Bol. Inst. Esp. Oceanogr. 14 (1 y 2). 1998: 5-18

Indicators of nutritional status of turbot *Scophthalmus maximus* (L., 1758) larvae

H. Paulsen[1], *N. E. Poulsen*[1], *J. Iglesias*[2], *M. Olmedo*[2], *B. Korsgaard*[3], *P. Lavens*[4] *and P. Burkhardt-Holm*[5,6]

[1] Danish Institute for Fisheries Research, P.O. Box 101, DK-9850 Hirtshals, Denmark
[2] Centro Oceanográfico de Vigo. Instituto Español de Oceanografía. Apartado 1552. E-36280 Vigo (Pontevedra), Spain
[3] Odense Universitet, Campusvej 55, DK-5230 Odense M, Denmark
[4] University of Ghent, Laboratory of Aquaculture and Artemia Reference Centre. Rozier 44, B-9000 Ghent, Belgium
[5] Zoologisches Institut der Universität Heidelberg. Im Neuenheimer Feld 230. D-6900 Heidelberg 1, Germany
[6] Current address: Interdisciplinary Centre for General Ecology. Unversity of Berne. Falkenplatz 16, CH-3012 Berne, Switzerland

ABSTRACT

Nutritional status indicators of larvae of turbot Scophthalmus maximus *(L., 1758) were investigated in feeding and starving turbot larvae from hatching up to an age of 53 days. The study included the whole-larvae parameters of length, dry weight and relative condition; the organ-related characteristics otolith diameter and intestinal histology; and the biochemical indicators fatty acids, ascorbic acid, RNA, DNA and total protein. Starvation was started at day 3, 9, 16, 23 and 27, and starved larvae were compared with feeding larvae from the same rearing group. After onset of starvation, lipid droplets in the intestinal epithelial cells disappeared within 24 h. Later, the influence of starvation also became apparent in most of the other indicators studied. Relative condition decreased by about 5%/day and relative otolith diameter increased by about 5%/day. A histologically observed total disappearance of chylomicrons and lipid droplets in the intestinal epithelial cells was in accordance with a drop in dry weight, protein content, and the fatty acids 18:1ω-9, 20:5ω-3 (EPA) and ω-3 HUFA (Highly Unsaturated Fatty Acids), as well as the RNA/DNA ratio in starved larvae. However, most of the indicators studied showed such a high variation between individual rearing groups that only the parameters lipid droplets in intestinal epithelial cells, relative condition and relative otolith diameter seem useful for detecting starvation in turbot larvae.*

Key words: Larvae, nutrition, starvation, turbot, *Scophthalmus maximus, Psetta maxima.*

RESUMEN

Indicadores del estado nutricional de larvas de rodaballo *Scophthalmus maximus* (L., 1758).

Se han analizado diferentes indicadores del estado nutricional de larvas de rodaballo Scophthalmus maximus *(L., 1758) alimentadas de hasta 53 días de edad y se han comparado con los de otras larvas sometidas a condiciones de inanición. Los indicadores estudiados fueron longitud, peso seco, condición relativa larvaria, diámetro del otolito, histología digestiva de las larvas, contenido en ácidos grasos, ácido ascórbico, RNA, DNA y proteínas. Los procesos de inanición se llevaron a cabo a los 3, 9, 16, 23 y 27 días de edad y los análisis de estas larvas fueron comparados con los de larvas alimentadas de las mismas edades. Durante las primeras 24 horas de inanición, las gotículas lipídicas en las células epiteliales desaparecen por completo del intestino. Después, la condición relativa disminuye aproximadamente el 5% por día y el diámetro relativo del otolito se incrementa en el 5% por día. Se producen también disminuciones en el peso seco, el contenido pro-*

teico, los niveles de ácidos grasos 18:1ω-9, 20:5ω-3 (EPA) y los de otros ácidos grasos altamente insaturados (ω-3 HUFA), y en la relación RNA/DNA. Sin embargo, la mayoría de los indicadores investigados muestran una alta variación, incluso entre las distintas experiencias de cultivo realizadas, por lo que se concluye que las gotículas de lípidos en las células epiteliales del intestino, la condición relativa y el diámetro relativo del otolito son las únicas variables que pueden ser útiles para detectar un proceso de inanición en larvas de rodaballo.

Palabras clave: Larva, nutrición, inanición, rodaballo, *Scophthalmus maximus, Psetta maxima.*

INTRODUCTION

Turbot *Scophthalmus maximus* (Linnaeus, 1758), also named *Psetta maxima* (Linnaeus, 1758), is considered one of the most promising species for marine fish-farming in Europe, and during recent years there has been an increase in the production of this species (Paulsen, 1989). One of the main problems in *S. maximus* rearing is the production of high quality larvae. It is therefore important to know whether the larvae are developing normally and are in good nutritional condition.

Malformations and malpigmentations are frequently observed in reared *S. maximus* larvae, and could be related to nutritional deficiencies, since supplementing their diet with polyunsaturated fatty acids and vitamins seems to reduce these problems (Reitan, Rainuzzo and Olsen, 1994).

