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Aquaculture 231 (2004) 469–487
Aquaculture
www.elsevier.com/locate/aqua-online

A method for radiolabeling *Artemia* with applications in studies of food intake, digestibility, protein and amino acid metabolism in larval fish

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Received 4 August 2003; received in revised form 7 September 2003; accepted 9 September 2003

Abstract

The present paper describes a method for radiolabeling *Artemia* that has potential applicability in the study of food intake, *Artemia* digestibility, protein and amino acid (AA) metabolism in early stages of marine fish larvae. *Artemia* nauplii were labeled by adding a uniformly labeled ^{14}C protein hydrolysate to its hatching or enrichment media. Both newly hatched and enriched nauplii assimilated rapidly the radiolabel and a very high proportion of the uptaken label was incorporated into the TCA precipitate (mostly protein) fraction after a short period of time — 84–91%, at the end of the hatching period and 81–87%, after 12 h of enrichment. In addition, it was observed that the amount of radiolabel per *Artemia*, as well as its distribution between the TCA precipitate and soluble (free AA) fractions, was relatively stable (up to 8 h) in conditions simulating transfer to larval feeding tanks. During the radiolabeling trials a substantial amount of the total initial radioactivity in the media was not recovered at the end of the hatching or enrichment period (38% and 29%, respectively). An experiment was designed to study the release of the radiotracer into the atmosphere, as $^{14}CO_2$. It was concluded that although the *Artemia* catabolism may be responsible for some release of the radiolabel into the atmosphere, bacterial activity in the enrichment media has a major impact, being responsible for the loss of 19.2% of the total initial radiolabel from the system. The digestibility and transfer rates of the labeled nutrients from *Artemia* to fish larvae was analysed "in vivo" by feeding radiolabeled newly hatched nauplii to herring (*Clupea harengus*) larvae and following the metabolic fate of the label. The results revealed a high digestibility (around 60%) of the *Artemia*, with 20% being retained in the body and 39% catabolised by the larvae, 24 h after feeding. The digestion process and subsequent absorption of the digested radiolabeled nutrients were quite

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doi:10.1016/j.aquaculture.2003.09.005

rapid, given that 40% was recovered in the body only 2 h after feeding and 6% was found in the CO_2 trap at this time.

Keywords: *Artemia*; Radiolabeling; Amino acids; Protein; Metabolism; Digestibility; Larval nutrition

1. Introduction

Satisfying the quantitative and qualitative nutrient requirements of the larval stages of marine fish with commercial potential is still a major constraint to their successful culture (Fyhn, 1989; Sargent et al., 1997). Although a significant amount of work has been carried out on the essential fatty acid requirements (Watanabe, 1982; Sargent et al., 1997), considerably less research has focused on the study of protein and amino acid (AA) metabolism, as well as on the quantitative requirements of the major nutrients (e.g. protein/lipid ratios) after the start of exogenous feeding. Amino acids are important catabolic substrates after the onset of first feeding and may account for 60% or more of the energy expenditure (Rønnestad et al., 1999). In addition, as the rapid growth rate of fish larvae (10–100% per day) primarily depends on an increase in body muscle mass through protein synthesis, it follows that there is a high dietary requirement for AA (Conceição, 1997; Rønnestad et al., 1999).

On the other hand, studies looking at the effect of diet composition on the ingestion rate of early larval stages are also lacking. Knowledge of the factors that control food intake in larva is incomplete and its study has been hampered by the small size of both the larvae and the food particles. In juvenile and adult fish several methodologies have been developed to quantify food intake. Although there are still limitations associated with these methodologies (Jobling et al., 2001), considerable progress has been made in the study of the effect of dietary protein and lipid levels, in terms of energy and/or essential nutrient content, on the regulation of food intake (Kaushik and Médale, 1994). Food intake studies are more easily conducted in juvenile fish as they are fed inert formulated diets, where it is simpler to manipulate its composition and quantify its consumption. However, the intensive larval rearing of the majority of marine fish continues to be based on the provision of live food such as *Artemia* sp. nauplii during the early larval stages (Cahu and Zambonino Infante, 2001).

Artemia is a continuous non-selective filter feeder (Reeve, 1963), which has aided the development of enrichment methodologies designed to improve its nutritional profile (Van Stappen, 1996a). On the other hand, nutrients can also be absorbed during the hatching process. This is due to the fact that aerobic metabolism, during hatching, promotes the conversion of the nutritive reserves of the cyst into glycerol, whose accumulation induces the absorption of water and its dissolved solutes into the embryo, which ultimately leads to the physical rupture of the cyst chorion (Clegg, 1964; Van Stappen, 1996b). The present work makes use of these particular characteristics of *Artemia* cysts and nauplii to develop a method for radiolabeling *Artemia* that can be applied to study food intake, digestibility, protein and AA metabolism during the early stages of marine fish larval development.

Artemia nauplii were labeled by adding a uniformly labeled [U-^{14}C] protein hydrolysate to its hatching or enrichment media, where the efficiency of incorporation, stability and distribution of the label was determined. In addition, the digestibility of the labeled nutrient was analysed "in vivo" by feeding radiolabeled newly hatched nauplii to herring (*Clupea harengus*) larvae and following its metabolic fate.

2. Materials and methods

Artemia cysts (EG type, Great Salt Lake, INVE, Belgium) were decapsulated and incubated during 24 h in 1 l cylindrical–conical plastic containers, under standard conditions (Van Stappen, 1996b). The newly hatched nauplii were harvested and washed on a 150-μm plankton net, transferred to clean seawater and their concentration was determined by counting the nauplii in 1 ml samples under a binocular microscope. Newly hatched nauplii were stocked (200 nauplii ml^{-1}) in 200 ml cylindrical–conical glass containers containing vigorously aerated seawater (33 ± 1 g/l), at 28 ± 1 °C. The standard enrichment emulsion ICES 50 (International Council for the Exploration of the Sea, ICES, Working Group on the Mass Rearing of Juvenile Fish) was used to enrich the nauplii for a period of 12 h, with a single dose of 0.3 g/l.

2.1. Radiolabeling of Artemia cysts during hatching

In this experiment, 0.3 g of decapsulated *Artemia* cysts were hatched in a single cylindrical–conical glass container containing 40 ml of seawater, to which 300 μl of a [U-^{14}C] protein hydrolysate (CFB25, 1.85 MBq/ml, Amersham Pharmacia Biotech, UK) was added at the start of the hatching period. The total amount of radiolabel in the system was determined from five 200-μl samples of the incubation media (including *Artemia* cysts and nauplii) that were placed in 20 ml scintillation vials (Packard Bioscience), at the start of hatching and every 4 h intervals afterwards. At 4 h and in the following sampling times, a 4-ml sample of the incubation media was transferred into a beaker and diluted (2 ×) with clean seawater. From this volume, four variable volume sub-samples were removed and the number of cysts and newly hatched *Artemia* were counted, rinsed in distilled water and transferred to a clean scintillation vial. The separate collection of non hatched cysts and newly hatched nauplii (four samples of each) was carried out after turning off the aeration, while illuminating the beaker from above. This allowed the newly hatched and actively swimming nauplii to remain in the upper water column; these were collected using a Pasteur pipette, washed and placed into three Eppendorf vials to which 500 μl of 6% trichloracetic acid (TCA) was added. The decapsulated nonhatched cysts which were on the bottom of the flask were treated in the same way. The remaining volume of incubation media was filtered and three 1-ml samples were transferred to clean scintillation vials. The *Artemia* (cysts and newly hatched nauplii) collected in the plankton net was thoroughly washed with distilled water and transferred to three Eppendorf vials, to which 500 μl of 6% TCA was added. After rotating the vials for 24 h at 4 °C (Rotamix, Heto, UK), each Eppendorf was vortexed and centrifuged (14,000 rpm) at 4 °C, and the TCA soluble supernatant pipetted into a clean scintillation vial. This was repeated twice with two

