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Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activities with a highly sensitive fluorescence technique

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Abstract

Based on conventional chromogenic methods for the analysis of enzyme activities, a highly sensitive, rapid, reproducible, and easy-to-handle fluorescence technique for the determination of tryptic enzyme activity in individual fish larvae is described. The application of Nα-carbobenzoxy-L-arginin-4-methylcoumarinyl-7-amide (CBZ-L-Arg-MCA) as a fluorogenic substrate results in an approximately 500-fold increase in sensitivity, as compared to conventional chromogenic methods. The sensitivity allows measurements of the tryptic enzyme activity in one herring yolk-sac larva (approximately 25 µg larval dry weight).

An example for an application to laboratory-reared larvae is given, showing that the nutritional state of individual fish larvae and the amount of variability can be determined.

This method should be a helpful tool in the field work on the nutritional condition of individual larvae and might be useful for testing the "starvation hypothesis".

Kurzfassung

Bestimmung des Ernährungszustandes von marinen Fischlarven durch individuelle Analyse der proteolytischen Enzymaktivitäten mit einer hochempfindlichen Fluoreszenztechnik

Auf der Basis konventioneller, chromogener Methoden zur Bestimmung von Enzymaktivitäten wurde eine hochempfindliche Fluoreszenztechnik zur Bestimmung von Trypsinaktivitäten entwikkelt. Damit steht eine schnelle, einfach anzuwendende und reproduzierbare Methode zur Bestimmung der Trypsinaktivität in einzelnen Fischlarven zur Verfügung. Die Verwendung von Nα-Carbobenzoxy-L-arginin-4-methylcoumarinyl-7-amid (CBZ-L-Arg-MCA) als fluorogenes Substrat führt zu einer ungefähr 500fachen Erhöhung der Empfindlichkeit, verglichen mit den konventionellen Methoden. Die Meßempfindlichkeit ist hoch genug, um die Trypsinaktivität in einer einzelnen Heringsdottersacklarve zu bestimmen (entsprechend einem Fischlarven-Trockengewicht von etwa 25 µg).

Am Beispiel von im Labor aufgezogenen Fischlarven wird gezeigt, daß mit dieser Methode eine individuelle Bestimmung der Ernährungssituation von Fischlarven und der Schwankungen innerhalb einer Gruppe möglich ist.

Die beschriebene Methode kann ein hilfreiches Instrument bei Arbeiten zur Bestimmung des Ernährungszustandes von *in situ* aufwachsenden Fischlarven sein und sollte bei der Aufklärung von Fragen zur „Verhungerungshypothese" beitragen.

Résumé

Détermination de la condition nutritive de larves individuelles de poissons marins par l'analyse de leurs activités d'enzymes protéolytiques avec une méthode fluorescente de haute sensibilité

Sur la base de méthodes chromogènes conventionelles, une technique très sensible, rapide, reproductible et facile a été developpée pour la détermination des activités d'enzymes tryptiques dans des larves de poisson séparées. L'utilisation de Nα-Carbobenzoxy-L-Arginin-4-Methylcoumarinyl-7-Amide (CBZ-L-Arg-MCA) comme substrat fluorogène rend une sensibilité 500-fois plus élevée que les méthodes conventionelles. Cette sensibilité suffit pour l'analyse de l'activité enzymatique de trypsine dans une seule larve d'hareng au sac vitelline (poids sec de 25 µg environ).

Un example de l'application de la technique aux larves élevées dans le laboratoire est donné, démonstrant que la condition nutritive de larves isolées peut être déterminée ainsi que la variabilité entre les larves.

La méthode présentée ici devrait servir à l'analyse de la condition nutritive *in situ* des larvés séparées, et elle pourrait être utile pour verifier «l'hypothèse d'affamation».

Introduction

Recruitment processes and the factors which influence growth and stock size are one of the major areas in fishery research. Studies on many different fish populations have shown that year-class strength can vary extremely from year to year without any relation to egg production and stock size. Lo (1985), for instance, found no relationship between egg production and corresponding year-class strength for a 24-year time series on northern anchovy.

Survival of fish larvae depends on the rates of predation and starvation (Hunter 1984). However, a lack of appropriate methods has impeded efforts to determine which of the two is the major factor dominating survival of the early ontogenetic stages of teleosts.