Several studies have described the effects of starvation, but only a few comparative studies involving many indicators have been conducted (Segner *et al.*, 1987; Segner and Braunbeck, 1988; Mani-Ponset *et al.*, 1994). A comparison of starvation indicators is important in order to get a better overall picture of the starvation process, and to select indicators of malnutrition for practical use in aquaculture. A suitable indicator should be specific, so that starved larvae can be readily distinguishable from well-nourished ones. It should also be highly responsive, so that conditions can be improved before a point of no return is reached, and it should be practical, so that it can be used routinely.

In the present study, results of changes during early development and effects of starvation have been obtained from simultaneous measurements of a number of parameters previously reported to be affected by starvation. These include length, dry weight, condition (Ehrlich, Blaxter and Pemberton, 1976), intestinal histology (O'Connell, 1976; Burkhardt and Storch, 1989), otolith diameter (Rosenberg and Haugen, 1982; Secor and Dean, 1989), fatty acids (Witt, Quantz and Kuhlmann, 1984; Tandler *et al.*, 1989), ascorbic acid (Dabrowski, 1992), RNA and DNA (Buckley, 1979; Clemmesen, 1987), and protein (Korsgaard, 1991).

MATERIAL AND METHODS

Turbot larvae were reared under laboratory conditions in Vigo, Spain and in large outdoor tanks in Hirtshals, Denmark. Larvae for laboratory rearing were obtained from the Vigo institute's own broodstock (Forés *et al.*, 1991). Two days after hatching larvae for laboratory rearing were transferred to 2 500 or 10 000 l cultivation tanks. The initial larval density varied from 20 to 40 larvae/l. The larvae were initially fed the rotifers *Brachionus plicatilis* (O. F. Müller, 1768), followed by newly hatched San Francisco Bay brine shrimp *Artemia* nauplii, at a concentration of 0.2-1 *A.* nauplii/ml, from day 7, and by 24-h-old *A.* nauplii from day 11. The nutritional value of the food organisms was improved by addition of the unicellular algae *Isochrysis galbana* (Parke, 1949) and a commercial fatty acid supplement, Superselco (Artemia Systems, Belgium).

For rearing in outdoor tanks, 2-day-old larvae were obtained from commercial

hatcheries in Norway (Tinfoss Aqua) and Scotland (Golden Sea Produce). They were checked for viability before release into 92-m^3 and 2 200-m^3 concrete tanks. Two weeks earlier the tanks had been emptied, cleaned and inoculated with phyto- and zooplankton (2-20 copepods/l). Larvae were added to a density between 0.1 larvae/l (92-m^3 tank) and 0.01 larvae/l (2 200-m^3 tank). The rearing technique used has been described in Paulsen and Andersen (1989).

The effects of starvation were studied by transferring larvae to food-free conditions. Results from fed and starved larvae of the same rearing batch were compared. Transfer to food-free conditions occurred at five different ages: day 3 (end of yolk sac stage), 9, 16, 23 and 27.

Starvation conditions in the laboratory system were achieved by cutting off the food supply to some tanks in a parallel series. In the outdoor tank system larvae were attracted by light during the night, and transferred –in water– to food-free conditions. Larvae were starved for a period of up to 7 days, with daily sampling for analysis.

Length and weight measurements were based on samples of at least 20 larvae selected randomly from each batch, and recorded for a total of five rearings in the laboratory system, and six rearings in the outdoor tank system. Length was measured as standard length (± 0.05 mm) immediately after sampling using a low-power microscope. Weight was determined after drying for 24 h at 60 °C (± 0.05 µg). A length-weight relationship was calculated from a regression of log weight on log length, for fed larvae. The relative condition of larvae was calculated as the measured weight multiplied by 100 and divided by the normal weight, calculated from the length-weight relation.

Larvae sampled for otolith analysis were preserved in 70 % ethanol. Maximum otolith diameter was measured on a total of 576 fed larvae and 256 starved larvae representing five batches, three in the laboratory system and two in the outdoor tank system. Of the six otoliths, the two largest (sagittae) were removed and mounted in Eukitt synthetic resin. Otolith measurements were made using a compound microscope equipped with a video monitor, at × 400 magnification.

Samples for intestinal histology were processed and fixed immediately for use in transmission electron microscopy, and studied as described by Segner *et al.* (1987). Samples for biochemical analysis were stored at –80 °C until they were freeze-dried. Fatty acids were quantitatively analysed by gas chromatography, as described by Léger, Bengtson and Sorgeloos (1989). For lipid extraction, the procedure of Ways and Hanahan (1964) was used. Esterification was done according to Lepage and Roy (1984). Ascorbic acid was quantitatively analysed using liquid chromatography, as described by Nelis, Sorgeloos and De Leenheer (1997). Total protein was determined according to Bradford's method (1976). RNA and DNA were determined according to the method of LePecq and Paoletti (1966), later modified by Karsten and Wollenberger (1972, 1977).

