

# Cholecystokinin and Trypsin Responses of Larval Red Drum (*Sciaenops Ocellatus*) to Soluble Components of Rotifers (*Brachionus Plicatilis*) and Algae (*Isochrysis Galbana*)

Ken Webb and Joan Holt

University of Texas Marine Science Institute

750 Channelview Drive, Port Aransas, TX 78373

Fax# (361)749-6749 E-mail: kenw@mail.utexas.edu

---

## Abstract

In an attempt to better understand the problems in weaning larval fish to artificial diets, our lab has begun to investigate the role of the digestive hormone cholecystokinin (CCK). While there are a number of other labs also investigating CCK and other digestive hormones such as bombesin, PPY, and gastrin; research into the roles of these hormones in fish is still in its infancy. Previous research with red drum larvae suggests that some component of rotifers and algae enable red drum larvae to more efficiently utilize microparticulate diets than when these are not included in the culture system. The current study investigated the impact of soluble components of rotifers and algae on the CCK and trypsin responses of larval red drum at 6 and 10 days post hatch (DPH). Introduction of homogenized rotifers was shown to significantly increase whole body CCK levels, CCK mRNA, and trypsin activity in 6 DPH red drum larvae, but not in 10 DPH larvae. Homogenates of *Isochrysis galbana* did not significantly affect CCK or trypsin at either age. This research suggests that there is a soluble component of rotifers that can upregulate digestive function in larval red drum, at least in 6 DPH larvae.

Keywords: *cholecystokinin, trypsin, digestive physiology*

## Introduction

Understanding the physiological controls of digestion in larval fishes is essentially in its infancy. While much of the knowledge gained from studying juveniles and adults can be translated to larvae, it most often comes with caveats and assumptions due to the relatively simple state of development in most marine larvae and the rapid organogenesis which marks the ontogenetic process. Considering that hormonal regulation of digestive processes is still very unclear in even adult fishes, there is a paucity of information on these processes in larvae. Only within the past fifteen years have researchers begun to look into the physiological controls of digestion in larval fish. This has been primarily driven by the burgeoning aquaculture industry around the world and the desire to reduce feed costs and improve growth and survival in marine larvae for growout. Of particular interest has been the desire to totally eliminate live feeds in the diets of marine larvae and replace them with formulated micro-particulate diets. This has proven difficult and while some success has been made (1-4), there is still no diet or rearing protocol which produces equivalent growth and / or survival that does not require at least some period of live prey.

What makes this need for co-feeding live prey particularly interesting is that there appears to be no obvious basis for this requirement. While growth and survival is decreased in larvae fed on microparticulate diets alone, sometimes, at least some of the fish are able to feed on the microparticulate diet and grow. In the case of the seabass (*Dicentrarchus labrax*), 35% of larvae fed from first feeding on a dry diet survived compared to 55% in a live prey control group (2). Growth of the fish in this experiment was slower, with fish fed the microparticulate diet taking 28 days to reach 3.4 mg verses 19 days for the live prey control, but the fish did grow. The same type of pattern has been seen in red drum (*Sciaenops ocellatus*) larvae that are fed zooplankton (rotifers, *Brachionus plicatilis*, and *Artemia salina* nauplii) supplemented with algae reaching approximately 4.1 mm in 14 days while those fed on microparticulate diets alone only reached 3.1 mm in the same time (4). Both the sea bass and red drum larvae possess functional pancreatic enzymes (5, 6) and these are clearly sufficient for at least some of the larvae to grow. What remains unknown is the factor which allows some fish to survive when fed microparticulate diets

alone while others in their cohort do not survive. This has led to the investigation of the physiological basis for these differences.

One area which has begun to receive attention is the regulation of pancreatic enzyme secretion. Marine larvae like the red drum depend heavily on pancreatic secretions until the development of a functional stomach (7-9) and one of the important controls of pancreatic enzyme secretion is the hormone cholecystokinin (CCK). Most work with CCK has been done in terrestrial vertebrates, but even among fish CCK is known to cause gall bladder contraction (10, 11), regulate gut motility (12), regulate ingestion (13), and regulate secretion of pancreatic enzymes (6, 14, 15). Due to the relative importance of pancreatic enzymes in early larvae, CCK is likely to be a major factor in the digestive physiology of these fish. Previous work in this laboratory (16) has shown that red drum possess CCK at first feeding and that in 18 day post hatch (DPH) larvae, CCK and trypsin respond to a volitional feeding event. What is not understood is how CCK secretion is stimulated.