washings of 500 μl of 6% TCA, to ensure complete recovery of the TCA soluble fraction. The TCA precipitate was transferred to a clean scintillation vial, to which 1 ml of tissue solubiliser (Solvable, Packard Bioscience) was added, as also to the vials containing incubation media with *Artemia* and to those containing only *Artemia*. These vials were then placed in an oven, at ca. 50 °C, for 2–3 h to solubilise the samples. After cooling, 10 ml of scintillation cocktail (Ultima Gold, Packard Bioscience) was added to each vial. In vials containing only water, 10 ml of Ultima Gold XR (Packard Bioscience) were added immediately. Samples were allowed to sit at room temperature for at least 1 h before being counted twice (disintegrations per minute—dpm) in a Tri-Carb 2300 (Packard) liquid scintillation counter. The amount of label found in the TCA precipitate and soluble fractions was expressed in terms of percentage of total counts.

2.2. Radiolabeling of Artemia nauplii during enrichment

In this trial 12,000 newly hatched *Artemia* nauplii were enriched once in 60 ml of seawater, to which 150 μl of the [U-^{14}C] protein hydrolysate was added. Five 1-ml samples were collected from the enrichment media, at time 0 and every 2 h afterwards, up to 12 h. Samples of enrichment media with or without nauplii and of counted or not counted (for TCA extraction) filtered and washed nauplii alone were collected and prepared as described above.

2.3. Artemia radiolabel stability

To test whether the *Artemia* composition would remain stable, i.e., without loss of the incorporated radiolabel after transfer to the larval rearing tanks, *Artemia* that had been radiolabeled during hatching or enrichment were washed and stocked in 200 ml of clean and well aerated seawater, at room temperature. The radioactivity in the whole *Artemia*, TCA precipitate, TCA soluble fraction and in the re-suspension seawater were determined at time zero and every 2 h afterwards, up to 8 h post hatching or enrichment. At each interval, four (after enrichment) or five (after hatching) replicates of 1 ml each were collected. The number of *Artemia* per sample were counted and solubilised in scintillation vials with 2 ml of Solvable, as mentioned previously. At the same time three 1-ml samples of filtered hatching or enrichment media were also collected. In addition, the filtered and washed *Artemia* were collected into three Eppendorf vials with TCA as outlined above. The samples were processed for scintillation counting as previously described. The amount of radiolabel in *Artemia* was calculated by subtracting the counts in 1 ml of filtered seawater from the counts in 1 ml of seawater containing *Artemia*, divided by the number of nauplii per milliliter.

2.4. Lipid extraction of the TCA precipitate

In order to verify whether some of the labeled free amino acids (FAA) were converted into lipid by the *Artemia*, therefore contributing to the counts found in the TCA precipitate fraction following a TCA extraction, a lipid extraction of the TCA precipitate was conducted, using a method modified from Bligh and Dyer (1959). *Artemia* metanauplii

were labeled as described above, over a period of 14 h. At the end of this period, three samples of *Artemia* were collected into eppendorfs and a 24-h TCA extraction was performed, as previously mentioned. The TCA precipitate fraction was homogenised during 3 min using a pellet pestle homogeniser (Kontes Glass Company, NJ, USA), after the addition of 0.8 ml of distilled water. Afterwards, 2 ml of methanol, 2 ml of chloroform and 1 ml of distilled water were added and the samples were vortexed a few times and kept at 4 °C overnight. After centrifugation and phase separation, the methanol + water, chloroform and the remaining precipitate were recovered and the counts in each fraction were determined, after evaporation of the solvents, through scintillation counting.

2.5. Artemia metabolism during enrichment

A part of the radiolabel that was added to the media at the start of hatching or enrichment could not be accounted for at the end of the radiolabeling period. In order to determine whether a loss of radiolabel as $^{14}CO_2$ into the atmosphere occurred from *Artemia* metabolism or bacterial activity, an experiment was conducted using metabolic chambers consisting of sealed incubation vials connected to a metabolic trap in which the CO_2 diffused into the air during *Artemia* incubation is retained by conversion to HCO_3^- (Rønnestad et al., 2001). Four treatments were tested: (1) *Artemia* enriched under standard conditions (described above); (2) *Artemia* enriched in disinfected seawater; (3) standard enrichment media without *Artemia*; and (4) enrichment media with disinfected seawater without *Artemia*. Seawater was disinfected with the antibiotic gentamicin sulphate (200 μl per 100 ml of seawater) 24 h prior to the trial. Eight replicates of 5 ml each were transferred to incubation vials connected through a plastic capillary to a metabolic trap containing 16 ml of KOH 0.5 M. Aeration to the incubation vials was provided through a syringe needle attached to a capillary and connected to an air supply. After 12 h, the content of the incubation vials was filtered and the *Artemia* was collected, rinsed and transferred to a clean vial, while the filtered media was resealed and returned to the incubation system. At this time, 1 ml of hydrochloric acid (HCl 0.1 M) was gradually added, resulting in a progressive decrease of pH causing the rapid diffusion of any remaining CO_2 from the water into the trap. The sampled *Artemia* were solubilised by adding 2 ml of Solvable, after which 10 ml of Ultima Gold was added. To the vials containing the incubation water and the KOH (metabolic trap), 10 ml of Ultima Gold XR was also added. The samples were counted on a liquid scintillation counter and the results calculated as a percentage of the counts found in each compartment in relation to the total counts (sum of all compartments—water, metabolic trap and *Artemia*).

