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The Propagation of Cod *Gadus morhua* L.

TRYPSIN AND TRYPSINOGEN AS INDICES OF GROWTH AND SURVIVAL POTENTIAL OF COD (*Gadus morhua* L.) LARVAE

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ABSTRACT

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Using a radioimmunoassay it could be demonstrated that the content of trypsin and trypsinogen in cod larvae increased markedly during the first 4 days after hatching. In the same period the trypsin activity, measured by the conventional method, was constant, but increased considerably on day 5. At that time the larvae were ready for the first feed intake. After day 5 the total quantity of trypsin and trypsinogen dropped again, independent of whether the larvae received any food or not.

Larvae offered food which supported growth, started to produce trypsin and trypsinogen, whereas, in starving larvae and those offered incomplete diets, the level of trypsin and trypsinogen remained very low. We have concluded that the radioimmunoassay is a convenient and sensitive method for the quantitative determination of trypsin and trypsinogen in very small samples, and that the level of trypsin and trypsinogen may be used as an index of the feeding status and growth potential of the larvae.

INTRODUCTION

Cod larvae are only 4-5 mm long when the yolk is exhausted and they become dependent upon exogenous nutrients (Theilacker and Dorsey, 1980). Even though larvae actively ingest dead particles at this stage, artificial food has proved to be inadequate to sustain growth throughout the remaining larval stages and beyond metamorphosis (Howell, 1979; Huse, 1980; Thompson and Riley, 1981).

Many theories have been proposed to explain the inability of most marine larvae to utilize dead food (Theilacker, 1980). One suggestion is that the digestive system of the larva is unable to transform the food particles into molecules, which the larvae can use for growth (Dabrowski, 1979; Hogendoorn, 1980; Huse et al., 1982; Szlaminska, 1980).

The first step in the digestion process in the larva is the enzymatic extracellular degradation of the food particles in the alimentary tract. Most fish larvae have no morphological (Fig. 1) or histological stomach (Tanaka, 1969, 1971). The entire extracellular digestion is therefore likely to occur in the intestine by pancreatic enzymes. The next step in the transformation of the ingested food is absorption of the degradation products through the intestinal wall. It has been demonstrated that intestinal cells of fish larvae are able to absorb protein macromolecules by means of pinocytosis (Iwai and Tanaka, 1968; Stroband and Kroon, 1981). This has led to the suggestion that pinocytosis and intracellular digestion in the larva compensate for a possible incomplete extracellular hydrolysis, in the absence of a functional stomach (Iwai, 1969; Noaillac-Depeyre, 1976; Stroband and Van der Veen, 1981).

The pancreas produces and stores digestive enzymes in the form of inactive proforms, which are then activiated when secreted into the gut. Since trypsin is the only pancreatic protease which can activate its own proform as well as the proforms of other proteases secreted from the pancreas (Corring, 1980), this enzyme has a key position in controlling the activity of the pancreatic proteases. For this reason analysis of trypsin content in the larvae was thought to be a suitable

tool for studying the digestive function and growth of larvae.

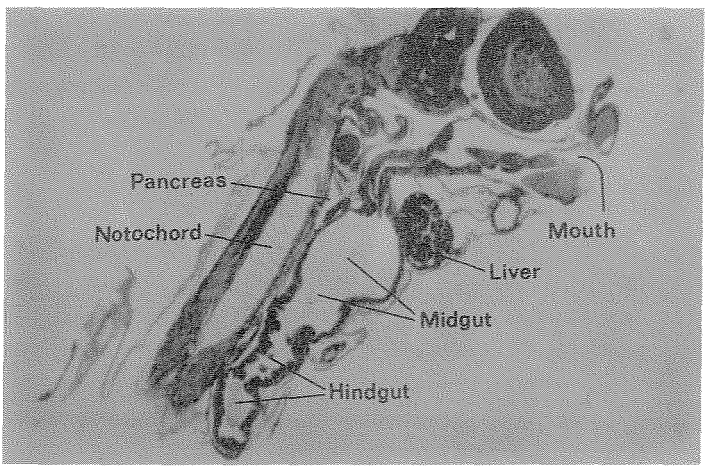

Fig. 1. Histomicrograph of a cod larva. The picture shows a longitudinal section of a larva.