Two of the major mechanisms suggested to stimulate the secretion of CCK are either through the mechanical expansion of the gut when food is ingested or through chemical stimulation by the food or a digestion product. Koven et al. (15) demonstrated that Atlantic herring (*Clupea harengus*) larvae responded with increased trypsin and CCK when tube fed solutions containing bovine serum albumin (BSA), free amino acids (FAA), or a combination of the two compared to fish that were fed the saline carrier only. This work suggests that gut fullness is not necessarily the most important factor. Further, differences in responses to the BSA and FAA treatments suggest that chemical differences may play a bigger role. In humans, products of fat and protein digestion are the most potent stimulatory compounds (17, 18) while in fish the story is mixed. In the herring, BSA or a mixture of BSA and FAA produced the fastest CCK response and the highest trypsin activity levels while FAA alone did not perform as well (15). In the sea bass however, CCK secretion was much higher in 42 DPH fish reared on a diet high in potato starch compared to fish reared on diets high in fish meal and / or hydrolyzed fish meal (6). Whether in response to products of protein or carbohydrate digestion, some chemical component of the diet produced differential responses in CCK secretion.

Previous work with red drum larvae has shown that including rotifers and / or algae along with microparticulate diets significantly increases growth and survival (4, 19, 20). While the rotifers are actively consumed and are nutritious by themselves, the ability of algae to increase survival and growth is very suggestive of some increased regulatory function promoted by the algae rather than a nutritional benefit of the algae itself. Both rotifers and algae contain significant fractions of compounds such as soluble polypeptides, FAA, and polyamines which are all commonly considered chemoattractants in larval feeds (21, 22) and these may be a factor in the ability of larval fish to utilize microparticulate diets better when either rotifers or algae are co-fed. The present study was therefore designed to test the hypothesis that some soluble component of rotifers or algae is responsible for up regulating CCK secretion in larval red drum.

## Methods

Three independent batches of red drum eggs were obtained from captive broodstock at the University of Texas Fisheries and Mariculture Laboratory (FAML) and raised on rotifers (*B. plicatilis*) in accordance with established protocols (3). For each batch of eggs, aliquots of the same three experimental treatments and a control were tested. The control (SW) consisted of seawater which was filtered to 5  $\mu\text{m}$  and then autoclaved. The three experimental treatments were homogenized rotifers (RO), rotifer culture water (RC), and homogenized Isochrysis (IS). The rotifers for the RO treatment were taken from the continuous culture maintained at FAML, enriched overnight with Algamac-3050, washed in saltwater to remove residual enrichment, and concentrated to approximately 1000 rotifers / mL. Rotifer culture water was taken from the FAML continuous culture. Each day, the rotifer cultures are cleaned using a collector fitted with a 40  $\mu\text{m}$  mesh. This mesh keeps rotifers on one side of the mesh but allows contaminants such as ciliates to be washed through into the second chamber. The RC sample was taken from the first rinse of a daily collection and represents pure rotifer culture water with all of the rotifers removed. The IS treatment was taken from a standing culture of *Isochrysis galbana* at FAML. Algae was taken from the culture tank and strained through a 40  $\mu\text{m}$  mesh to remove any contaminants and then enumerated (~600,000 cells / mL).

All treatments were collected fresh and processed on the same day. When not being processed, treatments were kept in a refrigerator at 4°C. All treatments, including the control, were processed by being homogenized in a commercial blender for a total of 10 minutes. In order to ensure the contents did not overheat, the blending vessel was removed after five minutes and placed briefly back in the refrigerator before being homogenized for another 5 minutes. Following homogenization, each treatment was filtered through a series of three fine porosity glass fiber filters (retention approximately 1 µm) before being filtered through a type HA membrane filter (0.45 µm pore size). After filtration, treatments were separated into aliquots and stored at -80°C until needed.

On the day before each sampling (days 5 and 9 post hatch), larvae were fed as normal (twice) in the morning but were not given any additional feed in the afternoon. Also, flow to the tanks was increased to approximately 30 - 40 L / h in order to flush any uneaten rotifers and algae out of the rearing tank. The tank overflow was fitted with a screen (400 µm mesh size) which kept the larvae in the tank but allowed even the largest rotifers to be washed out of the system. On the morning of sampling (day 6 or day 10 post hatch), the air and water flow to the rearing tank was turned off, causing the larvae to rise to the surface. After the larvae rose to the surface, they were dipped out using a beaker. From the collection beaker, 100 larvae were siphoned into one of three replicate 1 L beakers for each treatment (12 beakers in all) using a length of clear airline tubing which allowed the larvae to be counted as they passed. Once 100 larvae were in a treatment beaker, the volume was topped up to 1 L using water from the biofilter and an air stone with a very fine stream of bubbles was added in order to provide aeration. After all beakers were full, the larvae were allowed to acclimate to the beaker for 1 h, after which one of the four treatments was added to each beaker and larvae were left for 2 h. After 2 h the larvae were collected using a fine meshed (55 µm) sieve, washed with distilled water, transferred to glass vials, and placed directly into the -80°C freezer where they remained until analyzed. Each experiment (day 6 and 10 post hatch) was repeated three times using larvae from different egg batches.

