / 5.14 ± 3.40 / 1.30 ± 1.10) δείχνει ότι το Ενδιαίτημα Α είναι πλησιέστερο προς τις ενδεικνυόμενες DHA/EPA/ARA βέλτιστες τιμές 10/5/1 για την ανάπτυξη θαλάσσιων ιχθυονυμφών.

INTRODUCTION

Rotifers were successfully introduced as a live prey organism suitable for the culture of the early larval stages of the fish in the '60s by Japanese pioneers (Ito, 1960; Hirata and Mori, 1967; Hirano, 1969). There are several advantages to rotifers such as they are easy and cheap to cultivate in mass quantities on baker's yeast (Hirata and Mori, 1967; Kitajima et al., 1979; Hirata, 1980), they are of adequate size for the first stage of rearing of small fish larvae (Watanabe et al.,1983) and their high nutritional value and digestibility (Lubzens et al., 1985; Kestemont and Awaiss, 1989). In the following decades of the global aquaculture explosion, rotifers were used extensively as an early-starter live food (Hirata, 1980; Watanabe et al.,1983; Lubzens, 1987; Lubzens et al.,1989; Dhert et al., 2001) for a wide variety of very promising (at the time) potential marine finfish candidates for aquaculture, such as turbot (*Scophthalmus maximus*) (Bromley and Howel,1983); gobies (*Gobio gobio*) (Kestemont and Awais;1989); seabass (*Dicentrarchus labrax*) (Gatepouse and Luquet,1981); gillhead seabream (*Spaurus aurata*) (Chatain and Ounais-Guschemann, 1990; Divanach and Kentouri, 2000; Pousao-Ferreira et al., 2003); plaice (*Pleuronectes platessa*) (Howel, 1973; Bromley and Howell, 1983); and Ayu (*Plecoglossus altivelis*) (Oka et al.,1980; Teshima et al., 1987); Atlantic cod (Rosenlund and Halldorsson, 2007; Maehre et al., 2013) .

Despite efforts to substitute or eliminate the live feed stage in modern industrial practices (Chatain, 1997; Fernandez-Diaz and Yufera, 1997; Cahu and Zambonino Infante, 2001; Theodorou 2002) mainly due to its production cost and the instability of the available quantities, rotifers are still critical as a live feed organism. As live prey organisms of suitable size and palatability, stimulate the predation of the fish larvae, resulting to a better survival and fry quality (Pousao-Ferreira et al., 2003). The nutritional value of rotifers as live feed for fish larvae was studied intensively and continuously improved, as dietary value impacted larval growth and development (Oka et al., 1980; Rønnestad et al., 2013). Several rearing trials of rotifers fed with algal species and strains in various forms (fresh, frozen, dried, frozen-dried, concentrated, frozen concentrated, etc) have been carried out in order to support the population growth and survival of the rotifer cultures as well as improve the dietary value of *Brachionus plicatilis* (Scott and Baynes, 1978; Yufera and Pasqual, 1983; Yamasaki et al., 1984; Lubzens et al.,1995; Yufera and Navarro, 1995; Navarro and Sarasquete, 1998; Robert and Trintignac, 1997; Maruyama et al., 1997; Yoshimura et al., 1997; Tzovenis, et al., 2004; Palmtag et al., 2006; Tzovenis et al., 2009; Seychelles et al., 2009; Kotani et al., 2010). In addition, a range of artificial enrichments such as yeasts (Kitajima et al.,1979;Hirata, 1980, Kitajima et al., 1980a,b; Penglase et al.,2011), microcapsules (Jones et al., 1984; Walford and Lam, 1987; Langdon, 2003) and emulsions (Dhert et al., 1993; Haché and Plante, 2011) were continuously tested in an effort to improve and optimize the nutritional value (total lipids, fatty acids, trace elements, vitamins, amino acids) of the rotifers as they used as biocapsule-boosters to transfer nutrients and support fish larval growth (Lubzens et al., 1989; Coutteau and Sorgeloos, 1997; Rodríguez et al., 1996; Dhert et al., 2001; Lubzens and Zmora, 2003; Conceição et al., 2010).

Marine fish larvae have essential requirements for dietary ω-3 polyunsaturated fatty acids (Koven et al.,1990; Sargent et al., 1999 a,b, Tocher 2010), but rotifers are deficient in these, unless they are raised

on a lipid-rich diet (Fernandez-Reiriz et al., 1993; Naz, 2008; Demir and Diken, 2011a,b; Maehre et al., 2013). *Brachionus plicatilis* are commonly raised on baker's yeast, which does not supply adequate levels of ω-3 polyunsaturated fatty acids (PUFA). The fatty acid content of some microalgae species that can be used to feed rotifers may be adequate for this purpose (Ben-Amotz et al., 1987; Tzovenis et al., 2003a,b) but the quality coming out of large scale hatcheries is unreliable (Birkou et al., 2012). Several commercial artificial microencapsulated enrichment products has been developed to cover this gap. Rotifers were found to take up lipids from the microcapsules and increased their ω-3 PUFA content as a result of the incorporation of the 20:5ω3 and 22:6ω-3 (Walford and Lam, 1987). A feeding trial with two microencapsulated diets (Diet A and Diet B) of different manufacturing and nutrition formulas (Diet A and Diet B), is carried out in the present work in order to investigate their suitability for nutritional enhancement of the rotifers. The Diet A, following the manufacturer suggestions has a total lipids content about 30%. According to the Diet B product description, the enrichment diet contains fish oil (cuttlefish liver or cod fish oil) with high highly unsaturated fatty acids (HUFA) levels. This oil is capsulated with egg white peptide and has a 60% total lipids content of which 30% are HUFA (docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA)). In addition, a marine yeast type product (Diet C) with limited nutritional information available is tested for its efficiency as enrichment product.

