

Control and Efficiency of Digestive Function of Marine Fish Larvae

Ivar Rønnestad

Department of Zoology, University of Bergen, Allegt 41, N 5007 Bergen, Norway
Tel. + 47 55 58 35 86, Fax, +47 55 58 9276. ivar.ronnestad@zoo.uib.no

ABSTRACT

Recent downscaling and improvements of tube feeding techniques have allowed more detailed studies on the digestive and absorptive efficiency of larval fish, including the transfer kinetics of selected nutrients from the lumen of the digestive tract into the tissues of the body. Freely dissolved amino acids seem to be absorbed rapidly and with a high efficiency. There has also been some progress towards understanding how the digestive process is controlled in marine fish larvae. The peptide hormone cholecystokinin (CCK) has been targeted since it is believed to play an important role in controlling digestive function in vertebrates.

Key words: digestive function, cholecystokinin, absorption, amino acids, marine fish larvae

INTRODUCTION

When fish larvae commence exogenous feeding, the flow of nutrients formerly supplied only from yolk reserves becomes supplemented through the digestive tract. The majority of marine fish larvae currently targeted for cultivation hatch from pelagic eggs and their digestive system is still developing at the onset of exogenous feeding. A fully developed digestive tract, including gastric digestion, develops during metamorphosis. Although the larval gut is not completely developed at the onset of exogenous feeding, it is sufficiently efficient to support larval growth by digesting such prey as is available under natural conditions in the sea. The physiological constraints of the gut with respect to digestion of cultivated live prey and particularly formulated starter feeds still remain to be elucidated. This paper reviews some of our recent findings in the areas of control and efficiency of digestive function of marine fish larvae.

CHOLECYSTOKININ

The peptide hormone cholecystokinin (CCK) is a major regulatory hormone of digestion. CCK is produced in endocrine cells scattered among the epithelial cells lining the intestine (Liddle, 1995) which, when exposed to the appropriate stimuli in gut contents, release CCK into the circulation. The functions of CCK in vertebrates include (Figure 1): release of bile (1) and

pancreatic digestive enzymes (2), regulation of stomach emptying (3), and influencing peristaltic and motor activity (4). Specific studies on fish have demonstrated that CCK stimulates gall bladder motility *in vitro* (Aldman & Holmgren, 1987; Rajjo *et al.*, 1988; Andrews & Young, 1988) and *in vivo* (Aldman *et al.*, 1992). CCK also stimulates trypsin and chymotrypsin secretion into the gut (Einarsson *et al.*, 1997) and delays gastric emptying in rainbow trout (Olsson *et al.*, 1999).

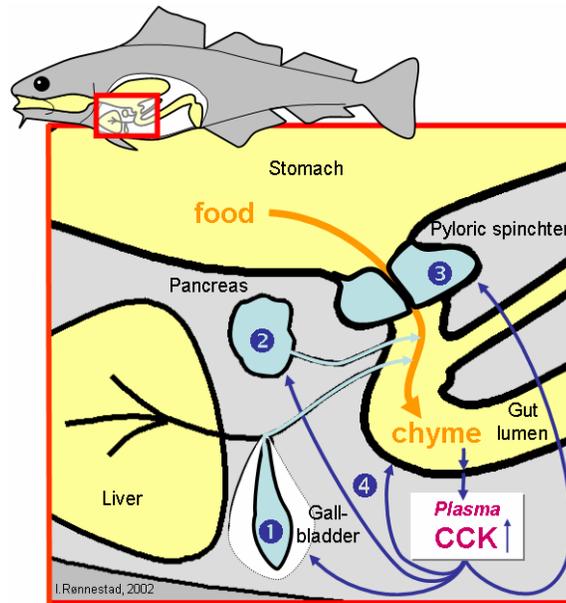


Figure 1. Roles of cholecystokinin (CCK) in digestion. Compiled from sources in the text.

The appearance and distribution of CCK-producing cells in the digestive tract of several developing larval fish were investigated by means of immunohistochemistry (Kamisaka *et al.*, 2001a,b, 2002), employing a primary antiserum against CCK that had been cloned for Japanese flounder, *Paralichthys olivaceus*. The distribution of CCK-immunoreactive (CCK-IR) cells was limited to the anterior intestine and pyloric caeca in fish with a looped alimentary canal (Atlantic halibut, and bluefin tuna, *Thunnus thynnus*), while CCK-IR cells were distributed all along the intestine in fish with a straight gut (herring, *Clupea harengus* and Ayu, *Plecoglossus altivelis*), as well as in the rectum in herring larvae. The localization of CCK-IR cells in the anterior midgut, particularly adjacent to the pyloric caeca in Atlantic halibut and bluefin tuna, agrees with the results of studies on Japanese flounder (Yoshida *et al.*, 1983; Kurokawa *et al.*, 2000) and rainbow trout (Barrenechea *et al.*, 1994).