All analyses were run on whole larvae and contain both neural and gastrointestinal CCK. Also, after some preliminary tests were run it was clear that the 100 fish in each replicate were

insufficient for analysis. Therefore, the three replicates for each treatment were pooled (300 larvae vs. 100 larvae) resulting in four treatments with each of the three independent spawning batches representing a replicate. Tubes containing the larvae were thawed on ice and pooled into a single tube. From each pool, 10 larvae were taken for trypsin analysis, 30 larvae for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and the remaining 260 larvae were used for determination of CCK peptide concentration using a competitive binding Enzyme-Linked ImmunoSorbent Assay (ELISA). All lab analyses were performed using assays adapted from previous work or developed in house (16). Homogeneity of variance in all samples was tested using Levene's test for homogeneity of variance. Values of CCK, Trypsin, and CCK mRNA were then tested using one-way analysis of variance using SPSS for Windows v. 16.0.0 (SPSS Inc., Chicago, IL) to find differences between treatments. Differences were considered significant at  $\alpha \leq 0.05$ . Data is expressed as Mean  $\pm$  S.E.

## Results and Discussion

CCK peptide concentrations (Fig. 1) were extraordinarily low in all fish samples with an overall mean in both treatments of 0.1058 fmoles / larva. In the 6 DPH fish, the only significant differences seen were between the SW control fish with  $0.085 \pm 0.015$  fmoles / larva and the fish in the RO treatment with  $0.153 \pm 0.025$  fmoles / larva. In the 10 DPH larvae, the overall range was approximately the same at  $0.067 \pm 0.017$  fmoles / larva in the RC treatment fish to  $0.126 \pm 0.022$  fmoles / larva in the RO treatment fish but no significant differences were seen between treatments.

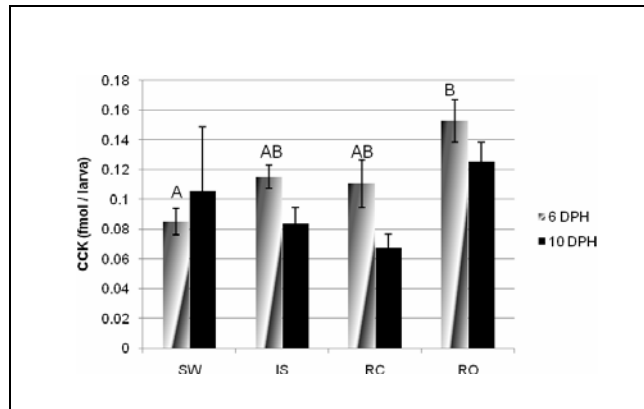


Figure 1. CCK response of 6 and 10 DPH red drum larvae. SW = Saltwater control, IS = Isochrysis galbana homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. Columns with different letters indicate significant differences among treatments within that time period. Results were considered different at  $\alpha \leq 0.05$ .

Error bars show standard error.

Trypsin activity was also variable, but not to the same degree as the CCK peptide concentrations. In the 6 DPH fish, the overall mean of trypsin activity was 29.41 U / larva with the highest activity seen in the fish in the RO treatment at  $47.73 \pm 9.02$  U / larva and the lowest activity seen in the RC treatment fish at  $21.59 \pm 7.10$  U / larva (Fig. 2). The trypsin activity of fish in the RO treatment was significantly higher than that of fish in all the other treatments. In the 10 DPH fish, the range was similar to that in the 6 DPH fish (from 34.47 to 57.78 U / larva in the SW and RO treatments, respectively) but the results were much more variable, and there were no significant differences among groups (Fig. 2).