2.6. Digestion and absorption of radiolabeled Artemia nauplii by herring larvae

An "in vivo" study was conducted to analyse the digestibility, absorption and metabolism of the radiolabeled *Artemia* (predominantly labeled protein and FAA) ingested by Atlantic herring (*C. harengus*) larvae. The experimental larvae (31–33 days after first feeding; DAFF) were reared at the High Technology Center of the University of Bergen and fed on natural zooplankton (Høie et al., 1999). Two days prior to the experiments larvae were collected from the rearing tanks, stocked in 3 l buckets (10 larvae l^{-1}) and

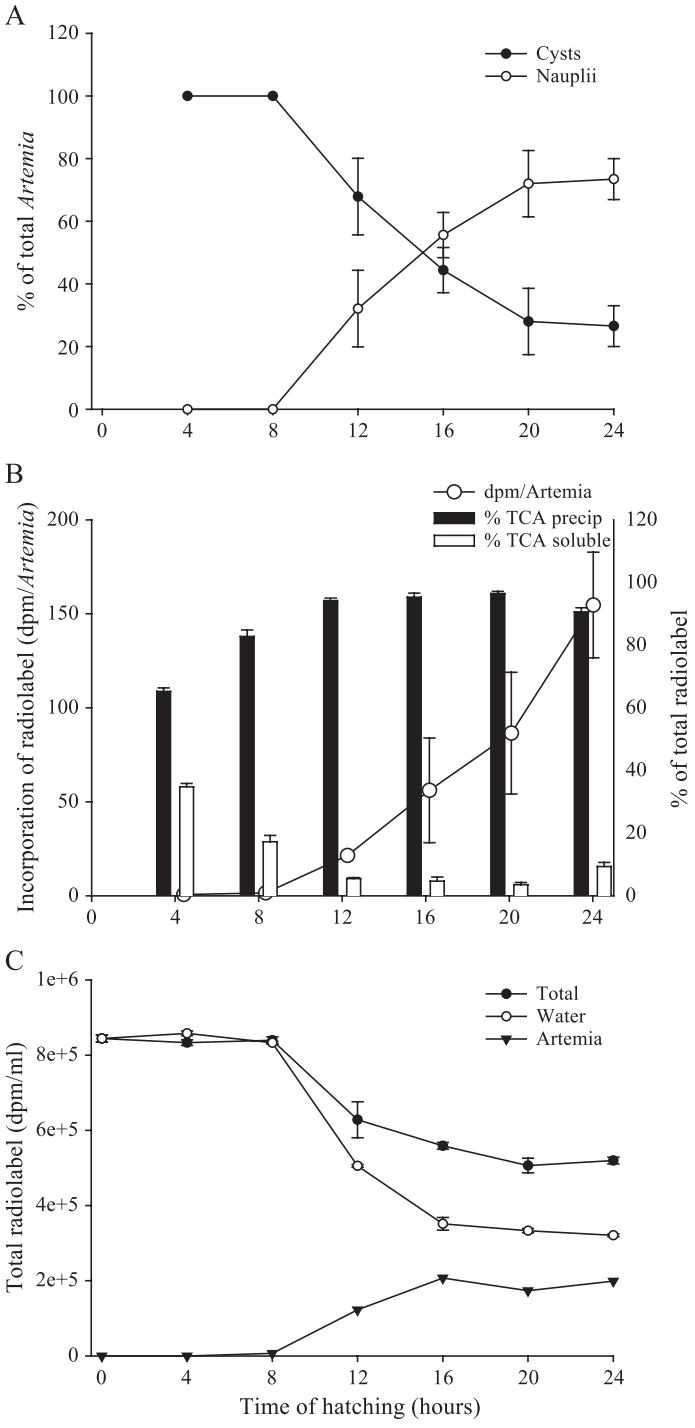
maintained in a temperature-controlled room at 12 ± 1 °C. The larvae were deprived of food 48 h prior to the experiment. *Artemia* nauplii were radiolabeled during hatching, using the methodology described above. At the end of the 24 h hatching period, washed *Artemia* nauplii were introduced into the larval containers (ca. 5 *Artemia* ml^{-1}) and the larvae were allowed to feed. If the transparent larvae, from periodic monitoring, showed the presence of nauplii in their digestive tract, they were removed from the bucket and transferred to a 500-ml container with clean seawater, where they were allowed to swim for approximately 1 min, to rinse any contaminating radiolabel. These larvae were then transferred (with as little water as possible) to an individual incubation chamber containing 8 ml of seawater, which was then sealed and connected to a metabolic trap (8 ml KOH 0.5 M), as described by Rønnestad et al. (2001). A gentle airflow was introduced into the vial above the incubation water, to avoid mechanical disturbance of the larvae. Not more than 30 min elapsed between start of feeding and transfer to the metabolic chambers. Larvae sampled from the vials in replicates of 6 at 2, 4, 6 and 24 h were rinsed in clean seawater and the gut separated from the body by dissection. Body and gut were placed in separate scintillation vials and solubilized in 1 ml of Solvable, after which 10 ml of scintillation cocktail was added. The incubation water and the $KOH-CO_2$ (metabolic trap) were prepared as previously outlined and the radioactivity in all samples was then counted. As in previous trials, results are presented as percent dpm found in the individual compartments (gut, body, water, metabolic trap) in relation to total dpm.

3. Results

3.1. Radiolabeling of Artemia cysts during hatching

The first newly hatched nauplii were detected at 12 h and hatching increased sharply up to 20 h, after which it stabilized at about 72% of the population (Fig. 1A). The radiolabel incorporation was analyzed separately in decapsulated nonhatched cysts and in newly hatched nauplii but, as it did not differ, Fig. 1 represents a homogeneous sample of the whole population. A gradual increase in radiolabel incorporation could be seen over time, becoming more marked after 12 h (Fig. 1B). At the end of the hatching period each *Artemia* had incorporated, in average, 154.7 ± 28.1 dpm. A high proportion (65.2 ± 1.1%) of the labeled protein hydrolysate was rapidly incorporated into the TCA precipitate fraction of the *Artemia* 4 h after start hatching, which increased steadily to 96.5 ± 0.7% at 20 h, decreasing slightly to 90.6 ± 1.3% at the end of the 24 h hatching period. Sampling of the whole media (total radiolabel in the system) showed that at the end of the hatching period the radiolabel in the system had been reduced to only 62% of the initial value (Fig.

Fig. 1. Radiolabeling of *Artemia* during hatching. (A) Hatching (% of newly hatched nauplii and % of nonhatched cysts) during the 24-h incubation period (mean ± S.D. of pooled samples, $n = 4$). (B) Radiolabel incorporation in *Artemia* cysts and nauplii (dpm/*Artemia*) (mean ± S.D. of pooled samples, $n = 4$) and in the TCA precipitate (mostly protein) and soluble (mostly FAA) fractions (% of total radiolabel) (mean ± S.D. of pooled samples, $n = 3$), over the hatching period. (C) Total amount of radiolabel (dpm/ml) in the whole system, including incubation water and *Artemia* (mean ± S.D. of pooled samples, $n = 5$).


A
Cysts
Nauplii
% of total Artemia
120
100
80
60
40
20
0
0
4
8
12
16
20
24
B
dpm/Artemia
% TCA precip
% TCA soluble
Incorporation of radiolabel (dpm/Artemia)
% of total radiolabel
200
150
100
50
0
120
100
80
60
40
20
0
C
Total
Water
Artemia
Total radiolabel (dpm/ml)
1e+6
8e+5
6e+5
4e+5
2e+5
0
Time of hatching (hours)

1C). At this time, 38% of the total radiolabel recovered in the system was incorporated by *Artemia*, while the remaining 62% remained in the water. No major radiolabel loss or incorporation into *Artemia* occurred in the first 8 h but a rapid transfer from the incubation water into *Artemia* was noted afterwards, particularly up to 16 h, after which it appeared to stabilize.

