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Cholecystokinin and the Ontogeny of Digestion in the Red Drum (*Sciaenops ocellatus*)

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CHOLECYSTOKININ AND THE ONTOGENY OF DIGESTION IN THE RED DRUM (*SCIAENOPS OCELLATUS*)

by

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DEDICATION

This work is dedicated to my wife Glenda who has supported me without question during the long journey to reach this point.

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CHOLECYSTOKININ AND THE ONTOGENY OF DIGESTION IN THE RED DRUM (*SCIAENOPS OCELLATUS*)

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While substantial progress has been made in replacing live prey with artificial diets in the feeding of marine fish larvae, it still remains impossible to successfully rear larvae on artificial diets without some period of co-feeding live prey or algae. This study investigated the presence and role of the gastrointestinal hormone cholecystokinin (CCK) in the red drum (*Sciaenops ocellatus*) to gain a better understanding of the factors limiting the utilization of artificial diets by red drum larvae. Work with other fish species has shown that CCK is the principal hormone which regulates the release of pancreatic enzymes into the gut lumen and emphasizes the potential importance of CCK in early red drum larvae. This work investigated the hypothesis that some signal present in the live prey or algae stimulates CCK and thereby initiates the digestive process in the larvae. First, the nucleotide and amino acid sequence of the putative red drum cholecystokinin

precursor was determined and the development of CCK immunoreactive cells was examined. This work showed that red drum CCK is highly similar to CCK in other vertebrates and can be detected in the digestive tract of larval red drum within three days after the initiation of exogenous feeding. Next; postprandial trypsin, CCK, and CCK mRNA responses were quantified in red drum juveniles and larvae over a three hour period. Both CCK and trypsin were increased within thirty minutes following feeding while CCK mRNA levels were increased within the next two to three hours. Finally, the trypsin, CCK, and CCK mRNA responses of red drum larvae to homogenates of live prey and algae were examined. Homogenized rotifers appeared to be sufficient to induce both the CCK and trypsin responses in larval red drum. These results suggest that in addition to other factors, some component of live prey may initiate the release of CCK and prime the digestive process. Understanding these factors and their effects in early larvae may allow us to formulate and produce a prepared diet which will support growth and survival to metamorphosis equal to that provided by live feeds.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION

Worldwide, the demand for aquaculture products has continued to increase at a steady pace to satisfy an increasing demand for fisheries products that can no longer be met by world's capture fisheries (FAO, 2007). In order to meet this demand, the aquaculture industry has had to develop new products and expand into new areas. There are a number of difficulties preventing the rapid expansion of the industry; greatest among these is the difficulty in rearing marine larvae. Due to their typically altricial nature, marine larvae currently require live foods for the first part of their lives which increases the difficulty and cost of raising the larvae to juveniles. Because of potential costs and other problems associated with supplying live feeds it is desirable to wean larvae to a formulated or inert diet as quickly as possible. Inert diets are preferable because they offer a feed with a consistent nutrient composition that can be frozen and remain in storage for relatively long periods. Inert feeds also remove the potential for contamination of the culture system by diseases which can enter the system with live feeds.

Inert feeds can also provide an alternative method for conducting research into the nutritional requirements of larvae. In larger fish, nutrient studies are traditionally performed in a dose-response fashion with groups of fish fed diets which provide varying levels of the nutrient of interest. For many nutrients, this is not currently possible in larval fish since the live feeds used already contain high levels of those nutrients or live feeds can possess a nutrient profile which is undesirable and

not subject to manipulation (Rønnestad et al., 2001). Thus, an inert diet which provides rapid growth and maximal survival from first feeding is highly desirable. A great deal of attention by both academic and commercial researchers has been focused on producing such an inert diet. This work has primarily focused on the production and testing of diets comprised of nutrients which have been ground to an extremely small size, mixed, and then bound together to form a microparticulate diet. Among the marine fish commercially cultured today however, none are reported to be capable of normal growth and survival on any inert diet from first feeding although seabass (*Dicentrarchus labrax*) and red drum (*Sciaenops ocellatus*) have come close (Holt, 1993; Cahu et al., 1998a; Lazo et al., 2000a).

Red drum larvae are well studied and present an excellent choice for studying the utilization of inert diets by larval marine fish since their development is rapid and the larvae will consume inert particles at first feeding. Holt (1993) reported that when red drum larvae were fed a microparticulate diet (BioKyowa, Kyowa Hakko Kogyo Co., Tokyo, Japan), alone, they consumed the inert feed as early as first feeding, but still failed to grow as well as the live prey fed control. However, those fish which were co-fed with live rotifers (*Brachionus plicatilis*) for 5 days plus the microparticulate diet (Fig. 1.1) were found to grow at the same rate as the live prey fed control and similar numbers survived through metamorphosis. She concluded that since the fish were found to ingest the microparticulate diet from day 3 post hatch (first feeding), poor growth in the fish fed the microparticulate diet alone might be explained by an inability to adequately digest or assimilate the prepared feed.

One of the potential explanations for increased survival and growth in larvae fed live prey is the supplementation of larval digestive enzymes with the enzymes provided by the prey animals. Assays using *in vitro* methods to examine protein digestibility in red drum larvae were used to see if such an exogenous enzyme supply was necessary (Lazo et al., 2002). Using these *in vitro* methods, the authors found that the larvae possessed sufficient digestive enzymes and were capable of digesting many of the common feedstuffs used in diet formulation. Also, in feeding trials with microparticulate diets, the potential influence of exogenous enzymes on red drum larvae has also been found to be insignificant, implying that some other factor may be at work (Lazo, 1999; Lazo et al., 2000a; Lazo et al., 2000b).

It has been shown that when fed microparticulate diets red drum larvae do not need zooplankton in order to thrive. Red drum larvae fed a commercial microparticulate diet grew as well as the live prey fed control when raised in a tank supplied with a marine algae (*Isochrysis galbana*) for the first five days (Lazo et al., 2000a). Additionally, when *Nannochloris occulata* was used instead of the *Isochrysis*, growth was significantly lower than the *Isochrysis* supplemented treatment, but higher than the microparticulate only diet (Holt, 2002). This type of "green water culture" is commonly useful in rearing many marine larvae although there is no clear explanation why this is of benefit, nor why one algal species is better than another. The fact that red drum larvae are able to survive and grow equally well when co-fed zooplankton or raised in green water systems implies that red drum larvae may have the potential to feed on an inert diet from first feeding if it were

possible to determine why algae or rotifers are required for the first five days. Supplementation of the microparticulate diet with either rotifers or live algae may affect the larval digestive system in such a way that it up-regulates the endogenous digestive enzymes. Determining the basis of such an up-regulation may allow formulation of microparticulate diets which contain the necessary stimulants for proper digestive function.

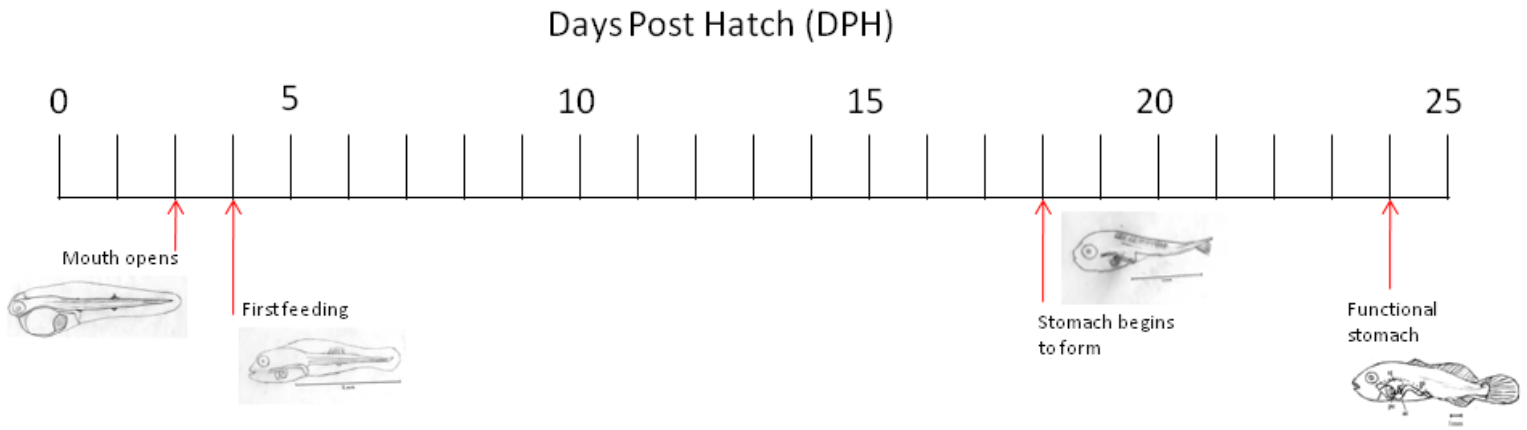
One of the newer fields of investigation in larval nutrition has been into the regulation of digestive processes. Cholecystokinin (CCK) has received a great deal of attention due to its role as a regulator of pancreatic enzyme secretion. Pancreatic enzymes are one of the primary means by which many larval fish including red drum (Lazo, 1999; Lazo et al., 2000b) and seabass (*Dicentrarchus labrax*) (Cahu et al., 1998b; Cahu et al., 2004) digest food until the development of an active stomach. CCK in fish is known to have a strong role in the regulation of gastrointestinal function and appears to function as part of a regulatory loop for pancreatic enzymes (Einarsson et al., 1997; de Pedro and Björnsson, 2001; Koven et al., 2002; Rønnestad, 2002; Rønnestad et al., 2003). CCK is a peptide which is found both in the central nervous system (CNS) and the gut (de Pedro and Björnsson, 2001). From research into humans, it is known that products of proteolysis and lipolysis stimulate the CCK (Endocrine I) cell to release CCK which then acts humorally on pancreatic acinar cells to release pancreatic enzymes (Klein et al., 1999; Liddle, 2000). Injection of exogenous CCK (porcine) produces a dose-dependent release of pancreatic proteases into the intestine of killifish (*Fundulus heteroclitus*) and Atlantic

salmon (*Salmo salar*) (Rajjo et al., 1988a; Einarsson et al., 1997). Endogenous CCK secretion has been shown to be stimulated in fish by bovine serum albumin (Koven et al., 2002), potato starch (Cahu et al., 2004), or free methionine (Koven et al., 2001). CCK therefore represents a likely candidate for investigation into the mechanism by which digestive function may be controlled in larval fishes.

The general hypothesis of the present work was that some component in live prey or algae increases digestive function in larval red drum through stimulation of endogenous CCK production. In order to test this hypothesis, a number of experiments were conducted. First, the presence of CCK immunoreactive cells in larval red drum during the first 26 days post hatch was investigated to ensure that CCK was present in the larvae and therefore capable of the hypothesized function. Second, the responses of larval red drum to volitional feeding were examined in order to elucidate if feeding on live prey was capable of eliciting CCK and pancreatic enzyme responses. Finally, the ability of soluble components of rotifers and *Isochrysis galbana* to induce CCK secretion was examined in order to determine if increases in growth could be a function of a non-nutritional component of the live prey.

Figure 1.1. Weaning protocol for red drum larvae using rotifers (*Brachionus plicatilis*) and BioKyowa microparticulate diet.

Protocol created by Holt (1993) using a five day period of decreasing concentrations of rotifers with increasing amounts of microparticulate diet.


Days Post Hatch (DPH)
0
5
10
15
20
25
Mouth opens
First feeding
Stomach begins to form
Functional stomach
Rotifers
Microparticulate Diet

CHAPTER 2: MOLECULAR CLONING AND IMMUNOHISTOCHEMICAL LOCALIZATION OF CHOLECYSTOKININ IN THE GASTROINTESTINAL TRACT OF LARVAL RED DRUM (*SCIAENOPS OCELLATUS*)

ABSTRACT

The current study sought to clarify the role of cholecystokinin (CCK) in the digestion of larval red drum (*Sciaenops ocellatus*) in order to better characterize the processes limiting the utilization of microparticulate diets at first feeding. The red drum CCK cDNA, isolated from adult anterior intestine and pyloric caeca, contains a 414 base pair (bp) open reading frame encoding a deduced amino acid sequence of 138 residues which is highly similar to preprocholecystokinin from other vertebrates. The mature CCK octapeptide has the same amino acid sequence as that found in mammals and in Atlantic herring (*Clupea harengus*). Tissue distribution analysis of adult and juvenile red drum using primers specific for red drum CCK mRNA revealed bright bands in samples from the brain and pyloric caeca with fainter bands seen in the anterior intestine and pancreas. Immunohistochemical analysis of larval red drum showed that CCK-immunoreactive (CCK-IR) cells were present as early as 3 days post hatch (DPH) in some fish and were present in all fish by 6 DPH. CCK-IR cells were found in the anterior midgut in early larvae and had spread to the first bend of the gut by day 6. In older larvae (18+ DPH), CCK-IR cells were found in large numbers in the anterior intestine and in the developing pyloric caeca. The sequence

and distribution of CCK mRNA along with the presence of CCK-IR cells in early red drum larvae suggest that CCK is present and may be capable of regulating pancreatic secretion in early stages of larval development in this species.

INTRODUCTION

Understanding the development of the digestive system in larval marine fish has become increasingly more important in order to replace live feeds with microparticulate diets. In the red drum (*Sciaenops ocellatus*), larvae fed microparticulate diets from first feeding die by 8 days post hatch (DPH) unless other live organisms such as rotifers (*Brachionus plicatilis*) and/or algae (*Isochrysis galbana*) are co-fed for at least the first 5 days of feeding (3 – 7 DPH) (Holt, 1993; Lazo et al., 2000a). This is perplexing since there are no major morphological changes in the gut of the larvae during this period (Lazo, 1999) and the larvae appear to possess sufficient digestive enzymes to digest the provided microparticulate diets (Lazo et al., 2000b). Further clouding the issue, substitution of the *I. galbana* with another species of algae (*Nannochloris occulata*) results in significantly decreased growth and survival which is still greater than that seen in fish which receive the microparticulate diet alone (Holt, 2002). The possibility exists that larval red drum require some stimulus present in the live organisms in order to properly stimulate digestion at first feeding. It is not well understood how such a

stimulus might act in altricial larvae such as those of the red drum, but cholecystokinin (CCK) may be a key hormone in this process.

Among its other functions in vertebrates, CCK is the single most potent regulatory hormone in the release of pancreatic digestive enzymes (Rehfeld, 2004). In altricial teleost larvae, the maternally supplied yolk and oil are exhausted before the fish has developed the complex digestive system that is present in the juvenile and adult form. Though the capture and digestion of prey is necessary to meet the high energy demands of these larvae following the transition to exogenous nutrition, they must depend upon this simple digestive system for all of their needs. The development of a functional (secretory) stomach is normally the last stage of gastrointestinal development in larvae (Govoni et al., 1986) and fish must rely upon other mechanisms for digestion of exogenous nutrients until this occurs. As most prepared diets focus on high protein content and protein digestion in larvae is potentially constrained by the lack of a functional stomach, there has been a great deal of research into protein availability and digestibility in larvae. Rønnestad et al. (2007) recently reviewed the ability of larvae to digest proteins, peptides, and amino acids and discussed this matter in detail. In short, larval fish are able to obtain some nutrients through pinocytosis but it has been suggested that the rate of nutrient absorption by this method is far too low to fuel the dramatic growth and differentiation of larvae during this period (Rønnestad et al., 2003). Developing larvae are therefore dependant on nutrients obtained through extracellular digestion of food particles. Until the formation of a functional stomach and the onset of acid

hydrolysis, extracellular digestion in larvae is limited to the digestive enzymes of the pancreas and brush border enzymes (Tonheim et al., 2004; Rønnestad et al., 2007). Of these enzymes, trypsin is perhaps the most important as it is the enzyme which is solely responsible for the activation of all the other pancreatic zymogens (Rinderknecht, 1993). The importance of trypsin during this period and the demonstrated relationship between CCK and trypsin secretion in fish (Iwai et al., 1987; Einarsson et al., 1997; Koven et al., 2002; Rojas-Garcia and Rønnestad, 2002; Kofuji et al., 2007) suggests that CCK may be the principal regulator of digestive activity in altricial larvae. Previous research with the larvae of red drum (*Sciaenops ocellatus*) has shown that red drum larvae do not possess a pyloric sphincter until approximately 18 DPH (Soto et al., 1998) nor a secretory stomach until 22 DPH though they do have a functional pancreas at first feeding (3 DPH) (Lazo, 1999; Lazo et al., 2000b). If CCK does play a role in the regulation of digestion in early red drum larvae, then CCK must be present at the onset of feeding.

The presence of gastrointestinal CCK-immunoreactive (CCK-IR) cells have been shown in a variety of larval fish species (Rajjo et al., 1988b; Jensen et al., 2001; Kamisaka et al., 2001; Kamisaka et al., 2002; Kurokawa et al., 2004; Lee et al., 2004; Murashita et al., 2006) and the appearance of these cells generally follows a pattern related to the presence or absence of a coiled gut (Rønnestad, 2002; Kamisaka et al., 2005; Rønnestad et al., 2007). In larvae which have a straight gut such as the Atlantic herring (*Clupea harengus*) or the ayu (*Plecoglossus altivelis*), CCK-IR cells are found in the gut at hatching (Kamisaka et al., 2003; Kamisaka et al.,

2005). In species which have a coiled gut, CCK-IR cells appear sometime after hatching but the exact developmental stage at which they appear can be variable. In some larvae with coiled guts such as those of the Japanese flounder (*Paralichthys olivaceus*) and bluefin tuna (*Thunnus thynnus*) CCK-IR cells appear before the onset of exogenous feeding (Kurokawa et al., 2000; Kamisaka et al., 2002), but in others such as the Atlantic halibut (*Hippoglossus hippoglossus*) and sea bass (*Dicentrarchus labrax*) CCK-IR cells are not detected until well after the onset of exogenous feeding (García Hernández et al., 1994; Kamisaka et al., 2001). While the timing of CCK-IR cell appearance seems to be somewhat varied, their distribution pattern appears to be closely related to the presence or absence of a coiled gut (Rønnestad, 2002; Kamisaka et al., 2005; Rønnestad et al., 2007). In the larvae of fish which have a coiled gut, CCK-IR cells are concentrated primarily in the anterior midgut and pyloric caeca of the larvae with none seen in the fore- or hindgut (Kurokawa et al., 2000; Kamisaka et al., 2001; Kamisaka et al., 2002). In fish like the Atlantic herring that have a straight gut, *in situ* hybridization has shown cells containing CCK mRNA scattered throughout the midgut with no significant areas of concentration (Kamisaka et al., 2005).