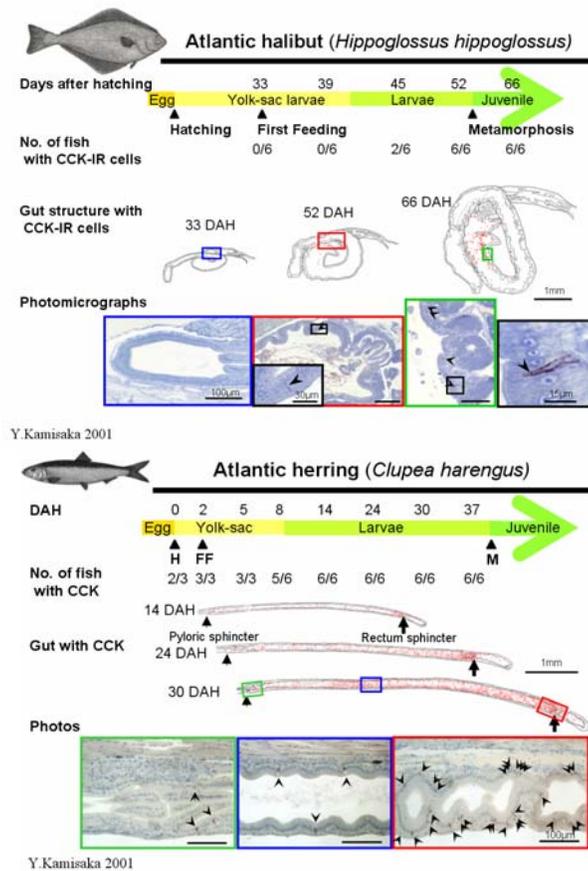


Figure 2. Ontogeny of CCK-IR cells in the digestive tract of larval Atlantic halibut (A) which has a rotated/looped gut and Atlantic herring (B) which has a straight gut. Adapted from Kamisaka *et al.* 2002.

The stomachless teleost, *Barbus conchoni*us, (Rombout & Taverne-Thiele, 1982), also showed a significant presence of CCK-IR cells adjacent to the entrance to the pyloric caeca. The distribution of CCK-IR cells is most likely linked to the requirement for close contact with the digesting food in order to properly regulate the release of digestive secretions. Such an arrangement would serve to optimize substrate-enzyme ratios in the intestinal lumen.

Rønnestad *et al.* (2000c) have proposed the participation of retrograde peristalsis in mixing chyme with digestive secretions in the region of the pyloric caeca. The retrograde contractions, which move in the opposite direction of normal propulsive waves, seem to be initiated from a specific location in the midgut curvature and spread anteriorly towards the pyloric sphincter (Figure 3).

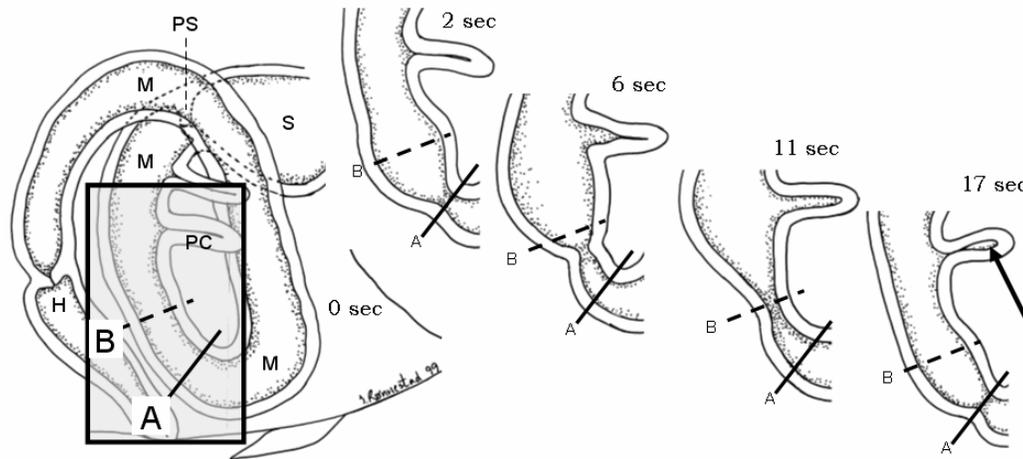


Figure 3. Retrograde peristalsis in post-larval Atlantic halibut. The wave starts caudad to the pyloric caeca (line “A”) and proceed cranially to become diminished in the region of the pyloric caeca (line “B”). The propulsion of one wave is shown in the sequence of figures (2 sec-17sec). S: stomach; M: midgut; PC: pyloric sphincter, H: hindgut. Adapted from Rønnestad *et al.*, 2000c.

This activity occurs in the area where the CCK-IR cells are located (Fig 2A; Kamisaka *et al.*, 2001a), supporting the idea that the pattern of distribution of CCK-IR cells have functional relevance to the control of the digestive processes including enzyme secretions and possibly intestinal mixing movements. Retrograde peristalsis was also demonstrated as a mechanism for filling the pyloric caeca in Atlantic halibut (Fig 3, Arrow at 17 sec; Rønnestad *et al.*, 2000c), a phenomenon that had formerly awaited confirmation for fish. A recent study has verified that retrograde peristalsis occurs in other larval fish as well (Holmgren *et al.*, 2001), pointing to a common phenomenon in fish larvae. Further documentation of peristaltic movements and motor activity in fish larva is currently being done in our laboratory.

INDICATIONS OF A REGULATORY LOOP FOR PANCREATIC SECRETIONS

The control of digestive functions in vertebrates is highly complex and involves many factors including neural pathways and peptide hormones. Our current understanding of the factors implicated in its regulation highlight CCK as an important hormone in the regulation of exocrine pancreatic secretions. Trypsin has been implicated in the feedback control of CCK release from the CCK-producing cells in the intestinal brush border. Studies on mammals have suggested that a luminal CCK-releasing factor is secreted into the gut where it stimulates the secretion of CCK, but only in the absence of trypsin, which proteolytically deactivates it (Figure 4; Lewis & Williams, 1990; Owyang, 1994; Liddle, 1995, 2000). Regulation is believed to occur when ingested protein out-competes CCK as substrates for trypsin thereby preventing the inactivation of the CCK-releasing factor (Owyang, 1994; Liddle, 1995, 2000).

