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Digestion and absorption of a pure triacylglycerol and a free fatty acid by *Clupea harengus* L. larvae

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The digestion, absorption and post absorptive metabolism of a radiolabelled triacylglycerol (TAG; triolein) and a free fatty acid (FFA; oleic acid), delivered by tube feeding, was studied in herring *Clupea harengus* larvae, using metabolic chambers and video analysis. In general, a large amount of the delivered lipid was evacuated. Most of the evacuation occurred between 2 and 6 h after tube feeding although a group of larvae responded by rapidly evacuating the lipid (>50% before 2 h). The volume of the tube-fed lipid affected its utilization. A small volume of triolein (9·2 nl, representing *c.* 6% of gut filling capacity) resulted in a lower proportion of fast evacuating larvae and improved utilization (lower evacuation and higher absorption: body incorporation and catabolism) compared with 50·6 nl (*c.* 17% of gut filling capacity). Increases in the volume of tube fed triolein enhanced only marginally label absorption and led to a steep rise in evacuation. At a comparable high volume (50·6 nl), oleic acid, which does not require digestion, was better absorbed and less evacuated than triolein. The video observation of the lipid digestive process revealed a considerable gut contractile activity that appeared effective in processing the tube fed lipid. Also, the gut wall seemed very sensitive to physical pressure. Signs of chemical degradation during lipid digestion were also noted. The metabolic studies, together with video image analysis, suggested that the limiting step for the utilization of high dietary lipid levels may have been the lipid absorption into the enterocytes and transport into the body, rather than lipid digestion. The results support the notion that the rate of lipid digestion and absorption in fishes is slower than that of mammals.

Key words: absorption; digestion; fish larvae; gut motility; lipid; nutrition.

INTRODUCTION

Lipids have multiple roles in marine animal nutrition: they are a main source of metabolic energy, important structural components of biological membranes and precursors of essential metabolites (Sargent *et al.*, 1993). Some fatty acids (FA) have been identified as essential nutrients and are required for the normal development and growth of marine fish larvae (Sargent *et al.*, 1997, 1999).

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Therefore, most of the research on larval nutrition has been conducted to determine the essential FA requirements of many commercially important species, while relatively few studies have examined their quantitative lipid requirements. Quantitative imbalances in diet composition, particularly excessive lipid content, have been suggested as a cause for poor growth and performance during larval development and it is believed to lower larval digestion ability and FA absorption efficiency (Kjørsvik *et al.*, 1991; Gara *et al.*, 1998; Olsen *et al.*, 2000).

Lipid digestion, absorption and transport in fishes, particularly in fish larvae, have been relatively poorly studied, although some similarities have been found in the digestive processes of fishes and mammals (Sire *et al.*, 1981; Honkanen *et al.*, 1985). Dietary triacylglycerol (TAG) may be partially hydrolysed to free fatty acids (FFA) and monoacylglycerol or completely hydrolysed to FFA and glycerol by pancreatic lipase in the gut lumen (Tocher & Sargent, 1984; Lie *et al.*, 1987; Gjellesvik, 1991; Koven *et al.*, 1994). The products of lipolysis are emulsified with bile components, forming mixed micelles, and the products of lipid digestion then diffuse into the intestinal mucosa cells, where they are mostly reesterified into TAG, before being incorporated into chylomicrons or very low density lipoproteins (VLDL) which are then released into the circulatory system (Noaillac-Depeyre & Gas, 1974; Sire *et al.*, 1981; Iijima *et al.*, 1990).

To date, there is no information concerning the maximal digestive and absorptive capacity for lipids and FA in marine fish larvae (Planas & Cunha, 1999). The present study focussed on the understanding of the mechanisms underlying and limiting lipid digestion and absorption in herring *Clupea harengus* L. larvae. A TAG, triolein, was used as the lipid source and the metabolism of a FFA, oleic acid, was also examined. In addition, different volumes of triolein were tube fed to the larvae in order to study the effect of lipid dose on digestion and metabolism. Finally, a visual description of the actions taking place in the gut during the passage of a pure lipid was made using video-tape recordings.

MATERIALS AND METHODS

FISH LARVAE AND TUBE-FEEDING PROCEDURE

Herring larvae from a single batch of eggs stripped from wild caught fish (March 2002) were reared at the High Technology Center, University of Bergen, and fed natural zooplankton, according to Høie *et al.* (1999). The experimental larvae were transferred from the rearing tanks into 5 l buckets containing clean sea water and deprived of food 24 h prior to its transfer to the Department of Biology, for tube-feeding. The experiments were conducted in a temperature-controlled cold room, at 9° C, range ±1° C, a salinity of 32 and with continuous illumination. The detailed description of the experimental set-up for *in vivo* tube-feeding and metabolic studies can be found in Rønnestad *et al.* (2001). Twelve larvae were tube fed per treatment and incubation period. Prior to tube feeding, each larva was sedated in 29·4 μg ml^{-1} of a MS-222 (Sigma Chemicals, St Louis, MO, U.S.A.) solution. After measuring total length (L_T) under a binocular microscope, the larvae were tube fed through a 0·19 mm plastic capillary tube (Sigma, St. Louis, MO, U.S.A.) and, using a nanolitre injector (World Precision Instruments, Sarasota, FL, U.S.A.), a known amount of the lipid (single droplet) was deposited in the foregut, just before the sphincter leading to the midgut. After rinsing, the larvae were transferred to individual incubation vials with 8 ml of clean sea water. Each incubation vial was sealed and connected to a metabolic trap (vial with 8 ml KOH 0·5 M), where the CO_2 diffused into the air during incubation was retained by conversion to HCO_3^-. Once the incubation

period was over and the fish had been sampled, the vial was resealed and 1 ml of acid (HCl 0·1 M) was added in a series of gradual steps, to cause the rapid diffusion of any remaining CO_2 from the water into the trap. After each incubation period the larval gut was separated from the body by dissection. Body and gut were placed on separate scintillation vials (Packard Bioscience, Gronungen, The Netherlands), dissolved in 2 ml of Solvable (Packard Bioscience), after which 10 ml of scintillation cocktail (Ultima Gold, Packard Bioscience) was added, for radioactive counting (dpm, disintegrations min^{-1}). The incubation water and the KOH-CO_2 (metabolic trap) were also prepared for radioactivity counting. The results are presented as a percentage of dpm found in each compartment in relation to total dpm.