The gut in red drum is straight at hatching, but forms a coil before the onset of exogenous feeding on 3 DPH. As in the Atlantic halibut, Japanese flounder, and bluefin tuna, the gut of red drum larvae is still relatively simple at this stage, but larval red drum have been shown to possess a fully functional exocrine pancreas, liver, and gall bladder at 3 DPH (Lazo, 1999). At the onset of exogenous feeding, the

yolk and oil globule have been mostly exhausted and are gone by 5 DPH. After this point, the size and complexity of the gut continues to increase but there are no major morphological changes in the gut until approximately 18 DPH. At this point, the pyloric sphincter and the pyloric caeca begin to develop though the stomach is not completely functional until approximately 22 DPH (Lazo, 1999). By 24 DPH, the formation of the gut is complete. Based on this morphology, it is expected that red drum CCK-IR cells will follow the developmental timing and pattern seen in those species with coiled guts rather than that seen in species with straight guts.

The primary purpose of this study was to characterize the development of CCK in larval red drum. In order to examine basic characteristics of red drum CCK and to validate the antibody used for IHC, we used molecular techniques to isolate a cDNA sequence which was then compared to sequences coding for CCK in other species. We also examined the distribution of CCK mRNA in various tissues of adult and juvenile red drum in order to better understand where CCK-IR cells should be found in larvae. Finally, we characterized the appearance and distribution of CCK-IR cells in the gastrointestinal tract of red drum larvae using immunohistochemistry (IHC). Understanding how CCK develops in larval red drum will allow future studies to investigate the impact of CCK on feeding and digestion in these fish.

MATERIALS AND METHODS

Specimens

Juvenile and adult red drum for the molecular cloning and tissue expression studies were collected from both the wild and from a local producer (Lonestar Aquafarms, Palacios, TX, USA). Larvae for the immunohistochemical analysis were raised from eggs obtained from captive broodstock maintained at the University of Texas Fisheries and Mariculture Laboratory (FAML). Eggs were placed into 400-L tanks provided with an internal biofilter where they remained throughout the course of the collection period. Salinity was maintained at 32 ± 2 g/L, temperature was kept at 27±1°C via ambient heating, and the photoperiod was maintained at 14 h light/10 h dark via fluorescent lighting. Water quality parameters remained optimal for growth of red drum larvae (total ammonia nitrogen and nitrite never exceeded 0.50 or 1.0 ppm, respectively) throughout the course of the sampling period and survival of the larvae was greater than 40%. Larvae were fed rotifers (*Brachionus plicatilis*) enriched with Algamac-3050 (Aquafauna Bio-Marine, Hawthorne, CA, USA) for the first nine days of feeding (days 3 – 11 post hatch), newly hatched brine shrimp nauplii (*Artemia* sp.) from days 11 – 14 post hatch, and enriched brine shrimp (newly hatched nauplii enriched overnight in Algamac-3050) from days 15 – 18 post hatch. Along with the live prey items, larvae were co-fed Otohime Marine Larvae Weaning Diet (Reed Mariculture, Campbell, CA, USA) of increasing size from days 3 – 18 post hatch. By day 19 post hatch, the fish had been completely weaned

to the microparticulate diet and received only prepared feeds for the rest of the sampling period.

Molecular cloning

Three adult red drum were euthanized via a combination of MS-222 (Tricaine Methanesulfonate, Argent Chemical Laboratories, Redmond, WA, USA) overdose and ice-bath immersion. This method of euthanasia has been approved by the University of Texas Institutional Animal Care and Use Committee under protocol #07043001. Following the ice bath, the spinal cord of the fish was cut and tissue was taken from the anterior intestine and pyloric caeca. This tissue was frozen on dry ice and stored at -80°C until analyzed. Total RNA was extracted from tissue samples by homogenization in TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription was performed using 1 µg of total RNA and 0.25 µg of oligo dT primer using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). Using this cDNA, a partial fragment of CCK mRNA was amplified by nested polymerase chain reaction (PCR) using Promega's GoTaq Green master mix (Promega Corporation, Madison, WI, USA) and degenerate primers designed from the conserved regions of known vertebrate CCK genes (Suzuki et al., 1999; Kurokawa et al., 2003; Kamisaka et al., 2005). The first round of PCR used degenerate forward primer-1 (DF-1, GGN ATC TGY GTR TGY GT) and degenerate reverse primer-1 (DR-1, CT SCG KCG NCC RAA RTC CAT CCA). The PCR parameters for the first round were 2 min at 95°C followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2

min. Products from this first round of PCR were separated using gel electrophoresis (1% agarose with ethidium bromide) and revealed multiple faint bands. The second round of nested PCR used the same degenerate reverse primer and the same PCR parameters as the first round but replaced DF-1 with a nested degenerate forward primer (DF-2, TGY GTR TGY GTN CTN YTG GCW GC). After gel electrophoresis, the sample yielded a band of the expected size (~350 bp) which was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified PCR product was ligated into the pCR®4-TOPO vector (Invitrogen) and five independent clones were sequenced at the University of Texas DNA Sequencing Facility using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Using the nucleotide sequence of the partial fragment, a gene specific forward primer (RDCCK-F1, CAG CGC TCC ATC TCT GCT CCT T) and a gene specific reverse primer (RDCCK-R1, CA GTC CGT TGC CTC TGC TGT) were designed. Total RNA was extracted from tissues as described above and 5' and 3' RACE (rapid amplification of cDNA ends) ready cDNA was prepared using the GeneRacer™ Kit (Invitrogen). For the 5' end, the GeneRacer™ 5' primer and RDCCK-R1 were used in a touchdown PCR reaction while the GeneRacer™ 3' primer and RDCCK-F1 were used for the 3' end. The parameters of the PCR were 2 min at 95°C; 5 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 2 min; 5 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 2 min; and 25 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2 min. This PCR yielded bands of approximately 400 bp (5' reaction) and 475 bp (3' reaction) which were gel purified and sequenced as described above. Finally, the gene specific

primers RDCCK-WF1 (GAA GAA GTG C TC TCC TCA CTC) and RDCCK-WR1 (TGG CAT ATG TAT ATT GCA TTA TTA CAG TTT) were used to obtain full-length cDNA sequences using the PCR conditions described for the original degenerate PCR described above.

Sequence analysis

Nucleotide sequences were compared with the GenBank nucleotide (nr/nt) database using the BLASTN algorithm (Altschul et al., 1997). AlignX (Vector NTI Software Suite 10.3.0, Invitrogen) was used to align both nucleotide and amino acid sequences. Deduced amino acid sequences were submitted to ClustalW2 (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the phylogenetic tree was constructed using all default settings (Chenna et al., 2003). NJplot 2.1 (Perriere and Gouy, 1996) was used to display the phylogenetic tree. SignalP (Emanuelsson et al., 2007) was used to determine the cleavage site for the signal peptide in the deduced amino acid sequence.

Tissue distribution

Three adult or juvenile red drum were euthanized as described above. Tissue samples from the brain, liver, stomach, pancreas, anterior intestine, white skeletal muscle (taken from the left dorsal trunk), pyloric caeca, and rectum of each fish were collected in RNA*later*® (Applied Biosystems, Austin, TX, USA) and stored at -80°C until

analyzed. Total RNA was extracted from tissue samples by homogenization in TRI Reagent®-RT RNA isolation reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse transcription was performed using 1 µg of total RNA and 0.25 µg of random hexamer primers using the ImProm-II™ reverse transcription system (Promega Corporation, Madison, WI, USA). PCR amplification was performed using GoTaq® Green Master Mix (Promega) and primers to specifically detect CCK (RDCCK-F2, CTG AAG CTC TCC TTG AGG CT; and RDCCK-R1) and 18S rRNA (18S-F, GTT AAT TCC GAT AAC GAA CGA GAC TC; and 18S-R, ACA GAC CTG TTA TTG CTC AAT CTC GTG) in 25 µL reactions. The PCR parameters were 2 min at 95°C followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 2 min. Following PCR, products were separated using gel electrophoresis (1% agarose with ethidium bromide) and visualized using a UV transilluminator and digital camera. 18S ribosomal RNA was used as a quality control check for RNA from each tissue type.

Immunohistochemistry

At 0, 3, 4, 5, 6, 9, 12, 18, and 26 DPH, larvae (N ≥ 5 fish at each sampling period) were removed from the culture system and fixed in 4% paraformaldehyde overnight at 4°C. After fixation, the samples were washed in 75% ethanol for 24h at 4°C and then dehydrated through a graded ethanol series. Samples were stored at 4°C in 100% ethanol until embedded in Paraplast® Plus (McCormick Scientific, St. Louis, MO, USA). Embedded larvae were cut into 10 µm thick serial sections that

were then fixed to Superfrost®/Plus (Fisher Scientific, Pittsburgh, PA, USA) slides. Tissue sections were deparafinized, rehydrated, washed in 1X phosphate-buffered saline (pH 7.4) for 5 min, incubated in 2% normal goat serum for 20 min at room temperature to block non-specific binding, then incubated overnight at 4°C in primary antibody diluted (1:8000) with 2% normal goat serum. A commercial CCK antiserum was used for the detection of CCK-IR cells. This antiserum (C2581, Sigma-Aldrich, St. Louis, MO, USA) was chosen because it is specific to the sulfated (100% cross-reactive) form of the CCK ocatapeptide (CCK-8S) but also binds with the non-sulfated form (15% cross-reactivity) while having a very low cross-reactivity with gastrin (≤ 1%). This ability to discriminate against gastrin is important as gastrin and CCK are structurally very similar. This ability to discriminate against gastrin was tested on selected slides by first blocking the antibody with either CCK-8S or with gastrin at concentrations equimolar to the diluted antibody for 20 min at room temperature. Following the overnight incubation in the primary antibody, slides were washed in 1X PBS and then incubated for 30 min at room temperature in an Alexa Fluor® 488 labeled secondary antibody (A-11008, Invitrogen) diluted (1:500) in 2% goat normal serum. Slides were washed in 1X PBS, dehydrated in an ethanol series, and mounted.

RESULTS

Molecular cloning and sequence analysis

The initial PCR reaction with degenerate primers DF-1 and DR-1 yielded a number of bands after agarose gel electrophoresis and several of these bands were near the expected size (350 bp). After running the nested PCR reaction with degenerate primers DF-2 and DR-1, there was a single clear band of approximately 350 bp. BLASTN analysis of this fragment showed high identity with CCK from yellowtail (*Seriola quinqueradiata*; 92%), Japanese flounder (91%), and spotted green pufferfish (*Tetraodon nigroviridis*; 85%). After RACE using the gene specific primers RDCCK-F1 and RDCCK-R1, bands of approximately 400 bp (5' reaction) and 475 bp (3' reaction) were isolated from the agarose gel, cloned and sequenced. The two sequences were aligned with the fragment from the degenerate PCR and the overlapping region was 100% homologous. Using primers RDCCK-WF1 and RDCCK-WR1, the sequence of the complete cDNA (Fig 2.1) was obtained indicating that the fragments originated from a single, complete mRNA. The sequence of the cDNA was compared with other nucleotide sequences in the GenBank database and again showed high identity with CCK 1 from yellowtail (91%), Japanese flounder (89%), and puffer (78%). The complete cDNA was found to be 635 bp in length and contained a 414 bp open reading frame encoding a deduced peptide that was 138 amino acids long and identified as the putative preprocholecystokinin of red drum. The

nucleotide sequence for this cDNA has been deposited in GenBank (accession number EU598150).

The complete putative red drum CCK precursor peptide was submitted to ClustalW2 (Chenna et al., 2003) for alignment along with eleven amino acid sequences for CCK precursor peptide Type 1 or 2 from seven other teleosts, three sequences for CCK precursor peptide Type 1 or 2 from two amphibians, and the CCK precursor peptide sequences from one chondrichthyan, one avian, one reptile, and three mammals. The peptide sequence for cionin, which is the oldest known homolog of cholecystokinin from the protochordate *Ciona intestinalis* (Johnsen, 1998; Rehfeld, 2004), was also included in the alignment, the results of which are shown in Figure 2.2. The resulting phylogenetic tree from this alignment (Fig. 2.3) groups the red drum CCK peptide with CCK Type 1 peptides, though the mature CCK-8 is identical to that of the Atlantic herring which is grouped in the CCK Type 2 cluster (Kurokawa et al., 2003; Kamisaka et al., 2005). SignalP analysis (Emanuelsson et al., 2007) revealed a cleavage site for the signal peptide between the 20th and 21st amino acid in the red drum sequence with the resulting twenty amino acid signal peptide showing 95% identity to the signal peptide in flounder CCK Type 1. The yellowtail amino acid sequence submitted to GenBank (AB205406) is incomplete, but the fourteen amino acid residues listed in the signal peptide are 100% identical to the red drum signal peptide. The mature CCK-8 peptide in red drum has the same sequence (DYMGWMDF) as that found in terrestrial vertebrates, unlike most fish that have a substitution at the sixth position from the C-terminus. The only other known

fish species that has a methionine residue in the sixth position is Atlantic herring (Kamisaka et al., 2005).

Tissue Distribution

PCR reactions to test for the presence of CCK mRNA in various tissues of red drum produced a band of expected size (approximately 210 bp) in the brain, pancreas, anterior intestine, pyloric caeca, and white skeletal muscle of all three fish examined though the intensity of the signal varied slightly between fish (Figure 2.4). The strongest signals were seen in the brain and pyloric caeca which produced substantially brighter bands than any other tissue. No signal was detected in the liver, stomach, or rectum of any of the fish studied. Control reactions with primers specific to 18S were positive in all samples. CCK primers used were also tested against genomic DNA without any amplification in the reaction.

Immunohistochemistry

The development of the gut in larval red drum is represented schematically in Figure 2.5. Antibody blocked with CCK-8S failed to react to slides from fish at any age while the antibody blocked with gastrin remained effective. CCK-IR cells in red drum larvae have the characteristic elongated triangular shape with the point facing into the lumen of the gut and the base touching the basal mucosa (Fig. 2.6, A and B).

Immunoreactive cells were first seen in red drum larvae at 3 DPH, although these were only seen in one of ten fish examined (Table 1). CCK-IR cells were seen in two fish at 4 DPH, four fish at 5 DPH, and in all fish examined from 6 DPH onward. In early larvae (<6DPH), only a few cells in total were seen and these were all located in the anterior midgut near the stomach anlage (See Fig. 2.5A). By 6 DPH (Fig. 2.5B), CCK-IR cells were more numerous and were seen to extend from the stomach anlage to just past the first bend in the midgut. In fish through 12 DPH, the location of CCK-IR cells remained relatively constant though the number of CCK-IR cells observed grew rapidly as the mucosal folds increased in complexity. By day 18, the pattern of CCK-IR cells began to change substantially. At this point, large numbers of CCK-IR cells were observed in the anterior portion of the midgut just behind the developing pyloric sphincter. By the time the formation of the stomach and pyloric caeca was complete (after 22 DPH), CCK-IR cells were highly concentrated in this region particularly within the pyloric caeca (Fig. 2.6C) and few were seen posterior to the first bend in the intestine.

Table 2.1. Distribution of CCK-IR cells in red drum larvae by gut section and age.

Presence of 5 or less cells/section is indicated by (+), between 5 and 10 cells by (++), and more than 10 cells/section by (+++). Absence of CCK-IR cells is indicated by (-) and (NP) indicates that the gut section is not present at that age.

	Age (Days Post Hatch)							
	3	4	5	6	9	12	18	26
Number of Larvae with CCK-IR Cells	1/10	2/5	4/5	5/5	5/5	5/5	5/5	5/5
Stomach	NP	NP	NP	NP	NP	NP	NP	-
Anterior Midgut	+	+	+	+	+	++	++	++
Pyloric Caeca	NP	NP	NP	NP	NP	NP	++	+++
Posterior Midgut	-	-	+	+	+	+	+	+
Hindgut	-	-	-	-	-	-	-	-

Figure 2.1. Nucleotide sequence of the cDNA and deduced amino acid sequence of cholecystokinin (CCK) from the pyloric caeca and intestine of adult red drum (*Sciaenops ocellatus*).

The darker shaded region indicates the signal peptide, the lighter shaded region indicates the mature CCK-8 peptide, and arrows indicate the primers used in this experiment. The * indicates the stop codon.