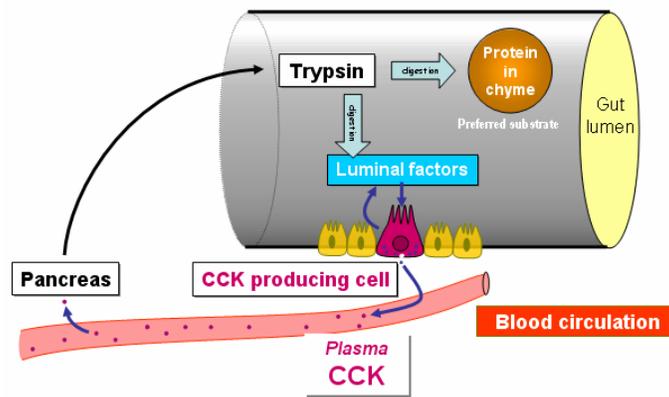


Figure 4. A proposed model for regulation of CCK release in mammals. Adapted from Lewis & Williams (1990) and Rojas-Garcia (2002).

Studies on humans have concluded that the most potent stimulants of CCK secretion are the partial digestion products of fat and protein, including di- and tri-peptides (Liddle, 2000). Studies on rats have shown that dietary stimulation of CCK release caused by intraduodenal administration of trypsin inhibitor, resulted in the transcription of new CCK mRNA (Liddle, 1994a). To complicate matters, the neuropeptide bombesin stimulates CCK secretion without modifying intestinal CCK mRNA levels (Liddle, 1994b). Thus, the secretion of CCK is not necessarily linked to its gene expression or its cellular content.

Where fish larvae are concerned, research is still in its infancy. A recent study on the first-feeding stages of Atlantic herring reported that soluble protein caused a more rapid and greater increase in CCK content (whole-body homogenate) than did free amino acids (FAA). In addition, tryptic activity increased in larvae fed protein, while no changes occurred in larvae fed FAA (Koven *et al.*, 2002). However, another study on seabass (*Dicentrarchus labrax*) found that trypsin secretion was stimulated in larvae fed a mixture of FAA while a protein hydrolysate (casein) actually reduced secretion (Cahu & Infante, 1995 a,b). In support of these findings, it has been demonstrated that digestive end-products like L-lysine can act directly on the pancreatic acinar cells to stimulate enzyme secretion (Grendell & Rothman, 1981). Tryptophan and phenylalanine are also potent AA for stimulating CCK secretion (Liddle, 2000). The two latter studies support the notion that FAA are themselves stimulants for pancreatic exocrine secretions.

The Atlantic herring larvae studied by Koven *et al.* (2002), which were tube-fed physiological saline, did not display a CCK response despite the presence of peristaltic movements and the apparent fullness of the gut. This indicates that distension of the gut wall is not a trigger for CCK synthesis. As previously stated the values reported by Koven *et al.* (2002) represent whole-body homogenate CCK and therefore it is not possible to differentiate between neural transmitter CCK in the brain and hormonal CCK in the digestive tract. In mammals, CCK present in the central nervous system (CNS), has been demonstrated to contribute to regulation of food intake and satiety. Using immunohistochemistry we have recently documented CCK IR cells in the brain of Atlantic halibut and herring (Y. Kamisaka, unpublished; Figure 5).

Our recent studies on eviscerated guts and bodies of developing Atlantic halibut larvae have shown that in these early stages neural CCK in the CNS may be the quantitative dominant form (Rojas-García & Rønnestad, 2002a; Rojas-García, 2002.).

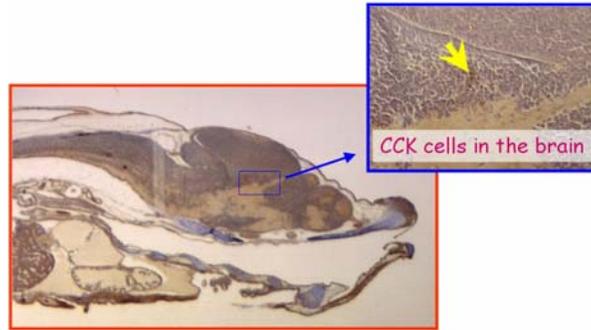


Figure 5. CCK-IR cells in the brain of Atlantic halibut larvae. Photo by Yuko Kamisaka, unpublished.

One week after the onset of exogenous feeding, the CCK content of the gut (analyzed by radio immunoassay RIA; Figure 6) represents 2% of the whole body CCK content, increasing to 62% at 4 weeks after first feeding (Rojas-García & Rønnestad, 2002a, Rojas-García, 2002.).