While it is difficult to simulate and to measure natural predation experimentally, it is readily possible to produce fed and starving larvae in the laboratory in order to obtain reference data with corresponding methods for field investigations. This approach requires rapid and reliable methods for the determination of the nutritional condition of the fish larvae.

Based on the "critical period concept" (Hjort 1914), various authors have investigated the starvation hypothesis using a variety of different methods. Length/weight relationships (Shelbourne 1957; Blaxter 1971), morphometric (Ehrlich et al. 1976) and chemical (Ehrlich 1974a, b, 1975) methods have been used to characterise the condition of larvae. These methods have limitations when applied to wild fish larvae, including allometric growth, metabolic changes during larval development and differences between laboratory reared and wild larvae. Histological methods which allow the detection of nutritional state by observing distinct cellular changes occurring in tissues of larval fish deprived of food (O'Connell 1976, 1981; Theilacker 1986) are helpful, but time consuming when used on a large number of samples. Besides, determination of the nutritional condition by histological methods is dependent on the experience of the observer.

Biochemical methods appear promising, as they should give information about the condition of fish larvae on the most sensitive cellular level, and are therefore compatible to histological assessments. But in contrast to the latter method large sample numbers can be investigated objectively, quantitatively and reproducibly once appropriate biochemical criteria are defined and routine methods for their analysis are developed.

Due to their essential role in metabolic reactions, enzymes can be good indicators for the condition of an organism. For fish larvae, the activity level of proteolytic enzymes is well suited as an indicator of the feeding activity, because protein is the major component of their diet. Trypsin, an endoproteinase which hydrolyses peptide bonds at the carboxyl groups of L-arginine and L-lysine, has been chosen as an indicator enzyme, because it is directly connected to the protein metabolism and is present in very young fish larvae (Alliot et al. 1977; Dabrowski 1979; Hjelmeland et al. 1984; Lauff and Hofer 1984; Ueberschär 1985; Pedersen et al. 1987).

Dabrowski (1982) was one of the first to demonstrate the dependence of the proteolytic enzyme activity in larvae of whitefish and rainbow trout on the amount of food ingested, and tried to identify the "point of no return" by using the proteolytic activity as an index. Hjelmeland et al. (1984) have shown that the amount of trypsin and trypsinogen in cod larvae is an indicator of their growth and survival potential.

Conventional chromogenic methods for analysis of tryptic enzyme activity (Erlanger

et al. 1961) do not allow to determine the enzyme activity of single larvae, especially of their youngest stages, 10–20 larvae must be pooled to provide sufficient tissue for analysis (UEBERSCHÄR 1985). Pooled samples give no information about the individual variability which is important for the interpretation of the nutritional situation actually existing for the individual fish larvae under in situ conditions.

HJELMELAND and JØRGENSEN (1985) have described a trypsin-radioimmunoassay, sensitive enough to determine the content of trypsin and its precursor trypsinogen in an individual larval fish. However, the method requires a large effort and needs to be completed by a sensitive tryptic enzyme activity assay (HJELMELAND and JØRGENSEN 1985).

The present study describes a simple, highly sensitive fluorometric method for the determination of tryptic enzyme activity in single fish larvae which can also be used aboard research vessels.

Material and methods

Larval material

Laboratory-reared herring and turbot larvae were used to develop and verify the new method. The larvae were kept under different feeding conditions and fed with *Brachionus plicatilis* and *Artemia*-nauplii. The laboratory conditions used for rearing of the larvae are described by UEBERSCHÄR (1985).

The larvae were conserved deep frozen (−74 °C) prior to analysis, to prevent any protein autolysis.

Extraction of tryptic-like enzymes

Single fish larvae were homogenized in an ice-cold POTTER-ELVEHJEM microhomogenizer, using 0.5 ml ice-cold TRIS-HCl buffer (0.1 molar, pH 8.00) containing 0.02 Mol $CaCl_2 \times H_2O$. The Ca^{2+}-ions prevent the formation of inert trypsin aggregations (MCDONALD and KUNITZ 1946). The total body homogenate was transferred into EPPENDORF-microtubes and centrifuged at 6000 rpm for 60 min. This crude purification step is necessary to remove tissue fragments. Temperature during centrifugation was kept near 0 °C. The supernatants of the homogenates were then used to measure tryptic enzyme activity.