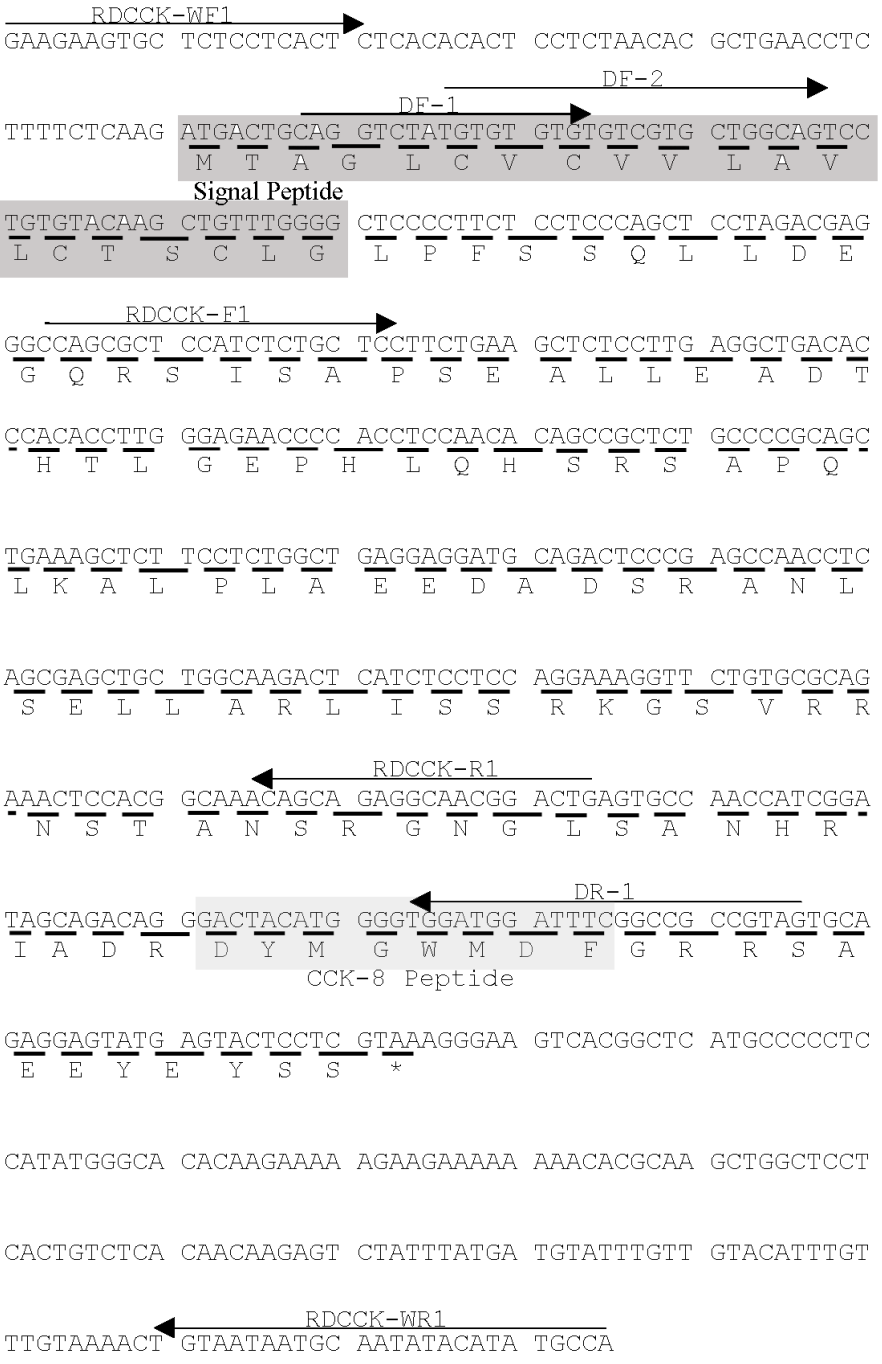

RDCCK-WF1
1 GAAGAAGTGC TCTCCTCACT CTCACACACT CCTCTAACAC GCTGAACCTC
DF-2
DF-1
51 TTTTCTCAAG ATGACTGCAG GTCTATGTGT GTGTGTCGTG CTGGCAGTCC
M T A G L C V C V V L A V
Signal Peptide
101 TGTGTACAAG CTGTTTGGGG CTCCCCTTCT CCTCCCAGCT CCTAGACGAG
L C T S C L G L P F S S Q L L D E
RDCCK-F1
151 GGCCAGCGCT CCATCTCTGC TCCTTCTGAA GCTCTCCTTG AGGCTGACAC
G Q R S I S A P S E A L L E A D T
201 CCACACCTTG GGAGAACCCC ACCTCCAACA CAGCCGCTCT GCCCCGCAGC
H T L G E P H L Q H S R S A P Q
251 TGAAAGCTCT TCCTCTGGCT GAGGAGGATG CAGACTCCCG AGCCAACCTC
L K A L P L A E E D A D S R A N L
301 AGCGAGCTGC TGGCAAGACT CATCTCCTCC AGGAAAGGTT CTGTGCGCAG
S E L L A R L I S S R K G S V R R
RDCCK-R1
351 AAACTCCACG GCAAACAGCA GAGGCAACGG ACTGAGTGCC AACCATCGGA
N S T A N S R G N G L S A N H R
DR-1
401 TAGCAGACAG GGACTACATG GGGTGGATGG ATTTCGGCCG CCGTAGTGCA
I A D R D Y M G W M D F G R R S A
CCK-8 Peptide
451 GAGGAGTATG AGTACTCCTC GTAAAGGGAA GTCACGGCTC ATGCCCCCTC
E E Y E Y S S *
501 CATATGGGCA CACAAGAAAA AGAAGAAAAA AAACACGCAA GCTGGCTCCT
551 CACTGTCTCA CAACAAGAGT CTATTTATGA TGTATTTGTT GTACATTTGT
RDCCK-WR1
601 TTGTAAAACT GTAATAATGC AATATACATA TGCCA

Figure 2.2. Alignment of the deduced preprocholecystokinin amino acid sequences from a variety of vertebrates and one protochordate.

Shaded areas indicate regions of conserved amino acids. GenBank accession numbers for the species in this alignment are: yellowtail (*Seriola quinqueradiata*; BAE16613), Japanese flounder (*Paralichthys olivaceus*; -1, BAA23734 and -2, AB086399), puffer (*Tetraodon nigroviridis*; -1, AB086401 and -2, AB086402), rainbow trout (*Oncorhynchus mykiss*; -L, CAA09907, -N, CAA09809, and –T, CAA09906), Japanese eel (*Anguilla japonica*; BAD01500), bullfrog (*Rana catesbeiana*; P80344), Xenopus (*X. laevis*; -1, P50144 and -2, P50145), ostrich (*Struthio camelus*; Q9PU29), red-eared slider turtle (*Trachemys scripta*; P80345), dogfish (*Squalus acanthias*; CAB10585), human (*Homo sapiens*; P06307), Norwegian rat (*Rattus norvegicus*; P01355), pig (*Sus scrofa*; P01356), *Ciona intestinalis* (cionin, P16240), goldfish (*Carassius auratus*; 093464), and Atlantic herring (*Clupea harengus*; AAQ17201). Where no amino acid sequence was available in GenBank, it was deduced from the available nucleotide sequence.

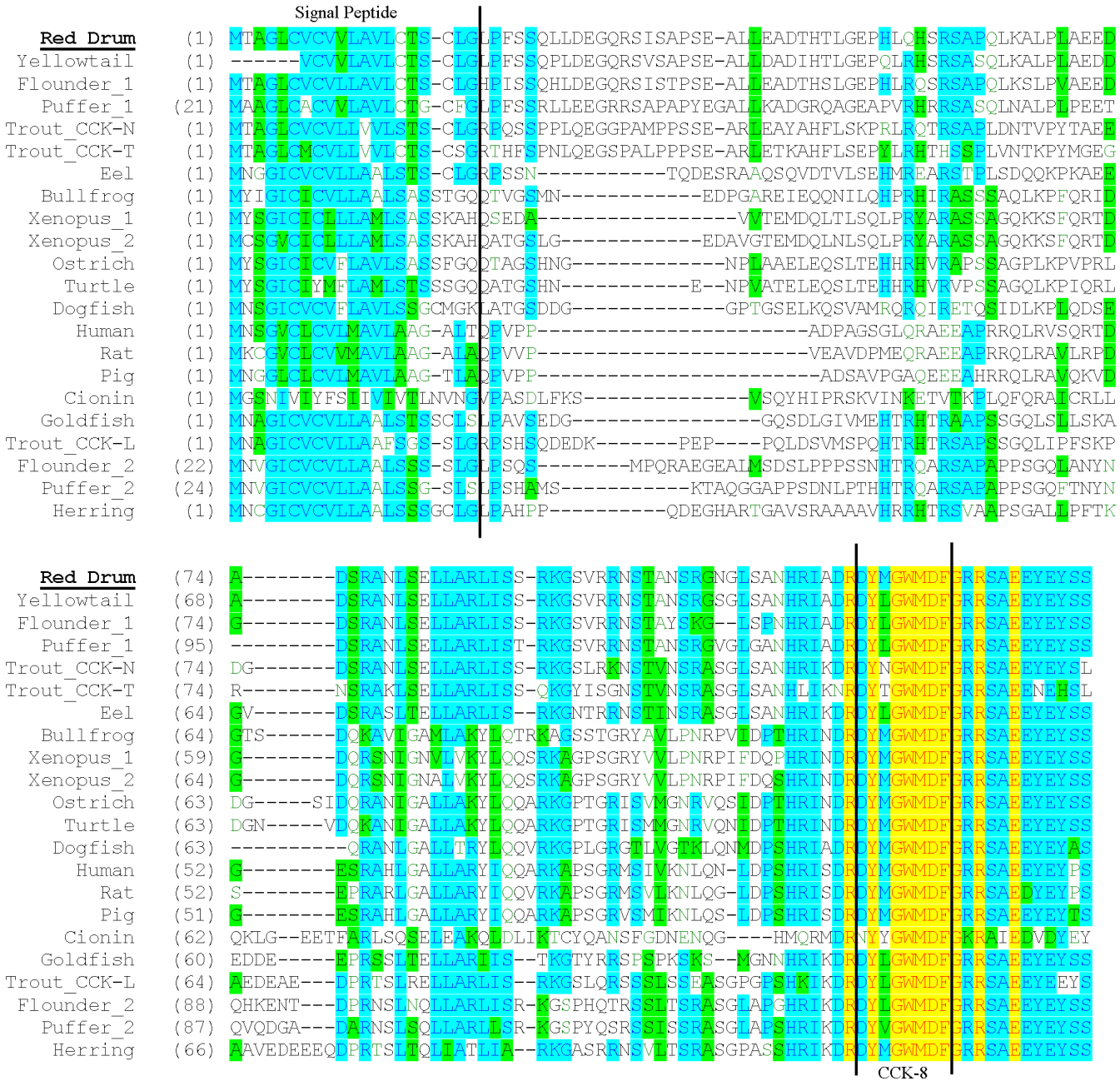

Signal Peptide
CCK-8

Figure 2.3. Unrooted phylogenetic tree of preprocholecystokinin peptides generated by ClustalW2 using the neighbor-joining method.

Numbers at each node represent the confidence analysis for 1000 bootstrap trials and the scale bar indicates the substitution rate per residue. GenBank accession numbers for all species can be found in Figure 2.

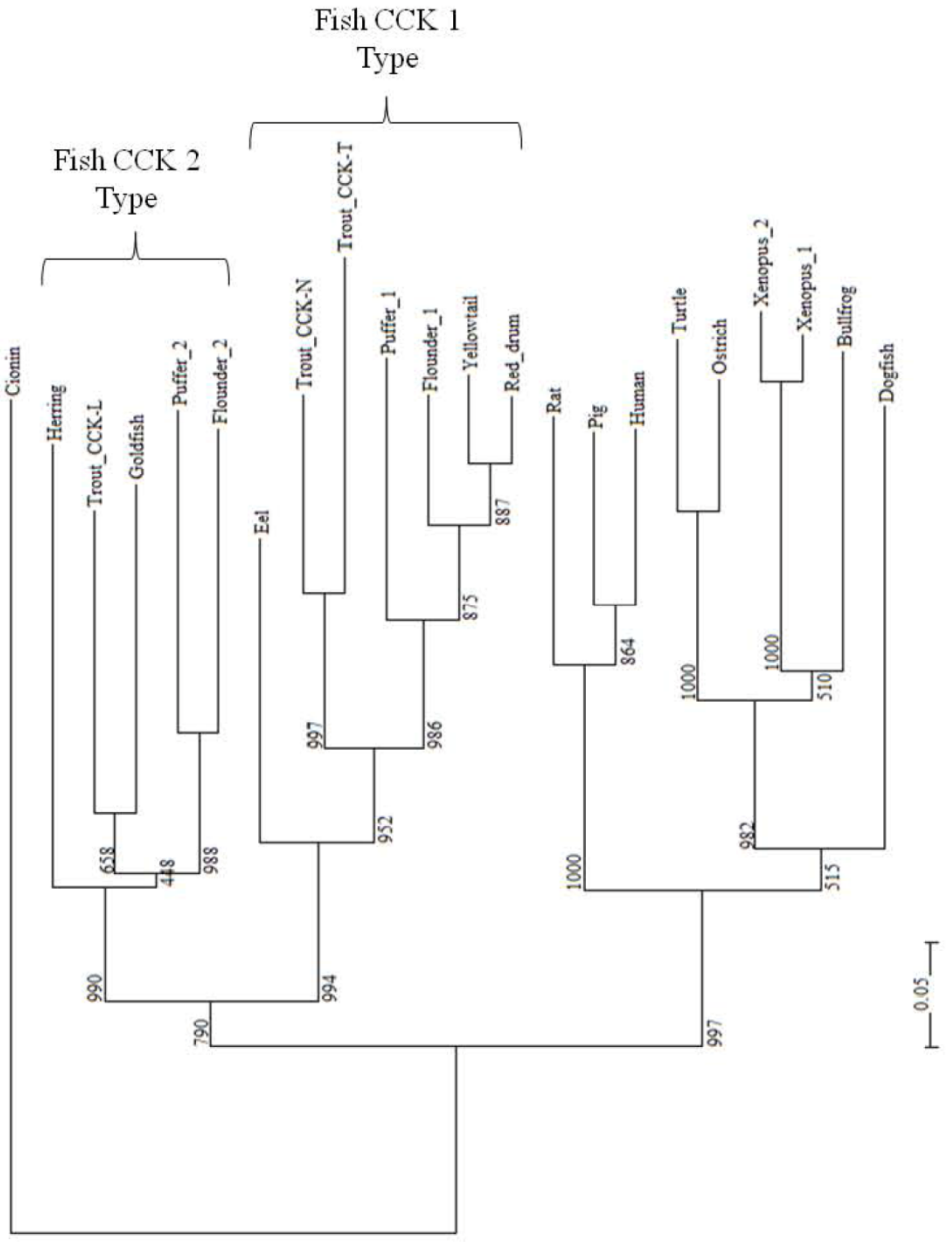

Fish CCK 1
Type
Fish CCK 2
Type
Cionin
Herring
Trout_CCK-L
Goldfish
Puffer_2
Flounder_2
Eel
Trout_CCK-N
Trout_CCK-T
Puffer_1
Flounder_1
Yellowtail
Red_drum
Rat
Pig
Human
Turtle
Ostrich
Xenopus_2
Xenopus_1
Bullfrog
Dogfish
658
448
988
952
997
986
875
887
990
994
790
1000
864
1000
510
982
515
997
0.05

Figure 2.4. Tissue distribution of CCK mRNA in adult red drum.

Image is a representative gel from a single red drum. B, brain; L, liver; S, stomach; P, pancreas; AI, anterior intestine; M, white skeletal muscle; PC, pyloric caeca; HG, hind gut.