3.2. Radiolabeling of Artemia nauplii during enrichment

A gradual increase was observed in the amount of radiolabel incorporated by *Artemia* nauplii, being this more marked between 2 and 6 h (Fig. 2A). At the end of the enrichment period *Artemia* nauplii had incorporated, in average, 85.9 ± 10.4 dpm. As in the previous case, the radiolabel was incorporated very rapidly into the TCA precipitate , with $71.4 \pm 3.8\%$ being already in this fraction after 2 h, increasing slowly up to $86.6 \pm 0.0\%$ at the end of the enrichment. The amount of radiolabel in the system was also monitored and it was determined that 29% of the radiolabel initially added to the enrichment media was no longer present in the system after 12 h and could not be accounted for (Fig. 2B). At the end of the enrichment 73% of the recovered radiolabel was still in the enrichment media, while 27% had been incorporated by *Artemia*.

3.3. Artemia radiolabel stability

The *Artemia* radiolabel content after transfer to clean water, as well as the amount of radiolabel being released into the clean seawater after hatching and enrichment are shown in Figs. 3 and 4, respectively. Considerable variability was noted in the radiolabel incorporation, particularly of newly hatched nauplii (Figs. 3A and 4A). In both cases, although more pronounced in the newly hatched nauplii, a small decrease in the radiolabel content of the *Artemia* was observed, together with a concomitant increase in the amount of radiolabel in the re-suspension water (Figs. 3B and 4B). It should be noted that when *Artemia* was radiolabeled during hatching a higher amount of radiolabel was already present in the re-suspension water at time zero (Fig. 3B). In addition, the increase in radiolabel concentration in the re-suspension water (Figs. 3B and 4B) appears more dramatic than the decrease in *Artemia*, as a consequence of the data being expressed in terms of activity of radiolabel per ml of water (containing several *Artemia*) in the first case and per individual *Artemia* in the second. As for the distribution of the radiolabel between the TCA precipitate and soluble fractions, it remained relatively constant over time, with only a very slight increase in the precipitate and a concurrent minor decrease in the soluble fraction (Figs. 3A and 4A).

3.4. Lipid extraction of the TCA precipitate

Quantification of the label distribution in the radiolabeled *Artemia* metanauplii, following a TCA and a lipid extraction, revealed that $3.6 \pm 0.27\%$ of the total label (or $4.0 \pm 0.29\%$ of the TCA precipitate counts) was found in the lipid (chloroform) fraction, $85.4 \pm 0.47\%$ in the remaining TCA precipitate (mostly protein) and 11.1 ± 0.56 % in the TCA soluble (mostly FAA) fraction.

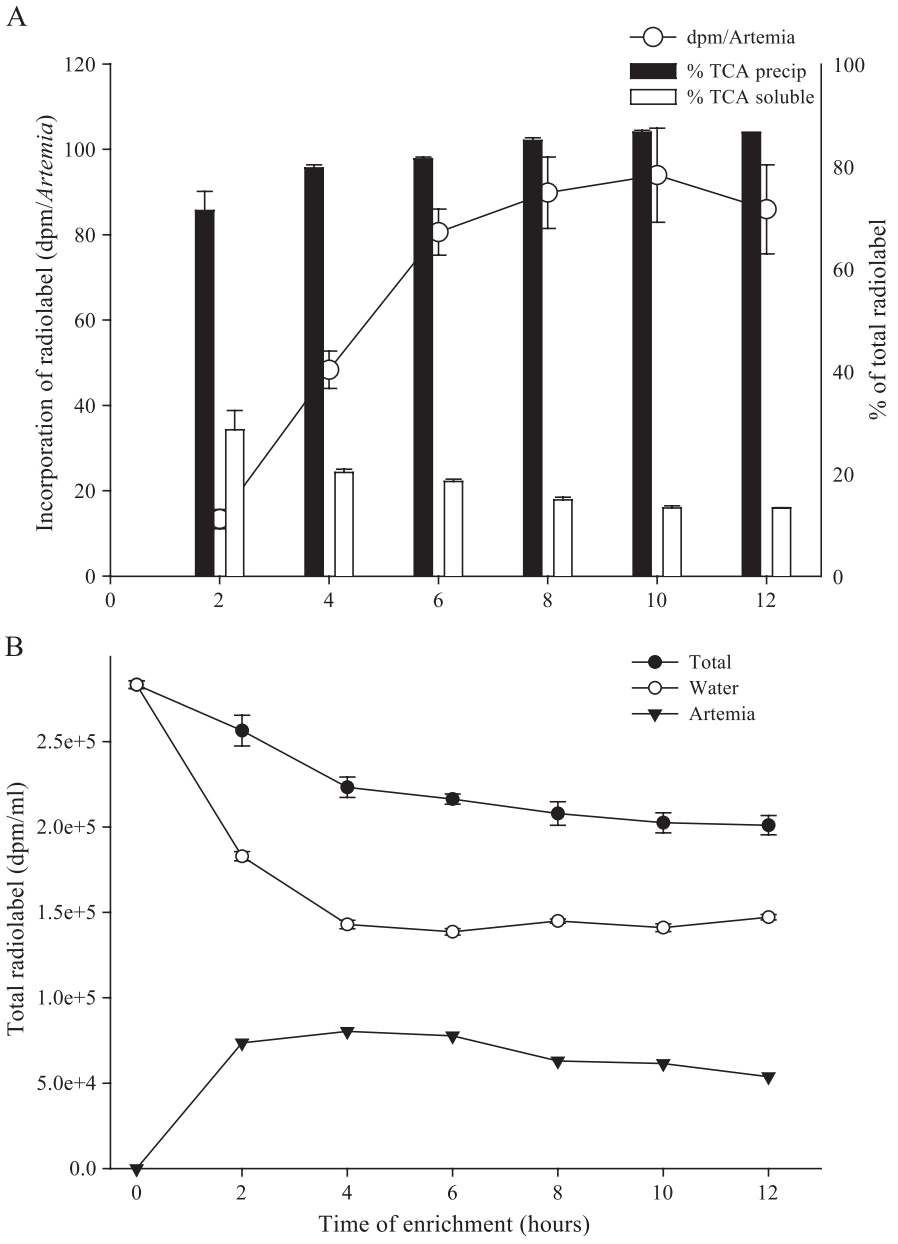


Fig. 2. Radiolabeling of *Artemia* during enrichment. (A) Radiolabel incorporation in *Artemia* nauplii (dpm/*Artemia*) (mean ± S.D. of pooled samples, $n = 4$) and in the TCA precipitate (mostly protein) and soluble (mostly FAA) fractions (% of total radiolabel) (mean ± S.D. of pooled samples, $n = 3$), over the enrichment period. (B) Total amount of radiolabel (dpm/ml) in the whole system, including incubation water and *Artemia* (mean ± S.D. of pooled samples, $n = 5$).