Trypsin-like activity is usually assayed with a model protein as the substrate at pH 7-9, or with a synthetic substrate (Reck, 1974). However, a crude enzyme-extract from the digestive tract, or of the whole organism, will contain other proteases than pancreatic trypsin which are able to react with both the model protein and the synthetic substrate at pH 7-9 (Barret, 1979). The trypsin level may therefore be overestimated. But two factors might cause an underestimation of trypsin assayed enzymatically; first, the presence of trypsin inhibitors in blood and tissue fluids, and secondly, because trypsin has no enzyme activity when existing in its proform, trypsinogen. These problems are circumvented by the use of an

immunological assay based on antibody recognition of both active and inhibited enzyme, as well as the inactive proform (Hjelmeland, et al., In prep.).

This paper presents the results of using this method for the analysis of trypsin content in cod larvae given living food organisms and dead artificial feed. The aim was to examine whether the amount of trypsin may be a useful index of growth and survival potential of the larvae, and whether they respond to different diets by producing different quantities of trypsin.

MATERIALS AND METHODS

Start-feeding of cod larvae

Fertilized eggs were collected from a pen with spawning cod and incubated and hatched in polythylene cylinders as described by Huse, et al. (1982). Five day old larvae were transferred to four 200 l conical experimental tanks with an initial larvae density of 20 per l. The experimental tanks were supplied with filtered and UV-treated sea water pumped from a depth of 55 m. The temperature varied between 5^{o}C and 8^{o}C during the experimental period. Feeding was started immediately after the first sampling on day 5. Larvae in group I were offered a standard diet based on hens egg, proteose peptone, cod liver oil, and fish protein autolysate with vitamins and minerals added (Huse, et al., 1982), group II a mixture of cultivated rotifers and wild living plankton, and group III a cod roe diet (Molvik et al., 1984). These larvae were fed three times daily. Larvae in group IV received no food.

Sampling and preparation of sample

Samples were taken from two different hatching groups. One group covers the period from day 0 to day 5 after hatching, while the second group represents larvae from 5 days on after hatching; the latter group was used in the feeding trial.

The random samples from the experimental tanks were used as

follows. Ten larvae from each sample were used for the estimation of trypsin by radioimmunoassay and by enzyme activity assay. The adhering sea water was gently removed from the larvae which were then frozen at -30°C in one small plastic tube. Later, a phosphate buffer saline with 0.2 % bovine albumen (PBSA) was added to the tube to give an average volume of 30 µl per larva. Thereafter, the sample was homogenized using a Branson Sonifier B-12X. The remaining larvae in the original sample (30-40 larvae) were preserved in 4 % formalin and used for determining dry weight.

Radioimmunoassay of trypsin in cod larvae

Details of the estimation of trypsin content in cod by radioimmunoassay will be described elsewhere (Hjelmeland, In prep.). Briefly, 50 µl of the sample (diluted 10 to 10.000-fold in PBSA) was mixed with 20 µl (0.5 ng) of ^{125}I-labelled purified cod trypsin (Hjelmeland and Raa, 1982) and 40 µl rabbit antibody against cod trypsin (diluted 20.000-fold in 2 % normal rabbit serum/PBSA). The precipitation antibody used was sheep anti-rabbit Ig diluted 5-fold in PBSA. All assays were done in duplicate.

Enzymatic activity assay of trypsin in cod larvae

Aliquots of 10 µl of the samples were mixed with 25 µl of 0.2 M Tris-buffer (pH 7.8) and 100 µl 1 mM solution of the chromogenic substrate Carbobenzoxy-Val-Gly-Arg-p-nitranilid-acetate (Boehringer Mannheim GmbH, W. Germany) in wells of a microtiter plate. After incubation at 23°C for 1 h the light absorption at 405 nm was recorded, using a Titertek Multiscan photometer (Flow).

RESULTS

The average amount of trypsin and its proform trypsinogen, measured by radioimmunoassay, in cod larvae increased markedly

during the first 4 days after hatching (Fig. 2). Before the first feeding between days 5 and 6 the content decreased. The larvae had a slight trypsin-like activity on the day of hatching, and there was no significant change in this activity during the following 4 days. On day 5, however, a sharp increase was found.