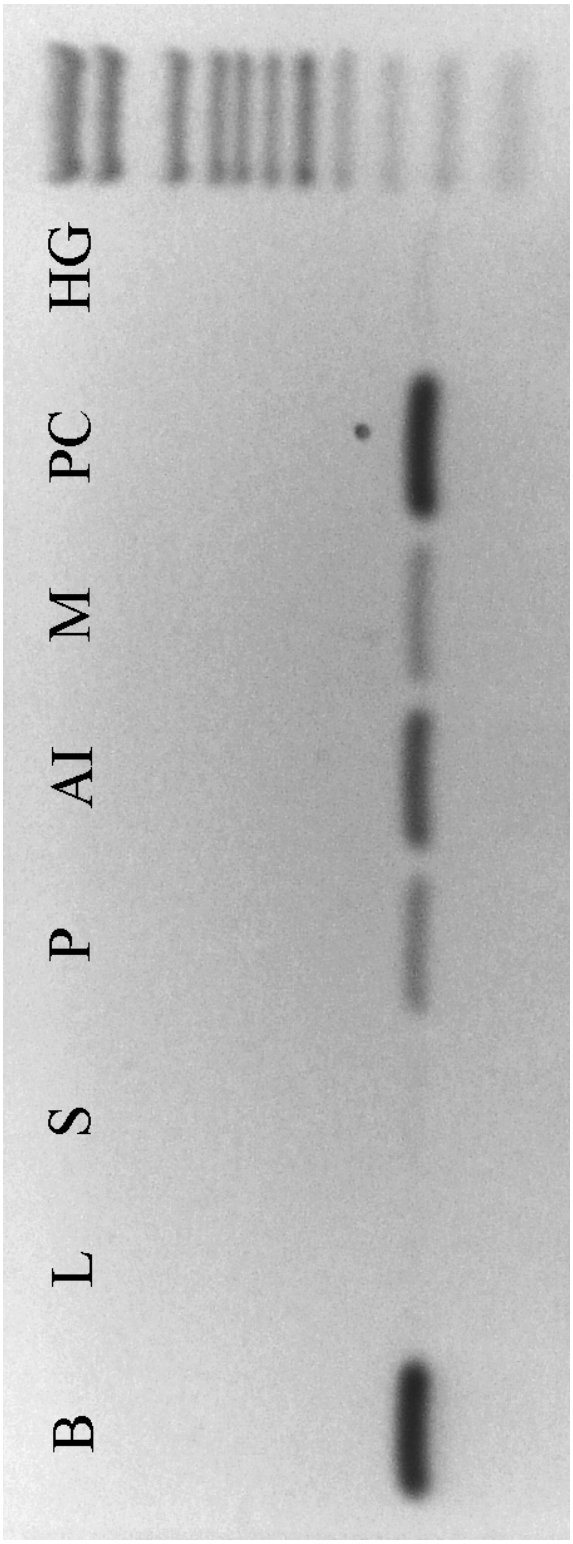
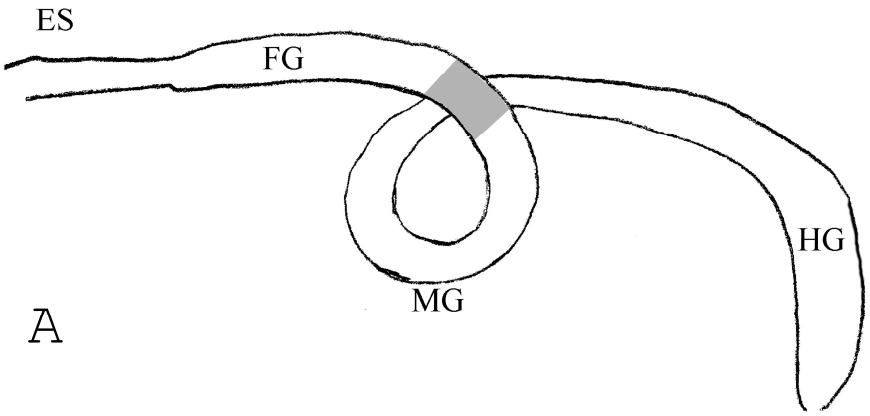
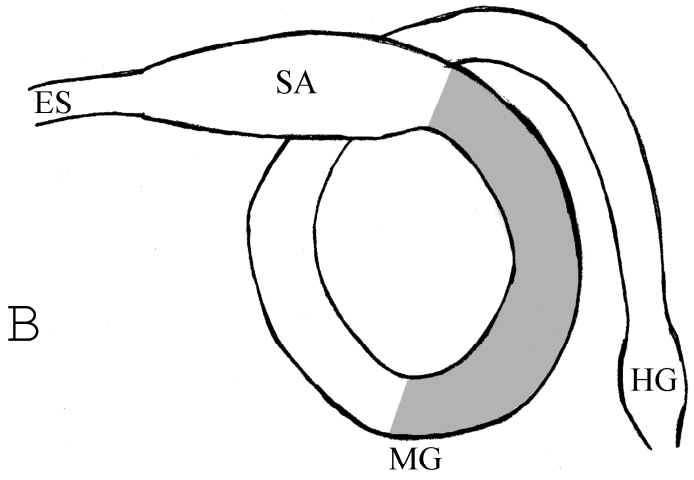
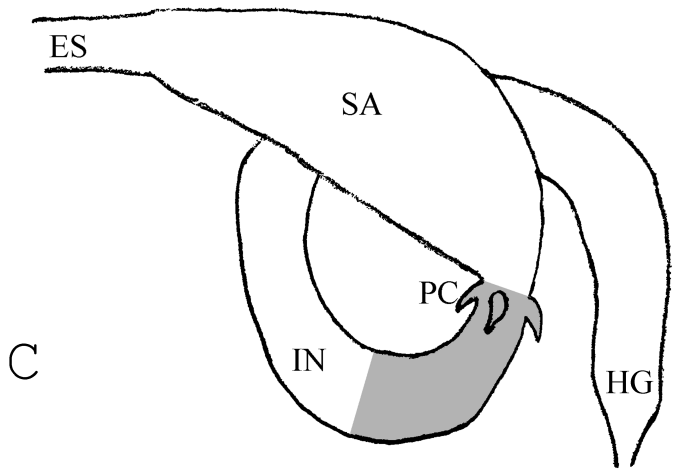

B
L
S
P
AI
M
PC
HG

Figure 2.5 A-D. Schematic representation of the developing gut in red drum larvae.

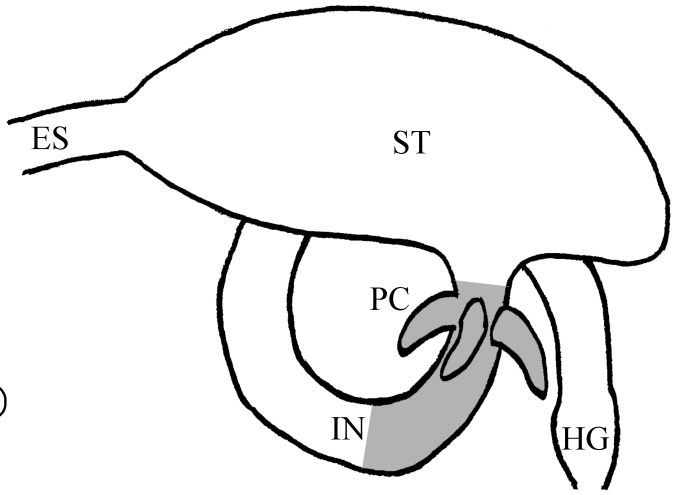
Drawings represent the guts of fish at 3 DPH (A), 6 DPH (B), 18 DPH (C), and 26 DPH (D). Morphology of the digestive tract was reconstructed from 10 µm serial histological sections. Grey shaded areas represent sections of the gut where CCK-IR cells were observed. ES: esophagus, HG: hind gut, IN: intestine, MG: mid gut, PC: pyloric caeca, SA: stomach anlage, and ST: stomach.


ES
FG
HG
MG
A


ES
SA
HG
MG
B



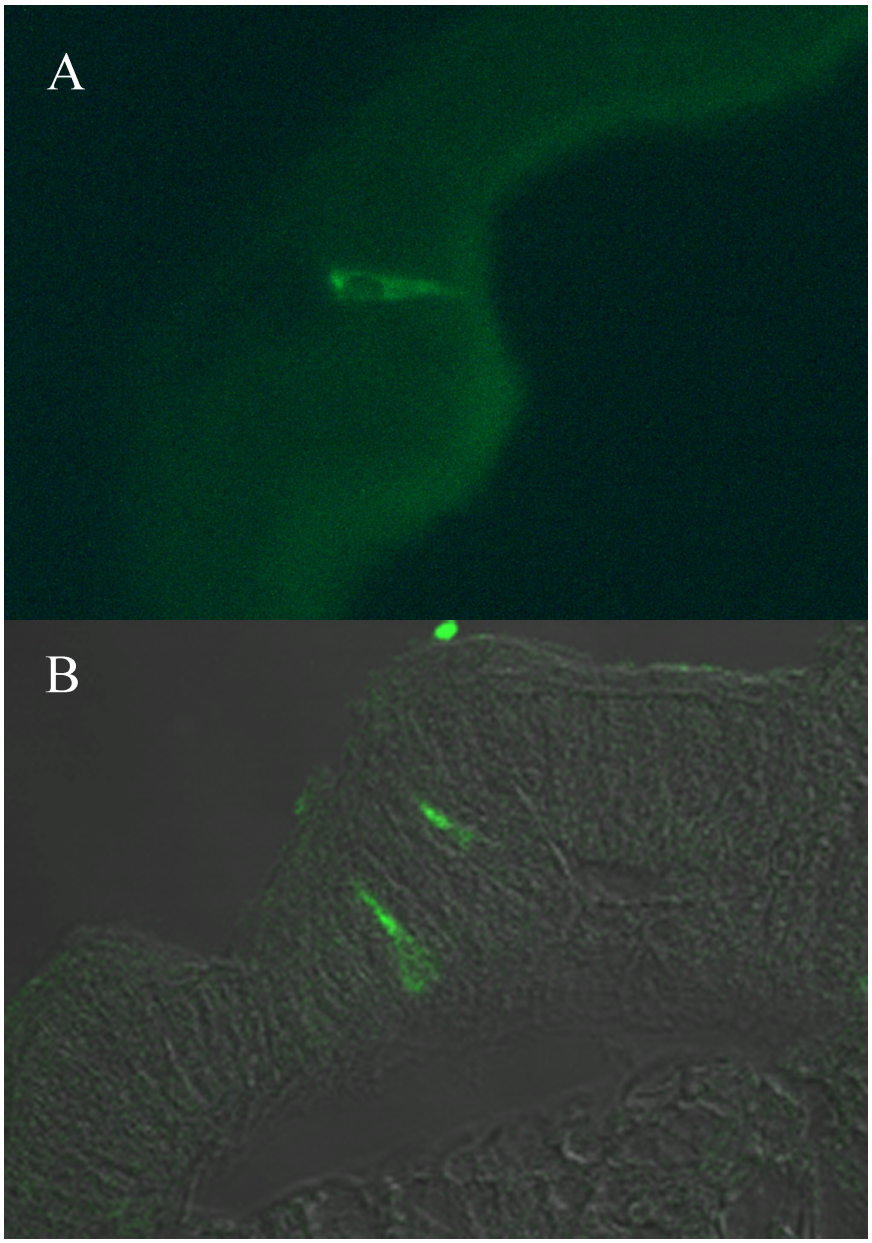
C

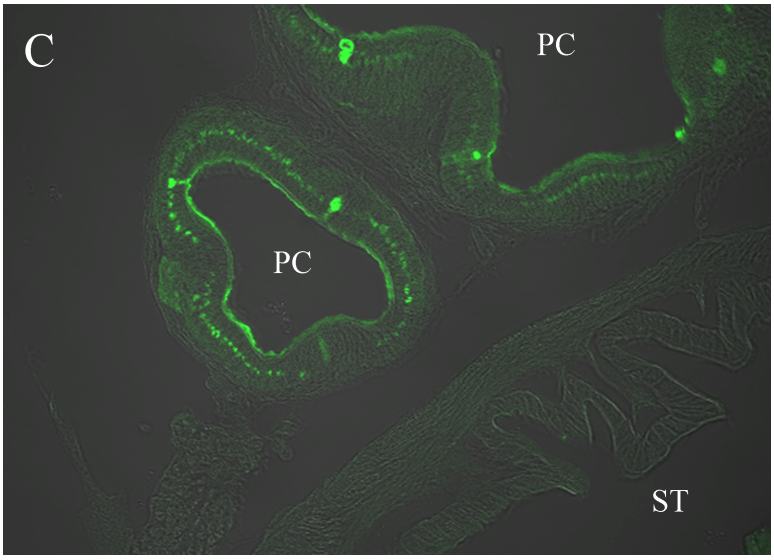


D

Figure 2.6 A-D. Cholecystokinin immune-reactive cells in the gut of larval red drum (Sciaenops ocellatus).

A: 400X image of a single CCK-IR cell in the anterior midgut of a 9 DPH red drum larva. B: 400X image of two CCK-IR cells in the anterior midgut of an 18 DPH red drum larva. C: 100X image of CCK immunoreactivity in the pyloric caeca of a 26DPH red drum larva. PC – Pyloric Caeca, ST – Stomach.


A
B


C
PC
PC
ST

DISCUSSION

The present work identified the nucleotide and amino acid sequence of CCK isolated from the anterior intestine and pyloric caeca of red drum. The red drum CCK sequence showed very high similarity with the CCK Type 1 sequences from yellowtail, Japanese flounder, and the spotted green puffer suggesting that the sequence isolated from red drum likely belonged to the CCK Type 1 family. This is further reinforced by the phylogenetic tree (Fig. 2.3) produced from the deduced CCK amino acid sequences of a number of vertebrates and a single protochordate which also groups red drum CCK with the CCK Type 1 group. This is predictable due to the tissue from which this RNA was extracted. Kurokawa et al. (2003) found that while the mRNA for CCK 1 and 2 were found in similar proportions in the brain of Japanese flounder, the mRNA for CCK 1 was found in clearly greater amounts in the intestine. As multiple CCK mRNAs have been found in other fish species, notably the rainbow trout (*Oncorhynchus mykiss*) which has three forms identified as CCK-N, CCK-L, and CCK-T (Jensen et al., 2001), it is likely that one or more additional CCK mRNAs remain to be discovered in red drum. The present work focused on the gastrointestinal role of CCK in red drum however and the pyloric caeca and anterior intestine was therefore chosen as the source tissue for the cloning work due to the high abundance of CCK Type 1 in these tissues in other species (Kurokawa et al., 2003). It is possible that if brain tissue was used as the source of the mRNA for the cloning work, we would have also been able to isolate red drum CCK Type 2.

Because the role of neural CCK in fish remains unknown, determining the sequence of CCK Type 2 remains very important for future work but was not within the scope of the present study.

It is interesting that while the red drum CCK octapeptide sequence was an exact match for the herring octapeptide, the phylogenetic tree grouped the herring as a CCK Type 2. This is not unheard of however since both the CCK Type 1 and Type 2 from the Japanese flounder share the same CCK-8 sequence. It is also interesting to note that the phylogenetic analysis grouped the dogfish with the higher vertebrates rather than with the fishes.

The tissue distribution of CCK mRNA was similar to that seen in other fishes. Major areas of CCK expression in red drum were the brain, pyloric caeca, and the anterior intestine. There are however differences among tissue expression in fish. One of the most interesting differences is the presence of CCK mRNA in the muscle. Similar to the present study, Murashita et al. (2006) found CCK mRNA in the white muscle of yellowtail. In contrast, studies with the Japanese flounder and the goldfish (*Carassius auratus*) reported no CCK mRNA in the muscle (Peyon et al., 1998; Suzuki et al., 1999). The role of CCK in the skeletal muscle of red drum is unknown, but in the guinea pig (*Cavia porcellus*) it has been shown that neurons which enervate the skeletal muscle have CCK-IR cells (Gibbins et al., 1987). It is possible that the neurons in the muscle of red drum and yellowtail produce CCK although there is no explanation as to why the neurons in muscle of Japanese flounder and

goldfish might be different. Further studies should be undertaken to examine the cellular distribution of CCK mRNA in muscle tissues utilizing *in situ* hybridization.

The development of the digestive system (Lazo, 1999) and of digestive enzymes (Lazo et al., 2000b) in red drum larvae is similar to that seen in many other marine species such as spot (*Leiostomus xanthurus*) (Govoni, 1980), Japanese flounder (Kurokawa et al., 2000), cobia (*Rachycentron canadum*) (Faulk et al., 2007), and bluefin tuna (Kamisaka et al., 2002). In these fish, the gut is initially straight at hatching with a sharp bend towards the anus. By the onset of exogenous feeding, the gut has looped and formed a coil which then persists as the organs of the digestive system continue to develop. While CCK-IR cells were detected as early as first feeding (3 DPH), this was a single cell in only one of ten fish sampled. While this single positive cell is not definitive, by 6 DPH all fish had CCK-IR cells in the anterior midgut. It is possible that the presence of CCK-IR cells in the guts of some of the early larvae and not in others is due to an inability of the immunohistochemical techniques to detect possible low peptide concentrations in these cells rather than an absence of CCK-IR cells altogether. This seems likely since the limited number of cells observed in fish at 3, 4, and 5 DPH show very weak staining. Since immunohistochemistry depends on the human eye to separate stained cells from background noise, weak staining due to low concentrations of CCK may very well be missed in these early fish. Lending credence to this possibility is the work of Kamisaka *et al.* (2005) in which the authors used *in situ* hybridization to examine the expression of CCK mRNA during the ontogeny of Atlantic herring and observed CCK

mRNA at hatching. While it is not certain that mature CCK peptide is being produced from RNA transcripts at these very early stages, it does suggest that the process is possible. Finally, detectable levels of CCK peptide have been observed using an extremely sensitive radioimmunoassay (RIA) technique in Atlantic halibut larvae 7 days after first feeding (40 DPH) (Rojas-Garcia and Rønnestad, 2002) and may have been present even earlier as this was the first time period reported by the authors. In contrast to this highly sensitive RIA, CCK-IR cells could not be detected in Atlantic halibut larvae until 45 DPH (Kamisaka et al., 2001). It is however possible that CCK-IR cells in red drum develop during this period when the midgut consists primarily of columnar epithelial cells with very few secretory cells (Lazo, 1999) and that mature CCK-8 is not produced until after the onset of exogenous feeding. Further studies will need to be performed in order to test this possibility.

The increase in CCK-IR cells seen in older red drum larvae was clear and followed the distribution pattern that was expected based on the coiled vs. straight gut model (Rønnestad et al., 2007). The concentration of the CCK-IR cells in the pyloric caeca and adjacent intestine suggest the importance of CCK to the function of this portion of the digestive tract. In addition to its role in pancreatic enzyme secretion, CCK has been shown to be important to the regulation of digesta flow through the intestine by both controlling the pyloric sphincter (Fisher et al., 1973) and regulating peristalsis in the intestinal muscle (Barthó et al., 1982). Release of CCK by the numerous CCK-IR cells in this area immediately adjacent to both the pyloric sphincter and the entrance to the pyloric caeca would therefore be ideal to

regulate the passage of digesta from the stomach and filling of the pyloric caeca. CCK might have a role in filling the pyloric caeca since it has been suggested as a stimulus for reverse peristalsis which could serve as a mechanism to move a food bolus into the caeca (Barthó et al., 1982; Rønnestad et al., 2000; Olsson and Holmgren, 2001). The presence of CCK in this area adjacent to the bile duct is also significant since the stimulation of gall bladder contraction and the release of bile salts is of critical importance in the proper digestion of dietary lipids.