The possible rotifers selectivity on certain fatty acids uptake was also investigated.

MATERIALS and METHODS

The schematic view of the feeding experimental procedure of the rotifers *Brachionus plicatilis* is presented in Figure 1. Details about the rotifers culture and the feeding trial are described below.

Rotifer culture

Rotifers (GS 74 strain) were cultured in a 75 L rectangular container at a room temperature of 26^{0}C, fed on baker's yeast (1gr/million) and maintained at a concentration 200 to 400 ind./mL in a 60 L total volume culture. Sufficient oxygen was provided by continuous mild aeration. Before harvesting, the air supply to the culture was stopped for a few minutes and bottom siphoning was used to remove any particles such as feces in order to prevent pollution and growth of ciliates. To control the water quality, the culture was concentrated daily at half of its volume and filtered sea water (0.2 μL) UV sterilised sea water was added to the initial volume.

Enrichment preparation

In order to enrich rotifers with the microencapsulated Diet A (containing 2400 X10^6 capsules), at a rate of 0.5 g/million rotifers, 1gr of the feed was diluted in 100 ml filtered (0.5 μ) UV sterilised sea water for 30 minutes under strong steering mixing then 30 ml was inoculated in each experimental flask (n=2). Similarly, 0.5 g microencapsulated Diet B was diluted in 50 mL filtered (0.5 μ) UV sterilised sea water. Then 20 mL of the solution (containing 0.2 g Diet B) was inoculated as feed in each flask with starved animals (n=2). Diet C was in a liquid form, and was given as a food, after a good shaking, at a rate of 4mL per million rotifers in the relevant experimental flasks.

Feeding trial

The 60 L rotifer culture was filtrated by a 40 μ sieve and gently washed with 0.5 μ filtered UV sterilized sea water. The sieve contents were gently poured (to avoid rafting of the rotifers) into a bucket then made up to 20L. Six x 20mL samples were counted to estimate the population of the rotifers, which was 217 ± 61 animals/mL. The culture was then reduced to 16L, at a concentration of 263 ± 56 animals/mL. No food added for 4h and 2 x 1mL samples were taken at the start and the end of this starvation period. 8 x 2 L conical experimental flasks were filled with the 2L each from the above suspended rotifer culture. Each flask was gently aerated by a glass pipette at a temperature of 18 ± 0.5 ^{0}C.

Six counts of 50μL were then taken to estimate the initial number of the rotifers per flask. These counts continued during the experiment in order to estimate the survival rate of the animals and the possible

toxicity effects of the diets, and took place prior to the harvesting of the animals for chemical analysis. As an earlier study by Budd (1989) indicated that a minimum of 5,000 rotifers would be required per sample for successful lipid extraction and analysis, after 4, 8 and 16h, 20mL and 30mL samples were removed from each flask for PUFA and total lipid estimation, respectively. The samples were gently poured into a 45μm sieve and washed with nearly 1L water to remove feces and uneaten food particles. The rotifers were pipetted with as little water as possible into 9mL aluminum-sealed glass vials, which had been rinsed with solvent. The vials for PUFA analysis were then almost filled with lipid extraction solvent, 2:1 chlorophorm:methanol(c:m), with butylated hydroxytoluene (BHT) as an antioxidant, and stored together with those for total lipids in a –20 ^{0}C freeze for subsequent analysis.

BIOCHEMICAL ANALYSIS

Total lipids analysis

The total lipids composition (as % dry weight) of the microencapsulated diets of known lipid content from the manufacturer were analysed in doublicate (n=2). More replicates (n=6) were carried out for the marine yeast type product as there is not any information about its lipids content.

Total lipids of the diets and the enriched rotifers were estimated colorimetrically by using a Test-Combination Total Lipids Kit from Boehringer Mannheim Gmbh Diagnostica (cat. No 124303). Analytically, 2 mL H_2SO_4 was added to test tubes containing 1mg dry weight samples weighed with the microbalance Cahn C-31. After shaking well and plugging with terylene wool, the mixture was stood in a boiling water bath for 10min. A standard series (usually triplicates) was prepared by adding 2 mL H_2SO_4 in tubes containing 0.05 mL of solution 1. The samplers were incubated as above, cooled in a cold water bath, and 0.10mL from each sample was inoculated into a dry test tube then 2.50 mL or 3 mL of solution 2 were added. After mixing well by using a shaker, they were kept at 20-25 ^{0}C for 30 min. The absorbance of sample and standard against a blank was measured spectrophotometrically in 530 nm. All counts took place within 30 min. Estimation of total lipids was completed using the following equation: Weight of lipids = (Optical Density (O.D.) sample/ Optical Density (O.D.) standards) x 0.5 mg % lipids = (Lipid Weight/ Sample Weight) x100.