Statistical analysis

The non-parametric Wilcoxon's signed rank test was chosen to avoid problems due to deviations from a normal distribution of the data. Possible general differences between fed and starved larvae were tested by the use of the non-parametric Kolmogorov-Smirnov two-sample test. Difference in length and weight between fed and starved larvae were tested using the Mann-Whitney *U*-test. Statistical analysis and regressions were performed with SYSTAT computer software.

RESULTS

Length-weight

A length-weight relation of *log* length (*log* L) to *log* weight (*log* W) produced a

good fit ($r^2 = 0.98$) to a linear regression model with the equation:

$$\log W \text{ (mg)} = 3.74 \times \log L \text{ (mm)} - 3.48$$
$$(n = 2\,217,\ r^2 = 0.98)$$

The weight growth rates for the individual batches, calculated as specific growth rates (%/day) over the rearing period, are shown in table 1.

Four laboratory rearings and four of the outdoor tank rearings included starvation periods. The effect of starvation on length and weight growth from day 16 and 23 in an individual experiment is shown in figure 1. During starvation the larvae rapidly started losing weight, whereas length seemed to remain constant. The relative condition, therefore, declined (figure 2). The confidence limits (95 %) of the regression line show that the decline in relative condition is significant after 2 days of starvation. The decline is on the order of 5 %/day, being dependent upon factors such as age and temperature. Since the relative condition declined during starvation, it was investigated whether the natural differences in growth rates among individuals in a batch also produced differences in relative condition. The results showed no indication of a lower relative condition in the slow-growing larvae.

Otolith diameter

The otolith diameter-body weight relationship could be described with a linear regression model. Differences between batches were significant, with larvae from the slowest-growing batch having larger otoliths in terms of length than larvae from faster-growing batches. The relative otolith size was calculated as the difference (in %) between the normal otolith diameter from the diameter-length regression, and the observed diameter. During starvation otolith growth was reduced, but the reduction was less than the reduction in length growth. The otoliths consequently became relatively larger in starving larvae, compared with feeding larvae of the same size, at a rate of approximately 5 %/day of starvation (figure 3). It was, therefore, decided to study whether the slow-growing fish in a batch also showed signs of starvation in the form of relatively larger otoliths. Figure 3 shows regressions of otolith diameter at

Table I. Mean specific weight growth rate (%/day) for five intensive laboratory and six outdoor tank rearings of turbot larvae. Length and weight growth rate (%/day) for fed and starved larvae. Samples of 20 larvae

Batch	Sampling (code) (days)	Total number period sampled	Weight growth of larvae	Starvation period (%/day) (days)	Length growth (%/day)		Weight growth (%/day)	
					Fed	Starved	Fed	Starved
Lab. 5	0-11	160	7.7	3-7	3.4	0.6	12.8	–6.8
				9-11	–1.0	1.0	0.0	–3.1
Lab. 12	0-25	220	21.2	16-21	7.5	4.3	31.1	8.1
Lab. 13	9-21	180	29.6	16-19	5.4	–2.6	23.1	–27.0
Lab. 53	0-25	200	22.6	16-20	5.7	0.4	25.7	0.0
Lab. 69	0-12	220	20.2					
Tank 3	10-35	380	14.1	16-20	2.3	–0.9	7.5	–7.5
				27-35	4.7	1.7	11.9	–0.8
Tank 4	0-7	100	7.4	3-7	3.4	1.2	68.5	3.5
Tank 5	5-28	120	41.7	16-18	12.3	0.0	48.2	–3.4
				23-28	6.1	0.4	16.3	–5.1
Tank 6	10-14	80	12.9					
Tank 7	3-53	180	10.5					
Tank 8	4-20	180	17.1					

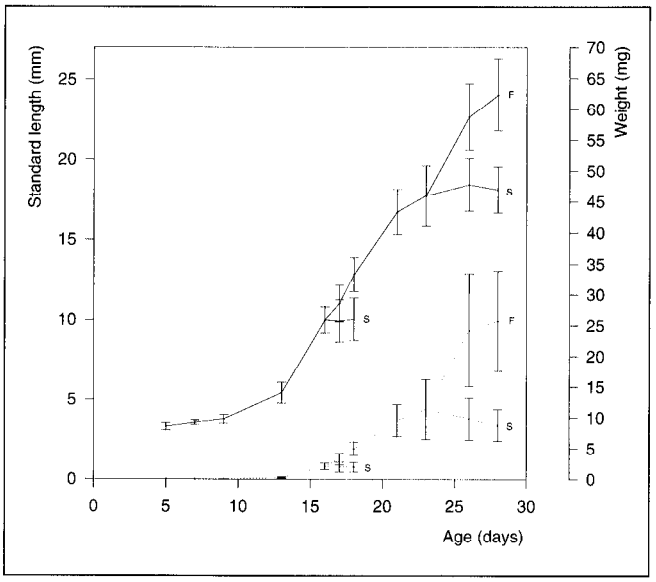

Figure 1. Effect of starvation on turbot larvae. Length at age (—) and weight at age (···) ± SD for 210 fed (F) and 80 starved larvae (S) from tank rearing 5. Starvation from day 16 and day 23

length for feeding and for starving larvae, and for feeding larvae at different ages. The results show that starving larvae have larger otoliths than feeding larvae of the same length. Calculation of confidence limits (95 %) for the regressions shows that the difference is significant. The results also show that for fed larvae of the same length, the oldest = slowest-growing larvae have larger otoliths than the youngest = fastest-growing larvae.