In mammals, it is believed that CCK release is stimulated by a "releasing factor" which is itself stimulated by a number of factors and is deactivated by trypsin (Miyasaka et al., 1989; Liddle, 1995; Rønnestad, 2002). There are a number of factors which have been shown to result in increased CCK secretion including long-chain fatty acids, protein digestive products, starches, and gastric acids (Benson and Reynolds, 2001; Koven et al., 2002; Cahu et al., 2004; Rehfeld, 2004; Swanson et al., 2004). Less is known about the role of CCK and its stimulation in fish, though CCK has been shown in species such as the Atlantic salmon (*Salmo salar*) to stimulate the contraction of the gall bladder and secretion of pancreatic enzymes similar to its effects in mammals (Einarsson et al., 1997). Also, it has been shown that tube-feeding soluble proteins (as BSA and BSA hydrolyzate) to larvae of the Atlantic herring produced both CCK and trypsin responses (Koven et al., 2002). All of this suggests that the concentration of CCK-IR cells at the pyloric valve and pyloric caeca of red drum larvae places it in a key position to regulate digestive processes and underscores the need for additional study.

The present study has shown that CCK is present in the digestive tract of red drum and analysis of the nucleotide sequence has shown it to be similar to the CCK Type 1 sequence from Japanese flounder and spotted green puffer. While the functional significance of CCK during the ontogeny of red drum larvae remains unknown, the present study has shown that CCK-IR cells can be detected in all fish within the first three days following first feeding and are concentrated in the anterior portion of the midgut in early larvae and in the pyloric caeca of larvae approaching metamorphosis. Further research is necessary to better understand the role that CCK plays in the regulation of digestive enzyme secretion and utilization of different feed types.

CHAPTER 3: POSTPRANDIAL CHOLECYSTOKININ AND TRYPSIN RESPONSES OF RED DRUM (*SCIAENOPS OCELLATUS*)

ABSTRACT

The desire to find a microparticulate diet capable of replacing live prey in the culture of marine larvae has led to the investigation of digestive physiology in larvae. Since most commercial marine larvae do not possess a stomach at the time of first feeding, pancreatic enzymes such as trypsin are of key importance in the digestive process. The peptide hormone, cholecystokinin (CCK) is a well studied regulator of pancreatic enzyme secretions in mammals. The current study investigated the CCK, trypsin, and CCK mRNA responses of juvenile and larval red drum following a meal. This was done in order to clarify if CCK and trypsin secretion are correlated as has been seen in mammals and in the Atlantic herring (*Clupea harengus*) as well as to investigate if transcription of the CCK gene could be induced by feeding. In both juvenile and larval red drum, CCK levels spiked within 30 min postprandial and trypsin was increased soon thereafter. CCK mRNA was increased in both the juveniles and larvae within 2.5 to 3 hours postprandial. These results suggest that CCK has a similar function in red drum that it has in mammals and is capable of regulating the secretion of trypsin. They also show that gene expression of CCK could be induced by feeding. This may have bearing on the inability of larvae to efficiently

utilize microparticulate diets and suggests future research into the ability of microparticulate diets to stimulate CCK secretion.

INTRODUCTION

So far, it has proven impossible to rear marine fish from first feeding on microparticulate diets alone without consequent reductions in survival and/or growth. This is important since one of the most labor and cost intensive steps in the production of marine larvae is the production and supply of live prey items for food. Replacing live food with high quality microparticulate feeds has proven to be difficult however. There have been successes in the development and use of microparticulate diets (Holt, 1993; Cahu et al., 1998a; Fontagne et al., 2000; Lazo et al., 2000a; Langdon, 2003; Fletcher et al., 2007), but at this time there is no microparticulate diet which performs as well as the live prey that it replaces. This difficulty has spawned a general investigation into the physiological basis for the reductions in growth and survival seen in larval marine fishes reared on microparticulate diets alone.

A series of recent studies have focused on understanding the regulation of digestive processes. In particular, cholecystokinin (CCK) has received attention due to its role as a regulator of pancreatic enzyme secretion in other species. Pancreatic enzymes are the primary means by which many larval fish including red drum (*Sciaenops ocellatus*) (Lazo et al., 2000b) and sea bass (*Dicentrarchus labrax*) (Cahu et al., 1998b) digest food until the development of an active stomach. CCK in fish is

known to have a strong role in the regulation of gastrointestinal function and provides evidence of a regulatory loop for pancreatic enzymes (Einarsson et al., 1997; de Pedro and Björnsson, 2001; Koven et al., 2002; Rønnestad, 2002; Rønnestad et al., 2007). CCK is a peptide hormone which has both a primary gut form and a carboxy-terminal octapeptide form and is found in both the central nervous system (CNS) and the gut (de Pedro and Björnsson 2001). Research into mammals has shown that products of proteolysis and lipolysis stimulate the CCK (Endocrine I) cell to release CCK which then acts humorally on pancreatic acinar cells to release pancreatic enzymes (Klein et al., 1999) either directly or through the action of a CCK releasing factor. In various organisms such as humans (Himeno et al., 1983; Beglinger et al., 1985), cattle (Swanson et al., 2004), and the Atlantic herring (*Clupea harengus*) (Koven et al., 2002) among others, ingested foods have been shown to have a direct impact on the secretion of CCK and thereby on the release of pancreatic enzymes.

The CCK and pancreatic enzyme response to feeding is not clear cut however. In the same study in which cattle were shown to increase secretion of the sulfated CCK octapeptide (CCK-8S) and pancreatic enzymes following the direct infusion of starch hydrolysate into the stomach, when casein was used it reduced the secretion of CCK-8 while still increasing the secretion of pancreatic enzymes (Swanson et al., 2004). In the rat, CCK secretion is regulated by a negative feedback system where active proteases inhibit CCK release by affecting the rate at which CCK mRNA is transcribed (Liddle, 1994b). In fish, there are indications that a similar regulation

occurs through the down regulation of a CCK-releasing factor by trypsin in the gut lumen (Rønnestad et al., 2003; Rønnestad et al., 2007). Ingested protein in the gut competes as a substrate for the trypsin preventing it from down-regulating the CCK-releasing factor. This results in an increased secretion of CCK which in turn stimulates the release of pancreatic enzymes including trypsin.

Postprandial CCK responses in mammals are fairly well characterized but at this time there has been very little work done to examine the postprandial response of fish. Work with tube feeding Atlantic herring has shown that free amino acids and bovine serum albumin both stimulate the release of CCK within two hours after being fed (Koven et al. 2002). While both BSA and the free amino acids stimulate the release of CCK, the level of CCK released in fish fed the free amino acids alone has been shown insufficient to increase the trypsin activity in the larvae. This implies that soluble proteins may have a stronger stimulatory effect on CCK than free amino acids. Live prey items which are commonly fed to larval marine fish in hatcheries such as copepods, rotifers, (*Brachionus* spp.), and brine shrimp (*Artemia* spp.) are known to contain large pools of soluble proteins (Scott and Baynes, 1978; Nagata and Whyte, 1992; Lie et al., 1997; Srivastava et al., 2006). Marine algae which are also commonly added to culture tanks either as live prey enrichment or as part of "green water" culture practices are also known to produce polyamines (Péres et al., 1997) which can function as stimulants for CCK secretion as well.

Red drum are capable of utilizing microparticulate diets at first feeding, but failure to co-feed live prey for at least the first five days leads to significant reductions

in survival (Holt, 1993). The need for live prey does not appear to be related to a physiological insufficiency on the part of the larvae. Early red drum larvae have been demonstrated to possess sufficient pancreatic enzymes to digest common microparticulate diet components (Lazo, 1999; Lazo et al., 2000b) but they still require the addition of live prey or algae to the culture system (Lazo et al., 2000a). Older red drum larvae do not have this need for the addition of live prey or algae, since it is possible to stop co-feeding the live prey and feed the microparticulate diet alone in larvae once they have passed 7 days post hatch (DPH) (Holt, 1993; Lazo et al., 2002). At least part of the reason microparticulate diets perform better in larvae that are co-fed live prey or algae may be related to the stimulation of pancreatic enzyme secretion in response to soluble proteins or some other soluble component which is not provided by microparticulate diets.

If some putative compound in live prey is the missing component in microparticulate diets which causes differential growth and survival in marine larvae, then two assumptions must be true. First, CCK must be present in larval fish at the time when this difference in performance is seen. In the previous chapter, it was shown both that the red drum produces CCK in tissues consistent with its role in other animals and that CCK immunoreactive (CCK-IR) cells could be detected in all larvae by 3 days after the start of exogenous feeding in red drum larvae. Second, if CCK is involved with differential utilization of microparticulate diets as hypothesized then it must be capable of induction by a feeding event and it must be correlated with an increase in digestive activity. The current study was therefore undertaken to

test the hypothesis that CCK and trypsin responses are related by examining the effect of a meal on the CCK peptide, trypsin, and CCK mRNA responses of both larval and juvenile red drum. Establishing the relationship between increased CCK levels following a feeding event and trypsin in red drum would further the understanding of digestive physiology in red drum and may provide insights on the processes limiting the use of microparticulate diets in red drum larvae.

MATERIALS AND METHODS

Fish and Culture Conditions

Juvenile red drum were obtained from Texas Parks and Wildlife's CCA/CPL Marine Development Center where they were raised in ponds to approximately 2 cm. Juvenile fish were brought to FAML and placed into 400 L tanks in a recirculating system. Juveniles were fed a commercial diet (2.0 mm pellets, Finfish Starter 50-15, Zeigler Bros., Gardners, PA, USA) by hand 5 times daily in order to habituate the fish to feeding in the presence of people.

Larval red drum were raised from eggs obtained from captive broodstock maintained at FAML. Eggs were hatched in 400-L tanks where the larvae remained throughout the course of the rearing period. Beginning with the onset of exogenous feeding at 3 days post hatch (DPH), water flow from a recirculating filter system was slowly increased until by 18 DPH water in the tank was being exchanged at least twice daily (approximately 1 L/min exchange). Larvae were fed rotifers (*Brachionus*

plicatilis) enriched with Algamac-3050 (Aquafauna Bio-Marine, Hawthorne, CA, USA) for the first nine days of feeding (3 – 11 DPH), newly hatched brine shrimp nauplii (*Artemia* sp.) from 11 – 14 DPH, and enriched brine shrimp nauplii (newly hatched nauplii enriched overnight in Algamac-3050) from 15 – 17 DPH. Live prey remaining in the tanks were enumerated and replaced (at 5 rotifers/mL and approximately 1 to 3 *Artemia* nauplii/mL) four times each day, adjusted so that no live prey was left in the tanks overnight. In addition to the live prey items fed to the larvae, live micro-algae (*Isochrysis galbana*) was added to the tanks once per day at approximately 40,000 - 60,000 cells/ml from 3 - 17 DPH.

Salinity in the recirculating system was maintained at 33 ± 1 g/L, temperature was kept at 27±1°C via ambient heating, and the photoperiod was maintained at 14 h light/10 h dark via fluorescent lighting. Total ammonia nitrogen and nitrite were checked periodically with a commercial test kit (Instant Ocean® Ammonia/Nitrite Test Kit, Spectrum Brands, Atlanta, GA, USA) and never exceeded 0.50 or 1.0 ppm, respectively throughout the course of the rearing period. Juveniles adapted readily to the pelleted feed and survival was high (> 95%). Larvae also did well (92% hatching, >35% survival to 18 DPH) and while growth was slightly slower than is normal under these conditions, it was within the normal variation seen in the lab.

Sampling

Prior to sampling, juveniles were fasted for 48 hours in order to ensure the gut was empty. On the morning of the sampling period, all fish were pooled and then split randomly between two treatment tanks. After one hour of acclimatization to the treatment tank, one tank of fish was fed by hand as much as they would eat during a five minute period (fed treatment) while the other tank received no feed (unfed treatment). Immediately following the feeding period, five fish were randomly selected from each tank for analysis as the "time 0" sample and any uneaten feed was removed from the fed tank. After this initial sampling, five random fish from each treatment were sampled at 0.5, 1, 1.5, 2, 2.5, and 3 hours postprandial. Sampled fish were euthanized via a combination of MS-222 overdose and ice-bath immersion and immediately processed to collect tissue samples.

On 17DPH, larvae were fed only once in the morning and allowed to fast overnight in order to ensure the gut was empty. On 18 DPH, all larvae were netted from their rearing tanks, randomly split into two pools, and placed into 20 L buckets provided with an air stone. After an hour to acclimate to the buckets, an initial sample of approximately 150 larvae was collected as described below and one bucket of fish was fed enriched *Artemia* nauplii in 1 L of culture water to reach a final concentration of approximately 50 nauplii/mL (fed treatment). This concentration was based on preliminary trials which showed that at this concentration of prey, greater than 99% of larvae had visible amounts of *Artemia* in their gut after 15

minutes. Seawater (1 L) taken from the same source as that used to enrich the *Artemia* was added to the bucket containing the other group of fish (unfed treatment) as a sham feeding. After fifteen minutes, both groups of fish were washed through a large 400 µm screen which allowed the *Artemia* nauplii to pass through without damage to the larvae. Previous trials with this washing methodology showed that very few *Artemia* remained after the wash (< 1 nauplii/ L) and that survival of the larvae was very high (>99% survival). After washing, both groups of fish were placed into clean 20 L buckets containing clean water (taken from the biofilter sump) where they remained for the rest of the collection period. As in the juvenile experiment, samples from each treatment were taken at 0.5, 1, 1.5, 2, 2.5, and 3 hours postprandial. Collection of the larvae differed from the juveniles primarily due to the differences in size and number of fish needed. Small groups of larvae were netted from the treatment buckets, rinsed thoroughly in distilled water, and then individually placed onto petri dishes resting on a block of dry ice where they were quickly frozen. Approximately 70 - 80 larvae from each treatment were collected at each sampling period and dishes containing these quick-frozen larvae were stored at -80°C until samples were processed and analyzed. Due to the large number of larvae collected at each sampling period, the time taken to collect the larvae ranged from 15 to 20 minutes.

Tissue Collection

In the juveniles, each fish was removed from the ice and measured for length and wet weight. Following these measurements, the gastrointestinal tract was removed and the region of the intestine from just posterior to the pyloric valve through the first bend was excised, washed in 1X PBS, and quick-frozen on dry ice. Efforts were made to exclude stomach contents but to maintain any digesta within the pyloric caeca or intestine itself. After collection, gut sections were placed individually into 1.5 mL microcentrifuge tubes and stored at -80°C until analyzed.

In the larvae, petri dishes containing frozen fish were processed on dry ice in order to keep the larvae frozen throughout the tissue collection. In order to develop a length vs. dry weight curve, 50 larvae from the initial time period were individually photographed using a stereomicroscope fitted with a digital camera (CoolSnap-Pro, Media Cybernetics, Bethesda, MD, USA), placed into individual microcentrifuge tubes, and then lyophilized using a Freezone® 4.5 freeze dry system (Labconco, Kansas City, MO, USA). Individual lengths were determined from the photographs using the ImageJ program (Abramoff et al., 2004) and dry weights of the lyophilized larvae were determined to the nearest ± 0.01 mg on a Denver Instruments M-220D microbalance (Denver Instruments, Denver, CO, USA). For all other larvae, the head was removed from each larva along a line running from the bottom of the eye through the top of the opercular opening using a fine bladed scalpel (Fig. 3.1). After

removal of the head, the trunks of 20 larvae were pooled in a single tube and kept at -80°C until further processed.

Sample Processing and Analysis

Following tissue collection, both the juvenile gut sections and the pooled larvae tissues were processed similarly. First, tubes containing the sample were thawed on ice and 200 µL of ice cold ultrapure water was added to each tube. Keeping the tube in ice, contents were homogenized using a handheld homogenizer with autoclaved pestles while being careful not to warm the tube contents with the handheld homogenizer. Immediately after homogenization, a small aliquot (50 µL) of the liquid homogenate was transferred to another microcentrifuge tube containing 750 µL of TriReagent®RT (Molecular Research Center, Cincinnati, OH, USA) and the remainder was spun briefly at 4°C in a centrifuge and returned to the -80°C freezer. The samples in the TriReagent were processed in accordance with the manufacturer's recommendation and total RNA was dissolved in 20 µL of nuclease-free water for later use in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

After being re-frozen, the larger portion of the tissue homogenate was processed for combined trypsin and CCK analysis as described by Rojas-Garcia et al. (2001). First, the samples were lyophilized and the dry weight of the resulting pellet was determined. Samples were then homogenized in 1 mL of methanol and allowed to sit on ice for at least 1 h. Following this incubation, tubes were centrifuged (6000

X g) at 4°C after which the methanol supernatant was carefully removed and transferred to new microcentrifuge tubes for later CCK analysis while the pellet containing the trypsin was left in the original tube. Following separation, methanol in both fractions was evaporated using a speedvac (Savant SC100). The extraction process was repeated twice in order to ensure maximal separation of the CCK and trypsin fractions.

CCK Peptide

The sulfated CCK octapeptide (CCK-8S) concentration was measured using a competitive binding Enzyme-Linked ImmunoSorbent Assay (ELISA). This ELISA was developed based upon the results of the molecular cloning information for red drum established in Chapter 2 and is highly specific for the CCK octapeptide common to red drum, Atlantic herring, and mammals (DYMGWMDF). The capture antibody chosen for use in this experiment was also specific for the sulfated form of CCK since this has been shown to have substantially greater potency in the gut than non-sulfated CCK (Grider, 1994) and since in rainbow trout (*Oncorhynchus mykiss*) all CCK peptides isolated from the gut were found to be fully sulfated (Jensen et al., 2001). The ELISA consisted of samples containing either unknown (extracted fish samples) or known (standards) amounts of CCK-8S being incubated in the presence of a labeled competitor in microtiter plates coated with a capture antibody. The CCK (both labeled and unlabeled) were captured by the antibody and the absorbance of

the labeled competitor measured on a microplate spectrophotometer. All components of the ELISA were prepared on site as they were needed.