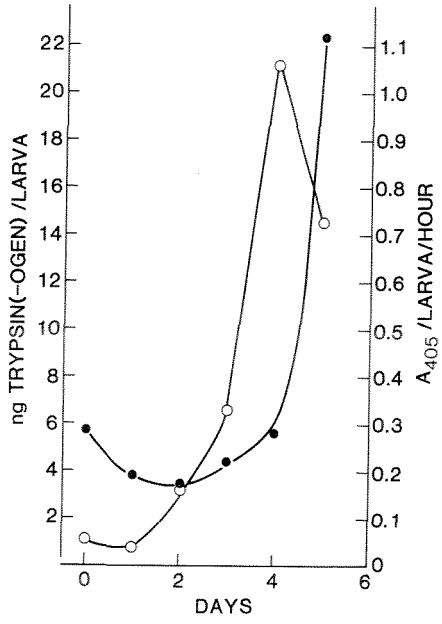

Fig. 2. Trypsin-like enzymatic activity (●) and trypsin/trypsinogen content (o) in cod larvae.
Abscissa expresses days after hatching.

Fig. 3 shows the results of trypsin/trypsinogen analyses by radioimmunoassay of larvae in feeding experiments. Also included are values for cod larvae from a pond where the larvae were feeding on natural plankton (Kvenseth and Øiestad, 1984). In all groups of larvae a drastic decrease in trypsin/trypsinogen content occurred during the first 3-4 days of feeding. The value of trypsin/trypsinogen on day 5 represents the average of as many as 60 larvae, while the other values are each based on samples containing 10 larvae. Larvae in the group

given a diet of living organisms (Calanus and Rotatoria) had an almost constant level of trypsin/trypsinogen from day 9 until day 18, when it started to rise very steeply.

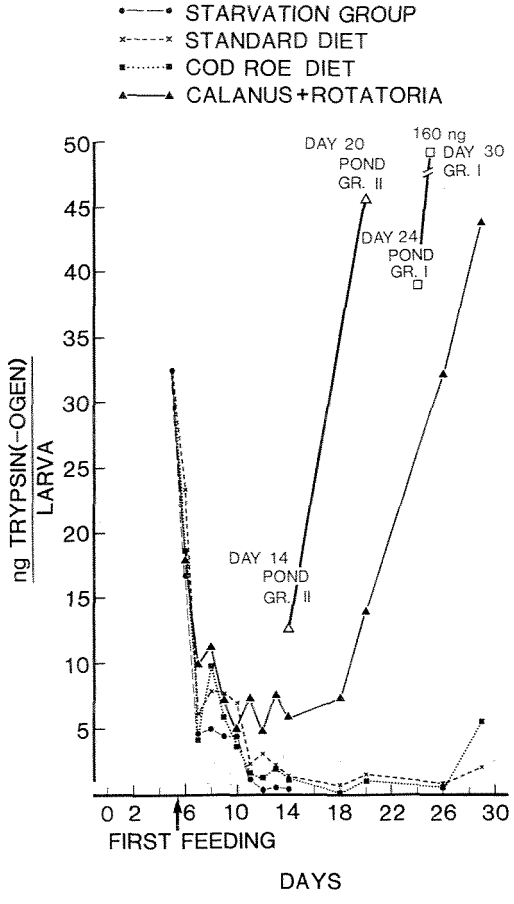

Fig. 3. Trypsin/trypsinogen content, estimated by radioimmunoassay, in cod larvae from a feeding experiment. Also included in the figure are values for larvae from a pond where they were feeding on natural plankton. The first sample (day 5) was taken 3 h before the larvae were offered any diet. Abscissa expresses days after hatching.

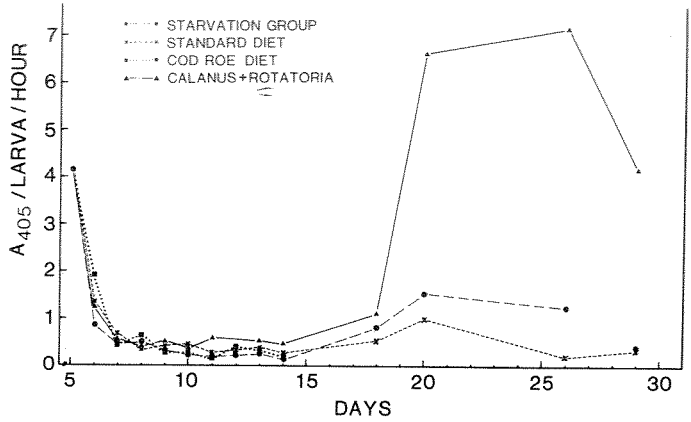

Fig. 4. Trypsin-like activity, estimated by an enzymatic assay, in cod larvae from a feeding experiment. Abscissa expresses days after hatching.