Chemicals

All chemicals used in the analytical procedure were analytical or research grade. Nα-carbobenzoxy-L-arginine-4-methylcoumarinyl-7-amide (CBZ-L-Arg-MCA), trypsin (twice cristallized, lyophilized, 40 U/mg) and trypsin-inhibitor (soybean, ca. 35 IU/mg) were obtained from SERVA, Heidelberg, West Germany.

Fluorescence assay

The fluorometric assay of tryptic enzyme activity depends on the use of a specific substrate, in this case an amide coupled with a fluorophore as the leaving group. Nα-carbobenzoxy-L-arginine-4-methylcoumarinyl-7-amide (CBZ-L-Arg-MCA), a derivative of L-arginine-4-methylcoumarinyl-7-amide, was used as the specific substrate for measuring tryptic enzyme activity. This substrate and its hydrolytic products are all fluorescent. However, when excited at 380 nm and measured at 440 nm, fluorescence intensity of the hydrolytic products is approximately 700-fold higher than that of the substrate itself so that the faint fluorescence of the substrate does not interfere with the fluorometric assay. The synthesis and fluorescent properties of L-arginine-4-methylcoumarinyl-7-amide and its derivatives have been described by KANAOKA et al. (1977).

The substrate concentration was as follows: 0.2 mMol CBZ-L-Arg-MCA (final concentration in the cuvette 0.17 mMol) in TRIS-HCL-buffer (0.1 Mol, pH 8.00), containing 0.02 Mol $CaCl_2 \times H_2O$. Because of the insolubility of the substrate in water, it was first dissolved in dimethylsulfoxide (DMSO, final concentration 0.5 %, v/v) and then diluted with buffer to the required concentration.

Assays of tryptic activity were typically performed as follows: To 500 µl substrate solution in the cuvette 100 µl trypsin standard solution or 100 µl larval homogenate were added and well mixed. When necessary, the larval homogenates were prediluted with buffer.

The assays were carried out in microcuvettes at 25 °C using a KONTRON spectral fluorometer (model SFM 25) with a temperature controlled automatic cuvette changer. The increase in emission at 440 nm (excitation 380 nm) was measured in intervals of 1.7 min. over a total of 15.3 min. (9 intervals). A flow chart of the analytical procedure is given in Fig. 1.

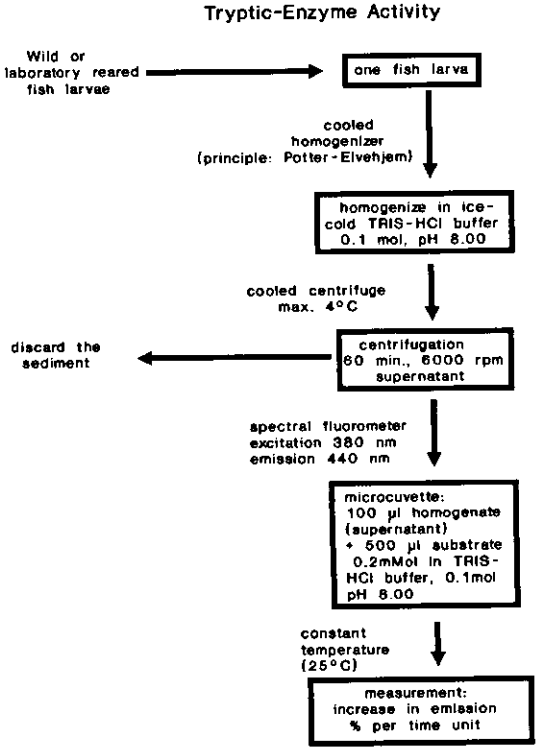


Fig. 1. Flow chart of analytical procedure

The fluorescence enhancement, resulting from the enzyme activity is given as the difference in emission at the beginning and at the end of an interval. The result was calculated as the mean value of several interval differences and expressed as the increase in emission per time interval. It is recommended to take time intervals in the middle of the measuring period because linearity is guaranteed in this range.

Results

Substrate concentrations

Several different substrate concentrations were incubated with either trypsin solution (constant concentration) or with larval homogenate (Fig. 2). The optimal substrate concentration, giving maximal reaction velocity (V_{max}), was within the range of 0.15–0.17 mMol for both the trypsin standard solution and the larval homogenate. For further analyses, an initial substrate concentration of 0.2 mMol was used (final concentration 0.17 mMol).