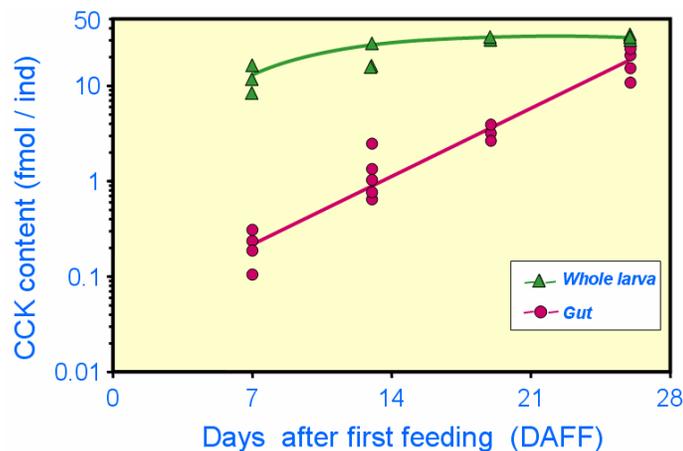


Figure 6. The CCK content of developing Atlantic halibut larvae (short-term fasted). Adapted from Rojas-García & Rønnestad, 2002a

The ontogeny and location of CCK in the brain of larval fish is currently being studied in our lab using *in situ* hybridization techniques. In contrast to the other species (Atlantic herring, Ayu, bluefin tuna) we have not been able to detect CCK in the digestive tract of Atlantic halibut at the stage when the larvae are offered live prey in aquaculture systems (Kamisaka *et al.*, 2001; Rojas-García & Rønnestad, 2002a). The mechanisms controlling the release of bile

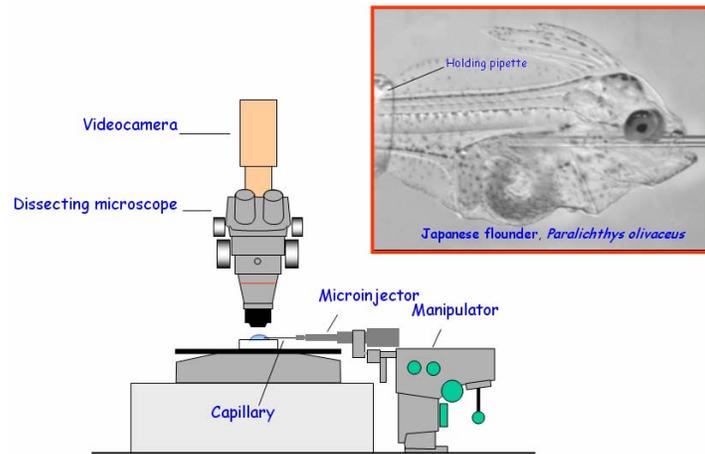
and pancreatic enzymes and peristalsis during the period between first feeding and the first detection of CCK in the gut remain to be clarified.

Clearly, more refined studies are required in order to understand the regulation of the digestive response in larval fish, in which the digestive system is still developing. These studies also demonstrate that it is necessary to separate neural and gastrointestinal sources of CCK in order to determine its alimentary role in fish larvae.

EFFICIENCY OF DIGESTIVE FUNCTION

Studies of digestive efficiency in marine fish larvae including absorption and nutrient assimilation are seriously limited by a number of factors. Fish larvae are very small at the onset of exogenous feeding with commercially important species such as turbot, *Scophthalmus maximus*, gilthead sea bream, *Sparus aurata* Senegal sole, *Solea senegalensis* and Japanese flounder have larvae that measure around 3 mm at the onset of exogenous feeding. Typical feed-particle sizes for these small stages range from 50 to 150 μm , which offers a major challenge to starter diet production technology. The acceptance of artificial diets by the larval fish is low and variable ingestion rates are obtained in feeding experiments. In some cases, first-feeding marine fish larvae ingest artificial feed but fail to grow, although recent advances have produced better results (Fernandez-Diaz & Yufera, 1997; Lazo *et al.*, 2000; Cahu & Zambonino-Infante, 2001; Koven *et al.*, 2001; Kolkovski, 2001). Nevertheless, in most species, problems arising from using formulated feeds from the onset of exogenous feeding persist.

A



B

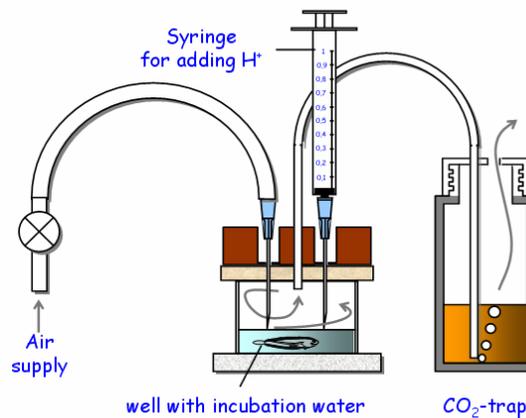


Figure 7. An *in vivo* method for controlled tube-feeding in fish larvae. A: the experimental set up. Insert shows first feeding Japanese flounder being tube-fed a colored solution. B: Metabolically produced $^{14}\text{CO}_2$ is entrapped through aeration and manipulation of the pH of the incubation water. Drawing B by S. Tonheim and adapted from Rønnestad *et al.*, 2001a.

The low acceptance and growth resulting from the use of formulated starter diets complicate studies of nutrient absorption, since traditional methods cannot be used. One possible route for quantifying the digestive capacity and assimilation of marine fish larvae is an *in vivo* method for controlled tube-feeding (Figure 7A; Rust *et al.*, 1993; Rust, 1995; Rønnestad *et al.*, 2001a). In the refinement of this procedure, metabolically produced $^{14}\text{CO}_2$ is entrapped through aeration and manipulation of the pH of the incubation water (Figure 7B; Rønnestad *et al.*, 2001a). This permits ^{14}C -labelled nutrients evacuated from the gut (or excreted elsewhere) to be distinguished from $^{14}\text{CO}_2$ originating from catabolism of the absorbed nutrients. In

combination with scintillation counting of isolated organs or body compartments (Figure 8), the system provides a useful framework for investigating features of gut absorption, oxidation and retention (assimilation) of nutrients.