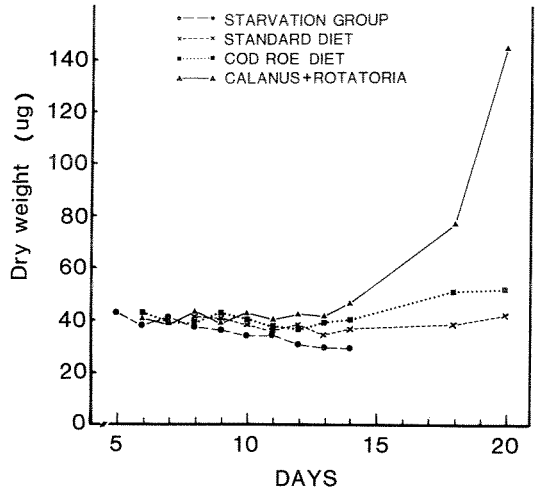

Fig. 5. Dry weight of cod larvae from a feeding experiment. Each value represents the average of 30-40 larvae, weighed individually. Abscissa expresses days after hatching.

The trypsin/trypsinogen content of the larvae which were given the artificial diets dropped to a stable low level on day 12. From then on and until day 26 the values were close to the lower detection limit of the method (0.3 ng).

At the end of the feeding trial 8 larvae remained in the group fed live food and 40-50 larvae in both groups fed artificial feed. The larvae in the starvation group were all dead on day 17 after hatching.

The trypsin-like activity in cod larvae was almost the same in all feeding groups during the period from day 5 until day 18 (Fig. 4). On day 18 there was a very steep rise in the trypsin-like activity in larvae feeding on live food. A slight increase in activity was also observed for the two other feeding groups. It is worth mentioning that the group given live food had a lower trypsin-like activity at the end of the experiment (day 29) than at day 20.

Fig. 5 shows the average dry weight of the larvae sampled from the four experimental tanks at Austevoll. Each value represents the average of 30-40 larvae, weighed individually. Until day 14 no significant difference in weight between larvae given live and dead food was registered, but after day 14 there was a significant increase in weight of those given live food. The larvae which were offered dead food did not gain any weight.

DISCUSSION

Due to its high sensitivity, the radioimmunoassay can be used to determine trypsin and trypsinogen in very small samples, for example a few cod larvae. Since the method is based on the very specific interaction between antibody and antigen (trypsin/trypsinogen), the enzyme content can be quantitatively determined even in crude extracts. This is unlike direct enzyme activity measurements, which will detect neither the proform of the enzyme nor any trypsin bound to tissue and serum inhibitors. Such inhibitors may be mixed with the gut trypsin during preparation of the sample (Hjelmeland and Raa, 1980; Hjelmeland, In press).

Using the radioimmunoassay it could be shown that the larvae rapidly mobilize trypsin/trypsinogen during the first 4 days after hatching. Since direct enzyme assays showed an almost constant low activity in the same period, it is likely that the trypsin recorded by the immunological assay was the inactive proform, trypsinogen. This is physiologically sound, since the larvae in this period are not feeding but conditioning themselves for the first feed intake a few days later. It might be of course that the constant low level of active trypsin during the first 4 days is due to inhibition by tissue inhibitors which become mixed with pancreatic trypsin during preparation of the sample. This, however, seems an unlikely supposition since a marked increase in trypsin activity was recorded on day 5, just at the time when the larvae had reached a stage of development when they were ready for their first feed intake (Ellertsen, et al., 1980). It is premature to speculate on the physiological significance of the increase in trypsin-like activity on day 5, and the simultaneous decrease of trypsin/trypsinogen as measured by the radioimmunoassay, before more experimental studies with larvae at this stage of development have been carried out. At any rate, on day 5 the larvae had no access to feed and trypsin activity can therefore not have been induced as a result of active feeding. Thus, the larvae apparently start to produce trypsin activity at a certain stage in development, somewhere between day 4 and 5, whether or not there is food present in the gut.