PUFA analysis

Fatty acids composition of each diet and rotifer treatment was estimated as percentage of total lipids dry weight of the total lipids using gas liquid chromatography.

The determination of the fatty acid composition of the rotifer sample was carried out using the method of Folch et al. (1957).

Samples from the vials were inoculated into centrifuge tubes. To ensure a single solvent (c:m) phase, further chlorophorm: methanol 2:1 (+BHT) was added to give a solvent:sample ratio of 15:1v/w. The tube was covered with aluminum foil and left to extract at 4 ^{0}C for 20 minutes. The sample was then filtered through a No 4 filter paper that had been prewashed in chlorophorm. To the filtrate was added 0.2 volume of 0.017% m/w aqueous magnesium chloride. The sample was then decanted into centrifuge tubes and centrifuged at 800g, for 5 minutes (2000 r.p.m.) by using MSE Centaur 1 centrifuge.

The upper layer (phase) was removed by aspiration and discarded. A little Folch upper phase solvent system (chlorophorm:methanol water, 3:48:47 v/v/v) was carefully layered on the top of the lower phase and swirled very gently. The upper layer was removed by aspiration and the process was repeated.

The lower layer (phase) was carefully pipetted into a rotovaporator flask, taking care not to transfer any remaining upper layer. The solvent was evaporated in the rotorevaporator (Buchi) at 40^{0}C and the sample was taken up in a small known volume (1 ml approx.) of chlorophorm.

A drop of lipids extract was placed in a 5mL reactivial followed by 3 mL of 2:1 14% boron trisulphate in methanol:dichloromethane. The samples were flushed with nitrogen, mixed thoroughly and heated for 1 at 100^{0}C.Then the samples were allowed to cool and 2 vol. pentane followed by 1 vol. of water were added. The tip phase was pipetted off and transferred

into a vial covered with an aluminum foil cup; the extraction was repeated and the top phase was kept.

The supernatant was pipetted off and was evaporated to dryness with nitrogen. It was taken up in 3mL of hexane.

To separate the fatty acids methylesters (FAME) were used a Pye model 104 (Pye-Unicam, Cambridge, England) gas liquid chromatograph fitted with a flame ionization detector. FAME were identified by using cod liver oil as a reference standard (Ackman and Burgher 1965).

Statistical analysis

The survival of the rotifers populations fed on the different diets were estimated through MANOVA between sampling treatments (n=6) on each sampling time during the experiment.The analysis of variance (ANOVA) was applied to test for significant differences in lipid percentage dry weight in the rotifers-diet trials. Furthermore, Fisher's LSD test was applied to check which animal-diet trials differed from each other (Zar, 1999).

The fatty acids were estimated as percentages of the total identified fatty acids in each sample.

To estimate the lipid selection of animals, Ivlev's electivity index (Ei) (Ivlev, 1961) was calculated from the mean percentage of lipid items found in the animal body contents and the diets, as follows:

Ei=(pi-Pi)/(pi+Pi), where pi is the percentage of lipid item i in the animal body, and Pi is the percentage of lipid item i in the diet.

RESULTS

Toxicity test of the diets

None of the diets tested in this trial produced toxicity effects on the rotifer populations compared with the starved animals. The MANOVA between rotifer populations fed on different diets and the starved animals used as a control during the experiment indicates that the rotifers density do not have any significant difference between treatments at each sampling time ($F=4.26$, $df=2.126$ $p=0.016$). However there is a decrease of the animal density through the time of the experiment 16 h. This may be occurred due to the natural mortality of the population. Hence there is no toxicity effect of diets on *Brachionus plicatilis*.

Total lipids and fatty acids composition of the diets

The total lipids content of the diets used in this trial is shown in Table 1. The total lipids percentage (%) of the microencapsulated Diet A (DA) (33.42 ± 3.00%) and Diet B (DB) (55.88 ± 3.50%) founded close to the manufacturers specifications (30% Diet A and 60% Diet B) that were quite high compared with those of the marine yeast-type Diet C (DC) (only 8.59±1.04%). The ω-3 PUFA content of Diets A and B was 21.61% and 34.90%, respectively, while Diet C was 37.60%. The EPA content in Diet C was 36.88% and Diet B 19.92%, while in Diet A it was reduced to 4.60%. The DHA levels of the microencapsulated Diets A and B were 5.56% and 8.85%, respectively while there was only a trace (0.04%) of them in the marine yeast-type Diet C. The essential polyunsaturated acids (EPA) content in Diet C was 36.88% and Diet B 19.92%, while in Diet A it was reduced to 4.60%. It has also to be mentioned that the HUFA (28.77%) content of the Diet B (DHA (8.85%) + EPA (19.92%)) founded to be close to the HUFA (30%) specifications of the manufacturer.