PREPARATION OF THE TEST LIPID MIXTURES

The test lipid mixtures were prepared by adding the solvent containing the radiolabelled compound to the 'cold' lipid, mixing and flushing nitrogen (N_2) to evaporate the solvent. All 'cold' reagents (*c.* 99%) were purchased from Sigma Chemicals, whereas the ^{14}C-radiolabelled triolein (1·85 MBq; 0·1 mCi ml^{-1}) (labelled in the three fatty acids) and oleic acid (1·85 MBq; 0·1 mCi ml^{-1}) were acquired from ARC (American Radiolabelled Chemicals, Inc., St Louis, MO, U.S.A.).

EFFECT OF LIPID TYPE (TAG OR FFA) ON METABOLIC UTILIZATION

In the first experiment two treatments were tested: triolein (TAG) and oleic acid (FFA). Preliminary tests had shown that oleic acid, when tube fed alone, 'solidifies' immediately after entering the foregut and, therefore, it could not be delivered in a pure form. Thus, a 'cold' mixture was prepared, with oleic acid being diluted in triolein (80 : 20 triolein and oleic acid, v : v), to which radiolabelled oleic acid was added, making up 3·5% (w : w) of the final mixture. In the triolein treatment, radiolabelled triolein was added to the 'cold' triolein, representing 12·9% (w : w). Larvae were tube fed a lipid volume of 50·6 nl and were sampled at 2, 6, 12 and 24 h after tube feeding. The experiment was performed at 29 days after first feeding (DAFF), when larvae had an mean ± S.D. L_T of 18·2 ± 1·00 mm in the triolein treatment and 18·4 ± 1·18 mm in the oleic acid treatment.

EFFECT OF TAG (TRIOLEIN) DOSE ON METABOLIC UTILIZATION

To examine the effect of reducing the lipid dose on its metabolic utilization, 9·2 nl of triolein was tube fed to 37 DAFF larvae (21·6 ± 1·41 mm L_T), which were sampled at 2, 6, 12 and 24 h after tube feeding. An additional trial was conducted in which a variety of volumes (within the 9·2–50·6 nl range) was tube fed to 42 DAFF larvae (23·3 ± 1·34 mm L_T). In this case, larvae were sampled only at 24 h after tube feeding. The amount of lipid that had been tube fed to each larva was back calculated by summing the counts in each compartment, taking into account the specific activity of the lipid delivered.

VIDEO OBSERVATIONS OF LIPID DIGESTION

Herring larvae have a straight digestive tube and are transparent (unpigmented) during the larval period. This allowed visual observation of the digestive processes, through the analysis of video tape recordings. At 33 and 34 DAFF (17·4–19·1 mm L_T), larvae were tube fed either 50·6 nl or 9·2 nl of triolein (to determine the effect of lipid volume on the digestive process), delivered as a single droplet deposited in the foregut, just before the sphincter leading to the midgut. Each larva was followed under a stereo dissecting microscope connected to a JVC TK-1070E colour video camera and a Panasonic

AG-5700 video cassette recorder, until the gut was empty. Image analysis was conducted using Adobe Premiere 5.0 (Adobe Systems San Jose, CA, U.S.A.).

STATISTICAL ANALYSES

The effect of lipid type on metabolic utilization was analysed through one-way ANOVA, at a significance level of $P = 0{\cdot}05$, after checking the assumption of homogeneity of variances using the Bartlett's test (Zar, 1996). The percentage of counts found in each compartment was $\arcsin(x^{0{\cdot}5})$ transformed and analysis was performed for each compartment, at each sampling point. The effect of triolein dose on metabolic utilization was also analysed through simple regression, where the significance of the regression for each compartment was tested by ANOVA, at a significance level of $P = 0{\cdot}05$. In addition, in order to compare the slopes of the regression lines obtained for each compartment, ANCOVA was performed, followed by the Tukey HSD multiple comparison test when the null hypothesis was rejected, at a significance level of $P = 0{\cdot}05$ (Zar, 1996). Statistical analyses were performed using Statistica 6.0 (StatSoft Inc, Tulsa, OK, U.S.A.).