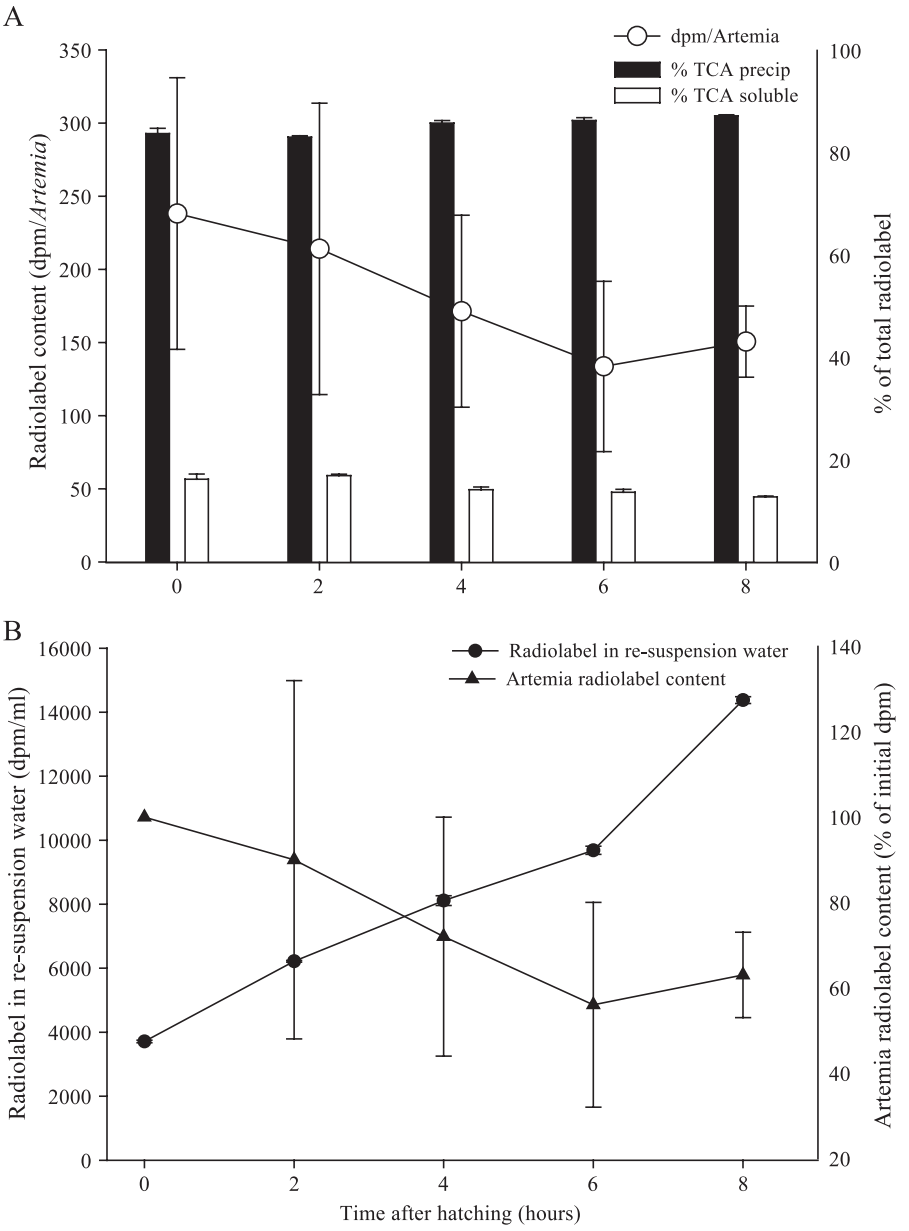


Fig. 3. Radiolabel stability of *Artemia* nauplii labeled during hatching, after transfer to clean seawater. (A) Radiolabel stability in *Artemia* nauplii (dpm/*Artemia*) (mean ± S.D. of pooled samples, $n = 5$) and in the TCA precipitate (mostly protein) and soluble (mostly FAA) fractions (% of total radiolabel) (mean ± S.D. of pooled samples, $n = 3$). (B) Total amount of radiolabel in the re-supension water (dpm/ml) (mean ± S.D., $n = 3$) and percentage of radiolabel in Artemia, relatively to the initial content (mean ± S.D. of pooled samples, $n = 5$).

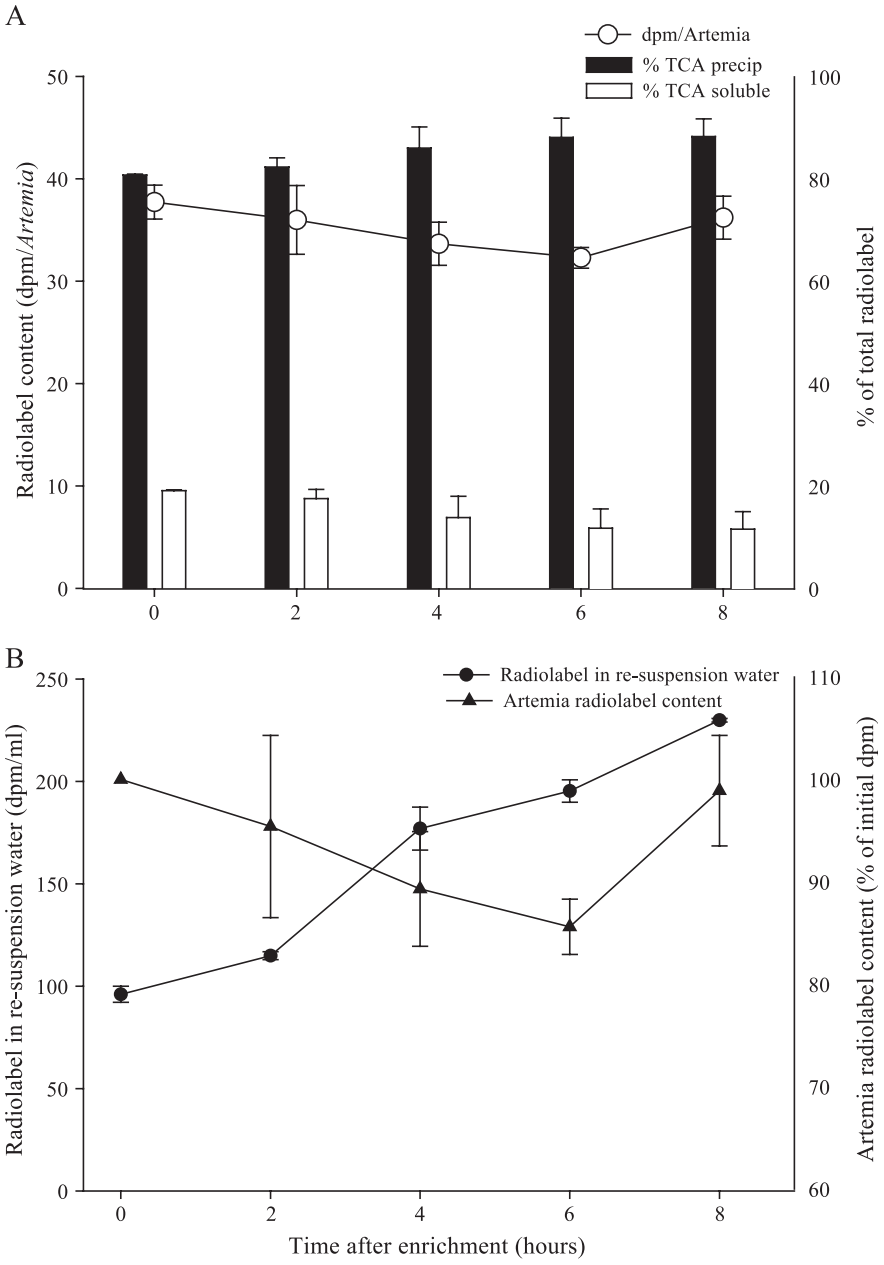


Fig. 4. Radiolabel stability of *Artemia* nauplii labeled during enrichment, after transfer to clean seawater. (A) Radiolabel stability in *Artemia* nauplii (dpm/*Artemia*) (mean ± S.D. of pooled samples, $n=4$) and in the TCA precipitate (mostly protein) and soluble (mostly FAA) fractions (% of total radiolabel) (mean ± S.D. of pooled samples, $n=3$). (B) Total amount of radiolabel in the re-supension water (dpm/ml) (mean ± S.D., $n=3$) and percentage of radiolabel in Artemia, relatively to the initial content (mean ± S.D. of pooled samples, $n=5$).