As an example, it can be observed (figure 4) that larvae reaching 19 mm at day 21 have a mean otolith diameter of 245 µm, whereas larvae reaching 19 mm at day 28 have a mean otolith diameter of 280 µm.

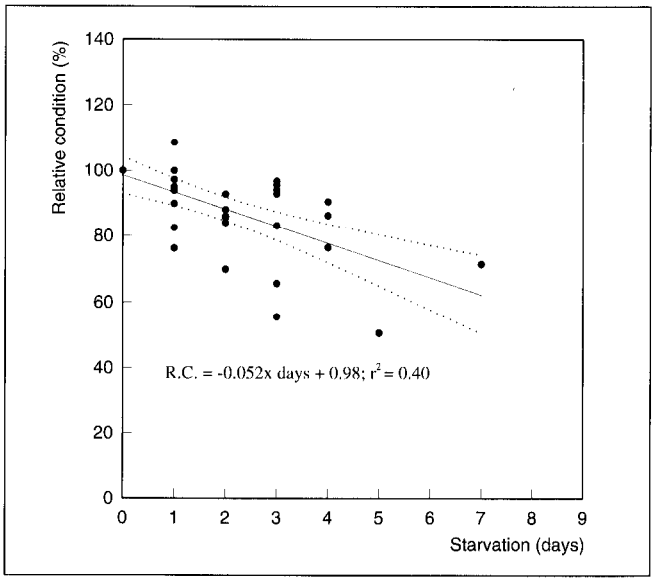

Figure 2. Relative condition of starved turbot larvae. Mean values from four rearings in laboratory intensive system and two rearings in outdoor tanks. Starvation started at day 9, 16, 23 and 27. Regression line with 95 % confidence limits based on 33 mean values from a total of 600 larvae. Relative condition: $-0.052x$ days + 0.98; $r^2 = 0.40$

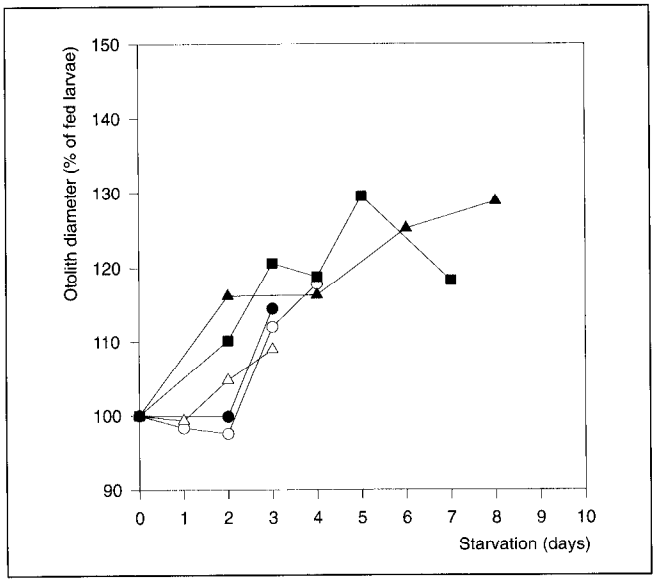

Figure 3. Mean otolith diameter of starved turbot larvae as percentage of diameter in fed larvae of same length. Larvae from laboratory rearing: Batch 53 day 16 (●), batch 12 day 16 (○), and from outdoor tanks: Batch 3 day 16 (△), batch 3 day 27 (■) and batch 5 day 16 (▲). Total 576 fed larvae and 256 starved larvae

Intestinal histology

Using histological criteria, the intestine can be divided into midgut, hindgut and rectum (Stroband, Meer and Timmermanns, 1979; Segner *et al.*, 1987). The ultrastructure of the intestinal epithelial cells was described by Segner *et al.* (1994). Signs of starvation are most evident in the midgut, the first part of the intestine proper.

The present study was therefore restricted to this part.

The midgut of fed larvae was characterised by lipid particles (chylomicrons) and lipid droplets in the absorptive epithelial cells (figure 5). The former were seen predominantly in small vesicles and cisternae of the endoplasmic reticulum, as well as in vacuoles of the Golgi apparatus and in the basolateral intercellular space. Where they occurred in high number, they were accompanied by lipid droplets, which were not membrane-bound. Areas which were fixed before the food bolus passed through did not show signs of lipid absorption.