Total lipids composition of the rotifers

Animals enriched with Diet B (RB) had the maximum percentage (%) of lipids content (25.78 ± 6.57), but it is not significant difference with those fed on Diet A (RA) (19.44 ± 2.92) ($p<0.05$). The lipids levels of RB and RA are significant different from the starved animals used as control (RS) (11.42± 2.49) indicating their uptake from the relevant diets ($p< 0.05$). In contrast, the rotifers fed on Diet C (RC) had poor lipid levels (10.46 ± 2.39), close to that of the starved rotifers RS (11.42± 2.49) ($p< 0.05$) (Table 2).

In all cases, the total lipid content of enriched rotifers was dependent on the total lipid content of the feed. The lipids absorption during the 16 h experiments are presented in Figure 2. The maximum lipids content of the enriched rotifers was 4 h after the enrichments with micro-encapsulated Diets A and B. These levels remained for 16 h of the experiment. In contrast animals fed with marine yeast haven't any increase of their lipids.

Fatty acid composition of the rotifers

Table 2 shows that the total PUFA content (%) of the rotifers fed on Diet A (RA) (32.70 ± 4.50%) and Diet B (RB) (31.50 ± 3.00%) are similar and have significantly improved compared with the starved animals (RS) 9.88 ± 0.90%. In contrast, Brachionus plicatilis fed on Diet C (RC) (12.90 ± 3.80%) had poor nutritional value, such as that of the starved rotifers used as Control (9.88 ± 0.90%). The RB have the highest EPA (20:5ω3) levels (14.60 ± 1.00%), followed by that of RA (8.18 ±1.10%). Non statistical differences were found between RC (5.15 ± 2.85%) and the control animals RS (2.61 ± 1.20 %). The DHA (22:6ω3) content in RA (10.70 ± 1.39%) was significantly higher than that of the RB (7.94 ± 3.10%), while it was absent in RC as in RS. The arachidonic acid ARA (20:4ω6) levels in animal treatments RA (2.08 ± 0.20%), RB (1.12 ± 0.40%) and RC (1.30 ± 1.10%) were similar with RS (1.83 ± 0.30%). The fatty acids selectivity absorbance by the boosted rotifers (RA, RB, RC) are presented in Figure 3. RB animals showed better ratios of ω-3/ω-6 (8.80) and EPA/ARA (13.03) than those of RA (3.30 and 3.94, respectively). Despite that, the DHA/EPA ratio was higher on the enriched rotifers with Diet A (1.31) than those fed on Diet B (RB) (0.42), and was absent in animals fed with Diet C (RC), as in the starved control (RS). Similar low values of ω-3/ω-6 were found in RC (1.60) and RS (1.40) animals.

DISCUSSION

The diets examined in this trial do not have any toxicity effects on the rotifer population. The gradual increase of the mortality during the 16 h sampling occurred due to the natural mortality of the population as there are not significant differences between fed and starved populations at the same sampling time.

The nutritional profile of the artificial diets showed that they affect the nutritional value of the enriched rotifers. Early investigations (Teshima et al., 1982; Walford and Lam, 1987) showed that rotifers can be reared and take up lipids from microcapsules. In this study, the total lipids content (%) of the boosted animals depended on the lipid content of the diet. Indeed a considerable and similar amount of lipid was gained by rotifers RA (19.44 ± 2.92%) and RB (25.78 ± 6.57%) fed with Diet A (33.42 ± 3.00%) and Diet B (55.88 ± 3.50%) microcapsules. Rotifers enriched with Diet A (RA) and Diet B (RB) took up 25% of the lipids in the diets. This indicates that the examined microencapsulated diets A and B has the same acceptability and stability. Rotifers (RC) fed with the marine yeast-type Diet C showed similar lipid levels to those of starved animals (RS). The poor lipids content (%) of the diet (8.59 ± 1.04%) does not help the animal increase its dietary value (10.46 ± 2.39).

The outcomes for the diets of the present feeding trial differs from the results of Demir and Diken (2011b) where rotifers fed with commercial products (Red Pepper Paste, Algamac and Spresso) of similar total lipid profile (13.50%, 56.20% and 32%, respectively) did not show any significant differences in the nutritional contents of the enriched rotifers. The authors suggested that the difference on the total lipid content of the commercial diets may not always result in a difference of the lipid content of the rotifers.

Rotifers in the present study uptake the total lipids from the diets up to its maximum within 4 hours and maintaining these levels up to 16 hours without significant changes of its concentrations. As a consequence, rotifers could be harvested after 4 hours after enrichment to used a live prey for fish larvae.

These results reveal also that the fatty acid composition of enriched rotifers is dependent on the fatty acid composition of the feed. Enriched animals with Diet B (RB) had the highest percentage (%) content of PUFA (32.70 ± 4.50%), similar (p=0.05) to those fed on Diet A (31.50 ± 3.00%). Rotifers fed on marine yeast-type Diet C (RC) had a limited or absent improvement on their nutritional value PUFA (12.90 ± 3.80%) compared with the starved animals (RS) used as a control (9.88 ± 0.90%). This is not surprising, as marine yeasts usually have poor HUFA constitution, and its recommended additional enrichment in order to be used as a feed for rotifers (Treece and Fox, 1993).