RESULTS

EFFECT OF LIPID TYPE (TAG OR FFA) ON METABOLIC UTILIZATION

The digestive utilization of triolein was compared with that of oleic acid, the FA which constitutes the triacylglycerol, in its free form. In both treatments, most of the lipid was evacuated from the gut into the incubation water between 2 and 6 h after tube feeding [Fig. 1(a), (b)]. At 2 h a very high variability was noted in the gut and water compartments [coefficient of variation (CV) = 46 and 47% in the gut; CV = 105 and 116% in the water, for the triolein and oleic acid treatments, respectively]. In the triolein treatment, 33% of the larvae (four out of 12) had released 59–83% of the radiolabel into the water, while 25% of the larvae tube-fed oleic acid (three out of 12) had evacuated 60–91% at 2 h after tube feeding. The oleic acid was also seen to cause an obstruction of the digestive tube at 6 h and onwards: at 6 h, 20% of the surviving larvae (two out of 10) had a 'blocked' gut, with 55% of the tracer still present in this compartment (CV = 67%), while at 12 h 44% of the surviving larvae (four out of nine) still presented 52 to 79% of total radiolabel in the gut (CV = 80%). At 24 h none of the sampled larvae had its gut obstructed but it was noted that all the dead larvae at this time had been unable to evacuate the lipid. The obstruction in the larval gut could be clearly noticed when examining the larvae under the binocular microscope and was probably the cause of a lower survival in the oleic acid treatment (100% survival at 2 h, 83·3% at 6 h and 75% at 12 and 24 h) when compared to triolein (100% survival at all incubation times). The results from larvae who responded by evacuating the lipid quickly (>50% of the tube fed label in the water compartment in the first 2 h) were not included in Fig. 1 as they clearly corresponded to a group which behaved differently in terms of evacuation. Furthermore, results from larvae tube fed the oleic acid which presented an obstructed gut (observed during dissection and results showing >50% of the tube fed label in the gut at sampling times >2 h) were not included. In addition, fast evacuating and gut-obstructed larvae were eliminated from the statistical analysis.

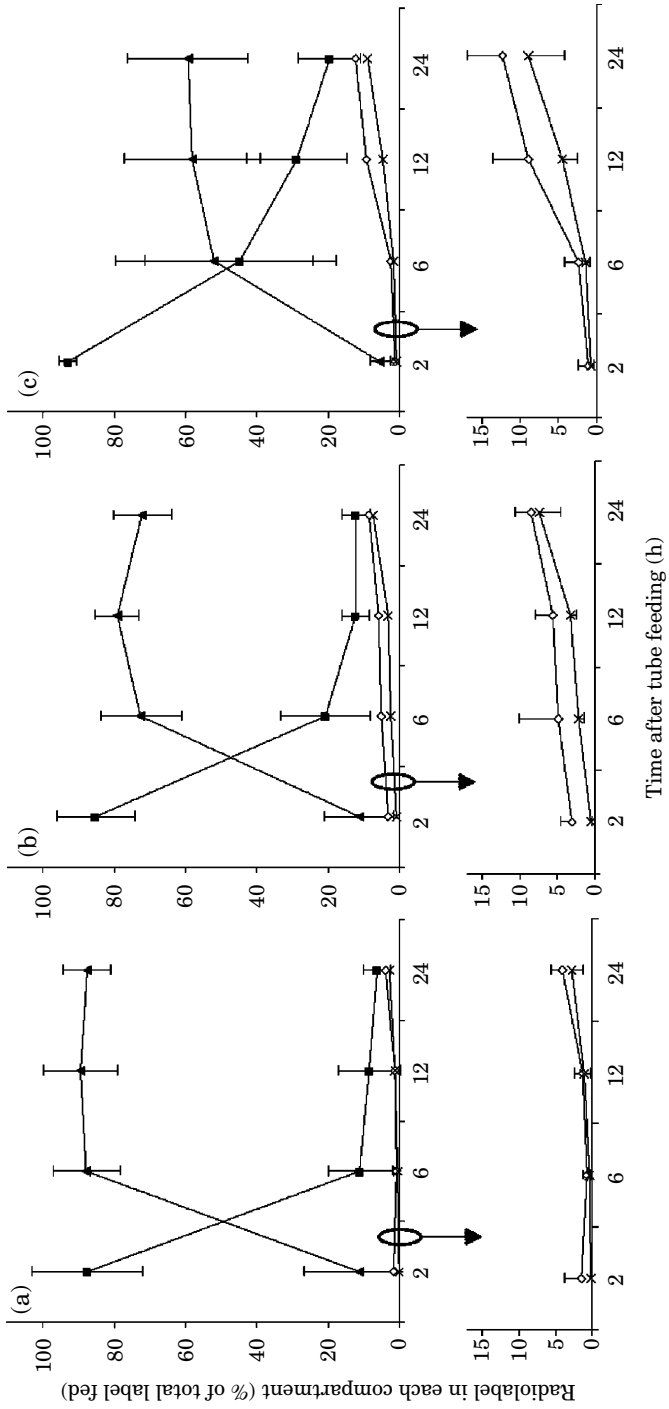

FIG. 1. Radiolabel in each compartment [water (▲), gut (■), body (○) and trap (×)] (per cent of total label fed), after tube feeding (a) 50·6 nl of triolein, (b) 50·6 nl of oleic acid mixture and (c) 9·2 nl of triolein. Data from 'fast evacuators' (>50% of label in the water) at 2 h and of larvae with an obstructed gut (>50% of label in the gut compartment) at 6 and 12 h are not included.

There was less evacuation in the oleic acid treatment with a significantly lower amount of radiolabel being found in the incubation water from 6 h onwards (75%, on average, compared with 88% for triolein) ($P = 0{\cdot}02$, $0{\cdot}04$ and $0{\cdot}002$, at 6, 12 and 24 h, respectively) [Fig. 1(a), (b)]. The amount of radiolabel in the gut compartment was comparable in both treatments at most sampling times but at 24 h it was significantly higher ($P = 0{\cdot}02$) with oleic acid (12 *v.* 6%). Despite the relatively low uptake into the body in both cases, oleic acid was significantly more ($P = 0{\cdot}03$ at 2 h and $P < 0{\cdot}001$ at 6, 12 and 24 h) incorporated than the digestion products of triolein at all sampling times (8 *v.* 4% at 24 h). Similarly, significant differences were found between treatments in the amount of label present in the metabolic trap (catabolized), with oleic acid being always significantly more ($P < 0{\cdot}001$) utilized for energy purposes than triolein (7 *v.* 3% at 24 h). Oleic acid was also catabolized faster, with traces (1%) of label being found in the metabolic trap 2 h after tube feeding, while in the triolein treatment signs of catabolism were only noted at 12 h.