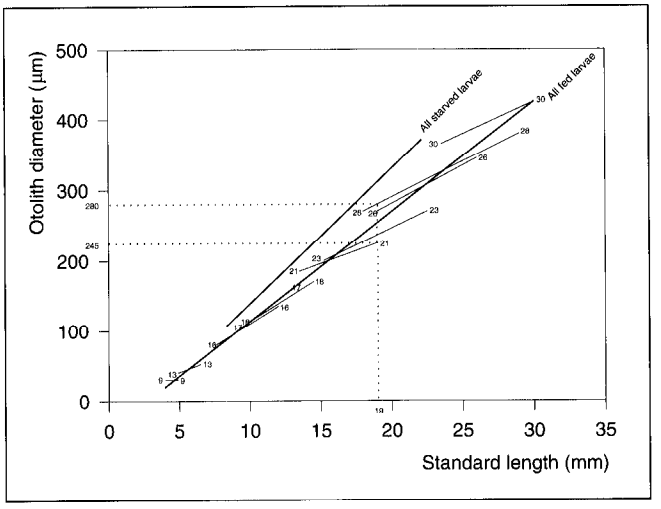

Figure 4. Regressions of otolith diameter on larval standard length from outdoor tank batch 5. Numbers indicate endpoints of length range for larvae of same age. E.g. 28-28 shows regression of 28-day-old larvae between ca. 17 mm and 29 mm. Total 183 fed larvae and 79 starved larvae

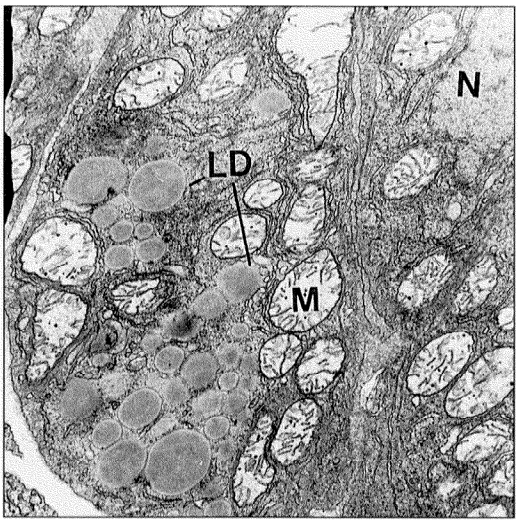

Figure 5. EM photo from the proximal part of the intestine in a recently fed larva. (LD): lipid droplets; (M): mitochondria; (N): nucleus. ×1 100 magnification

After onset of starvation (figure 6) a picture of 'no absorption activity' emerged, with a total absence of chylomicrons and later of lipid droplets, as well, along the entire length of the intestine. As starvation proceeded, the microvillus border in the proximal part of the intestine reduced in height and the number of myelin-like structures increased. These structures were found in the cytoplasm, in mitochondria and, less often, in cisternae of the Golgi apparatus and endoplasmic reticulum. Lysosomes were often multiplied, enlarged, and had a dark and heterogeneous appearance. The presence of crystal-like inclusions in some lysosomes was noteworthy. The endoplasmic reticulum was broken in short cisternae and vesicles and a condensation of the cytoplasm and a dilation of the endoplasmatic cisternae and the nuclear membranes were often seen. Finally, a widening of the intercellular space and loosening of cell contacts could be seen. In several specimens, an invasion of lymphocytes and macrophages into the base of the epithelium was observed.

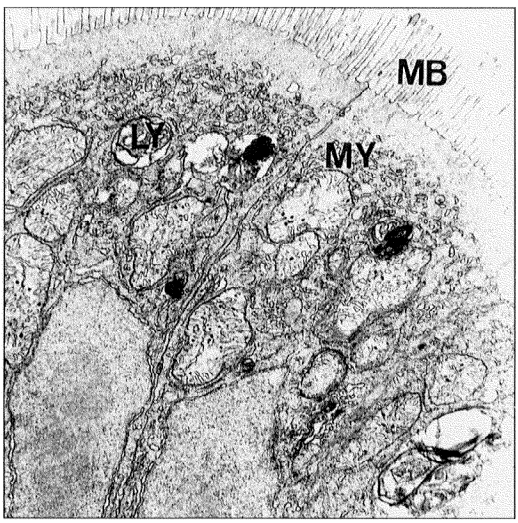

Figure 6. EM photo from the proximal part of the intestine in a state of no absorption activity. (MB): microvillus border; (MY): myelin-like structures; (LY): lysosomes. ×9 500 magnification

Fatty acids

Feeding and starving larvae were analysed for content of fatty acids (18:1ω-9, 18:3ω-3, 18:3ω-6, 20:4ω-6, 20:5ω-3 (EPA), 22:6ω-3 (DHA); total ω-6, total ω-3; highly unsaturated fatty acids (HUFA); and total lipids).

A comparison of fed and starved larvae from the same batch and age, using Wilcoxon's signed rank test, showed that the content of 18:1ω-9, 18:3ω-3, 20:4ω-6, EPA, HUFA and the ratio EPA/DHA was significantly reduced ($p < 0.05$).