Walford and Lam (1987) showed that rotifers cultured with baker's yeast fed secondary with AR 121 Frippak microcapsules (21.70% total lipids) at a

concentration of 4 million caps/mL had a maximum ω-3 PUFA (17.10%) 8 h (rather than for 24 h and 48 h) from the start of the experiment. The present data indicates that animals fed with Diet A (33,42 ± 3,00 % total lipids) and Diet B (55,88 ± 3,50% total lipids) for 16 hours improve the ω-3 PUFA to higher levels (31.50 ± 3.00 % and 32.70 ± 4.50%, respectively) than the AR121 capsules.

The limited concentration of EPA in starved animals RS (2.61 ± 1.20%), positively changed in enriched rotifers with Diet B (RB) (14.6 ± 1.00%) and Diet A (RA) (8.18 ± 1.10%) compared to Diet C (RC) (5.14 ± 3 .40%). As a result of incorporation of these fatty acids (EPA) from boosters, the EPA-rich marine yeast Diet C (36.88%) shows poor accumulation compared to the microencapsulated Diet B (19.92%) and Diet A (4.60%).

In the case where the EPA levels were higher in enriched rotifers with Diet A than that of the relevant microcapsules, the DHA included in this booster could possibly be more easily transformed to EPA (Hache and Plante, 2011).

The ARA levels of the rotifers are not significantly affected by the nutritional enrichment in both treatments. Hache and Plante (2011) found similar results for Algamac 2000™ and DC Selco ™ enrichments, while DHA Protein Selco ™ as an ARA-rich product showed better ARA accumulation by increasing the rotifers' ARA levels. However. the animals fed with Diet B (13.03) showed the highest EPA/ARA ratio, followed by that of Diet A (3.94).

The DHA/EPA ratio is used as an indicator to select the optimum boosting strategy for larval feeds, as showed in a range of previous works (Estevez et al., 1999; Copeman et al., 2002; O'Brien-MacDonald; Plante et al., 2007; Hache and Plante, 2011). In the present trial, it was shown to be significantly higher in animals enriched with Diet A (1.31) rather than those fed on Diet B (0.42) and Diet C (absent), and was closer to the suitable 2/1 DHA/EPA ratio found in the natural food of a range of marine fish larvae (Sargent et al., 1997). In addition, a later study of Sargent et al.(1999a) suggested the DHA/EPA ratio as an indicator could be further improved if ARA is also taken into account (as a precursor, similar to EPA, of eicosanoids that are involved in a range of structural and physiological larval processes and demonstrated by Izquierdo et al., 2000; Bell and Sargent, 2003; Koven et al., 2003) by transforming the relation as DHA/EPA/ARA with optimal value 10/5/1 approximately for marine fish larval growth. In our case DHA was absent in the rotifers fed on Diet C (0 / 5.14 ± 3.40 / 1.30 ± 1.10), indicating its unsuitability as a direct booster for rearing marine fish. Rotifers fed on Diet B (6.20 ± 2.30 / 14.60 ±1.00 / 1.12 ± 0.40) had a higher EPA content than the indicative. As eicosanoids originated by EPA are less biologically active, a lower EPA/ARA ratio could result in better larval growth (Sargent et al., 1999) and increase the survival and resistance to the stress in fresh water fish (Czesny et al., 1999). Animals enriched with microcapsules of Diet A (10.70 ± 1.60 / 8.18 ± 1.10 / 2.08 ± 0.20) are closer to the indicative optimal value of the rate DHA/EPA/ARA, proving the suitability of Diet A as a booster for potential further use in marine fish nutrition. In this case, the higher percentage of the total lipid content of Diet B (55.88 ± 3.50%) compared to that of Diet A (33.42 ± 3.00%) may be not efficiently used by rotifers as shown in animals enriched with Diet B (RB) (25.78 ± 6.57%) and Diet A (RA) (19.44 ± 2.92%) and also discussed in similar studies (Demir and Diken; 2011).

CONCLUSION

The microencapsulated Diets A and B showed better enrichment results than the marine yeast-type Diet C. Rotifers fed with Diets A and B have similar total lipid content, while Diet A, according to the critical ratio values of DHA/EPA and DHA/EPA/ARA, is more suitable for secondary marine larval feeding.