EFFECT OF TAG (TRIOLEIN) DOSE ON METABOLIC UTILIZATION

In the second experiment herring larvae were tube fed a smaller dose of triolein (9·2 nl) [Fig. 1(c)]. Survival during incubation was 90% in this experiment. Fast evacuating larvae could still be detected: 17% of the larvae evacuated 63–93% of the TAG in the first 2 h after tube feeding (CV = 167% in the water compartment) (two out of 12) and at 6 h 44% of the larvae had released 56–98% of the radiolabelled triolein into the incubation water (CV = 51%) (four out of nine). Results from the group of larvae which had evacuated most of the lipid at 2 h after tube feeding are not included in Fig. 1(c). The evacuation of a smaller dose also appeared to occur mostly between 2 and 6 h after tube feeding but a high percentage of larvae still had not evacuated a high proportion of the lipid at 6 h. No substantial differences were found between the utilization of 9·2 [Fig. 1(c)] and 50·6 [Fig. 1(a)] nl of triolein in the gut and water compartments at 2 h after tube feeding but afterwards a lower dose of triolein resulted in a lower evacuation of label into the water (59% at 24 h) and a higher retention in the gut (19% at 24 h). Some differences were also found in label absorption, with a higher amount being incorporated into the body of larvae tube fed 9·2 nl of triolein dose, from 6 h onwards (12% at 24 h), and a larger amount of radiolabel being found in the metabolic trap of these larvae, at all incubation times (9% at 24 h).

The results obtained from tube feeding a range of volumes, after a 24 h incubation period, are shown in Fig. 2. Survival at the end of the incubation period was 72% in this trial. The amount of label incorporated into the body and catabolized were considered together, as the absorbed fraction. The results show that as the volume of tube fed lipid increased there was an increment in the evacuated, gut retained and absorbed fractions. The increase in the evacuated fraction was quite sharp, however, compared to the small rise observed in the percentage remaining in the gut or absorbed. The slopes of the three regression analysis were significantly different from zero and the analysis of covariance revealed that the slopes were significantly different from each other ($P < 0{\cdot}001$);

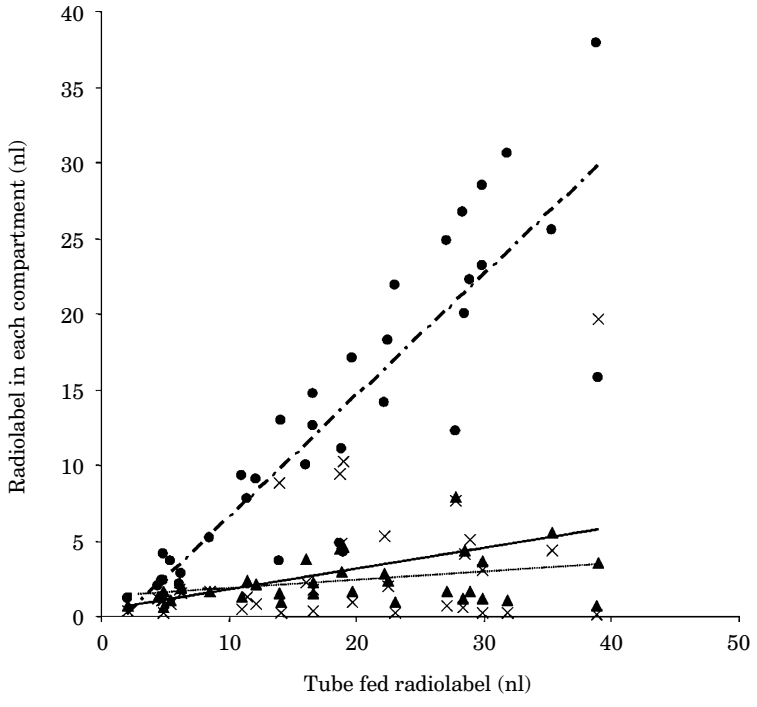

FIG. 2. Volume of radiolabel in each compartment [water (●, ·-·), gut (×, –) and body plus trap (▲, . . .)] 24 h after tube feeding a range of triolein volumes. Lines correspond to linear regression lines fitted to the data points (water: $y = 0{\cdot}8061x - 1{\cdot}6735$; gut: $y = 0{\cdot}1391x + 3{\cdot}933$; body plus trap $y = 0{\cdot}0547x + 1{\cdot}2802$). The Ho: ß = 0 was rejected by ANOVA, for the three analysed compartments. Water: $r^2 = 0{\cdot}769871$, $P < 0{\cdot}001$; gut: $r^2 = 0{\cdot}134500$, $P = 0{\cdot}030$; body plus trap: $r^2 = 0{\cdot}134473$, $P = 0{\cdot}030$. ANCOVA: $P < 0{\cdot}001$, with the slope of 'water' regression being significantly different from 'gut' and 'body plus trap'.

the regression for the water compartment was found to be significantly different from the regressions for the gut and body plus trap, while no significant differences were found between these two compartments.