Factors such as batch differences and age had a clear effect on the content, and only for 18:1ω-9, EPA and HUFA could a general significant difference be observed (Kolmogorov-Smirnov two-sample test). Figure 7 shows the effect of starvation time on the content of these three lipids. The data for the other fatty acids are not presented.

Ascorbic acid

Ascorbic acid content was measured in starved larvae from hatching until day 7.

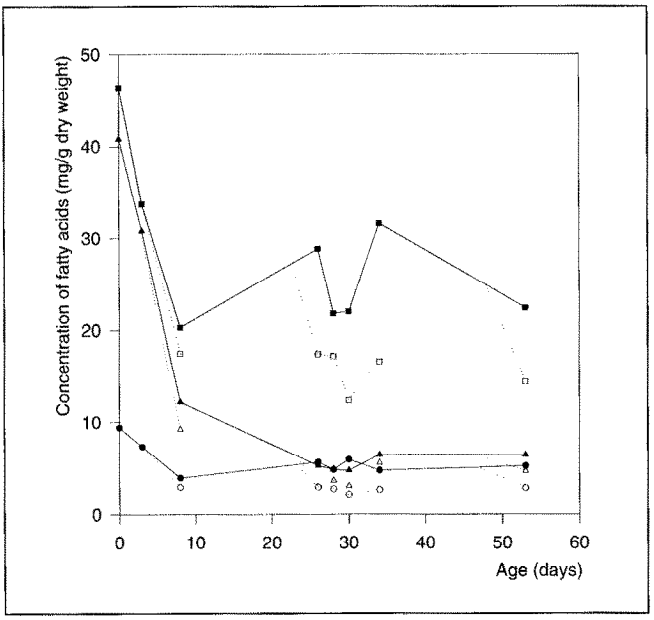

Figure 7. Concentrations of fatty acids in fed and starved turbot larvae from outdoor tank rearings. 18:1ω-9: (▲): fed, (△): starved; 20:5ω-3 (EPA): (●): fed, (○): starved; ω-3 HUFA: (■): fed, (□): starved

The results indicated a decline from a level of 400-600 µg ascorbic acid/g dry weight at hatching to less than 300 µg ascorbic acid/g dry weight after day 3 (figure 8).

RNA, DNA and protein

The concentration of RNA, DNA and protein expressed as mg/g dry weight are presented in figure 9. The concentration of RNA and DNA seemed to decline gradually after day 7, while the concentration of protein remained relatively constant.

Comparing starving and feeding larvae from the same rearing group and age showed a general tendency for a higher concentration of all three substances in starving larvae, but the difference was not significant (Wilcoxon's signed rank test $p > 0.05$).

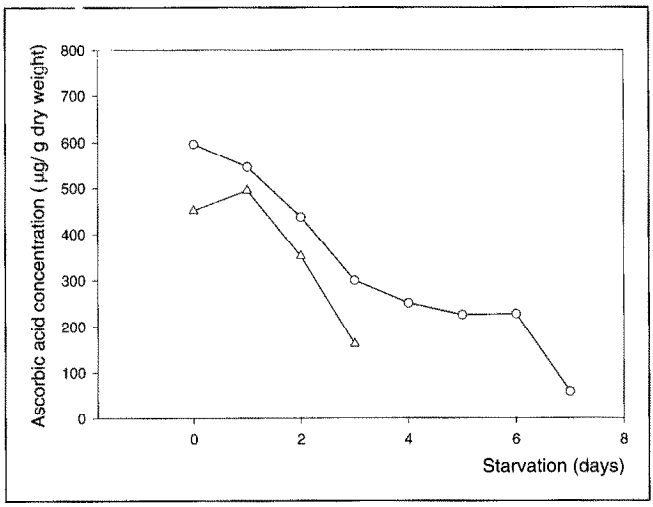

Figure 8. Concentration of ascorbic acid in starved turbot larvae: (△): outdoor tank; (○): laboratory rearing

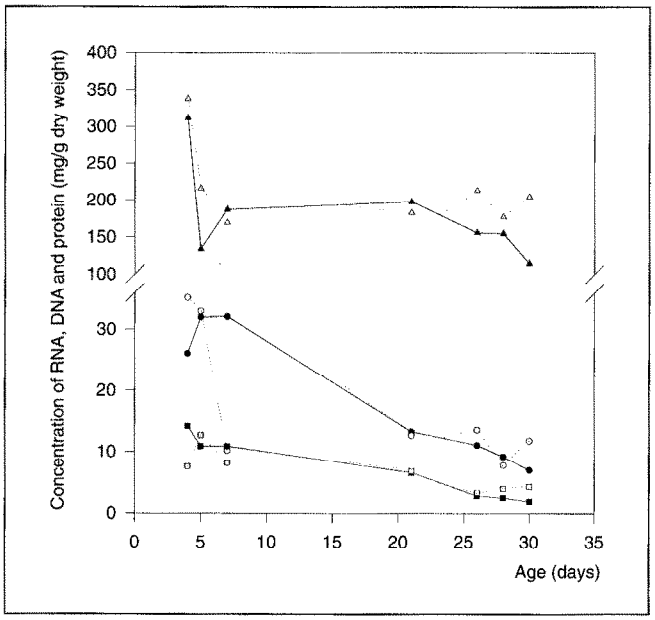

Figure 9. Concentrations of RNA, DNA and protein in fed and starved turbot larvae from outdoor tank rearings. Protein: (▲): fed, (△): starved; RNA: (●): fed, (○): starved; DNA: (■): fed, (□): starved