3.5. Artemia metabolism during enrichment

Results from the experiment quantifying the losses of radiolabel from the enrichment system and examining possible effects of bacterial activity are shown in Fig. 5. Under standard enrichment conditions, 7.0 ± 1.8% of the total radiolabel was incorporated by the *Artemia* while 62.2 ± 1.8% remained in the water. The remaining 30.9 ± 1.2% was catabolised and consequently lost from the system, in the form of $^{14}CO_2$. On the other hand, when the *Artemia* enrichment was conducted with disinfected water, 96.4 ± 0.5% was still in the water and only a residual fraction was either incorporated by the *Artemia* or catabolised (1.1 ± 0.2% and 2.4 ± 0.5%, respectively). The radioactivity found in the untreated enrichment media containing no *Artemia* showed that 19.2 ± 1.6% of the total initial radiolabel was catabolised, being this reduced to 0.2 ± 0.03% when the water was disinfected.

3.6. Digestion and absorption of radiolabeled Artemia nauplii by herring larvae

Herring larvae were fed newly hatched radiolabeled *Artemia* with an activity of 129–141 dpm/*Artemia*, of which 84–91% was present in the TCA precipitate (mostly protein) and the remaining 9–16% in the TCA soluble (mostly FAA) fraction (Fig. 6). Two hours after feeding, 45 ± 6% of the radiolabel was found in the gut compartment, while 40 ± 7% was recovered in the body. A lesser level of 9 ± 3% had been evacuated and 6 ± 2% was catabolised. During the first 2–6 h after feeding the amount of radiolabel found in the body remained relatively constant (40–43%) while the catabolised fraction increased from 6% to 16%. Throughout this period there was a gradual decrease in the radioactivity of the

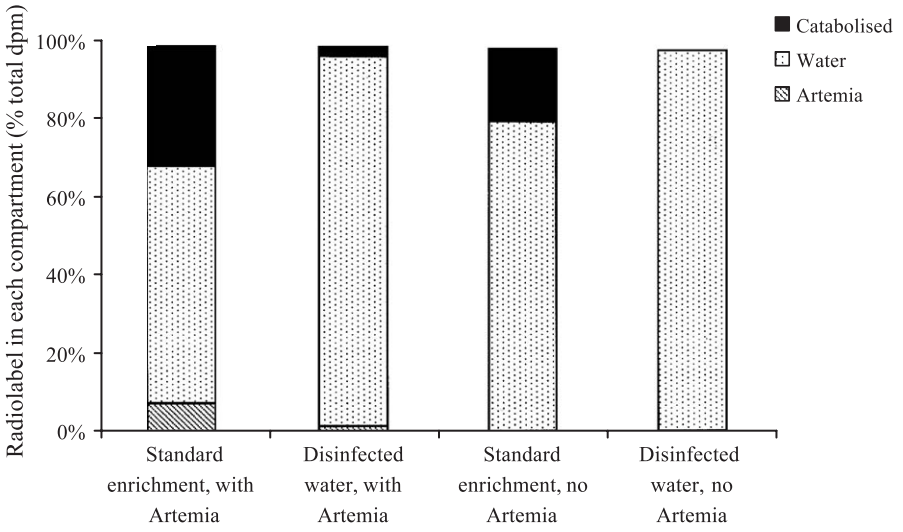


Fig. 5. Radiolabel (% of total dpm) catabolised, found unabsorbed in the water media and incorporated by the *Artemia*, at the end of a 12-h enrichment period (data are means of eight samples).

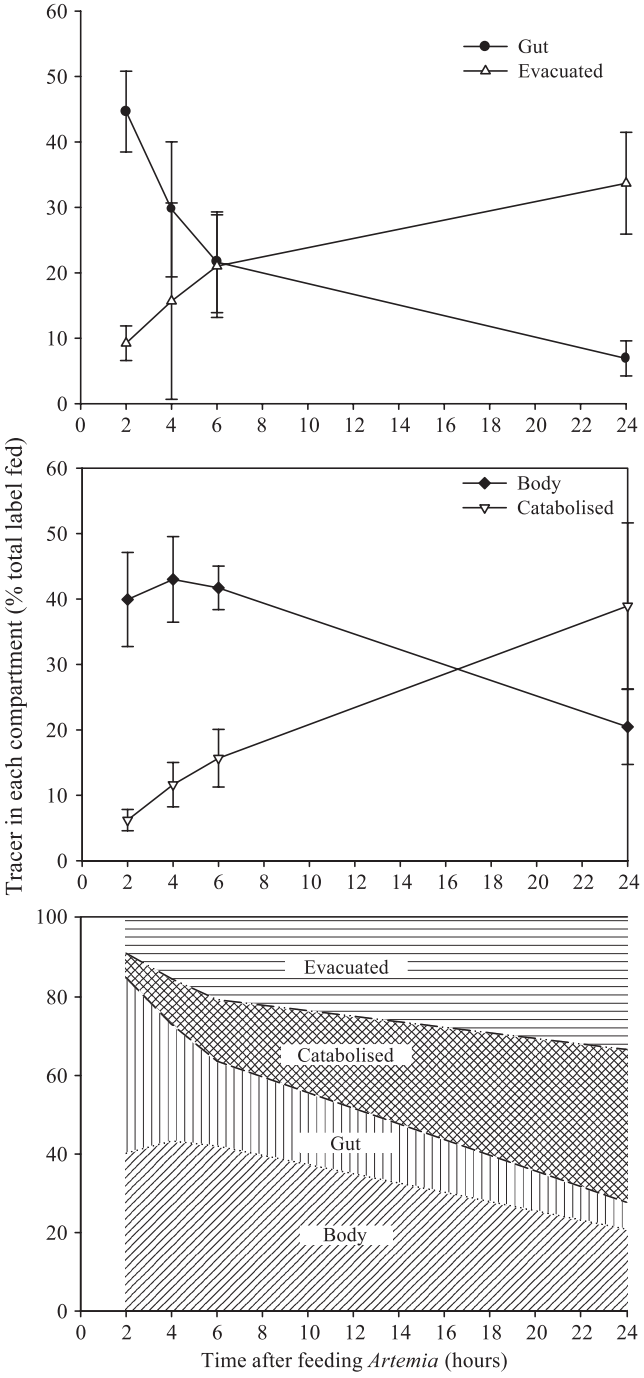


Fig. 6. Radiolabel (% of total dpm) evacuated, catabolised or found in the body or gut tissues of herring larvae fed radiolabeled newly hatched *Artemia*, at 2, 4, 6 and 24 h after feeding (mean ± S.D., $n = 6$).

gut compartment and a coincident increase in the evacuated fraction (45–22% and 9–21%, respectively). The digestibility of the *Artemia* protein and FAA fraction was about 60%, with 20 ± 6% being retained in the body and 39 ± 13% catabolised following 24 h of feeding. At this time, 7 ± 3% of the radioactivity was detected in the gut, possibly in the mucosa of the digestive tract as the gut lumen of all sampled larvae appeared empty, and 34 ± 8% had been evacuated into the incubation water.