VIDEO OBSERVATIONS OF LIPID DIGESTION

When larvae were tube fed a 50·6 nl droplet of triolein (17% of the midgut volume, on average) [Fig. 3(a)] very strong contractions of the midgut wall, close to the anterior sphincter, started almost instantly, causing the lipid droplet to move caudally. The duration and periodicity of these anterograde contractions during the first minutes after tube feeding were quite variable; two to five localized contractions per minute could be observed, with duration varying between 3 and 22 s. The interval between consecutive contractions was also highly variable, with 1–23 s intervals being noted. The strong contractions in the anterior region of the midgut [Fig. 3(b)] were responsible for the division of the large lipid droplet into smaller ones which started to be actively dispersed throughout the midgut; shortly after tube feeding ($\leq$10 min) there were already

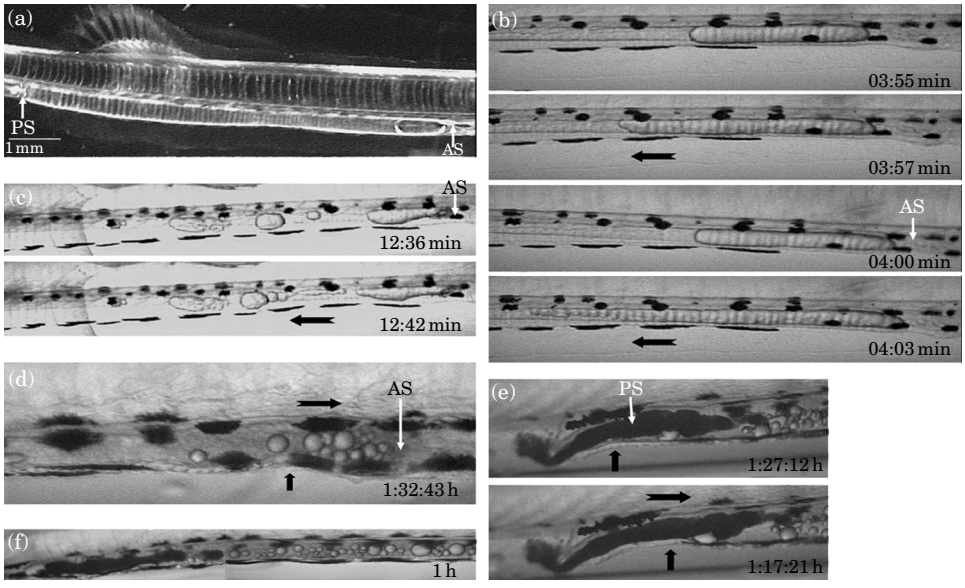

FIG. 3. Lipid digestion and gut motility in one herring larva, (a) immediately after tube feeding 50·6 nl of triolein and (b)–(f) over time. The anterior (AS) and posterior (PS) midgut sphincters are indicated (⇩), ⬆, the approximate location of origin of the contraction; ➡, the direction of the movement.

smaller droplets being formed and dispersed [Fig. 3(c)]. During the first 30 min to 1 h most of the lipid volume was contained in the anterior part of the midgut and the predominant movements were localized close to the anterior sphincter, with an anterograde (caudal) direction. When droplets were dispersed through the entire midgut, contractile activity was observed predominantly close to the anterior and posterior sphincters, depending on where the biggest concentration of larger droplets was found [Fig. 3(d)]. Some contractions were also seen to start in the mid midgut but only when there were voluminous droplets concentrated in this region. With time, the bulk of the lipid droplets were moved to the posterior region and strong retrograde (with an oral direction) contractions could then be seen, pushing the droplets in the direction of the anterior sphincter which appeared quite strong, withstanding considerable pressure and not allowing any lipid droplets to return to the foregut. As for the posterior sphincter, it was sometimes open at 1 h after tube feeding, allowing transfer and mixing between the hindgut and midgut, aided by strong retrograde contractions of the hindgut [Fig. 3(e)]. At 1 h after tube feeding the lipid was already considerably processed, with small droplets being mixed and dispersed throughout the whole midgut [Fig. 3(f)]. Some of the lipid droplets still conserved a translucent and clear appearance but there were also some particles with an opaque whitish look. At this time the contractions occurred throughout the entire midgut, causing considerable mixing in both directions; five to six contractions per minute were recorded, with duration of 6–13 s. These movements were fast, one following another with an interval of 1–4 s. At *c.* 1·5 h after tube feeding, the posterior midgut retrograde contractions were normally quite fast (up to seven to eight contractions per minute), with a duration of 3–13 s and a short

interval between them (1–7 s). Evacuation was seen to start as early as 1 h and 20 min after tube feeding and almost complete evacuation occurred at 2–2·5 h. A curious observation was made regarding the emulsification capacity of the tube fed lipids. In one larva, 1·5 h after tube feeding, retrograde contractions were seen to temporarily divide lipid droplets into smaller ones but these fused again when coming into contact with each other.

When larvae were tube fed 9·2 nl of triolein (occupying *c.* 6% of the midgut volume) [Fig. 4(a)], gut contractions were much more subtle and were mainly inferred from movements of the droplet. Nonetheless, they were still recorded [Fig. 4(b)] and were responsible for releasing smaller droplets from within the large droplet, thereby exposing them to the gut digestive secretions. In the minutes immediately following tube feeding, the periodicity of the contractions close to the midgut anterior sphincter was also quite variable, with two to five contractions per minute being recorded. These had a duration of 3–12 s and the