DISCUSSION

Growth

The length-weight data showed highly variable growth between batches, with a tendency to higher variation in the outdoor tanks than in the laboratory system (table I). The differences may be caused by differences in food availability, food quality, temperature and other unknown factors. These results are in good agreement with the results of other investigations.

In outdoor tanks, Paulsen and Andersen (1989) observed a growth rate of 20-34 %/day at day 4-31, and Danielsen, Haugen and Øiestad (1990) of 32-36 %/day at day 2-37 and 51 %/day at day 14-17. In laboratory culture, Olesen and Minck (1983) observed a growth rate of 35 %/day at day 2-10 and 26 %/day at day 10-24; Gatesoupe (1990) of 29 %/day at day 3-10; and Støttrup and Attramadal (1992) of 18-30 %/day at day 2-13.

Also within the individual batches, there was a high variation in growth rate between fast-growing individuals and slow-growing individuals. As an example of this, figure 4 shows that fast-growing individuals had a body length almost twice that of slow-growing individuals sampled the same day. Calculation of relative condition for fish from slow-growing rearings and fast-growing batches, and for slow- and fast-growing individuals in the same batch, provided no indication that slow growth was associated with a reduction in relative condition. Larvae subject to starvation showed a reduction in relative condition of about 5 %/day (figure 2), suggesting that fish larvae experiencing food shortages generally get smaller, but maintain their condition, probably until food intake approaches the requirements for basic metabolism. Calculation of relative condition may therefore be useful for identifying starvation or serious food deprivation.

Otolith diameter

The results showed a high correlation ($r^2 = 0.98$) for a linear relationship between otolith diameter and standard length of the larvae. The relationship was, however, different for the different batches, with the slowest-growing batch having the largest otoliths in terms of length. In order to observe deviations in otolith length, a relative otolith size was calculated as the differ-

ence in % from the diameter at length regression models. In starved larvae, an increase on the order of 5 %/day could already be observed after 1-2 days starvation (figure 3). Within a batch or a cohort of larvae there were normally considerable differences in individual growth rates. When the results of slow- and fast-growers were compared, the former had relatively larger otoliths (figure 4). The results, therefore, seem to support recent reports of growth effects on otolith diameter, where slow-growing fish have relatively larger otoliths (Secor and Dean, 1992).

Several explanations for this relationship between otolith size and somatic growth rate have been suggested. Mosegaard, Svedang and Taberman (1988) and Wright (1991) suggested a relationship to metabolism in general, whereas Secor and Dean (1992) suggested that otolith growth relates to age. The present study showed that even during starvation there was otolith growth, while body length remained constant and weight declined. Otolith size was therefore related to metabolism, and calculation of relative otolith size may be useful for evaluation of past growth rate or recent starvation.

Intestinal histology

The first part of the intestine is known to absorb nutrients (Segner *et al.*, 1987; Burkhardt and Storch, 1989). The first signs of starvation of cod *Gadus morhua* L., 1758 larvae are also found in this part of the gut (Kjorsvik *et al.*, 1991). The restriction of the lipid droplets and chylomicrons to the area where the food bolus just passed by can be explained by the quick reaction of these specialised intestinal absorptive cells. When the lipids are transported to the lymph and blood vessels, the cells are again in a state of no absorption activity.

The ultrastructural changes that were observed in the first part of the intestine of starved *S. maximus* larvae seem to be similar to observations of other species during food deprivation (Gas and Noailliac-Depeyre, 1976; Segner *et al.*, 1987; Burkhardt and Storch, 1989; Kjorsvik *et al.*, 1991). The first sign of starvation is the reduction in lipid droplets and chylomicrons; this is in accordance with the drop in lipid and dry weight of starved larvae. With ongoing starvation, a fragmentation of the epithelial microvilli leads to a reduction of the absorptive surface (Burkhardt and Storch, 1989) and often to a decrease in enzyme activities (Gas and Noailliac-Depeyre, 1976). A decrease of the height of the intestinal epithelium during starvation has also been confirmed by image analysis (McFadzen, Lowe and Coombs, 1994). Further, an increase in lysosomes, their enlargement and content of electron-dense debris was often observed which indicates an increased turnover of cellular components in starved carp. The association of lysosomes with myelin-like whorls was described by Gas and Noailliac-Depeyre (1976). The widening of the intracellular space and condensation of the cytoplasm goes along with the observed change in the ratio of protein and DNA, indicating a decrease in cell volume. An invasion of macrophages and lymphocytes in the intestinal epithelium was observed under starvation conditions as well as under pathological conditions, pointing to an inflammatory reaction.