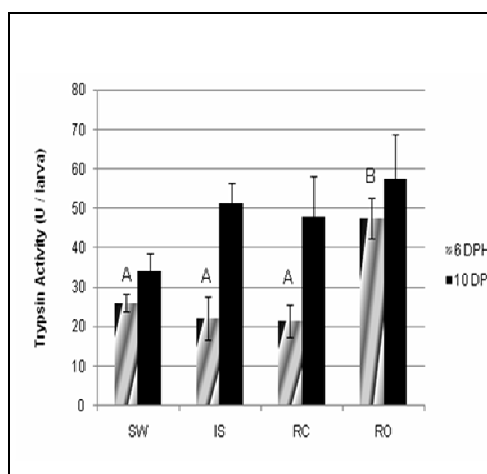


Figure 2. Trypsin response of 6 and 10 DPH red drum larvae. SW = Saltwater control, IS = Isochrysis galbana homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. Columns with different letters indicate significant differences among treatments within that time period. Results were considered different at  $\alpha \leq 0.05$ .

Error bars show standard error.

Copies of CCK mRNA showed a similar pattern to that seen in the CCK peptide concentrations and trypsin activities. In the 6 DPH fish, the highest number of copies was seen in fish in the RO treatment with  $143.74 \pm 13.93$  copies /  $\mu\text{g}$  and the lowest number was seen in the SW control fish with  $86.61 \pm 10.31$  copies /  $\mu\text{g}$  and these two treatments were the only ones found to be significantly different (Fig. 3). In the 10 DPH fish, CCK mRNA was highly variable resulting in no significant differences among treatments which ranged from  $24.72 \pm 5.82$  to  $91.52 \pm 19.50$  copies /  $\mu\text{g}$  in the IS and RC treatment fish, respectively (Fig. 3).

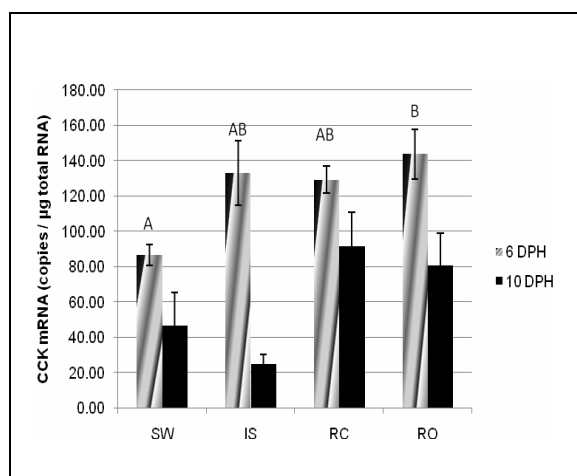


Figure 3. CCK mRNA response of 6 and 10 DPH red drum larvae. SW = Saltwater control, IS = Isochrysis galbana homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. Columns with different letters indicate significant differences among treatments within that time period.

Results were considered different at  $\alpha \leq 0.05$ . Error bars show standard error.



## Conclusions

The present study demonstrated that some soluble component of rotifers elicited CCK, trypsin, and CCK mRNA responses in 6 DPH red drum larvae but was insufficient to elicit the same response in 10 DPH fish. The general complexity of the red drum digestive tract increases during the period between 6 and 10 DPH, but there are no major histological changes which occur during this time (19). Previous work with red drum has shown that the number of CCK immunoreactive cells in the intestine also increases gradually during this time period, but there is no major expansion of CCK-IR cells (16). It is possible that these relatively minor increases in CCK-IR cell concentrations in 10 DPH fish over 6 DPH fish are a factor, but it seems likely that increased numbers of CCK-IR would lead to a greater response rather than a lack of response. The results of this study do suggest that while there are no clear ontogenetic changes occurring in red drum larvae during this time period, some fundamental change does occur between days 6 and 10 post hatch which alters the effect of the RO treatment on the CCK, trypsin, and CCK mRNA responses of the larvae. It is possible that this change does not lie with the CCK secreting cells directly, but perhaps with some CCK stimulatory mechanism which has not yet been considered.

The current work has successfully shown that larval red drum are capable of responding to the soluble fractions of at least one of the treatments used. The exact mechanism by which the RO treatment stimulated CCK, trypsin, and CCK mRNA responses is unclear. The fact that the RO treatment produced a response in 6 DPH and not in 10 DPH fish further supports the hypothesis that some physiological change takes place during the period between first feeding and weaning to microparticulate diets at 11 DPH. These findings may eventually assist in developing a microparticulate diet which can completely replace live prey without any decline in survival or growth.