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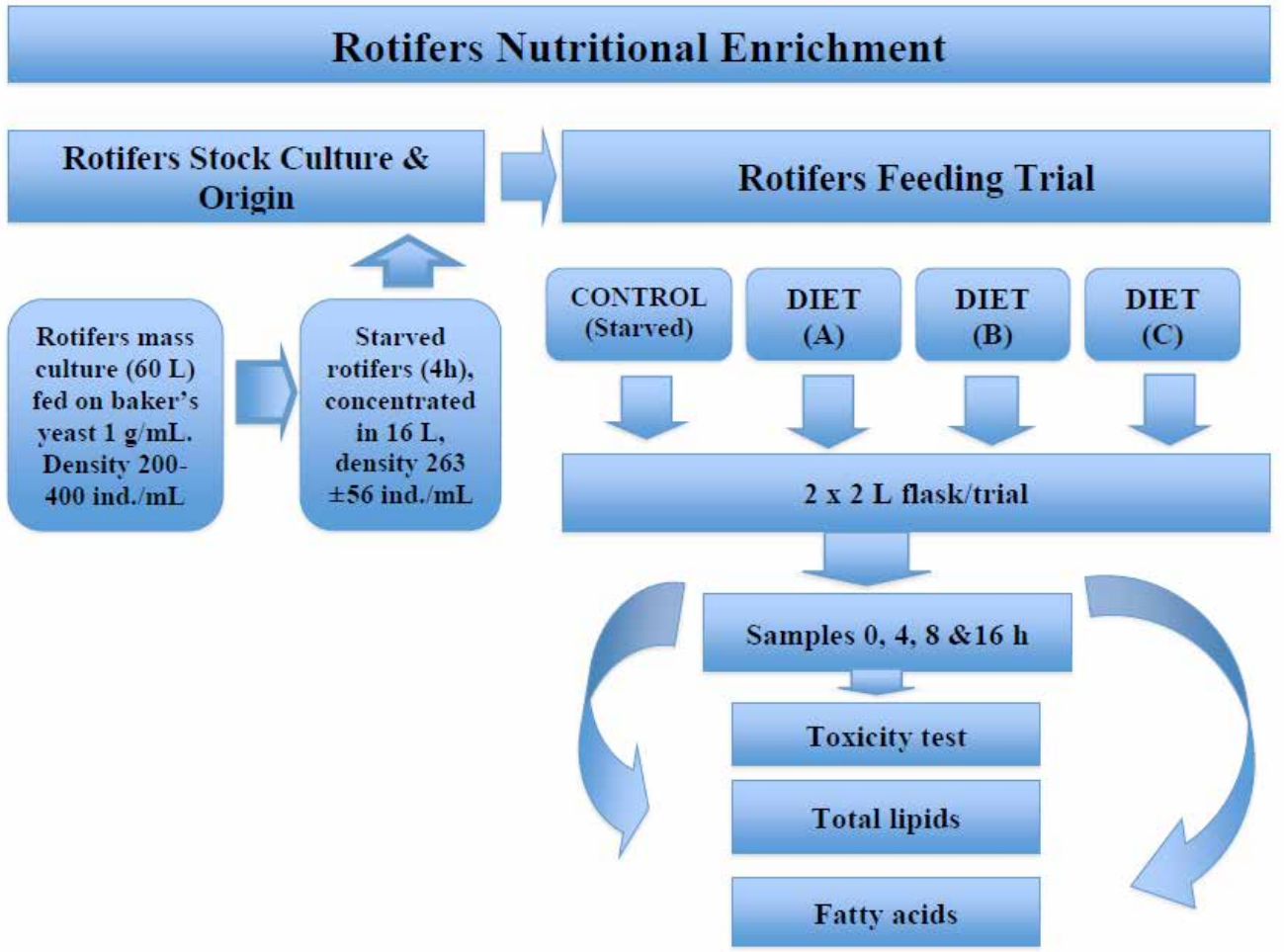

Figure 1. Schematic view of the experimental procedure of the nutritional enrichment of the rotifers *Brachionus plicatilis*.

Table 1. Total lipids (% dry weight) and fatty acids content (% dry weight) of the commercial rotifers enrichment diets (A, B and C) used during the feeding trial.

Enrichment	Type	Feeding Rate	Total Lipids (%)	Total Lipids (%)	ω-3 PUFA (%)	DHA (%)	EPA (%)	ARA (%)	DHA/ EPA	EPA/ ARA
Diet A	microcapsules	0.50 g/million rotifers	30	33.42±3.00 (n=2)	21.61	5.56	4.60	1.35	1.20	3.40
Diet B	microcapsules*	0.50 g/million rotifers	60	55.88±3.50 (n=2)	34.90	8.85	19.92	0.69	0.44	28.86
Diet C	marine yeast	4.00 mL/ million rotifers	not available	8.59±1.04 (n=6)	37.60	0.04	36.88	6.41	0.001	5.75

* *total HUFA (DHA, EPA): 30% of the total lipid content according to the manufacturer*

Table 2. Fatty acid compositions (% mass of total fatty acid) of the starved rotifers (RS) *Brachionus plicatilis* (% mass of total fatty acid) enriched with a range of microencapsulated Diet A (RA) and Diet B (RB) and a marine yeast product Diet C (RC), respectively, during the 16h feeding experiment (n=3).