Quantification of histological and cytological criteria to assess the nutritional status can be done morphometrically (McFadzen, Lowe and Coombs, 1994; Hall and Bellwood, 1995). However, since this is rather expensive in terms of time and hardware, we suggest a qualitative histological evaluation of the midgut of starved larvae based on ordinary light microscopy. The best method is probably application of histochemical lipid detection in cryostat sections, as described by Segner and Braunbeck (1988).

Fatty acids

The fatty acid composition in newly hatched larvae reflects the content in the eggs, which again is determined by mater-

nal nutrition (Chou *et al.*, 1993). During larval development some general tendencies could be observed, but it should be noted that the rearing conditions and growth rates also influence fatty acid composition. The first week of development is characterised by the consumption of the yolk sac and relatively passive feeding on small live food. Later the fatty acid composition is primarily determined by the composition in the food. During the first week a decrease in the lipid content was observed, with the possible exception of 20:4ω-6, which seemed to be maintained. The literature generally reports a decrease in 18:1ω-9, whereas the results for other lipids are conflicting (Witt, Quantz and Kuhlmann, 1984; Rainuzzo, Reitan and Jørgensen, 1992).

Comparing fed and starved larvae, the results showed a general tendency for reduced lipid content in the starved larvae. For 18:1ω-9, EPA and HUFA there was an overall significant difference between fed and starved larvae ($p < 0.05$) (figure7). Comparing fed and starved larvae from the same batch and day, the difference was also significant for 18:3ω-3, 20:4ω-6 and DHA/EPA. Since at this time the protein content was relatively constant (see below), it indicates that lipids are the prime source of energy during this period. Lipids are the most efficient nutrients for supplying energy for movement, and during early postlarval development the intestinal epithelium serves as a fat depot (Tanaka, 1972).

Generally it seemed that ω-9 loss was higher than ω-6 loss in *S. maximus* larvae, and that the essential fatty acids were conserved under starvation. Our findings coincide with the earlier results of Tandler *et al.* (1989) on red seabream *Pagrus major* (Temminck and Schlegel, 1843).

The DHA/EPA ratio showed a clear increase during starvation, indicating that DHA is better conserved than EPA; this may be due to selective incorporation of this essential fatty acid into membranes and neural tissues (Sargent *et al.*, 1993). The conservation of DHA under starvation conditions has also been shown for cod (Fraser, Gamble and Sargent, 1988).

RNA, DNA and protein

In feeding larvae, the concentration of protein, DNA and RNA dropped (figure 9), probably indicating synthesis of relatively higher amounts of non-protein substances during ossification.

The ratio of protein to DNA is a measure of the relationship between cell mass (total protein) and cell number (DNA). The increase observed for feeding larvae in the protein/DNA ratio indicates that larval growth from days 20-30 is primarily a result of an increase in cell volume. During prolonged starvation (day 21, day 28 and day 30) this ratio decreased, indicating that cell mass is reduced compared with feeding larvae. This observation is in accordance with reports by Clemmesen (1987) on turbot larvae and by Raae *et al.* (1988) on cod larvae.

Expressing results in terms of the RNA/protein ratio is assumed to give an index of protein synthesis intensity (McMillan and Houlihan, 1992). Thus, high concentrations of RNA will result in a greater stimulation of protein synthesis, leading to an increase in body protein deposition, and, consequently, in growth. A gradual decrease in the RNA/protein ratio was observed after termination of the yolk sac phase. Starvation may change activity as well as numbers of ribosomes in a cell.

The RNA/DNA ratio has been used as an indicator of starvation. A ratio of 2.5 for larvae older than 20 days has been suggested as critical (Clemmesen, 1987). In the present study, starved *S. maximus* larvae subject to prolonged starvation (day 7, day 21, day 28 and day 30), showed indications of a lower RNA/DNA ratio than fed larvae. The results thereby support results obtained by Clemmesen (1994) on herring *Clupea harengus* L., 1758 larvae.

The differences found in our study were not statistically significant. This may be attributed to the limited number of observations available.

The present investigation has shown such a high natural variation in most of the parameters that their value as indicators of starvation seems to be limited. Histological

evaluation, relative otolith diameter and relative condition seem to be the most useful for identification of starved larvae from an unknown sample.

It is recommended that system- and species-specific reference values be established for larvae of commercially important fish species. The values should relate growth rate to temperature, condition, otolith size, total lipid content, ω-3 HUFA content and 18:1ω-9 content. Individual batches of larvae should be compared to these reference values for early detection of deviations from normal development in order to improve rearing methodologies.

ACKNOWLEDGEMENTS

The present study was supported by the EU Commission under the research programme Fisheries and Aquaculture Research (FAR), project AQ-1-60. The participants are most grateful to the European Commission, and particularly to Dr Victor Bye from the Commission for his support for the project and his suggestions.

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Received October 1997. Accepted December 1998.