4. Discussion

The present study has demonstrated the viability of radiolabeling newly hatched and enriched *Artemia* nauplii by introducing a [U-^{14}C] protein hydrolysate (a mixture of radiolabeled FAA) into either the hatching or the enrichment media. Moreover, in conditions simulating transfer to larval rearing tanks, the *Artemia* radiolabel content, as well as its distribution between the TCA precipitate and soluble fractions, remains relatively stable for up to 8 h. These results indicate a possible application of this methodology in studies of food intake, digestibility and absorption in larval fish. In fact, Conceição et al. (1998) had previously used a radiolabeled FAA (phenylalanine) to label newly hatched *Artemia* and were able to successfully estimate food intake of African catfish larvae. This methodology may thus be quite useful for conducting studies with very small sized larvae which prey upon newly hatched nauplii. When working with larger larvae feeding on *Artemia* metanauplii, radiolabeling can also be performed during the enrichment phase. Furthermore, and more importantly, the use of enriched *Artemia* opens the possibility of studying the effect of nutritional parameters, such as lipid composition and dietary protein/lipid (total energy) levels on the regulation of larval food intake and/or live prey digestibility and protein and amino acid metabolism.

One constraint observed with this approach was the variability found in the incorporation of the label per *Artemia* despite thorough washing of the samples. This was more pronounced with newly hatched nauplii compared to the more uniform labeling found in enriched *Artemia*. However, this variability can limit the accuracy of calculating ingestion rates, particularly in cases where a small number of *Artemia* are ingested per unit time, suggesting that this approach may be more useful in studies requiring instar II nauplii.

Although a good radiolabeling was always accomplished and the general patterns of variation during trials of incorporation and stability after hatching or enrichment were consistent, preliminary trials showed considerable variability in *Artemia* radiolabel content, despite the same amount of radiolabel being added to the media. The *Artemia* stage of development was not assessed at the end of the hatching period and before the start of enrichment. Although it is current practice to harvest the nauplii 24 h after the start of the incubation, hatching can be quite asynchronous (Van Stappen, 1996a). In general, when incubated in 33 g/l seawater at 25 °C, the first nauplii should appear after 12–16 h and the last nauplii should have hatched within 8 h afterwards (Van Stappen, 1996b). In the present study, the first nauplii were sampled at 12 h and the hatching percentage stabilized at 20 h, which conforms to what is commonly observed. After a 24-h incubation period, the *Artemia* may be found at two different stages of development. The nauplii stage (instar I) is still dependent on its endogenous reserves, its digestive tube is not yet

completely formed and the mouth and anus are closed. After about 8 h the animal molts into the metanauplii I (instar II) stage, when they start filter feeding small food particles (1–50 μm) (Van Stappen, 1996a). Thus, the proportion of actively feeding *Artemia* at the start of different enrichment trials most probably varies and this might affect the amount of radiolabel that is incorporated, on average, by the *Artemia* population. Moreover, workers have reported considerable variability in essential fatty acid content after *Artemia* enrichment despite attempts to standardize protocols (Merchie, 1996). On the other hand, *Artemia* radiolabeling was successfully accomplished during hatching, with non-feeding nauplii and no differences were observed in radiolabel incorporation between non hatched decapsulated cysts and newly hatched nauplii. Thus, uptake and incorporation of the radiolabel must also occur through diffusion. Pavillion and Tan Tue (1981) have already reported an active absorption of ^{14}C amino acids dissolved in the medium by eggs and nauplii of *Artemia*, with histo-autoradiographic studies showing the incorporation of these substances in the proteins of embryonic tissues. One possible explanation for the relatively lower variability in radiolabel incorporation found after a 12-h enrichment period could be that incorporation through filter feeding, compared to absorption, might be prone to less variability.

In the stability experiments there was a high amount of radiolabel in the re-suspension water immediately after transfer to clean seawater, being this particularly noticeable in newly hatched nauplii. It is suspected that the radiolabel was released very rapidly between rinsing the *Artemia* and start of sampling (about 30 min time lag). The higher amount of radiolabel present in the re-suspension water after hatching was probably a consequence of higher *Artemia* densities being maintained in this case, in addition to the higher radiolabel incorporation that was achieved in newly hatched *Artemia* in this trial.

During the radiolabeling trials a substantial amount of the total initial radioactivity was not recovered at the end of the hatching or enrichment period (38% and 29%, respectively). One possible way for the radiotracer to leave the water media was through release to the atmosphere, as $^{14}CO_2$. It is likely that the *Artemia* nauplii catabolise some of the absorbed FAA, thus releasing $^{14}CO_2$ into the atmosphere. On the other hand, the hatching and enrichment media provides very good nutritive conditions for bacteria proliferation (Van Stappen, 1996b). Therefore, a fraction of the radiolabeled protein hydrolysate was possibly catabolised by bacteria growing in the water. When radiolabeling was conducted in standard enrichment conditions, an average "loss" of 30.9% of the total initial radiolabel was measured. On the other hand, when antibiotic-disinfected water was used, only 2.4% of the total initial radiolabel was catabolised after 12 h. However, in this case, the antibiotic may have affected the *Artemia* metabolism, as only 1.1% of the total radiolabel was incorporated by the *Artemia* population compared to 7.0% during standard conditions, leaving most of the radiolabel remaining in the enrichment media (96.4%). Pavillion and Tan Tue (1981) suggested that the use of antibiotics may alter the membrane permeability to organic molecules and therefore block the process of absorption. Another hypothesis is that bacterial gut microflora and/or the ingestion of bacteria from the culture media could be contributing to the *Artemia* radiolabel intake, as bacteria have been reported to contribute to the nutrition of *Artemia* by being a source of protein and amino acids (Gorospe et al., 1996). However, this does not seem to be of quantitative importance, given that in the present study the relative label incorporation is similar in newly hatched

nauplii, who are still dependent on their endogenous reserves and have their mouth and anus closed, and in metanauplii. Figs. 1C and 2B show that *Artemia* had incorporated nearly 24% of the total initial radiolabel after 24 h of hatching and around 19% after a 12-h enrichment period, respectively. A better estimation of bacterial activity was made by comparing the radioactivity found in the metabolic trap of incubation vials containing no *Artemia* when the enrichment media used disinfected (0.2%) or non-disinfected water (19.2%). Therefore, it is concluded that although the *Artemia* catabolism is responsible for some release of radiolabel into the atmosphere, bacterial activity has also a major impact. The loss of radiolabel during hatching was not examined but a similar situation is likely to be occurring. The higher amount of radioactive product that could not be accounted for at the end of the hatching period compared to the enrichment trial might simply be a consequence of the higher incubation time used for hatching.