## References

1. Cahu, C. and J.Z. Infante, 2001. Substitution of live food by formulated diets in marine fish larvae. *Aquaculture* **200**(1-2): p. 161-180.
2. Cahu, C., et al., 1998. Preliminary results on sea bass (*Dicentrarchus labrax*) larvae rearing with compound diet from first feeding. Comparison with carp (*Cyprinus carpio*) larvae. *Aquaculture* **169**(1-2): p. 1-7.
3. Holt, G.J., 1993. Feeding larval red drum on microparticulate diets in a closed recirculating water system. *J World Aquaculture Soc.* **24**(2): p. 225-230.
4. Lazo, J.P., et al., 2000. Co-feeding microparticulate diets with algae: toward eliminating the need of zooplankton at first feeding in larval red drum (*Sciaenops ocellatus*). *Aquaculture* **188**(3-4): p. 339-351.
5. Lazo, J.P., G.J. Holt, and C.R. Arnold, 2000. Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus*. *Aquaculture Nutrition* **6**(3): p. 183-192.
6. Cahu, C., et al., 2004. Expression and activities of pancreatic enzymes in developing sea bass larvae (*Dicentrarchus labrax*) in relation to intact and hydrolyzed dietary protein; involvement of cholecystokinin. *Aquaculture* **238**(1-4): p. 295-308.
7. Govoni, J., G. Boehlert, and Y. Watanabe, 1986. The physiology of digestion in fish larvae. *Environ. Biol. Fish.* **16**(1): p. 59-77.
8. Ribeiro, L., et al., 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture* **179**(1-4): p. 465-473.
9. Yufera, M. and M.J. Darias, 2007. The onset of exogenous feeding in marine fish larvae. *Aquaculture* **268**(1-4): p. 53-63.
10. Rajjo, I.M., S.R. Vigna, and J.W. Crim, 1988. Actions of cholecystokinin-related peptides on the gallbladder of bony fishes in vitro. *Comparative Biochemistry and Physiology, C* **90C**(1): p. 267-273.
11. Einarsson, S., P.S. Davies, and C. Talbot, 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 Toxicol. Pharmacol.* **117**(1): p. 63-67.
12. Olsson, C. and S. Holmgren, 2001. The control of gut motility. *Comp. Biochem. Physiol. Physiol.* **128**(3): p. 479-501.
13. Gelineau, A. and T. Boujard, 2001. Oral administration of cholecystokinin receptor antagonists increase feed intake in rainbow trout. *Journal of Fish Biology* **58**(3): p. 716-724.
14. Rojas-Garcia, C.R. and I. Rønnestad, 2002. Cholecystokinin and tryptic activity in the gut and body of developing Atlantic halibut larvae: evidence for participation in the regulation of protein digestion. *J. Fish Biol.* **61**(4): p. 973-986.
15. Koven, W., et al., 2002. Stimulatory effect of ingested protein and/or free amino acids on the secretion of the gastro-endocrine hormone cholecystokinin and on tryptic activity, in early-feeding herring larvae, *Clupea harengus*. *Marine Biology* **140**(6): p. 1241-1247.

16. Webb, K.A., 2008. Cholecystokinin and the Ontogeny of Digestion in the Red Drum (*Sciaenops ocellatus*). University of Texas at Austin, Port Aransas, TX, USA, 168.
17. Liddle, R.A., 1997. Cholecystokinin Cells. Annual Review of Physiology **59**(1): p. 221-242.
18. Liddle, R.A., 2000. Regulation of cholecystokinin secretion in humans. Journal of Gastroenterology **35**(3): p. 181-187.
19. Lazo, J.P., 1999. Development of the digestive system in red drum (*Sciaenops ocellatus*) larvae. Ph.D. Dissertation, The University of Texas at Austin, Austin, 212.
20. Lazo, J.P., G.J. Holt, and C.R. Arnold, 2002. Towards the development of suitable microdiets for substitution of live prey in the rearing of red drum (*Sciaenops ocellatus*) larvae: Applications of studies on digestive physiology. Int. Commemorative Symp. -- 70th Anniversary of the Japan Society of Fisheries Science, Yokohama (Japan), 1-5 Oct 2002: p. Vol. 68, suppl. 1, p.
21. Srivastava, A., et al., 2006. Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): With emphasis on the water soluble fraction. Aquaculture **254**(1-4): p. 534-543.
22. Hamana, K. and M. Niitsu, 2006. Cellular polyamines of lower eukaryotes belonging to the phyla Glaucophyta, Rhodophyta, Cryptophyta, Haptophyta and Percolozoa. The Journal of General and Applied Microbiology **52**(4): p. 235-240.