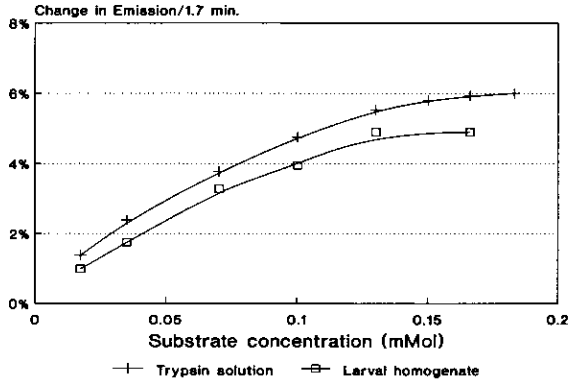


Fig. 2. Increase in emission in relation to the substrate concentration (abscissa gives the final concentrations) with a trypsin standard solution and with larval homogenate. Substrate CBZ-L-Arg-MCA in TRIS-HCl buffer, pH 8.0, 25 °C, excitation 380 nm, emission 440 nm, $\delta t = 1.7$ min.

Specificity of the chosen substrate to tryptic enzymes in a larval homogenate

It is assumed that the total body homogenate of a fish larva contains proteolytic enzymes other than trypsin in unknown and changing amounts, and that these may participate in hydrolysing the fluorogenic substrate. To test what fraction of the total proteolytic enzyme activity is due to trypsin alone, the homogenate was treated with a specific trypsin inhibitor (0.1 mg/ml). The enzyme activities of these two samples, total activity and trypsin-inhibitor added sample were then measured as described above (Table 1).

Table 1 shows that the proportion of tryptic enzyme activity is about 93 % of the total proteolytic activity measured. To determine the effectiveness of the inhibitor, a trypsin

Table 1. **The relative share of non-tryptic enzyme activity in larval homogenate after adition of a trypsin specific inhibitor (soybean)**

Homogenate of 27-day-old herring larvae. Total proteolytic enzyme activity without inhibitor = 100 %, substrate CBZ-L-Arg-MCA, 0.20 mMol in Tris-HCl buffer, pH 8.0, 25 °C, δt = 1.7 min., excitation 380 nm, emission 440 nm

	Trypsin standard solution 3.3×10^{-4} U	Larval homogenate
Without inhibitor, rel. emission	100 %	100 %
With inhibitor, rel. emission	1.78 %	7.02 %

standard solution was measured for comparison. It can be seen that the inhibition effect for trypsin is nearly 99 % (Table 1). The inhibitor did not interfere with the fluorescence measurement.

Proportionality

A linear increase in emission was observed when a larval homogenate was incubated with the substrate over a time period of about 15 minutes (Fig. 3). A linear correlation is given between the increase in fluorescence per time interval and eight different concentrations of trypsin standard solution (expressed in Units) in the assay solution (Fig. 4). The same was found when different quantities of a larval homogenate were incubated (Fig. 5).

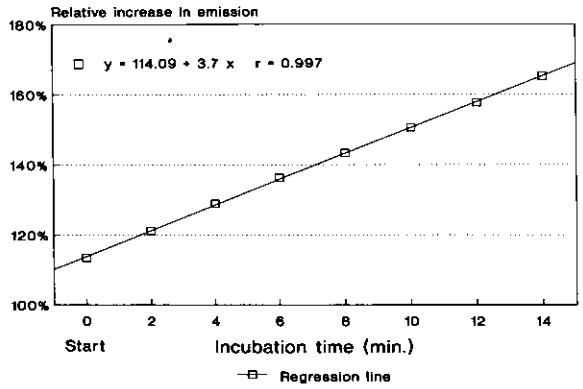


Fig. 3. Relative increase in emission during a total measuring period of 15 min., larval homogenate. The curve was fitted by linear regression. Substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer, pH 8.0, 25 °C, excitation 380 nm, emission 440 nm