It is a significant phenomenon, demonstrated with all larval groups, that the total quantity of trypsin/trypsinogen, measured by radioimmunoassay, decreases sharply after day 5, independent of whether the larvae are offered feed or not (Fig. 3). Accordingly, we feel confident that the high trypsin/trypsinogen content in the larvae on day 5 reflects an imprinted physiological event in their development, and that this occurs independently of their later destiny. A drop in average trypsin/trypsinogen level in a sample of larvae would also have been recorded if a high proportion of the larvae after days 4-5 were non-functional, or "losers". But accepting this as the main reason for the drop in trypsin/trypsinogen

after day 5 (Fig. 3), would be to imply that the majority of the larvae were actually physiologically dead. This was definitely not the case with the larvae from the pond, which were also hatched in the laboratory, where a survival beyond metamorphoses of 70 % was obtained (Kvenseth and Øiestad, 1984). Another interpretation of the decline in trypsin/trypsinogen after day 5 could be that the larvae actually were beginning to starve at that time, and that pancreatic proteins like trypsin therefore were degraded to mobilize energy (O'Connell, 1976). However, since the successful pond experiment (Kvenseth and Øiestad, 1984) was also done with larvae hatched artificially and transferred to the pond on day 5, any starvation before day 5 cannot seriously have affected the survival potential of the larvae. Nevertheless, our experiments should be supported with measurements of trypsin/trypsinogen in larvae hatched under natural conditions, where soluble nutrients and small particles may be taken up passively by the larvae before they start active feeding. In addition, experiments where feed is offered before day 5 should be carried out.

There was a fairly good correlation between dry weight of the larvae and the analytical figures on trypsin activity and trypsin/trypsinogen content. When the larvae did not commence growth they did not produce trypsin; when they grew they did.

With the radioimmunoassay it was possible to record a significant increase in trypsin/trypsinogen in growing larvae when the weight gain was significant. Trypsin/trypsinogen is accordingly a useful and significant index of the nutritional status as well as the growth capability of young larvae. It also seems to be a useful tool in screening potential starter feeds for larvae, since good feeds should be able to induce an increase in trypsin/trypsinogen. The only artificial feed which so far has supported growth of the cod larvae is one based on cod roe, and the larvae responded to this feed by producing more trypsin/trypsinogen (Molvik, et al., 1984).

Since cod larvae have no stomach, it is debatable whether the larvae are able to digest feed protein completely to amino acids in the gut. Trypsin and chymotrypsin alone are,

according to their substrate specificity (Walsh and Wilcox), 1970), definitely not able to degrade proteins to free amino acids. This poses the question of whether the larvae take up peptides and even complete particles and perform intracellular digestion (Stroband and Kroon, 1981). Our data do not relate directly to this question, but indirectly by showing that the trypsin content present in one larva of 40 µg is sufficient to degrade 25 µg protein an hour. This figure derives from the fact that 1 ng trypsin from fish is able to degrade about 8 µg muscle protein at 25°C and pH 7.2 (Gildberg, 1982), or about 0.8 µg at 6-8°C. Such a high trypsin activity in the gut suggests a major role for extracellular digestion in rendering the feed available for growth of the larvae, but that complete hydrolysis to amino acids may involve intracellular digestion.

ACKNOWLDGEMENTS

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REFERENCES

Barret, A.J., 1979. Introduction to the history and classification on tissue proteinases. In: A.J. Barret (Editor), Proteinases in mammalian cells and tissues. North-Holland Publishing Co., Amsterdam, pp. 1-55.

Corring, T., 1980. The adaptation of digestive enzymes to the diet: Its physiological significance. Reprod. Nutr. Develop., 20: 1217-1235.

Dabrowski, R.K., 1979. The role of proteolytic enzymes in fish digestion. In: European mariculture society, No. 4. Cultivation of fish fry and its live food, pp. 107-126.

Ellertsen, B., Moksness, E., Solemdal, P., Strømme, T., Tilseth, S., Westgård, T. and Øiestad, V., 1980. Some biological aspects of cod larvae (*Gadus morhua* L.). Fisk Dir. Skr. Ser. HavUnders., 17: 29-47.

Gildberg, A., 1982. Autolysis of fish tissue - General aspects. Thesis, University of Tromsø, 112 pp.