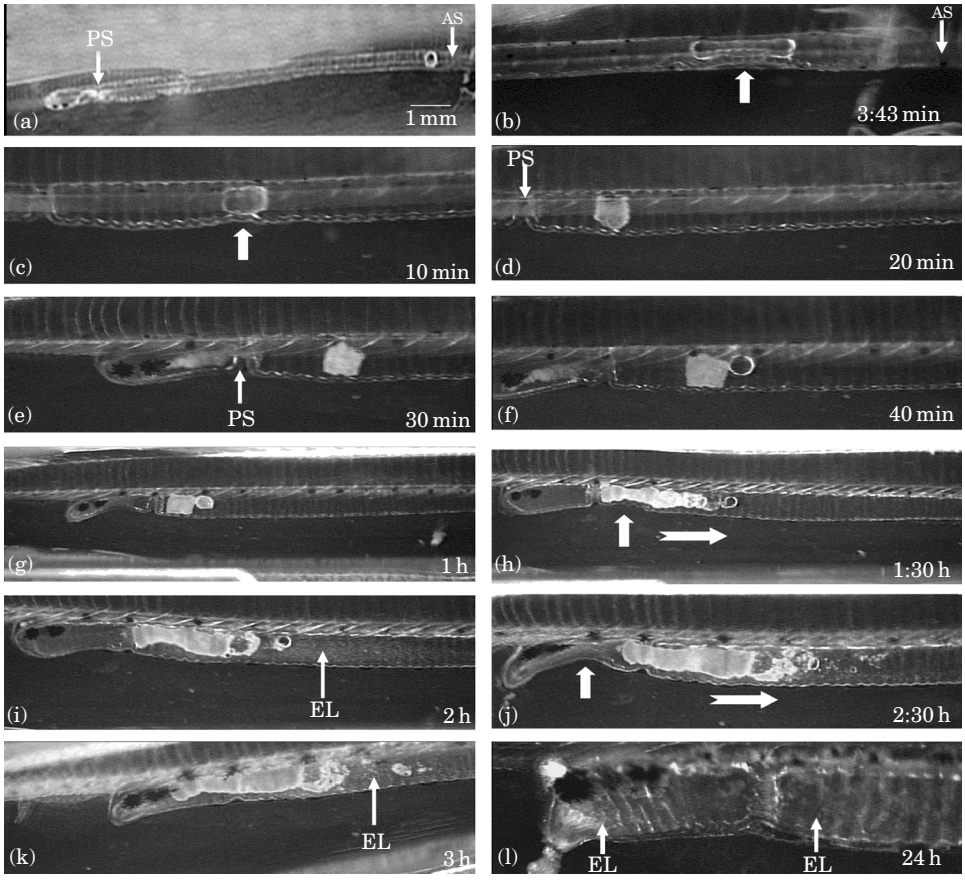

FIG. 4. Lipid digestion and gut motility in one herring larva, (a) immediately after tube feeding 9·2 nl of triolein and over time (b)–(l). ⇧, the approximate location of origin of the contraction; ⇨, the direction of the movement. ⇳, the approximate location of the anterior (AS) and posterior (PS), midgut sphincters. EL, emulsified lipid.

interval between them varied from 2 to 25 s. At *c*. 10 min after tube feeding the lipid droplet was already very close to the posterior midgut sphincter (in the last third of the midgut) [Fig. 4(c)]. At this time the contractions were very small and spaced between each other. As time went by, the lipid droplet lost its translucent aspect and became more opaque [Fig. 4(d)–(l)]. At 1–1·5 h after tube feeding, both the hindgut and the posterior midgut (close to the sphincter) regions were quite active, showing periodic and strong retrograde contractile activity [Fig. 4(g), (h)]. Three to four contractions per minute were registered at this time, with a duration of 3–16 s and an interval between contractions of 1–15 s. These contractions were responsible for slowly pushing and dispersing the droplet orally, releasing smaller droplets from within the opaque big droplet [Fig. 4(h)–(j)]. At 2 h very small, apparently emulsified droplets (with a cloudy appearance), could be clearly observed in the midgut [Fig. 4(i)]. At this time the posterior sphincter was opened and mixing occurred between the hindgut and the midgut, aided by hindgut contractions [Fig. 4(j)]. The video observations of larvae tube fed a lower volume of triolein indicated a slower evacuation given that, in this case, almost complete evacuation was seen to start only after 3 h and apparently emulsified lipid could sometimes still be seen in the hindgut and midgut 24 h after tube feeding [Fig. 4(l)].

DISCUSSION

The objective of the present work was to gain some knowledge on the digestive and absorptive capacity of marine fish larvae in relation to a pure lipid. Despite being an artificial situation that can only be experimentally provoked, the analysis of how a pure lipid is utilized by the larvae may give valid insight as to how lipids are digested and metabolized by larvae when processing a complete diet. It has been shown that the digestive tract of larval herring is able to digest and absorb lipids in pure form, either as TAG (triolein) or FFA (oleic acid), although a major fraction was evacuated into the water. It is likely, however, that in natural conditions herring larvae do not ingest such high lipid doses in a single meal, at least not in a pure form.