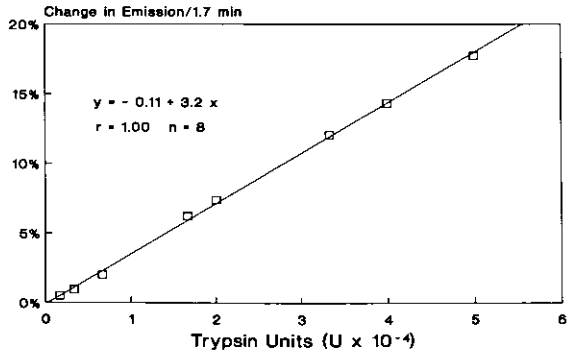


Fig. 4. Trypsin standard curve using different amounts of trypsin standard solution, expressed in units (U). The curve was fitted by linear regression. Substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer, pH 8.0, 25 °C, δt = 1.7 min., excitation 380 nm, emission 440 nm

Rates of non-enzymatic hydrolysis in relation to pH of the solvent (TRIS-HCl buffer)

The slight non-enzymatic hydrolysis (autohydrolysis) of the substrate varies with changing pH. To minimize this effect, the change in emission per time unit was determined at six different pH levels in the range from pH 6.16–10.03. With increasing pH-values, the autohydrolysis of the substrate increased considerably (Fig. 6). For pH values from 6–8 the

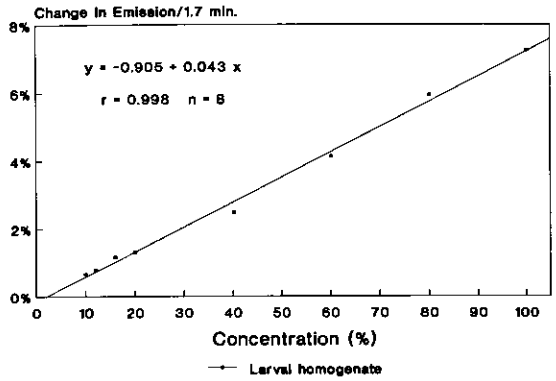


Fig. 5. Relationship between change in emission per time unit and the amount of larval homogenate. 100 % concentration means 100 µl undiluted larval homogenate in the assay solution. The curve was fitted by linear regression. Substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer, pH 8.0, 25 °C, $\delta t = 1.7$ min., excitation 380 nm, emission 440 nm

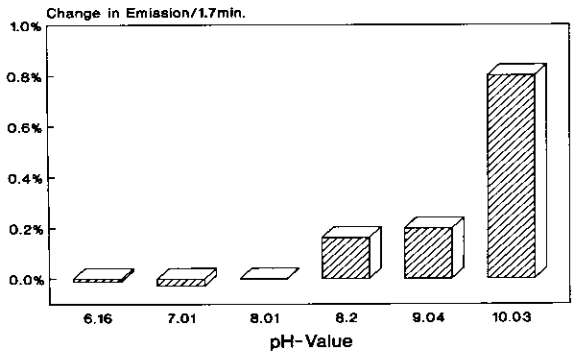


Fig. 6. Rates of non-enzymatic hydrolysis of the substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer in relation to different pH-values in the assay solution, TRIS buffer 0.1 Mol, 25 °C, $\delta t = 1.7$ min., excitation 380 nm, emission 440 nm

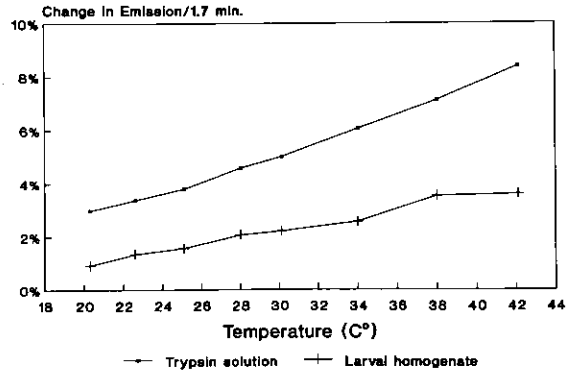


Fig. 7. Change in emission in relation to the temperature of the assay solution. Eight different temperatures in the range from 20 °C–42 °C, trypsin standard solution, and larval homogenate were tested. Substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer, pH 8.0, $\delta t = 1.7$ min., excitation 380 nm, emission 440 nm

non-enzymatic hydrolysis was nearly zero. This pH-range includes the optimal pH-value of maximum tryptic enzyme activity found in herring and turbot larvae (UEBERSCHÄR 1985). Therefore a pH of 8.00 (± 0.02) was adjusted in the buffers and solvents used in this investigation.