ELISA plates were prepared by coating polystyrene microtiter plates with the CCK specific capture antibody (C2581, Sigma-Aldrich, St. Louis, MO, USA) via adsorption. Before coating the plates, a carbonate buffer (15 mM Na_2CO_3 and 34.8 mM $NaHCO_3$, pH 9.6) and a blocking buffer (5 g BSA/L of carbonate buffer) were prepared and filter sterilized through a 0.45 µm membrane filter. The capture antibody was diluted in carbonate buffer (200 µg/mL) and 100 µL of the resulting solution was pipetted into each well of the microtiter plate. The plate was then covered with an impermeable adhesive cover and placed on a shaker plate at 4°C overnight (14 - 16 h). The following morning, the plate was washed 4 times with blocking buffer using a Nunc-Immuno™ Wash 8 (Thermo Fisher Scientific). Each well was then filled with 300 µL of blocking buffer and incubated for 2 h at room temperature. After the blocking reaction was complete, plates were washed thoroughly using a wash bottle containing blocking buffer to remove any weakly bound antibody and then placed upside down to air dry. After the plates were dry, they were stored in plastic bags at -20°C until used.

In order to concentrate the very small amounts of CCK-8S and remove any interfering contaminants from the tissue samples, it was necessary to purify the CCK-8S through immunoprecipitation of the samples. First, the lyophilized CCK sample was rehydrated in 1 mL of a buffer containing 137 mM NaCL, 20 mM Tris HCL (pH adjusted to 8.0), 2 mM EDTA, 10% glycerol, 1% Triton X-100, and 1 µg/mL Aprotinin

(#A6103, Sigma Aldrich). Immediately after rehydration, the sample was processed using a Pierce Seize® X Protein A immunoprecipitation kit (#45215, Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's directions. After immunoprecipitation, the CCK-8S antigen was eluted from the spin column using 190 µL of the elution buffer and then neutralized by adding 10 µL of 1 M Tris acetate. Previous experiments with the immunoprecipitation step showed that small but sometimes measurable quantities of CCK-8S could be recovered with a second elution so this was done and the two eluted aliquots were pooled into a single 400 µL sample.

Before it was possible to analyze samples with the ELISA, it was necessary to determine the concentration of the competitor antibody which yielded 80% of the maximum obtainable signal on the prepared ELISA plates. First, biotinylated CCK-8S was prepared using EZ-Link® Sulfo-NHS-Biotin (Pierce Biotechnology) to label purified CCK-8S (#C2175, Sigma Aldrich) in accordance with the manufacturer's directions. Then, eight 2-fold dilutions of the competitor were prepared using a diluent solution (1 g BSA and 0.5 mL of Tween-20 in 1 L of 1X PBS) and an aliquot (50 µL) of each of these dilutions was loaded into triplicate wells, covered with foil, and incubated at 4°C on a shaker plate for 2 h. Following this incubation the plate was developed using the following procedure. First, the plate was washed four times with a washing solution (0.5 mL Tween-20 in 1 L of 1X PBS). Then 50 µL of a dilute streptavidin-horseradish peroxidase (SA-HRP) solution (1:20,000 SA-HRP in the diluent solution) was added to each well, the plate was covered with an impermeable adhesive cover

and incubated in the dark on a plate shaker for 30 min at room temperature in order to bind the SA-HRP to any labeled competitor bound to the capture antibody. Following this incubation, The plate was again washed four times with the washing solution and incubated with 100 µL of a chromogen substrate solution (32 µM o-phenylenediamine in a buffer containing 50 mM sodium citrate, 50 mM boric acid, 0.4 ppt H_2O_2, and 0.25 mM thimerosal) for 1 h on a plate shaker. After an hour, the chromogenic reaction was stopped by adding 100 µL of a dilute (0.5 M) sulfuric acid solution. The plate was then read on the microplate spectrophotometer at 450nm. Using these readings, the competitor concentration which yielded 80% saturation was determined and that concentration was used until new plates or competitor was needed.

For each plate of unknown samples to be analyzed, a standard curve was prepared by taking 0.5 mg purified CCK-8S diluted in 1 mL of diluent solution and preparing eight 2-fold dilutions. To analyze samples, 50 µL of the unknown or standard dilution was added to triplicate wells of the microtiter plate along with 50 µL of the diluted competitor antibody, covered with an impermeable adhesive strip and incubated on a shaker plate at 4°C overnight. Following the overnight incubation, plates were developed and measured as described above. CCK concentration was reported in fmol/mg of dry weight.

Trypsin

Trypsin assays generally followed the procedure of Faulk et al. (2007). The lyophilized pellet was rehydrated and homogenized in 200 µL of homogenization buffer (20 mM Tris-HCl, 1mM EDTA, and 10mM $CaCl_2$, pH 7.5) and allowed to incubate on ice for 30 minutes. Following incubation, the sample was centrifuged (4000 x *g*) at 4°C for 10 minutes and the supernatant was removed. While the samples were incubating on ice, a fresh analysis buffer containing 1 mM of BAPNA (*N*α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride), 50 mM Tris acetate, and 20 mM $CaCl_2$ adjusted to a pH of 8.2 was prepared and warmed to 30°C in a water bath. An aliquot (50 µL) of supernatant from each sample along with a positive control (purified trypsin, 12 µg/mL) and a plate blank (homogenization buffer only) was added to triplicate wells in a 96-well plate. 100 µL of the preheated analysis buffer was rapidly added to each well using a multichannel pipettor and the plate was placed within a heated (30°C) microplate spectrophotometer (Spectramax 190 spectrophotometer, Molecular Devices Corp., Sunnyvale, CA, U.S.A.). The production of *p*-nitroanaline was monitored at a wavelength of 410 nm with absorbance measurements obtained every 30 s for 30 min. The spectrophotometer temperature was maintained at 30° C. Trypsin activity is reported in activity units (U) per mg of dry sample weight (U/mg) where one unit represents one µmole of BAPNA hydrolyzed per minute. Activity was calculated as:

$$Trypsin\ activity\ (U) = \frac{\frac{\Delta A}{\Delta T}}{\varepsilon} \times \frac{TV}{SV} \times 10^6$$

where ΔA is the change in absorbance, ΔT is the period of linearity in minutes, ε is the molar extinction coefficient of *p*-nitroanaline (8800 M^{-1} cm^{-1}), TV is the total volume of the reaction and SV is the volume of the sample aliquot.

CCK mRNA

CCK mRNA was quantified using a two-step qRT-PCR method where RNA was first reverse transcribed to cDNA and then aliquots of the cDNA synthesis reaction were used in a quantitative PCR to determine number of copies based on a standard curve. Before cDNA synthesis, the total RNA concentration in each sample was measured using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Contamination of the RNA sample with proteins was assessed at this time by examination of the optical density ratio (260nm/280nm). Samples in which the 260/280 ratio was <1.8 were precipitated with isopropanol, washed with 75% ethanol, dissolved in 20 µL of nuclease water and re-measured. This procedure was repeated until the RNA had a 260/280 ratio >1.8. The cDNA synthesis reaction was performed using approximately 1 µg of total RNA and 0.5 µg of random hexamer primers in a 20 µL reaction using the ImProm-II™ reverse transcription system (Promega Corporation, Madison, WI, USA). First, the RNA and the primers were mixed in a 5 µL reaction and incubated at 70°C for five minutes and then placed

immediately on ice for another 10 minutes in order to remove any secondary structure and anneal the primers. Then, 15 µL of a master mix containing the reaction buffer, $MgCl_2$ (3 mM final concentration), dNTPs (0.5 mM final concentration of each), RNasin ribonuclease inhibitor (20u), and the reverse transcriptase was added to the reaction. Reverse transcription was performed with a five minute annealing step at 25°C, a one hour extension step at 42°C, and a 15 minute incubation at 70°C to inactivate the reverse transcriptase. The resulting cDNA was then diluted 2:1 before use in the PCR reaction. One cDNA synthesis reaction was performed on each RNA sample.

The quantitative PCR (qPCR) was run on a Stratagene MX3000P (Agilent Technologies, Santa Clara, CA, USA) using the Maxima™ SYBR Green qPCR system (Fermentas, Burlington, Ontario, CAN). Primers for the PCR reaction (RD-qCCKF2: TCT CCT CCC AGC TCC TAG A and RD-qCCKR2: GAT AGC AGA CAG GGA CTA CAT GG) were designed using the Primer3 program (Rozen and Skaletsky, 2000) and amplified a product 294 bp long. The forward and reverse primers were combined into a qPCR primer mix which contained each primer at 5 µM. Efficiency tests with both plasmids containing the CCK insert and cDNA from native tissues showed an excellent amplification efficiency (97.2%) across a wide range of copy densities (3 x 10^{-1} - 3 x 10^5 copies, Fig. 3.2). These primers were also tested in the qPCR reaction with both genomic DNA and RT^- controls without any detectable signal and the products were run on a 1% agarose gel to confirm the absence of any bands. Each qPCR reaction contained 12.5 µL of the qPCR master mix, 1 µL of the primer mixture,

and 5 µL of the diluted cDNA in a 25 µL reaction. Cycling parameters of the PCR were: 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30s. Following the PCR reaction, a single-cycle melting curve from 55 to 95°C was performed (Fig. 3.3). In order to quantify the number of CCK cDNA copies in each sample, a standard curve was constructed using 10-fold dilutions of a solution containing plasmids with a red drum CCK insert as described in the Applied Biosystems technical guide, "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR". Each cDNA sample was run in duplicate and the resulting average copy count was used for quantifying CCK mRNA. CCK mRNA concentrations in each sample were standardized and reported as the number of copies per microgram of total RNA (copies/µg).

Statistical Analysis

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 at each time point similarly to the method used by Koven et al (Koven et al., 2002). Differences were considered significant at $\alpha \leq 0.05$. Data is expressed as mean ± S.E.

RESULTS

Juveniles

Due to a very small pool of fish from which to sample, there was a relatively wide range in fish sizes used for the juvenile experiment (Fig. 3.4). Lengths ranged from 3.32 to 6.50 cm with a mean of 4.72 ± 0.80 (SD) and weights ranged from 0.63 to 5.59 g with a mean of 1.91 ± 1.03. All fish however were well developed juveniles with fully functional stomachs and clearly identifiable pyloric caeca. All fish in the fed treatment were very willing to feed despite the recent handling and continued to feed throughout the allotted five minute feeding period. At time 0, all fish in the fed treatment had clearly engorged stomachs which were full of partially digested feed pellets while none of the fish in the unfed treatment had any identifiable stomach contents. Throughout the remainder of the sampling period, this remained consistent; fish in the unfed treatment had nothing in their stomachs while fish in the fed treatment had food in their stomachs. By the 0.5 h sampling period, fed fish were observed to have digesta in the anterior intestine and the pyloric caeca and this was also present in all subsequent fish in the fed treatment group.

The CCK peptide levels in fish in the fed treatment were highly variable in most time periods (Fig. 3.5). Initially, CCK levels were similar in both treatment groups (35.3 ± 12.5 fmol/mg in the unfed vs. 15.4 ± 5.8 fmol/mg in the fed) but by 0.5 h postprandial fish in the fed treatment group had significantly higher CCK levels (569.9 ± 146.6 fmol/mg) than did fish in the unfed group (20.9 ± 6.3 fmol/mg) and

this continued throughout the course of the sampling period. Trypsin levels followed a similar pattern, but were slightly out of phase with the CCK results (Fig. 3.6). Trypsin levels were not significantly different between treatments at either the 0 or the 0.5 h sampling periods but by the 1 h sampling period the fed group had significantly higher (111.6 ± 22.0 U/mg) levels than did the unfed group (29.1 ± 9.3 U/mg) and this significance remained throughout the rest of the sampling period. CCK mRNA levels in the two treatment groups followed a different pattern (Fig. 3.7). At time period 0, fish in the unfed treatment group possessed significantly greater numbers of CCK mRNA copies (666.4 ± 182.0 copies/µg) than did the fish in the fed treatment group (207.1 ± 72.6 copies/µg) but the difference was relatively small and most likely represented a statistical anomaly. After that, mRNA levels remained fairly low in both treatment groups and were not significantly different until the 2.0 h sampling period. At that point, the fish in the fed treatment group had significantly greater numbers of CCK mRNA copies than the fish in the unfed group (1221.1 ± 366.2 vs. 334.8 ±65.0 copies/µg) although the mean difference was still relatively small. By the last sampling period (3.0 h postprandial) however, the CCK mRNA levels in the fed fish had spiked and there was a large difference in mean values between the fed and unfed groups (5536.3 ± 1627.7 vs. 384.4 ± 153.3 copies/µg, respectively).

Larvae

Like the juvenile study, no effort was made to cull under- or over-sized fish for this experiment and thus the size of individual larvae varied widely (Fig. 3.8). Lengths of larvae ranged from 4.05 to 7.28 mm and dry weights ranged from 0.13 to 1.56 mg. All of these fish were taken before the start of the feeding trial and the weight numbers therefore reflect the fasted dry weight of the fish. All larvae measured had commenced notochord flexion which typically begins at around 4.0 mm and none had reached the size at which gastric development normally begins (~8.0 mm), thus all fish were considered to be in the same developmental state despite differences in size.

As in the juveniles, the CCK peptide response of the 18 DPH larvae (Fig. 3.9) was significantly different between treatments at the 0.5 h sampling period (1.1 ± 0.2 vs. 0.2 ± 0.1 fmol/mg in the fed vs. unfed treatments). This significant difference remained through the 1.5 h sampling period, but from the 2.0 h sampling period onward there were no significant differences between the two treatments. The trypsin response of the larvae (Fig. 3.10) was extremely variable and the only significant differences were in the 1.0 and 1.5 h time periods when the fish in the fed treatment (19.0 ± 1.2 and 43.1 ± 12.4 U/mg, respectively) had significantly more trypsin than did the fish in the unfed treatment (10.6 ± 1.8 and 13.0 ± 3.6 U/mg, respectively). CCK mRNA levels in the larvae were also extremely variable (Fig. 3.11) and the only significant difference was seen in the 3.0 h time period when fish in the

fed treatment (51.1 ± 11.7 copies/µg) had significantly higher CCK mRNA levels than did the fish in the unfed treatment (16.3 ± 4.1).

DISCUSSION

The present study has demonstrated that postprandial differences in CCK peptide, trypsin, and CCK mRNA responses occur in both juvenile and larval red drum. Allowing the fish to feed naturally rather than being tube fed a measured dose may have contributed to the large amounts of variability in all of the analytes due to the differences in both ingestion and timing, but the present study has shown the validity of this method for red drum juveniles and advanced larvae.

The selection of a portion of the gastrointestinal tract as the source for the tissue in the juvenile experiment was made to maximize the recovery of the three analytes measured in this study. This was based on previous work that showed this area contains the highest concentration of CCK immunoreactive (CCK-IR) cells in the intestine (Chapter 2). Additionally, the region around the pyloric caeca contains the diffuse pancreatic tissue which is the source of trypsin (Lazo, 1999) and tissue distribution of CCK mRNA in adult and juvenile red drum (Chapter 2) showed a very high concentration of CCK mRNA in these tissues as well. Because of the relatively small size of the 18DPH larvae, it was not possible to isolate this portion of the intestine in the larval samples. This was due to the time spent to capture, wash, and

freeze the individual larvae as well the fact that number of fish necessary to reach minimum sample sizes given extracted guts would have been prohibitively large.

While including the body tissues likely decreased the concentration of the analytes in the sample somewhat it was far more important to remove the brain from the sample. While the brain would not have increased the levels of trypsin, it could have dramatically altered the CCK and CCK mRNA levels measured in the samples. The tissue distribution work in Chapter 2 illustrates just how much CCK mRNA there is present in the brain of red drum (Fig. 2.4) and while it was not quantified, the level of the CCK peptide is likely quite high. This has been seen in other animals such as the spiny dogfish (*Squalus acanthias*), where the relative concentration of the CCK peptide has been reported to be more than twice as high in the brain as in the intestine (Johnsen et al., 1997) and in larval Atlantic halibut (*Hippoglossus hippoglossus*) where neural CCK is the dominant form (Rojas-Garcia et al., 2001; Rojas-Garcia and Rønnestad, 2002; Rønnestad et al., 2003).

Oddly, juveniles in the unfed treatment had significantly higher CCK mRNA levels initially than did the unfed fish. This was unexpected since the fish had only five minutes to feed. Other than this initial difference in the juveniles, the pattern of CCK expression between the juveniles and larvae is quite similar given the relative differences in gut passage time between the two ages. As stated previously, the juvenile red drum in the present study had food in their stomach throughout the course of the entire three hour sampling period. As the presence of chyme or digesta has been shown to stimulate CCK secretion in a number of species (Liddle et al.,

1988; Benson and Reynolds, 2001; Rønnestad, 2002; Leray et al., 2003; Rehfeld, 2004), the release of digesta from the stomach over the course of the sampling period is likely the cause of the elevated CCK levels in the fed treatment that lasted throughout the course of the experiment. However, while a large number of the 18 DPH larvae (>50%) in the fed treatment did have some visible *Artemia* residue present in the posterior midgut and hindgut through the last time period, the foregut and anterior midgut in these fish were empty by the 2.0 h postprandial time period (Fig. 3.12). It appears that most of the food bolus has passed the bend in the intestine which marks the end of where CCK-IR cells were principally found (Chapter 2) and thus even though there is still material in the gut, it may have passed the point at which it is stimulating CCK secretion. This is not unexpected since the 18 DPH larvae did not possess functional gastric glands nor did they have a functional pyloric valve, which does not become functional in red drum larvae until approximately 7 - 8 mm (Soto et al., 1998). This also matches intra-laboratory data which shows that lower densities of prey can extend the time it takes red drum larvae to evacuate their gut, but that it still normally occurs within 105 to 125 minutes (G.J. Holt, pers. com.). The lack of digesta in the areas where CCK-IR cells have been shown to be located would suggest that CCK levels in the larvae should decrease after 2 h and matches what was seen in the current experiment. The influence of CCK on gut passage time in larval fish is unknown making this finding worthy of future consideration as well.