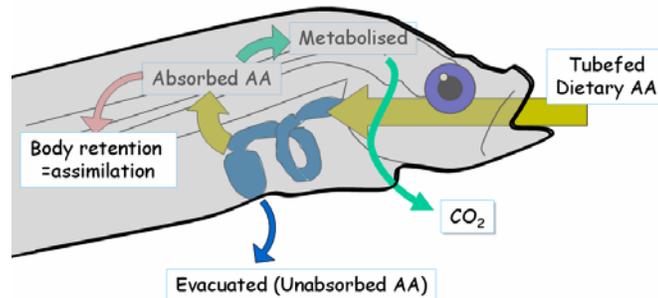


Figure 8. Using ¹⁴C-labelled nutrients (eg amino acids) the fraction evacuated from the gut (or excreted elsewhere) may be distinguished from ¹⁴CO₂ originating from catabolism of the absorbed nutrients.

Rust (1995), using an early version of this tube-feeding setup and ³⁵S labelled AA, concluded that fish larvae without a stomach at first feeding, initially assimilated simple forms of AA more efficiently than more complex nutrients (assimilation order: FAA > peptides > protein). His results further revealed that differences between the assimilation efficiencies of the three sources of AA were reduced as the larvae approached metamorphosis.

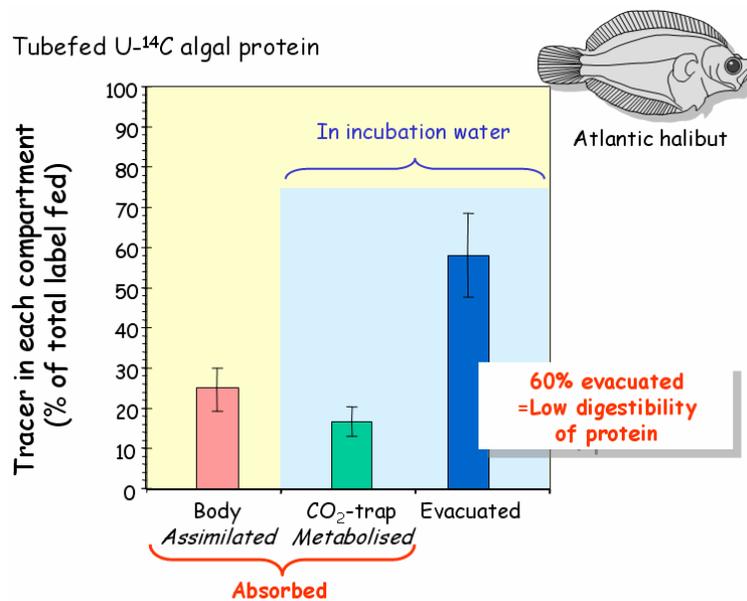


Figure 9. Atlantic halibut post larvae (46 Days post first feeding) tube-fed a ¹⁴C-labelled algal protein. Adapted from Rønnestad *et al.* (2001a)

More recent data support the idea of low absorption of AA from protein. Using ¹⁴C labelled algal protein, we demonstrated low absorptive capacity for protein from the digestive tract in

postlarval Atlantic halibut (Figure 9) supporting the data of Rønnestad *et al.* (2000a) and Rojas-García & Rønnestad (2002b), as well as previous data for juvenile and adult fish (Atlantic cod, Berge *et al.*, 1994; Atlantic salmon, *Salmo salar*, Espe *et al.*, 1993, 1999; rainbow trout, *Oncorhynchus mykiss*, Yamada *et al.*, 1981) and other vertebrates including man (Metges *et al.*, 2000).

In contrast to protein, FAA seem to be absorbed with a high efficiency. In postlarval Senegal sole, the AA tested were absorbed with similar efficiency (>97%; Figure 10). A small fraction (12 - 15%) of the indispensable AA lysine and arginine were catabolised and a high proportion was retained in the body (81-86%). For the dispensable AA tested, more were catabolised (ca 40 and 65% for glutamate and alanine, respectively) and less was retained (33% and 56%). Comparable values have also been found for Atlantic herring (Conceição *et al.*, 2002)

A potential pitfall with the use of dietary FAA is overloading of the metabolic systems by a high AA flow from the gut. Absorbed dietary AA are either used for protein synthesis or are otherwise processed by being channelled to energy production, gluconeogenesis, lipogenesis (Berge *et al.*, 1994), or may even be lost as intact molecules through the urine or gills. Urinary loss is not a major route of AA excretion in older fish (Ng *et al.*, 1996). Kolkovski (2001) has suggested that most of the FAA and peptides in micro-diets will be flushed out of the digestive system. However, the data we have collected thus far suggest that only a small proportion (<3% in Senegal sole, see above) of the FAA supplied to fish larvae as a single pulse by tube feeding is lost in intact form. Whether differences in FAA loading to the gut or other experimental procedures may explain these differences is currently being studied in our lab. These experiments will also be targeted towards further describing and quantifying the roles and fates of amino acids in marine fish larvae as follow-ups to our previous work (e.g. Fyhn, 1989; 1990; Rønnestad & Fyhn 1993; Rønnestad *et al.*, 1992a,b; 1994; 1999; 2000a,b,c; 2001a,b; 2002; Finn, 1995a,b,c; Terjesen *et al.*, 2000, 2002, Conceicao *et al.*, 2002a,b).