Tube fed larvae were seen to have two distinct lipid utilization patterns. Some larvae, here referred to as 'fast evacuators', appeared to evacuate most of the lipid (>50%, up to 90%) during the first 2 h after tube feeding. Nonetheless, the majority of the larvae seemed to evacuate most of the tube fed lipid between 2 and 6 h, independently of the dose and nature of the tube fed lipid. Tube feeding a FFA label did not appear to affect evacuation time in most larvae. Additionally, a slightly lower but comparable proportion of the larvae showed fast evacuation of the tube fed oleic acid (25 *v*. 33% with triolein). Nevertheless, with oleic acid there was an inverse problem: although diluted in triolein (80 : 20 triolein : oleic acid mixture), oleic acid appears to have induced physico-chemical changes of the test mixture in the gut lumen. From 6 h after tube feeding onwards (in some cases even earlier), the tube fed lipid acquired a 'waxy' and solid appearance that seemed to obstruct the larval gut, causing problems of evacuation. A possible explanation may be the formation of insoluble soaps inside the gut lumen, caused by the presence of a long chain monounsaturated FFA (oleic acid) in the tube fed mixture. Divalent cations (*e.g.* calcium and

magnesium) originating from drinking sea water are present in the intestine of larvae and may combine with FFA, forming insoluble soaps and limiting the absorption of certain fatty acids (Lied & Lambertsen, 1982; Lie *et al*., 1987; Olsen *et al*., 1998). In mammals, the strength of this soap formation increases with chain length and decreases with unsaturation (Hayes *et al*., 1994).) Nonetheless, in spite of these problems, some interesting results could be obtained by analysing the larvae in which the gut was not obstructed.

EFFECT OF TAG (TRIOLEIN) DOSE ON METABOLIC UTILIZATION

When larvae were tube fed a lower triolein volume (9·2 nl), the percentage of 'fast evacuators' in the population was greatly reduced (17% at 2 h) and evacuation was much slower and variable. The analysis of video images supports the evacuation findings obtained from the metabolic studies. In addition, a lower volume of triolein was more efficiently absorbed, with a higher fraction of the label being found in the body compartment and in the metabolic trap. Increasing the amount of tube fed triolein increased only slightly the amount of lipid digestion products that were absorbed (incorporated into the body and catabolized) and retained in the gut 24 h after tube feeding, while the most noticeable result appeared to be a steep (and significantly different) rise in evacuation. The effects of food type and ration size on absorption efficiencies are poorly known in fish larvae (Govoni *et al*., 1986). Ration size has been positively related with evacuation rate and it has been suggested that the absorption efficiency increases at a lower ration, through an increase of the residence time of food particles (Werner & Blaxter, 1980; Ryer & Boehlert, 1983; Boehlert & Yoklavich, 1984). The rapid and massive evacuation that was observed in some larvae may be due to an inefficient digestion or absorption of the lipid and appeared to be related to the amount of lipid that was tube fed. In humans, for instance, cases of 'diarrhea' have been reported in association with an impaired lipid hydrolysis and malabsorption (Hopman *et al*., 1984).

EFFECT OF LIPID TYPE (TAG OR FFA) ON METABOLIC UTILIZATION

Herring were also tube fed a mixture containing labelled oleic acid, in order to investigate whether the low utilization of the TAG (triolein) could be attributed to an insufficient larval digestive capacity. The oleic acid label was significantly evacuated less and significantly more retained in the gut, as well as significantly more and faster incorporated into the body and catabolized, compared to the products of triolein digestion. In both treatments some incorporation into the body could already be detected at 2 h but it was higher with oleic acid at all times. Additionally, traces of free oleic acid catabolism were found in the metabolic trap as early as 2 h after tube feeding, compared to 12 h in the triolein treatment. Many authors believe that marine fish larvae have a poorly developed digestive system, with a limited digestive capacity (Dabrowski & Glogowski, 1977; Lauff & Hofer, 1984; Walford & Lam, 1993; Kolkovski *et al*., 1997). Also, the lipolytic activities of neutral lipase and phospholipase A2 have been shown

to be stimulated in response to their substrate level but only up to a certain level, when a plateau is attained and this has been suggested to indicate the existence of a maximal capacity for enzyme synthesis (Zambonino Infante & Cahu, 1999). Several studies, however, have found significant levels of pancreatic and intestinal enzymes and nutrient absorption capacities in fish larvae (Pedersen *et al.*, 1987; Cahu *et al.*, 1995; Ribeiro *et al.*, 1999*a*, *b*). In the present study, when comparing the utilization of tube fed triolein and oleic acid, it appears that a low enzymatic capacity (*i.e.* a low rate of lipolysis) cannot explain entirely the low lipid absorption and, conversely, the elevated and rapid gut emptying of TAG. Tube feeding oleic acid did indeed result in a significantly higher FA absorption but a substantial percentage of the FA was still found in the incubation water after 24 h (72 *v.* 88% with triolein). It remains unclear whether the higher amount of tracer found in the body and trap compartments in the oleic acid treatment corresponds to its more efficient use or if it simply reflects a more rapid incorporation of a FFA, compared to a lipid that still has to be hydrolyzed. Nonetheless, the higher percentage of radiolabel still present at 24 h in the gut of larvae tube fed oleic acid indicates a higher utilization potential. Therefore, although an inadequate digestive capacity could not fully explain the results, the utilization of a FFA, rather than a TAG, did improve absorption efficiency. The differences in absorption efficiency appeared to be mostly explained by a dose effect, given that reducing the triolein volume from 50·6 to 9·2 nl was sufficient to achieve a higher retention in the gut, incorporation into the body and catabolism and, conversely, a more reduced evacuation, than in the oleic acid treatment (50·6 nl).