	Treatments					
Fatty Acids	RS	RA	RB	RC	Sig.	LSD test groups
14:00	3.75(2.40)	3.26(1.10)	5.40(1.40)	2.12(1.10)	ns	RS=RA=RB=RC
15:00	0(00)	0.23(0.40)	0(0)	0.53(0.90)	ns	RS=RA=RB=RC
16:00	7.40(5.70)	11.50(1.60)	15.20(5.80)	15.2(2.30)	ns	RS=RA=RB=RC
17:00	0.00	0.00	0.00	0.00		
18:00	7.80(1.30)	4.32(0.80)	3.17(1.80)	6.62(0.90)	*	RS=RC>RC=RA>RA=RB
19:00	0.00	0.00	0.00	0.00		
20:00	0.00	0.00	0.00	0.00		
ΣFA	18.90(2.30)	19.30(2.80)	23.80(8.50)	24.50(5.10)	ns	RS=RA=RB=RC
16:1ω7	26.30(2.60)	11.9(0.90)	14.80(3.80)	21.90(1.60)	*	RS=RC>RA=RB
18:1ω7	10.60(2.00)	7.24(1.70)	8.12(0.50)	13.20(4.50)	ns	RS=RA=RB=RC
18:1ω9	26.60(1.30)	21.40(0.90)	12.40(1.70)	22.50(1.40)	*	RC<RS>RC=RA>RB
20:1ω7	0.00	0.06(0.10)	0.00	0.70(1.20)	ns	RS=RA=RB=RC
20:1ω9	6.01(2.70)	5.62(0.80)	0.00	3.82(1.50)	ns	RS=RA=RB=RC
22:1ω11	1.36(2.30)	1.83(0.20)	0.00	0.17(0.20)	ns	RS=RA=RB=RC
ΣMUFA	71.0(1.70)	48.10(0.60)	38.40(4.40)	62.40(7.00)	*	RS>RC>RA>RB
18:2ω6	2.23(1.00)	5(0.50)	2.22(0.50)	3.63(0.20)	*	RS=RB<RC<RA
18:3ω3	1.70(0.70)	1.54(1.10)	0.93(0.30)	0.49(0.40)	ns	RS=RA=RB=RC
18:4ω3	1.50(0.30)	2.38(1.10)	6.08(2.00)	2.38(1.50)	*	RS=RA=RC<RB
19:3ω3	0.00	0.00	0.00	0.00		
19:4ω3	0.00	0.00	0.00	0.00		
20:2ω6	0.00	0.08(0.10)	0.00	0.00	ns	RS=RA=RB=RC
20:4ω6 (ARA)	1.83(0.30)	2.08(0.20)	1.12(0.40)	1.30(1.10)	ns	RS=RA=RB=RC
20:5ω3 (EPA)	2.61(1.20)	8.18(1.10)	14.6(1.00)	5.14(3.40)	*	RS=RC<RA=RC<RB
22:4ω6	0.00	0.23(0.30)	0.00	0.00	ns	RS=RA=RB=RC
22:5ω3	0.00	1.36(0.50)	1.46(0.40)	0.00	*	RS=RC<RA=RB
22:6ω3 (DHA)	0.00	10.70(1.60)	6.20(2.30)	0.00	*	RS=RC<RA=RB
ΣPUFA	9.88(0.90)	31.5(3.00)	32.70(4.50)	12.90(3.80)	*	RS=RC<RA=RB
Σω3	5.81(0.70)	24.1(2.40)	29.30(5.10)	8.01(2.70)	*	RS=RC<RA=RB
Σω6	4.06(1.30)	7.39(0.80)	3.35(0.60)	4.93(1.20)	*	RS=RC=RB<RA
ω3/ω6	1.40	3.30	8.80	1.60		
DHA/EPA	0.00	1.31	0.42	0.00		
EPA/ARA	1.43	3.94	13.03	3.96		
Total Lipids (%)	**11.42(2.49)**	**19.44(2.92)**	**25.78(6.57)**	**10.46(2.39)**		

Mean value and standard deviation (in brackets) of fatty acids percentage; Sig: statistically significant difference (*), no statistically significant difference (ns) by ANOVA at P=0.05; LSD test groups: homogeneous group by post hoc LSD test at P=0.05. ΣMUFA, ΣPUFA and ΣFA are the sum of MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids) and FA (fatty acids), respectively. Total lipids are given as mean values of percentage dry weights.