Another interesting issue posed by the observed presence of food still in the gut three hours after the last meal is the possibility that high CCK levels in the intestine extended the time necessary for the food bolus to pass through the gut. It has been postulated that CCK may play a role in the mixing of digestive secretions with a food bolus and the filling of the pyloric caeca through the stimulation of reverse peristalsis (Rønnestad, 2002; Rønnestad et al., 2003). Thus the observed extension in gut passage time may be related to elevated levels of CCK inducing reverse peristalsis. As increased gut retention of microparticulate particles would allow increased time for hydration and digestion, this could be an area of importance in designing microparticulate diets for first feeding larvae and merits further investigation.

The increase in trypsin activity in both the juveniles and the larvae lagged behind the increase in CCK levels by approximately 30 minutes. This is consistent with the model of CCK inducing the secretion of trypsin in red drum and is similar to what was seen in postprandial work with Atlantic herring by Koven et al. (2002), They reported that CCK was significantly increased within 15 minutes after tube feeding BSA to Atlantic herring but trypsin was not significantly greater than the control until 1 h postprandial (the next sampling period) The authors did report a slightly different pattern however when the herring larvae were fed a 1:1 ratio of BSA and free amino acids (FAA). In this case, the herring larvae had significantly increased CCK and trypsin levels at the first sampling period (15 min postprandial). It is possible that the combined BSA and FAA mixture more closely resembled natural prey. If this were

the case however, the live prey used in the current study should more closely match the pattern seen in the BSA:FAA mixture than the pattern seen when the herring were fed BSA alone. It is likely that since the *Artemia* consist of both nitrogenous compounds as well as a mix of other nutrients, unlike the purified solutions used by Koven et al., there are interactions which have not yet been considered affecting the results in the current study. Other possible explanations for the differences between the two studies are the increased sensitivity of the RIA used by Koven et al., differences in species or temperature, differences between the volitional feeding of the present study and the tube feeding of the previous work, or just the large variability in the CCK assay in both experiments.

In the red drum larvae; when the elevated CCK levels decreased in the 2.0 h sampling period, trypsin had decreased as well. This agrees with the regulatory loop model for pancreatic secretions suggested by Rønnestad (Rønnestad, 2002). This model, based on research in mammals, suggests that food stimulates CCK indirectly through a stimulation of a luminal CCK-releasing factor (CCK-RF) which acts upon endocrine cells within the intestinal mucosa that release CCK into the gut lumen. This secretion of CCK then stimulates trypsin/pancreatic enzyme secretion. While food remains present in the gut, it protects CCK-RF from degradation by trypsin by outcompeting the CCK-RF as a substrate. As the food is processed, there is less to protect the CCK-RF from inactivation by trypsin. Inactivation of the CCK-RF then shuts down the secretion of CCK into the lumen and thus down-regulates the secretion of trypsin into the lumen as well. It is possible that ingested food acts

directly upon the endocrine cells in the intestinal lumen to directly stimulate the secretion of CCK into the intestine, but whether directly or indirectly stimulated the current research supports the model of a regulatory loop. Perhaps the most exciting finding of the current research is the increase in CCK mRNA in both juveniles and larvae after only 2.5 - 3.0 h postprandial. While the transcriptional regulation of CCK synthesis by dietary factors has been previously established in the rat (*Rattus norvegicus*) (Liddle et al., 1988; Liddle, 1994a; Liddle, 1994b; Cousins, 2007), this is the first known example of feeding induced transcription in fish. There is the possibility that the results of the present study do not show an increase in CCK mRNA expression, but instead show a return to basal state following a fasting period. It has been demonstrated in rats that a period of fasting reduces CCK mRNA levels (Yamada et al., 1997), possibly through the action of somatostatin (Kanayama and Liddle, 1991), and since both the juveniles and larvae in the present study were fasted prior to the start of the experiment this possibility must be considered. Still, whether an increase over basal levels due to feeding or a return to basal levels following the end of a period of fasting, the current work demonstrates that CCK mRNA transcription in red drum can be modified through dietary components. Understanding how CCK mRNA can be affected by feeding has implications not just within the context of the present work, but as a tool for assessing the feeding state of red drum larvae in the wild as well. Future research into this area will clarify the response of red drum to feeding and fasting over a period of time.

This work has shown that CCK, trypsin, and CCK mRNA levels were all increased following volitional feeding in red drum juveniles and larvae. Patterns of postprandial expression of these three analytes suggest that CCK plays a similar role in the digestive process of red drum as it does in other animals and that it has a direct influence on the secretion of trypsin. This is significant since it has been hypothesized that the presence of some component of live feeds is responsible for the increased utilization of microparticulate diets in larval fishes. The results of this study show that CCK and trypsin are both present in red drum larvae and responsive to feeding within a short time frame. Since it is still unknown if early larvae will display the same responses, future studies should investigate larvae at an earlier time period. Also, future work should attempt to separate olfactory, gustatory, and intestinal signals in order to better understand the mechanism by which feeding induces CCK secretion.

Figure 3.1. Representation of the cut made to remove the head in 18 DPH red drum larvae.

The cut was made with a fine bladed scalpel in a line which ran from the lower edge of the eye through the top of the opercular opening.

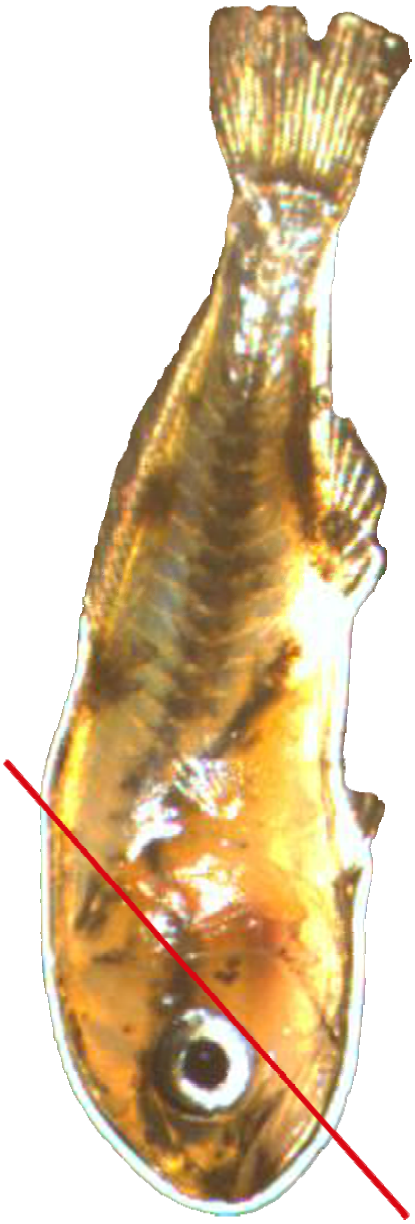


Figure 3.2. Efficiency of the qRT-PCR reaction.

Standards were derived from 10-fold dilutions of a sample of plasmids containing the CCK insert. Efficiency of the reaction is 97.2%.

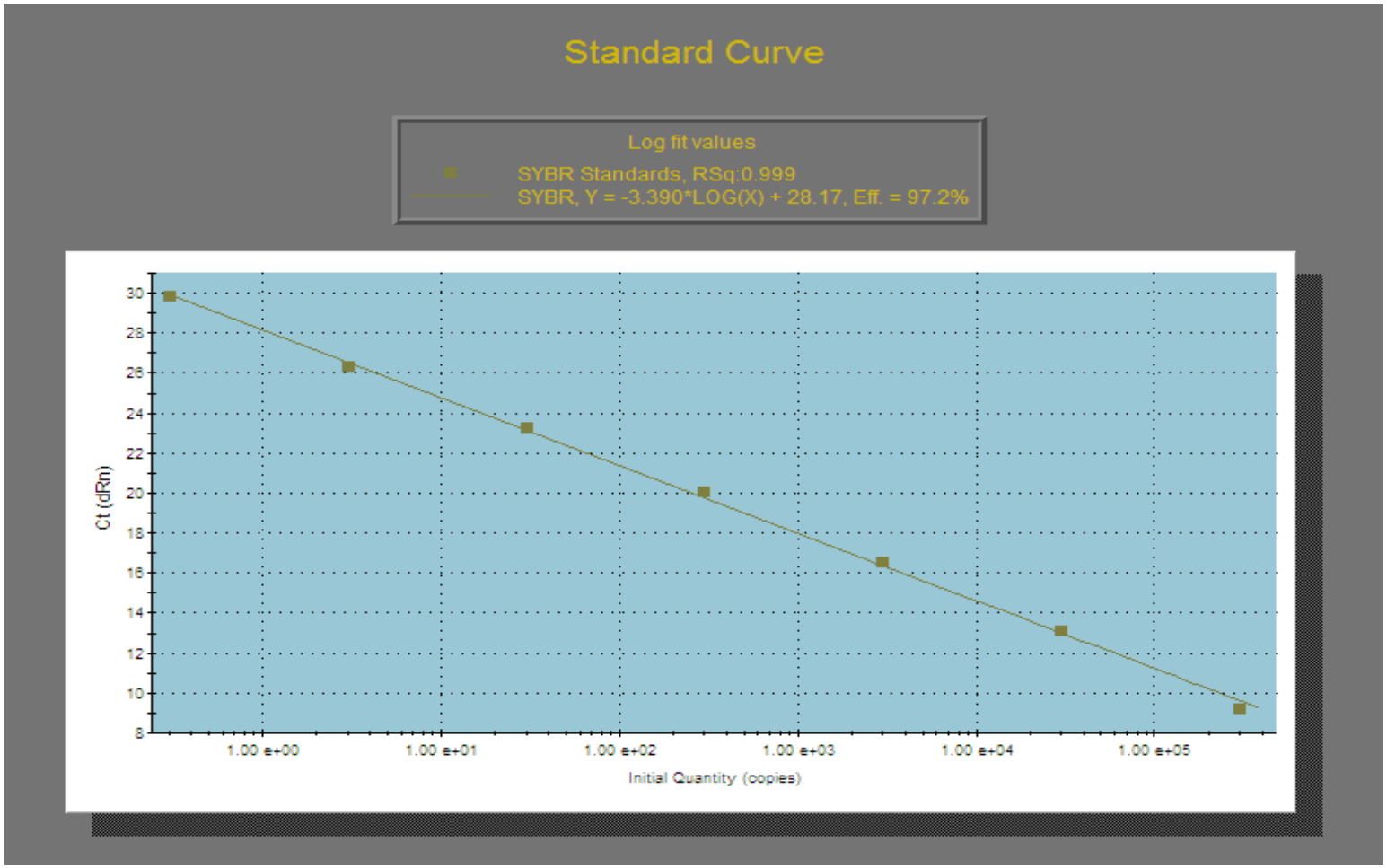

Standard Curve
Log fit values
SYBR Standards, RSq:0.999
SYBR, Y = -3.390*LOG(X) + 28.17, Eff. = 97.2%
Ct (dRn)
30
28
26
24
22
20
18
16
14
12
10
8
1.00 e+00
1.00 e+01
1.00 e+02
1.00 e+03
1.00 e+04
1.00 e+05
Initial Quantity (copies)

Figure 3.3. Representative melting curve of the qPCR products.

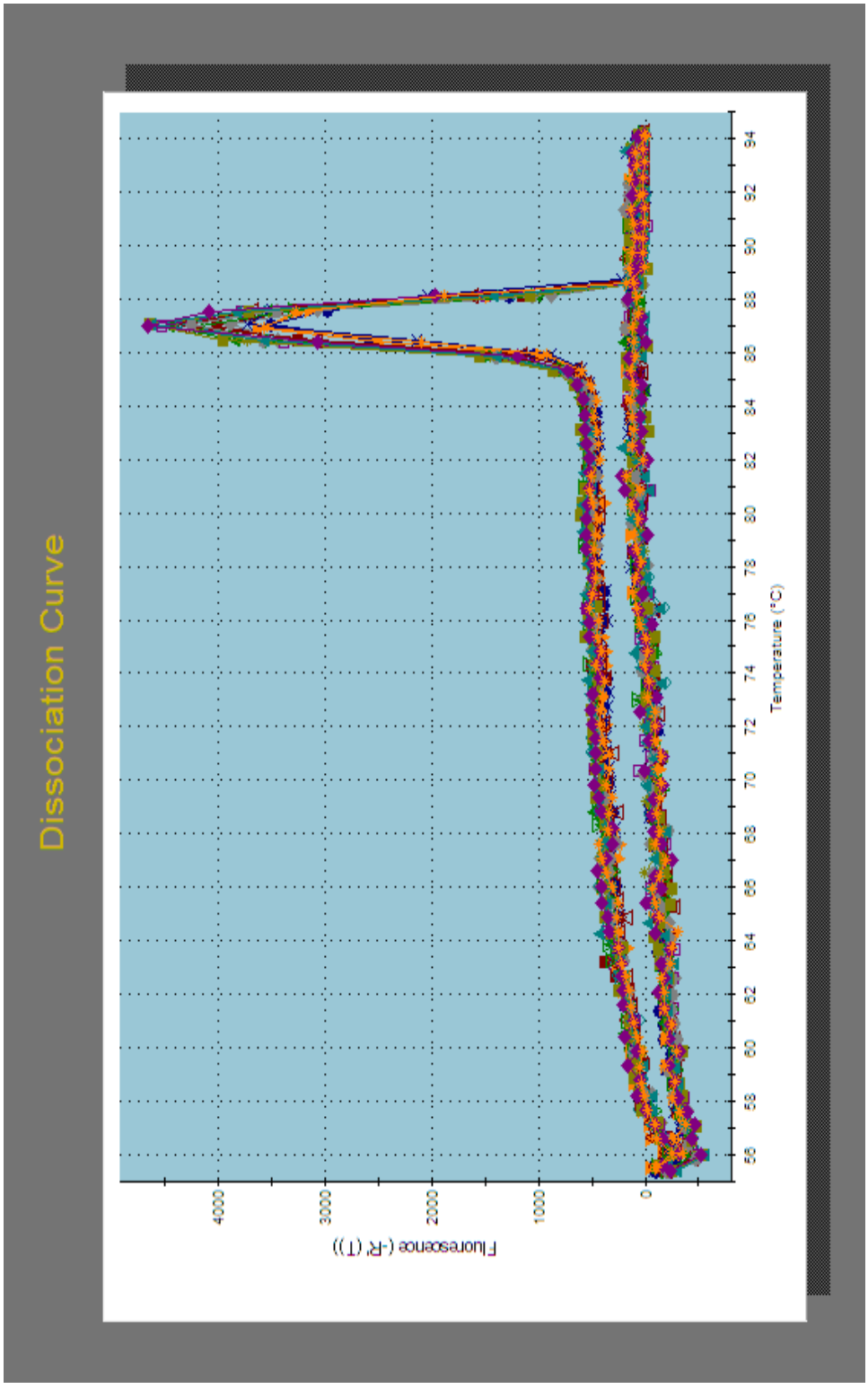

Dissociation Curve
Fluorescence (-R' (T))
4000
3000
2000
1000
0
Temperature (°C)
56
58
60
62
64
66
68
70
72
74
76
78
80
82
84
86
88
90
92
94

Figure 3.4. Length vs. Weight curve of red drum juveniles used in the postprandial experiment.

All fish were well developed juveniles and possessed fully functional stomachs and clearly identifiable pyloric caeca.