VISUAL OBSERVATION OF LIPID DIGESTION AND GUT MOTILITY

Analysis of the video tape recordings indicates that herring larvae have a certain degree of digestive capacity to utilize, at least partially, a pure lipid. The gut mechanical action appeared quite effective in processing the tube fed lipid. Contractions of the gut walls were responsible for the mechanical breakdown of the large lipid droplet into smaller ones which were actively dispersed throughout the midgut and most probably insured considerable mixing between the lipid droplets and the bile salts and pancreatic secretions being released in the anterior midgut region, close to the anterior sphincter (Pedersen, 1984). As the bulk of the lipid droplets were moved to the posterior region of the midgut, strong retrograde contractions could be seen. These movements probably increased the retention time in the gut but also allowed a better emulsification and mixing with bile and pancreatic secretions. Gut motility in vertebrates is controlled by the presence of food, by autonomic nerves and by hormones (Olsson & Holmgren, 2001; Holmberg *et al.*, 2003). Feeding and the presence of food in the digestive system initiates contractions of the stomach wall and subsequently gastric emptying, peristalsis, migrating motor complexes and other patterns of mobility, with the objective of transporting the ingested food at an optimal rate and allowing mixing of food with the best possible exposure to digestive enzymes (Olsson & Holmgren, 2001). The most common gastrointestinal motility patterns are standing contractions and peristaltic movements (in an

anterograde or a retrograde direction). These have been identified and described in fishes, such as in Atlantic halibut *Hippoglossus hippoglossus* (L.) juveniles (Rønnestad *et al*., 2000) and in zebrafish *Danio rerio* (Hamilton) larvae (Holmberg *et al*., 2003). In the present study, the most obvious pattern of motility appeared to be of the standing contraction type which is responsible for mixing the gastrointestinal contents through local, unpropagated smooth muscle contractions. The midgut wall appeared to be particularly sensitive to the presence of lipid, with gut contractions being mainly concentrated on the biggest lipid droplets. This was probably a result of the stimulation of sensory nerve endings located in the mucosa or within the muscle layers which initiate enteric reflexes. The sensory fibres are stimulated chemically by substances such as hypertonic salt solutions, FA, lipids and bile salts, or mechanically by distension of the gut wall (Olsson & Holmgren, 2001).

The video images also revealed signs of chemical digestive processes taking place during lipid digestion. For instance, the fact that the smaller droplets which are released from the larger ones by gut wall contractions do not fuse back together indicates the presence of bile salts in the gut lumen. In addition, the gradual change in the appearance of the lipid droplets, from a translucent and clear to an opaque and whitish look, was most probably a result of the chemical mechanisms occurring during digestion, such as the diffusion of protonated long-chain FA from the bulk of the micelles to the surface of the TAG droplets (where they become partially ionized) or the transfer of biliary lipids to the surface of the emulsions. These chemical changes occurring at the emulsion coat markedly increase its surface and, together with the mechanical emulsification, create smaller stabilized particles (Carey *et al*., 1983). In some cases, however, the larvae may have not been able to emulsify efficiently very high lipid loads. At least in one occasion, in a larva fed 50·6 nl of triolein, 1·5 h after tube feeding, retrograde contractions were seen to temporarily divide lipid droplets into smaller ones but these fused again when coming into contact with each other. This observation indicates that bile salts may have been completely used up at this time and that no further emulsification and digestion was possible. This fact could partially explain the apparently precocious evacuation that was noted in some larvae. It is conceivable that when the larval emulsifying and digestive capacity is exhausted evacuation can be accelerated.

LIPID ABSORPTION

A high amount of the absorbed (*i.e*. non-evacuated) radiolabel was retained in the gut for a relatively long period of time. At 6, 12 and 24 h the radiolabelled lipid fraction found in the gut compartment was relatively high and decreased gradually over time, while the incorporation into the body tissues and catabolism was low and increased slowly. Given the small size of the herring gut, it was not possible to remove its contents and separate the non-digested and non-absorbed fraction from the fraction stored within the enterocytes. Visual observations under the binocular microscope during gut dissection and video recording, however, suggest that most of the radiolabel found in the gut at longer incubation periods (6, 12 and 24 h) was not in the gut lumen and was therefore stored in the enterocytes. Studies conducted on lipid assimilation in humans have

revealed that maximum absorption occurs within 6 to 8 h (Jenkins *et al.*, 1994). The absorption of lipid and secretion of lipoproteins into the lymph by fish intestine are basically similar to mammals, although the process has been described as being slower (Sire *et al.*, 1981; Honkanen *et al.*, 1985). The present study confirms the idea of a slow absorption of lipid digestion products in fishes. This slow lipid processing causes a temporal separation in intestinal tissue absorption and assimilation and the enterocytes may function as temporary lipid storage sites when the rate of absorption exceeds the synthesis of lipoproteins (Noaillac-Depeyre & Gas, 1974; Sire & Vernier, 1981; Sire *et al.*, 1981; Olsen *et al.*, 1999).

The digestive capacity of herring larvae appears to be greatly dependent on the amount of lipid which is ingested in a single meal; reducing the lipid dose from 50·6 to 9·2 nl resulted in a lower evacuation and better FA absorption. Therefore, there appears to be a limit to the lipid inclusion level above which a further increment will lead to an increased 'waste' (evacuation into the water) without corresponding benefits in terms of lipid absorption. The metabolic studies, together with video image analysis, suggest that the limiting step for the utilization of high dietary lipid levels may be more a question of lipid absorption into the enterocyte and posterior transport into the body than of lipid digestion. Emulsification was apparently effective, even when a high lipid volume was tube fed, although the data does not allow conclusions on the larval enzymatic capacity to utilize these high lipid loads; it might be hypothesized that evacuation can be accelerated when the larval digestive capacity is exhausted. Endogenous phospholipids, together with cholesterol and bile salts were the only emulsifiers present in the larval gut. It should be noted, however, that feeding a 'complete' diet, *i.e.* a diet containing not only a pure neutral lipid but also phospholipids, proteins and carbohydrates, will most probably extend the optimal lipid inclusion level by providing additional potential emulsifying agents. Finally, the results support previous studies that showed that the rate of lipid digestion and absorption in fishes is slower than that of mammals.

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