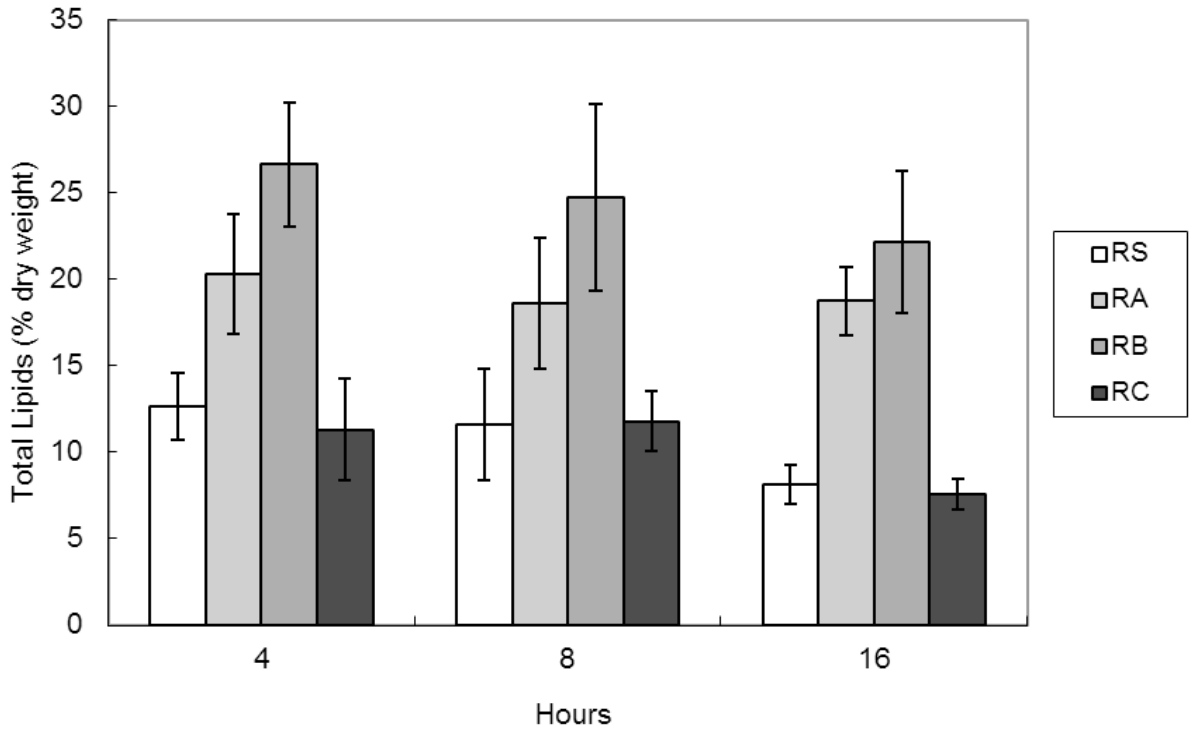

Figure 2. Total lipids content (% dry weight) of the starved (RS) rotifers used as control and enriched rotifers (RA, RB and RC) with Diet A, B and C respectively (n= 3).

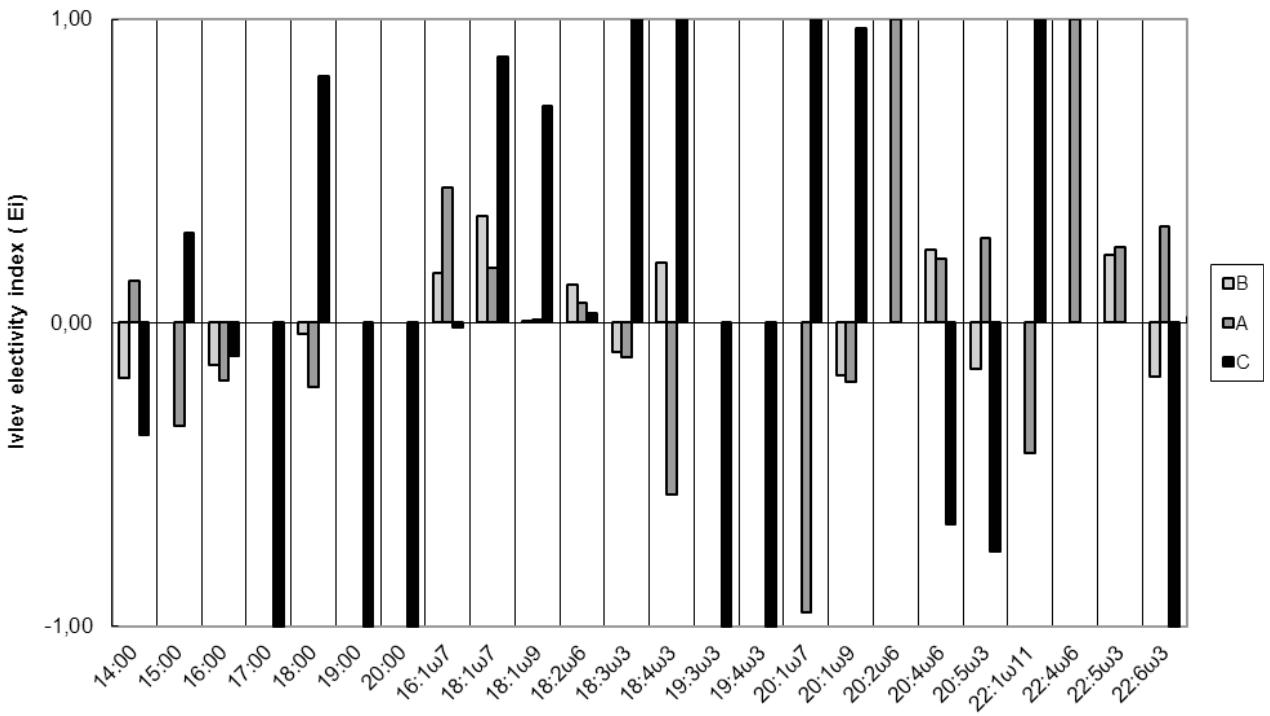

Figure 3. Fatty acids selectivity index of the enriched rotifers RA, RB and RC with Diet A, B and Diet C, respectively.

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