Hjelmeland, K. and Raa, J., 1980. Fish tissue degradation by trypsin type enzymes. In: J.J. Connell (Editor), Advances in fish science and technology. Fishing News Books Ltd., Farnham, Surrey, England, pp. 456-459.
Hjelmeland, K. and Raa, J., 1982. Characteristics of two trypsin type isozymes isolated from the arctic fish capelin (*Mallotus villosus*). Comp. Biochem. Physiol., Vol. 71B, 4: 557-562.
Hjelmeland, K., 1983. Proteinase inhibitors in the muscle and serum of cod (*Gadus morhua*). Isolation and characterization. Comp. Biochem. Physiol.
Hogendoorn, H., 1980. Controlled propagation of the African catfish, *Clarias lazera* (C. & V.) III: Feeding and growth of fry. Aquaculture, 21: 233-241.
Howell, B.R., 1979. Rearing larval cod (*Gadus morhua* L.) on cultured foods. Coun. Meet. Int. Coun. Explor. Sea, 1979 (F:17) 1-4, 2 Figs (Mimeo.).
Huse, I., 1980. An attempt to start feed cod larvae with artificial diets. Coun. Meet. Int. Coun. Explor. Sea, 1982 (F:14), 1-3 (Mimeo.).
Huse, I., Opstad, I., Klungsøyr, L. and Walther, B., 1982. Endocrine and nutritional factors affecting the first food uptake by cod larvae. Coun. Meet. Int. Coun. Explor. Sea, 1982 (F:17), 1-7, 5 Figs (Mimeo.).
Iwai, T., 1969. Fine structure of gut epithelial cells of larval and juvenile carp during absorption of fat and protein. Arch. histol. jap. 30: 183-199.
Iwai, T. and Tanaka, M., 1968. The comparative study of the digestive tract of teleost larvae - III. Epithelial cells in the posterior gut of halfbeak larvae. Bull. Jap. Soc. scient. Fish., 34: 44-48.
Kvenseth, P.G. and Øiestad, V., 1984. Large scale rearing of cod fry in an enclosed pond on the natural food production. In: E. Dahl, D.S. Danielssen, E. Moksness and P. Solemdal (Editors), The propagation of Cod *Gadus morhua* L. Flødevigen rapportser., 1, 1984: 645-655.
Molvik, G., Hjelmeland, K., Ringø, E. and Raa, J., 1984. Properties of a new artificial diet for fish larvae, including cod (*Gadus morhua* L.). In: E. Dahl, D.S. Danielssen, E. Moksness and P. Solemdal (Editors), The Propagation of Cod *Gadus morhua* L. Flødevigen rapportser., 1, 1984: 203-211.
Noaillac-Depeyre, J. and Gas, N., 1976. Electron microscopic study on gut epithelium of the tench (*Tinca tinca* L.) with respect to its absorptive functions. Tissue & Cell, 8: 511-530.
O'Connell, C.P., 1976. Histological criteria for diagnosing the starving condition in early post yolk sac larvae of the northern anchovy, *Engraulis mordax* Girard. J. exp. mar. Biol. Ecol., 25: 285-312.
Reck, W., 1974. Trypsin. In: U.H. Bergmeyer (Editor), Methods of Enzymatic Analysis, Vol. II, Academic Press, New York, pp.1013-1024.
Stroband, H.W.J. and Kroon, G.A., 1981. The development of the stomach in *Clarius lazera* and the intestinal absorption of protein macromolecules. Cell & Tissue Res., 215: 397-415.

Stroband, H.W.J. and Van der Veen, F.H., 1981. Localization of protein absorption during transport of food in the intestine of the grasscarp *Ctenopharyngodon idella* (Val.), J. Exp. Zool., 218: 149-156.

Szlaminska, M., 1980. A histochemical study of digestive enzymes in pike larvae. Fish. Mgmt., 11: 139-140.

Tanaka, M., 1969. Studies on the structure and function of the digestive system in teleost larvae - II. Characteristics of the digestive system in larvae at the stage of first feeding. Jnp. J. Ichthyol., 16: 41-49.

Tanaka, M., 1971. Studies on the structure and function of the digestive system in teleost larvae - III. Development of the digestive system during postlarval stage. Jpn. J. Ichthyol., 18: 164-174.

Theilacker, G.H., 1980. Rearing container size affects morphology and nutritional condition of larval jack mackerel *Trachurus symmetricus*. Fish. Bull., U.S., 78: 789-791.

Theilacker, G. and Dorsey, K., 1980. Larval fish diversity, a summary of laboratory and field research. In: G. Sharp (Editor), Workshop on the effects of environmental variation on the survival of the larval pelagic fishes. UNESCO, Intergovernmental Oceanographic Commission, Workshop Report 28, pp. 105-142.

Thompson, B.M. and Riley, J.D., 1981. Egg and larval development studies in the north sea cod (*Gadus morhua* L.). Rapp. P.-v. Reun. Cons. int. Explor. Mer, 178: 553-559.

Walsh, K.A. and Wilcox, P.E., 1970. Serine proteases. In: E.G. Perlmann and L. Lorand (Editors), Methods in Enzymology, Vol. XIX, Academic Press, New York, pp. 31-41.