Radioactive tracers have already been used by a number of authors to estimate food intake and diet assimilation efficiencies in fish larvae (Govoni et al., 1982; Boehlert and Yoklavich, 1984; Kolkovski et al., 1993; Rust, 1995; Conceição et al., 1998). However, what distinguishes this methodology is the fact that a very high proportion of the label is shown to be incorporated into the TCA precipitate (mostly protein) fraction of *Artemia*, after a short period of time. This is true for both newly hatched (84–91%, at the end of the hatching period) and for enriched nauplii (81–87%, after 12 h of enrichment). Furthermore, the amount of radioactivity in the TCA precipitate remains relatively stable for up to 8 h in clean seawater. The lipid extraction performed on the TCA precipitate has shown that this fraction is made up essentially of protein, with only around 4% of the labeled FAA having been converted into lipids. Label incorporation into the carbohydrate fraction was not quantified but it can be assumed as negligible. According to García-Ortega et al. (1998), the proximate composition of newly hatched nauplii is 56.2% DW protein, 17.0% DW lipid, 3.6% DW carbohydrate and 7.6% DW ash. Given the low carbohydrate content of *Artemia*, an eventual conversion of the label into carbohydrates is expected to be even lower than into lipids, i.e., insignificant. In view of the lack of precise methods to look at protein metabolism in small larvae feeding on live prey, these characteristics render this methodology a potential tool with high applicability in protein and AA metabolism studies. For instance, it allows the integrated analysis of the number of ingested prey (i.e., estimation of the total energy, protein or AA intake) and the metabolic fate of dietary protein or AA.

In order to examine the digestibility of the radiolabeled prey (mostly protein and FAA), herring larvae were fed radiolabeled newly hatched *Artemia* nauplii and their metabolism was studied up to 24 h after prey ingestion. The results revealed a high digestibility (around 60%) of the *Artemia* protein, with 20% being found in the body and 39% catabolised by the larvae, 24 h after feeding. This confirms previous studies in which a considerable proportion of the absorbed protein was catabolised for energy (Govoni et al., 1982; Rønnestad et al., 2001). The digestibility of this protein source was comparatively higher than the ^{14}C-labeled algal protein used by Rønnestad et al. (2001) in Atlantic halibut post-larvae, in which 58% was evacuated unabsorbed into the water, 25% was retained in the body and 17% catabolised and released into the water as CO_2, 13 h after tube feeding. Bovine serum albumin (BSA) has also been used as a protein source, with an average absorption of about 58% being measured in Senegal sole post-larvae and 32.5% in

post-larval Atlantic halibut, 8 h after tube feeding (Rønnestad et al., 2000; Rojas-García and Rønnestad, 2003a). In the present case, when looking at the end sampling point, it would appear that a comparably lower percentage of the protein was retained in the body. However, if the previous experiments had been prolonged up to 24 h, a fraction of the absorbed protein could have been further catabolised, as observed in the present study. Furthermore, as discussed by Rojas-García and Rønnestad (2003b), the results based on the use of BSA need verification with uniformly labeled protein since methylated BSA as the test protein may underestimate the proteolytic capacity. On the other hand, considerable differences may be found between different species and stages of development. Rust (1995), for instance, when tube feeding different altricial-gastric species with a ^{35}S-labeled *Escherichia coli* protein, found average values of protein assimilation of 29–62% in striped bass (19–47 DAFF), 42–58% in walleye (10–40 DAFF) and 64–94% in zebrafish (5–13 DAFF).

García-Ortega et al. (1998) analysed the protein digestibility of *Artemia* decapsulated cysts and newly hatched nauplii and found a higher protein digestibility, between 77.8% and 82.8%, than in the present study. However, these authors used an "in vitro" multi-enzyme assay employing acid digestion and which does not take into account evacuation. However, herring larvae do not possess a completely developed stomach, suggesting that digestion takes place at a higher pH, which complicates comparing the results in these studies. In addition, in the present study, partially digested protein may have been evacuated before absorption was complete.

The digestion process and subsequent absorption of the digested radiolabeled nutrients was quite rapid, given that 40% was found in the body only 2 h after feeding while 6% was recovered after respiration in the metabolic trap. Similar rapid proteolysis after 2 h was also reported by Rønnestad et al. (2000) and Rojas-García and Rønnestad (2003a) using BSA. In the case of the post-larval Atlantic halibut (Rojas-García and Rønnestad, 2003a), however, the degradation products seemed to have been more slowly and gradually transferred into the body.

Some variability was found in the way the larvae "handled" the diet, being this particularly noticeable in the gut and evacuated fractions, at 4 and 6 h. This might be partly explained by individual differences in gut emptying rates; at 2 h the majority of the larvae (86%) still had full guts, while at 4 and 6 h the percentage of larvae with food in the gut was 33% and 14%, respectively; at 24 h all larvae had their guts empty. On the other hand, some variability was also noticed in the amount of diet that was utilized by the larvae (absorbed into the body and catabolised). Meal size might have induced some additional variability, given that larvae were allowed to feed voluntarily and the number of prey ingested could not be controlled and varied considerably. In fact, these factors should not be considered independently, as a direct relationship has been found between the amount of ingested preys, gut emptying rate and nutrient assimilation efficiency; ration size has been positively related with evacuation rate and it has been suggested that the assimilation efficiency increases at a lower ration, through an increase of the residence time of food particles (Werner and Blaxter, 1980; Boehlert and Yoklavich, 1984).

Contrary to the lipid composition, the manipulation of the total protein content and of the AA profile in live preys has not been commonly attempted, particularly in *Artemia.*

However, Tonheim et al. (2000) have shown that the FAA pool of Artemia can be selectively enriched with methionine. The present technique of *Artemia* enrichment with selected radiolabeled AA can be a good complement to studies of *Artemia* supplementation with FAA or proteins. It may allow further insight into the efficiency of absorption of each AA, as well as the use of AA for energy or other purposes, and the AA profile of the proteins being synthesised by the larvae. In particular, these methods will enable quantitative *in vivo* studies of digestion and absorption rates of dietary protein and FAA from onset of exogenous feeding and through metamorphosis in fish.

Acknowledgements

Sofia Morais and Luis Conceição acknowledge financial support from the "Fundação para a Ciência e a Tecnologia", Portugal (grants SFRH/BD/4902/2001 and SFRH/BPD/7149/2001). Ivar Rønnestad acknowledges support from Research Council of Norway (141990/120; ELHMF publ # 197). The authors would like to express their deepest gratitude to Dr. Bill Koven, for his thorough revision of the manuscript and for greatly improving the paper with his comments. The arguments presented by an anonymous reviewer were also very important in improving the discussion of the results and for this the authors are thankful.

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