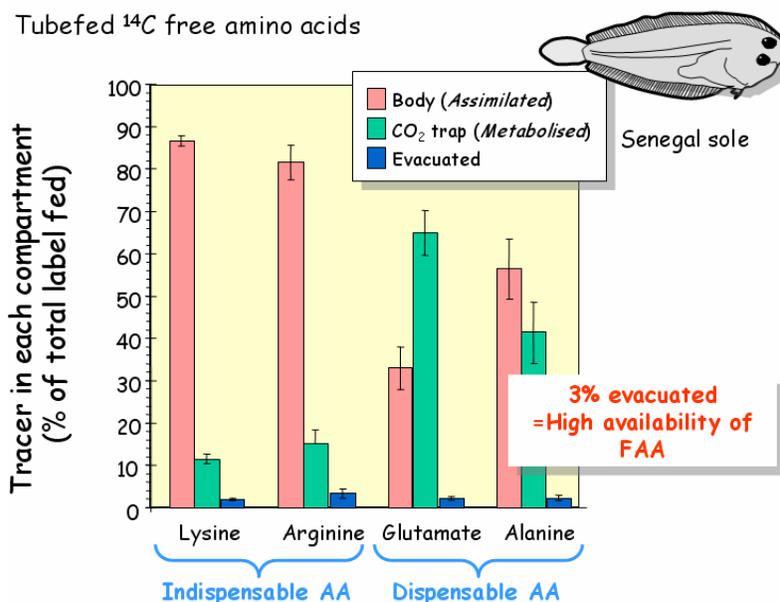


Figure 10. Senegal sole post larvae (30 days post first feeding) tube fed a mixture of free amino acids containing either ^{14}C labeled lysine, arginine, glutamate or alanine. (Adapted from Rønnestad *et al.*, 2002)

CONCLUSIONS

Research is still in its infancy regarding the understanding of the control of digestion and the efficiency of digestive and absorptive process in larval fish. Such data will serve as the basis to developing dry formulated feeds which are easily accepted and digested by the larvae from onset of exogenous feeding.

ACKNOWLEDGEMENT

This paper is based on collaborative work with many scientists and students. I am pleased to acknowledge their skills and friendship. Work supported by Research Council of Norway (141990/120, 138382/140). Contribution no. 131 from the Marine Fish Locus at the University of Bergen.

REFERENCES

- Aldman, G., Grove, D., Holmgren, S., 1992. Duodenal acidification and intra-arterial injection of CCK-8 increase gallbladder motility in the rainbow trout, *Oncorhynchus mykiss*. Gen Comp. Endocrinol. 86: 20-25
- Aldman, G., Holmgren, S., 1987. Control of gallbladder motility in the rainbow trout, *Salmo gairdneri*. Fish Physiol. Biochem. 4: 143-155
- Andrews, P. O. L. R., Young, J. Z., 1988. The effect of peptides on the motility of the stomach intestine, intestine and rectum in the skate (*Raja*). Comp. Biochem. Physiol. C 89: 343-348
- Barrenechea, M. A., Lopez, J., Martinez, A., 1994. Regulatory peptides in gastric endocrine cells of the rainbow trout *Oncorhynchus mykiss*: general distribution and colocalizations. Tissue Cell. 26(3): 309-21.
- Berge, G. E., Lied, E., Espe, M., 1994. Absorption and incorporation of dietary free and protein bound ($U^{14}C$)-lysine in Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. 109A, 681-688.
- Cahu, C. L., Zambonino Infante, J. L., 1995a. Effect of the molecular form of dietary nitrogen supply in sea bass larvae: Response of pancreatic enzymes and intestinal peptidases. Fish Physiol Biochem 14: 209-214
- Cahu, C. L., Zambonino Infante, J. L. 1995b. Maturation of the pancreatic and intestinal digestive functions in sea bass (*Dicentrarchus labrax*): effect of weaning with different protein sources. Fish Physiol Biochem 14: 431-437
- Cahu, C. L., Zambonino Infante, J. L., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200, 161-180.
- Conceição, L. E. C., Rønnestad, I., Tonheim, S. K., 2002a. Metabolic budgets for lysine and glutamate in unfed herring (*Clupea harengus*) larvae. Aquaculture. 206, 305-312
- Conceição, L. E. C., Grasdalen, H., Rønnestad, I., 2002b. Amino acid requirements of fish larvae and post-larvae: new tools and recent findings. Aquaculture (in press)
- Einarsson, S., Davies, P. S., Talbot, C., 1997. Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the Atlantic salmon, *Salmo salar* L. Comp. Biochem. Physiol. C 117C, 63-67.
- Espe, M., Lied, E., Torrissen, K. R., 1993. Changes in plasma and muscle free amino acids in Atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolysed cod muscle protein. Comp. Biochem. Physiol A 105, 555-562.
- Espe, M., Sveier, H., Høgøy, I., Lied, E., 1999. Nutrient absorption and growth of Atlantic salmon (*Salmo salar* L.) fed fish protein concentrate. Aquaculture 174, 119-137.
- Fernández-Díaz, C., Yüfera, M., 1997. Detecting growth in gilthead seabream, *Sparus aurata* L.; larvae fed microcapsules. Aquaculture 153, 193-102.
- Finn, R.N., Fyhn, H.J., Henderson, R.J., Evjen, M.S., 1995a. Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). I. Respiration and nitrogen metabolism. Mar. Biol. 124, 355-369.
- Finn, R.N., Rønnestad, I., Fyhn, H.J., 1995b. Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp. Biochem. Physiol. [A] 111, 647-671.
- Finn, R. N., Widdows, J., Fyhn, H. J., 1995c. Calorespirometry of developing embryos and yolk-sac larvae of turbot (*Scophthalmus maximus*). Mar. Biol. 122, 157-163.
- Fyhn, H. J., 1989. First feeding of marine fish larvae: Are free amino acids the source of energy? Aquaculture, 80, 111-120.
- Fyhn, H. J., 1990. Energy productin in marine fish larvae with emphasis on free amino acids as a potential fuel. In: Mellinger, J. (ed.) Comparative physiology. Animal nutrition and transport processes. 1. Nutrition in wild and domestic animals. Vol. 5. Karger, Basel. pp. 176-192.
- Grendell, J. H., Rothman, S.S., 1981. Digestive end products mobilize secretory proteins from subcellular stores in the pancreas. Am. J. Physiol. 241, G67-G73.
- Holmgren, S., Holmberg, A., Fritsche, R., Pelster, B., Schwerte, T., 2001. Control of gut motility in larval fish and amphibians. Second Int. Conf. Comp. Physiol. Biochem. in Africa. How Animals Work. Chobe National Park, Botswana, Aug 18-24, 2001.
- Kamisaka, Y., Kurokawa, T., Suzuki, T., Tagawa, M., Tanaka, M., Totland, G.K., Rønnestad, I., 2001. Ontogeny of cholecystokinin producing cells in Atlantic halibut (*Hippoglossus hippoglossus*) larvae. Gen. Comp. Endocrinol. 123, 31-37.