Temperature

Molecular fluorescence is quite sensitive to changes in temperature, the fluorescence output decreases with increasing temperature (GUILBAULT 1973) and autohydrolysis of the substrate correlates positive with increasing temperature. On the other hand, the rate of enzyme-catalyzed reactions generally increases with temperature, thus raising the sensitivity of the method. This is shown for tryptic enzyme activity in standard solution as well as in larval homogenate, expressed as an increasing change in emission per time intervall (Fig. 7). A logarithmic relationship normally exists between temperature and enzyme activity (BERGMEYER 1977). The results in Figure 7 rather show a linear relationship, due to the effect of decreasing fluorescence yield with increasing temperature and beginning protein denaturation. Constant temperature conditions are necessary for the precision and reproducibility of this method. Because of the minor autohydrolysis of the fluorogenic substrate below 28 °C (Fig. 8), 25 °C was found to be a good compromise.

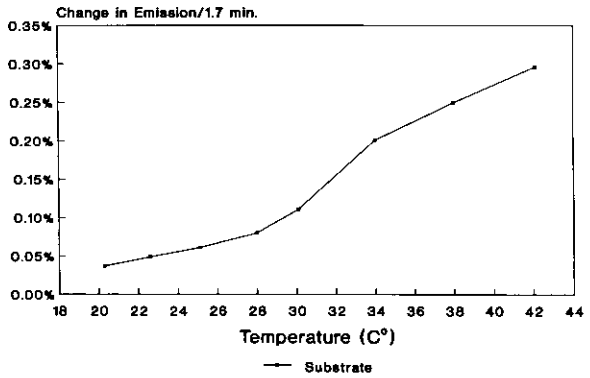


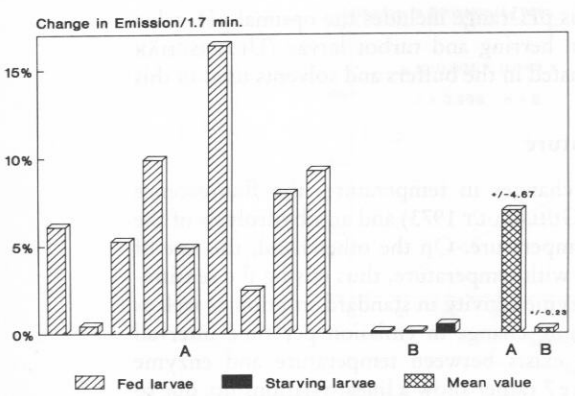
Fig. 8. Rates of autohydrolysis of the substrate CBZ-L-Arg-MCA, 0.20 mMol in TRIS-HCl buffer in relation to temperature in the assay solution. TRIS-HCl buffer 0.1 Mol, pH 8.0, $\delta t = 1.7$ min., excitation 380 nm, emission 440 nm

Measurement of enzyme activity in fish larvae

First application of the described analytical procedure to fish larvae reared under defined conditions in the laboratory produced the results given in Fig. 9 and Fig. 10. The analysis of tryptic enzyme activity in 25-day-old fed and starving turbot larvae and in 30-day-old fed and starving herring larvae shows significant differences between the fed and starved groups.

Precision of the measurement procedure

The coefficient of variability was 4.75 % (emission in percent/1.7 min.) when 26 aliquots (larval homogenate) were measured twice each.



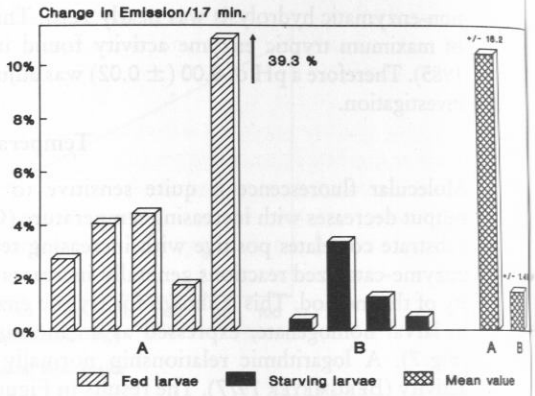


Fig. 9, left. Tryptic enzyme activity of 25-day-old fed and starved turbot larvae reared in the laboratory. Each bar gives the enzyme activity of an individually measured larva. The starving larvae were deprived of food for 7 days. Analytical procedure as described above

Fig. 10, right. Tryptic enzyme activity of 30-day-old fed and starved herring larvae reared in the laboratory. Each bar gives the enzyme activity of an individually measured larva. The starving larvae were deprived of food for 5 days. Analytical procedure as described above

Discussion

Evaluation of starvation as a cause for the mass mortality in the early life stages of teleosts has been hampered by the lack of methods enabling the assessment of the nutritional condition of a large number of individual fish larvae. Earlier work on the development of enzymes in young herring larvae has shown that starving larvae possess an average lower trypsin level as compared to fed larvae (Ueberschär 1985). These results were found by using a conventional chromogenic method which requires pooled larvae samples.

The method presented here has been proven to be a very sensitive tool to determine the tryptic enzyme activity in individual fish larvae. Compared with formerly used chromogenic methods, the fluorescence technique results in an approximately 500-fold increase in sensitivity. The lowest detectable limit corresponds to the tryptic activity in one yolk-sac herring larva (about 25 µg of larvae dry weight). When using the catalytic activity to determine the amount of trypsin in a larval homogenate it is of methodical advantage that the increase in fluorescence can be read per time unit directly. Changing self-fluorescence of the homogenate, substrate or other fluorescence sources in the assay solution need not to be taken into consideration as long as they remain constant during the measurement. This would not be possible when a photometrical one-point measurement is used.

Proteolytic enzymes other than trypsin could also be suitable as indicators of nutritional condition. However, using trypsin as an indicator enzyme is of advantage for several reasons. Trypsin is the best investigated digestive enzyme in young fish larvae and has been shown to be the most important proteolytic enzyme during the first weeks after hatching in turbot, as well as in herring larvae (Ueberschär 1985).

Trypsin-like enzymes have also been found in the food organisms (zooplankton) of fish larvae (Hirche 1979; Lauff and Hofer 1984). The analytical procedure described here does not distinguish between exogenous and endogenous trypsin. Low autolysis and reabsorption rate of ingested proteolytic enzymes in the hindgut of fish larvae (Hofer and Schiemer 1981; Lauff and Hofer 1984) raise the level of the measurable enzyme activity in larvae which have just fed on zooplankton. Therefore the difference in enzyme activity

between fed and starving larvae increases. Part of the difference in enzyme activity between fed and starving larvae may thus be attributed to exogenous trypsin.

The release of trypsinogen into the gut (where it is rapidly converted into the active form trypsin) is triggered by the ingestion of adequate food organisms (FÄNGE and GROVE 1979; PEDERSEN et al. 1987) so that the amount of trypsin in a larva depends on the amount of ingested food. Since the activation of trypsinogen to trypsin is a covalent one-way regulated process (LEHNINGER 1977), trypsin cannot be transformed back into the respective zymogens, and remains as the active form in the digestive tract.

The above-mentioned properties of trypsin and its significance in larval digestive processes make this enzyme the best-suited indicator enzyme. The tryptic enzyme activity is not only a sensitive instrument to detect starving larvae but also an indicator of the current feeding level (short-term variation) (PEDERSEN and HJELMELAND 1988).

HJELMELAND and JØRGENSEN (1985) presumed that, when using a total body larval homogenate, inhibitor effects to trypsin might cause an underestimation of tryptic enzyme activity and disturb the measurement. But in earlier investigations dealing with this problem in herring and turbot larvae, no inhibitor effects were found (UEBERSCHÄR 1985). This is valid for the species investigated, but before applying the method on other species, this potential problem must be taken into consideration.

An exemplary application of the described procedure to laboratory reared fish larvae (Fig. 9 and 10) under known nutritional conditions gives first indications to the usefulness of this method to distinguish fed and starving larvae. It was also demonstrated that the amount of individual variation within the groups is considerable, emphasizing the need for individual analyses, if questions on trypsin production in fish larvae are to be answered. The given examples consider only the simplest case (well-fed versus starving larvae). Ongoing investigations will yield information about the effect time since the last meal, and meal size have on tryptic acitivity.

Following a method by GLAZER and STEER (1976) the described assay will be modified to determine the total tryptic enzyme potential as the sum of active trypsin and its inactive precursor trypsinogen. This will be a sensitive instrument to determine the survival potential even of starving larvae, and will help to find their "point of no return".

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