- Kamisaka, Y., Kurokawa, K., Suzuki, T., Totland, G. K., Rønnestad, I., Tagawa, M., Tanaka, M. 2002a. Ontogenetic appearance and distribution of the digestive hormone cholecystokinin (CCK) in fish. *Fish. Sci.* (in press)
- Kamisaka, Y., Kaji, T., Masuma, S., Tezuka, N., Kurokawa, T., Suzuki, T., Totland, G. K., Rønnestad, I., Tagawa, M., Tanaka, M., 2002b. Ontogeny of cholecystokinin - immunoreactive cells in the digestive tract of bluefin tuna, *Thunnus thynnus*, larvae. *Sarsia* (in press)
- Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles - implications and applications to formulated diets. *Aquaculture* 200, 181-200.
- Koven, W., Kolkovski, S., Hadas, E., Gamsiz, K.T.A., 2001. Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. *Aquaculture* 194, 107-121.
- Koven, W., Rojas-García, C. R., Finn, R. N., Tandler, A., Rønnestad, I., 2002. The stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone, cholecystokinin (CCK) and the protease, trypsin, in first feeding herring larvae, *Clupea harengus*. *Marine Biology*. 140: 1241-1247.
- Kurokawa, T., Suzuki, T., Andoh, T., 2000. Development of cholecystokinin and pancreatic polypeptide endocrine systems during the larval stage of Japanese flounder, *Paralichthys olivaceus*. *Gen Comp. Endocrinol.* 120(1): 8-16.
- Lazo, J. P., Dinis, M. T., Holt, J. G., Faulk, C., Arnold, C.R., 2000. Co-feeding microparticulate diets with algae: towards eliminating the need of zooplankton at first feeding red drum (*Sciaenops ocellatus*). *Aquaculture* 188, 339-351.
- Liddle, R. A., 1994a. Regulation of cholecystokinin gene-expression in rat intestine. *Ann. New York Acad. Sci.* 713, 2-31
- Liddle, R. A., 1994b. Regulation of cholecystokinin synthesis and secretion in rat intestine. *J. Nutr.* 124, S1308-S1314
- Liddle, R. A., 1995. Regulation of cholecystokinin secretion by intraluminal releasing factors. *Am. J. Physiol.* 269 (Gastrointest. Liver Physiol 32): G319-G327
- Liddle, R. A., 2000. Regulation of cholecystokinin secretion in humans. *J. Gastroenterol.* 35, 181-187
- Metges, C. C., ElKhoury, A. E., Selvaraj, A. B., Tsay, R. H., Atkinson, A., Regan, M. M., Bequette, B. J., Young, V. R., 2000. Kinetics of L-[1-C-13]leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein. *Am. J. Physiol.-endocrinol. Metabol.* 278, E1000-E1009.
- Ng, W. K., Hung, S. S. O., Herold, M. A., 1996. Poor utilization of dietary free amino acids by white sturgeon. *Fish Physiol. Biochem.* 15, 131-142.
- Olsson, C., Aldman, G., Larsson, A., Holmgren, S., 1999. Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* 202, 160-170.
- Owyang, C., 1994. Negative feedback-control of exocrine pancreatic-secretion - role of cholecystokinin and cholinergic pathway. *J. Nutr.* 124, S1321-S1326
- Rajjo, I. M., Vigna, S. R., Crim, J. W., 1988. Actions of cholecystokinin-related peptides on the gallbladder of bony fishes *in vitro*. *Comp. Biochem. Physiol. C* 90: 267-273
- Rojas-García, C. R., 2002. Intestinal function in marine fish larvae: Digestion and absorption of proteins and amino acids; efficiency and relation to cholecystokinin and trypsin. Dr. Thesis, University of Bergen, Bergen Norway
- Rojas-García, C. R., Rønnestad, I., Ueberschäer, B., 2001. Combined sensitive analytical methods for cholecystokinin levels and tryptic activity in individual fish larvae. *J. Exp. Mar. Biol. Ecol.* 265, 101-115
- Rojas-García, C. R. Rønnestad, I., 2002a. Cholecystokinin and tryptic activity in the gut of developing Atlantic halibut (*Hippoglossus hippoglossus*): evidence for participation in the regulation of protein digestion. *J. Fish Biol.* (in press)
- Rojas-García, C.R., Rønnestad, I., 2002b. Assimilation of dietary free amino acids, peptides and protein in post-larval Atlantic halibut (*Hippoglossus hippoglossus*). *Marine Biology*. DOI 10.1007/s002270100675. Published online (in press)
- Rombout, J. H., Taverne-Thiele, J. J., 1982. An immunocytochemical and electron-microscopical study of endocrine cells in the gut and pancreas of a stomachless teleost fish, *Barbus conchonioides* (Cyprinidae). *Cell. Tissue Res.* 227(3): 577-93.

- Rønnestad, I., Fyhn, H. J., 1993. Metabolic aspects of free amino acids in developing marine fish eggs and larvae. *Rev. Fish. Sci.* 1, 239-259.
- Rønnestad, I., Finn, R. N., Groot, E. P., Fyhn, H. J., 1992a. Utilization of free amino acids related to energy metabolism of developing eggs and larvae of lemon sole *Microstomus kitt* reared in the laboratory. *Mar. Ecol. Progr. Ser.* 88, 195-205.
- Rønnestad, I., Fyhn, H. J., Gravningen, K., 1992b. The importance of free amino acids to the energy metabolism of eggs and larvae of turbot (*Scophthalmus maximus*). *Mar. Biol.* 114, 517-525.
- Rønnestad, I., Koven, W. M., Tandler, A., Harel, M., Fyhn, H. J., 1994. Energy metabolism during development of eggs and larvae of gilthead sea bream (*Sparus aurata*). *Mar. Biol.* 120, 187-196.
- Rønnestad, I., Thorsen, A., Finn, R.N., 1999. Fish larval nutrition: Recent advances in amino acid metabolism. *Aquaculture* 177, 201-216.
- Rønnestad, I., Conceição, L. E. C., Aragão, C., Dinis, M. T., 2000a. Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). *J. Nutr.* 130, 2809-2812.
- Rønnestad, I., Pérez Dominguez, R., Tanaka, M., 2000b. Ontogeny of digestive tract functionality in Japanese flounder, *Paralichthys olivaceus* studied by in vivo microinjection: pH and assimilation of free amino acids. *Fish Physiol. Biochem.* 22, 225-235.
- Rønnestad, I., Rojas-García, C. R., Skadal, J., 2000c. Retrograde peristalsis, a possible mechanism for filling the pyloric caecae? *J. Fish Biol.* 56, 216-218.
- Rønnestad, I., Rojas-García, C. R., Tonheim, S. K., Conceição, L. E. C., 2001a. *In vivo* studies of digestion and nutrient assimilation in marine fish larvae. *Aquaculture* 201, 161-175.
- Rønnestad, I., Conceição, L. E. C., Aragão, C., Dinis, M. T., 2001b. Assimilation and catabolism of dispensable and indispensable free amino acids in post-larval Senegal sole (*Solea senegalensis*). *Comp. Biochem. Physiol.* C. 130: 461-466
- Rønnestad, I., Tonheim, S. K., Fyhn, H. J., Rojas-García, C.R., Kamisaka, Y., Koven, W., Finn, R. N., Terjesen, B. F., Barr, Y., Conceição, L. E. C., 2002. The supply of amino acids during early feeding stages of marine fish larvae: A review of recent findings. *Aquaculture (in press)*
- Rust, M. B., 1995 Quantitative aspects of nutrient assimilation in six species of fish larvae. Dr. Thesis. School of Fisheries: University of Washington, USA.
- Rust, M. B., Hardy, R. W., Stickney, R. R., 1993. A new method for force-feeding larval fish. *Aquaculture* 116, 341-352.
- Terjesen, B. F., Finn, R. N., Norberg, B., Rønnestad, I., 2002. Kinetics and fates of ammonia, urea, and uric acid during oocyte maturation and ontogeny of Atlantic halibut (*Hippoglossus hippoglossus*). *Comp. Biochem. Physiol. A* 131: 443-455.
- Terjesen, B. F., Rønnestad, I., Norberg, B., Anderson, P. M., 2000. Detection and basic properties of carbamoyl phosphate synthetase III during teleost ontogeny: a case study in the Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comp. Biochem. Physiol. [B]* 126, 521-535.
- Yamada, S., Simpson, K. L., Tanaka, Y., Katayama, T., 1981. Plasma amino acid changes in rainbow trout (*Salmo gairdneri*) force-fed casein and a corresponding amino acid mixture. *Bull. Jap. Soc. Sci. Fish.* 47, 1035-1040.
- Yoshida, K., Iwanaga, T., Fujita, T. 1983. Gastro-entero-pancreatic (GEP) endocrine system of the flatfish, *Paralichthys olivaceus*: an immunocytochemical study. *Arch. Histol. Jpn.* 46(2): 259-66.