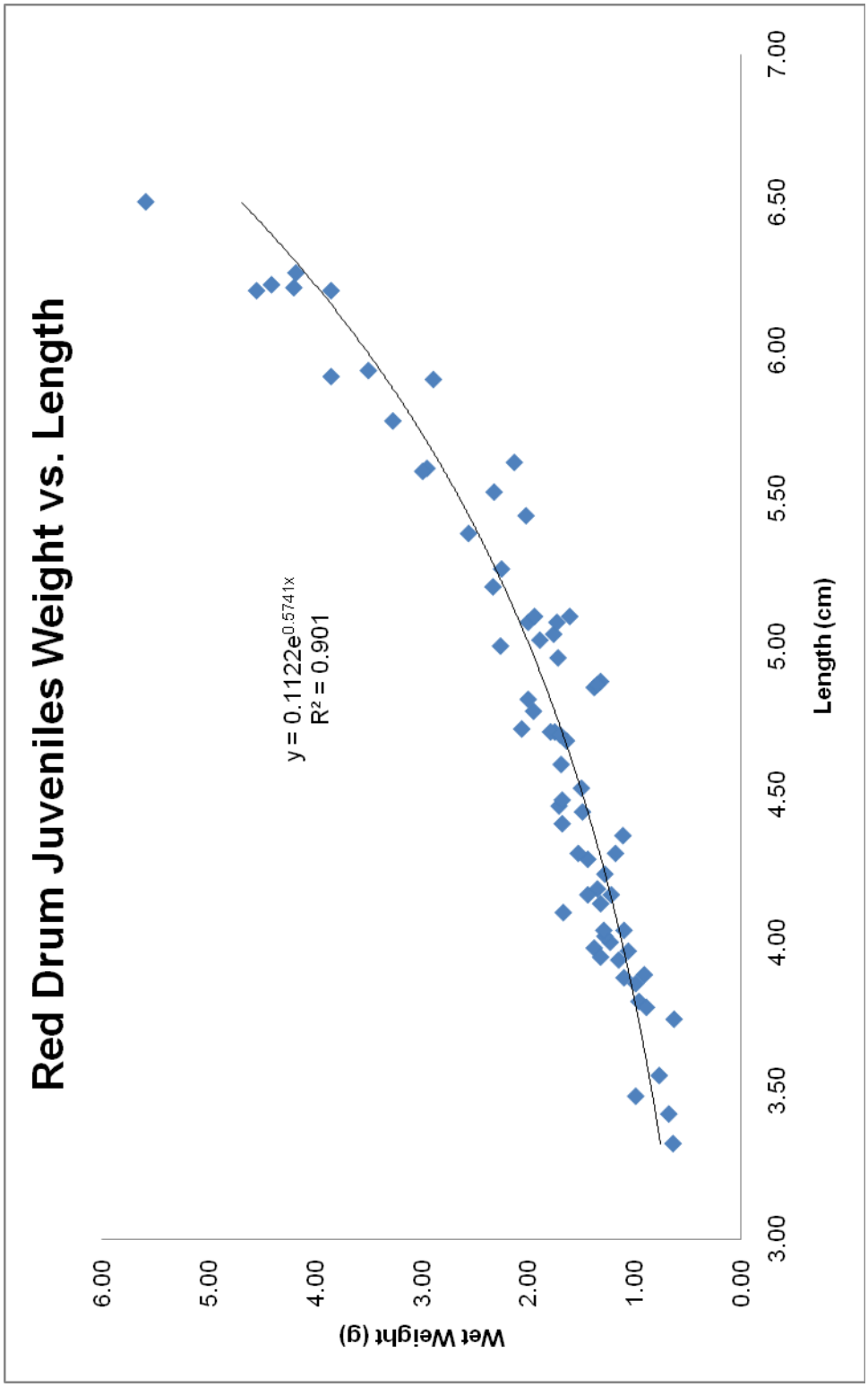

Red Drum Juveniles Weight vs. Length
y = 0.1122e^0.5741x
R² = 0.901
Wet Weight (g)
Length (cm)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00

Figure 3.5. Postprandial CCK peptide response of juvenile red drum.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

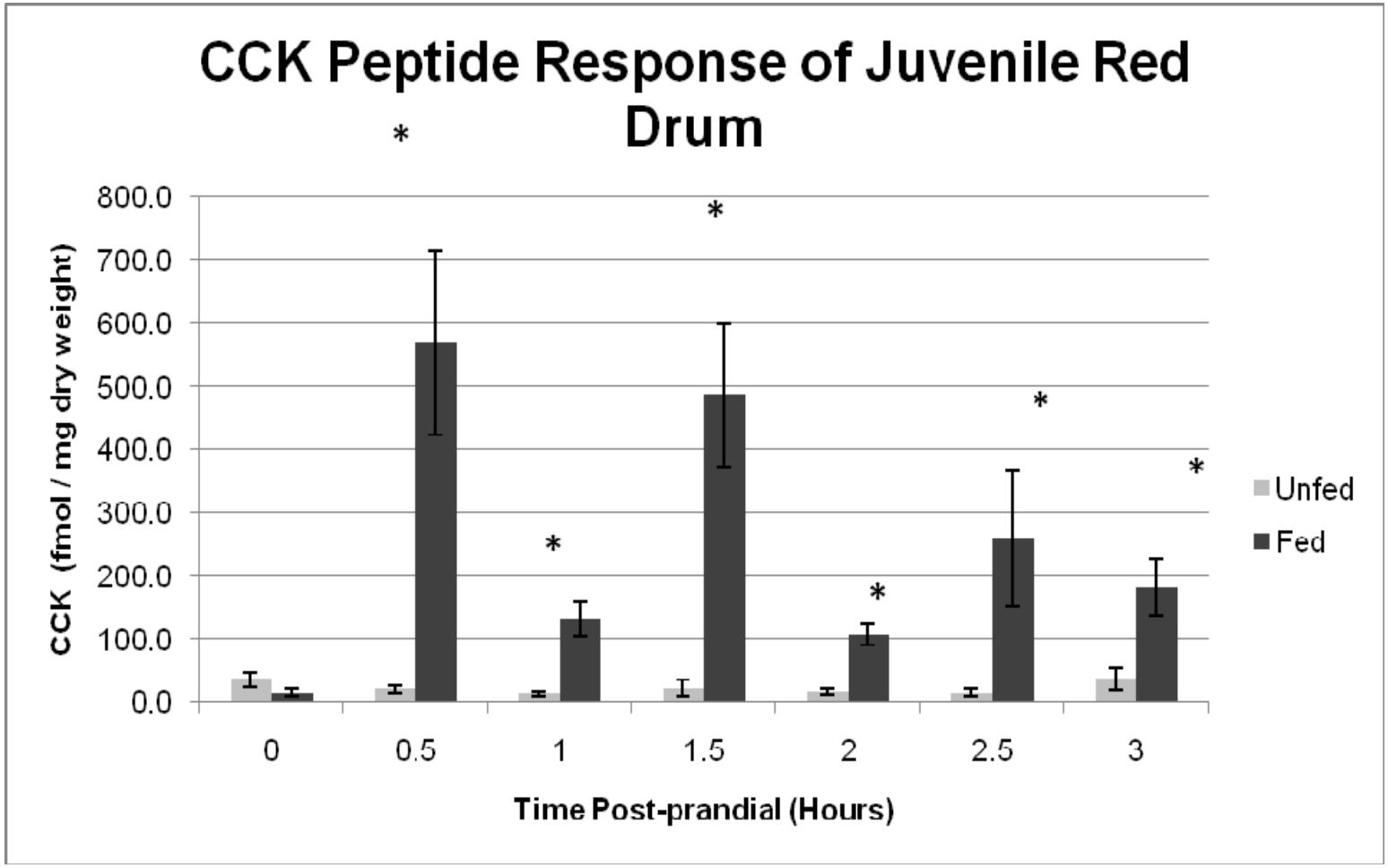

CCK Peptide Response of Juvenile Red Drum
CCK (fmol / mg dry weight)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed
*

Figure 3.6. Postprandial trypsin response of juvenile red drum.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

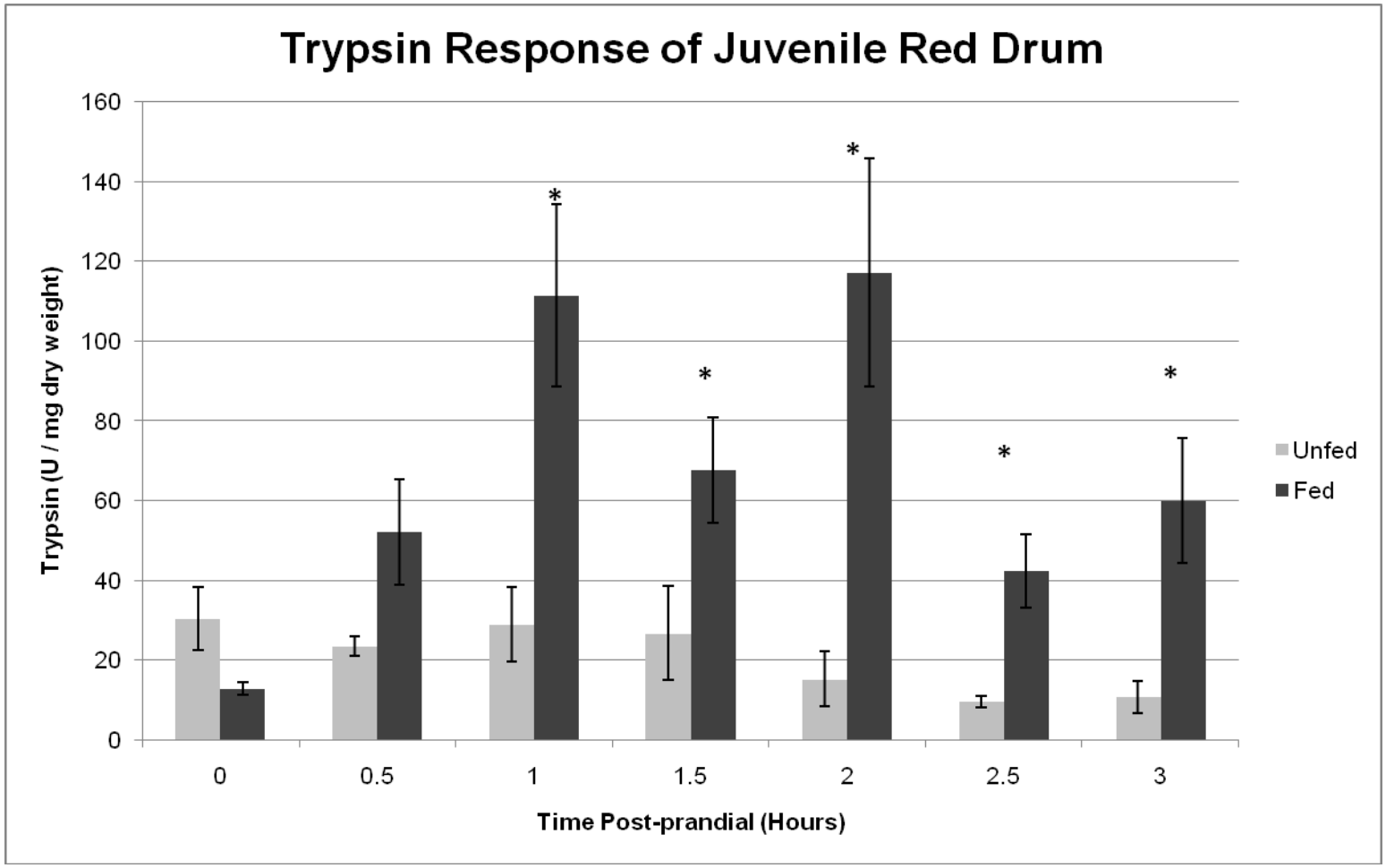

Trypsin Response of Juvenile Red Drum
Trypsin (U / mg dry weight)
160
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed
*
*
*
*
*

Figure 3.7. Postprandial CCK mRNA response of juvenile red drum.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

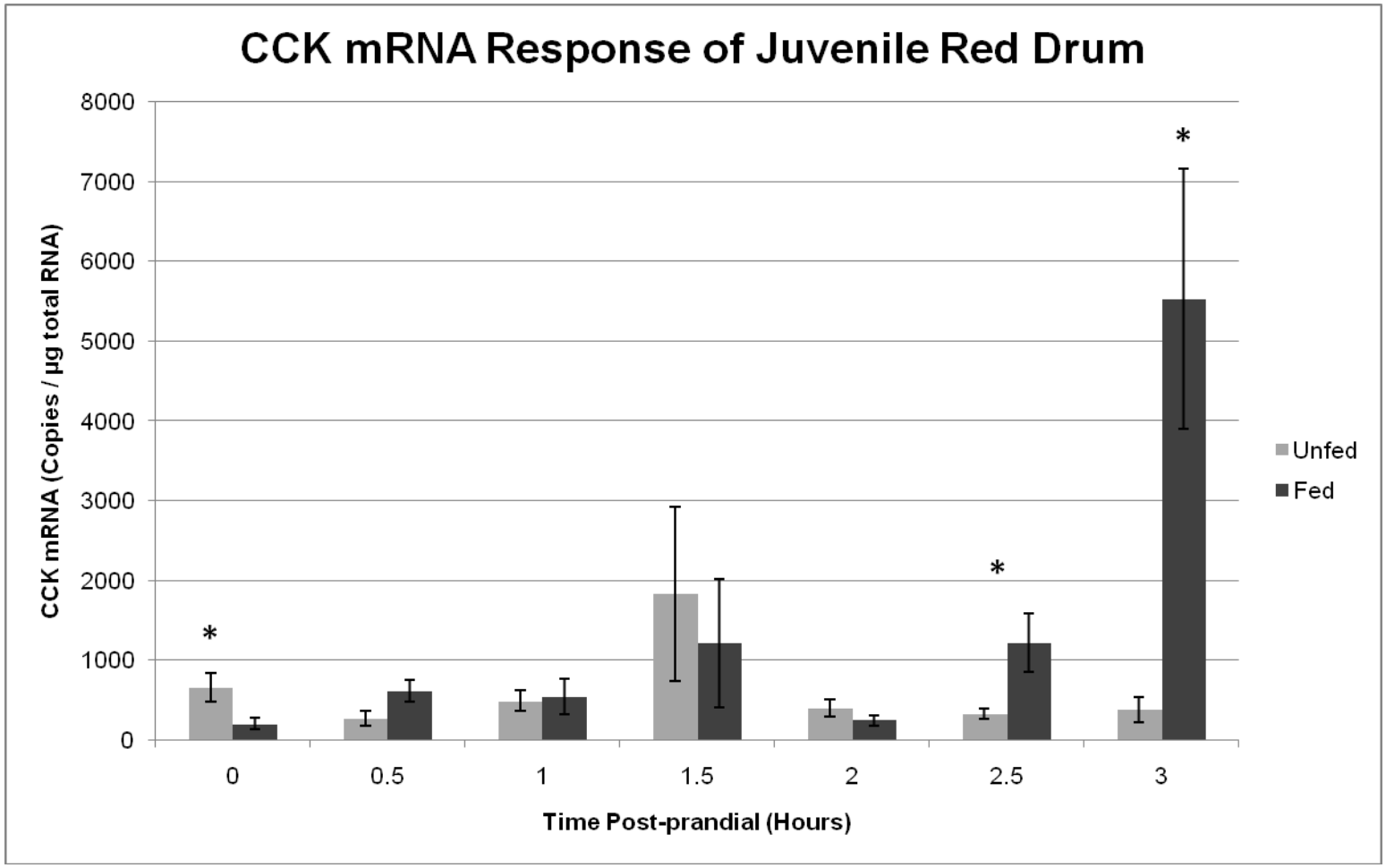

CCK mRNA Response of Juvenile Red Drum
CCK mRNA (Copies / µg total RNA)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed
*
*
*

Figure 3.8. Length to dry weight relationship of 18 DPH red drum larvae.

Data is from a sub-sample of 50 representative larvae.

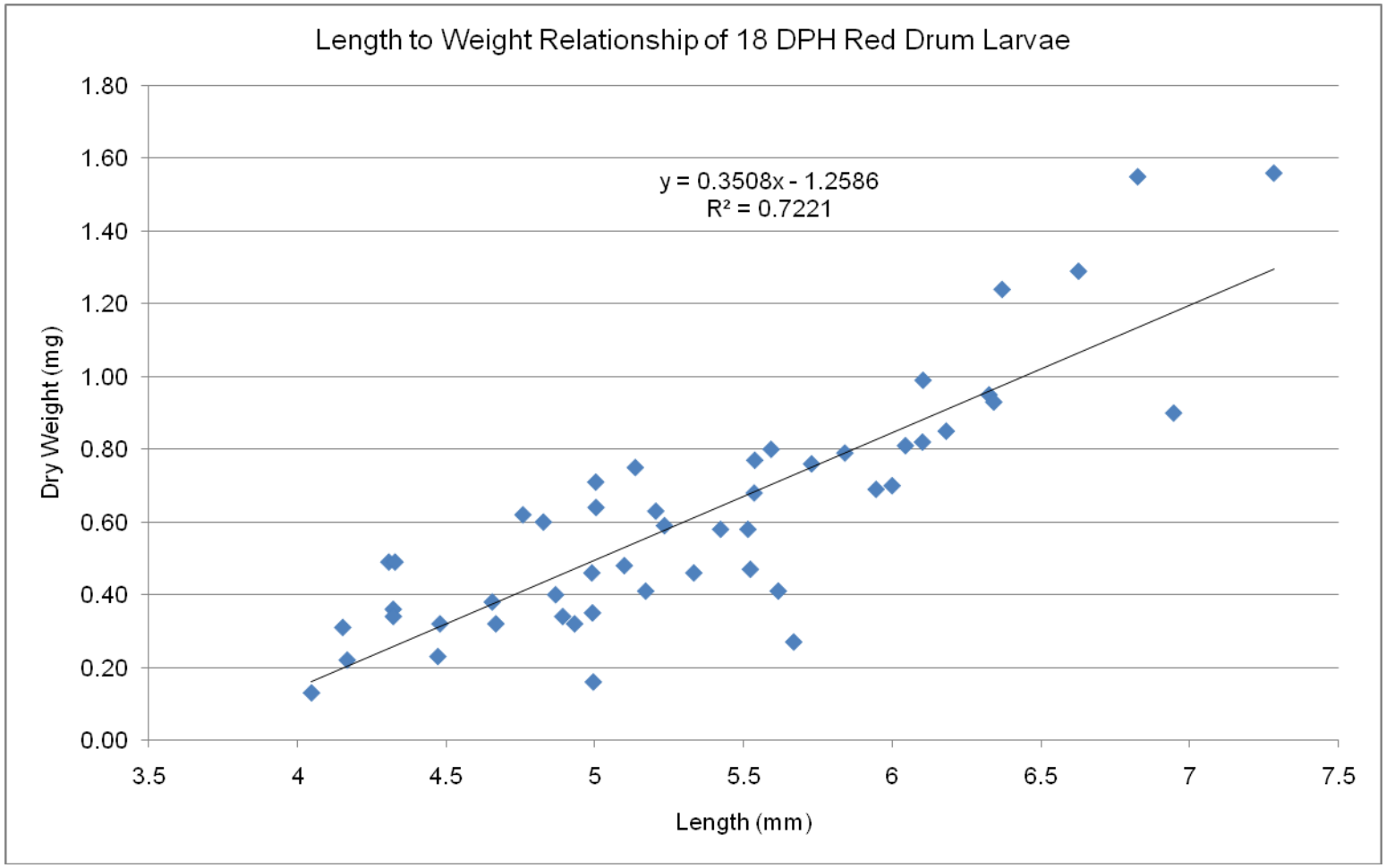

Length to Weight Relationship of 18 DPH Red Drum Larvae
y = 0.3508x - 1.2586
R² = 0.7221
Dry Weight (mg)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
3.5
4
4.5
5
5.5
6
6.5
7
7.5
Length (mm)

Figure 3.9. Postprandial CCK peptide response of 18 DPH red drum larvae.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

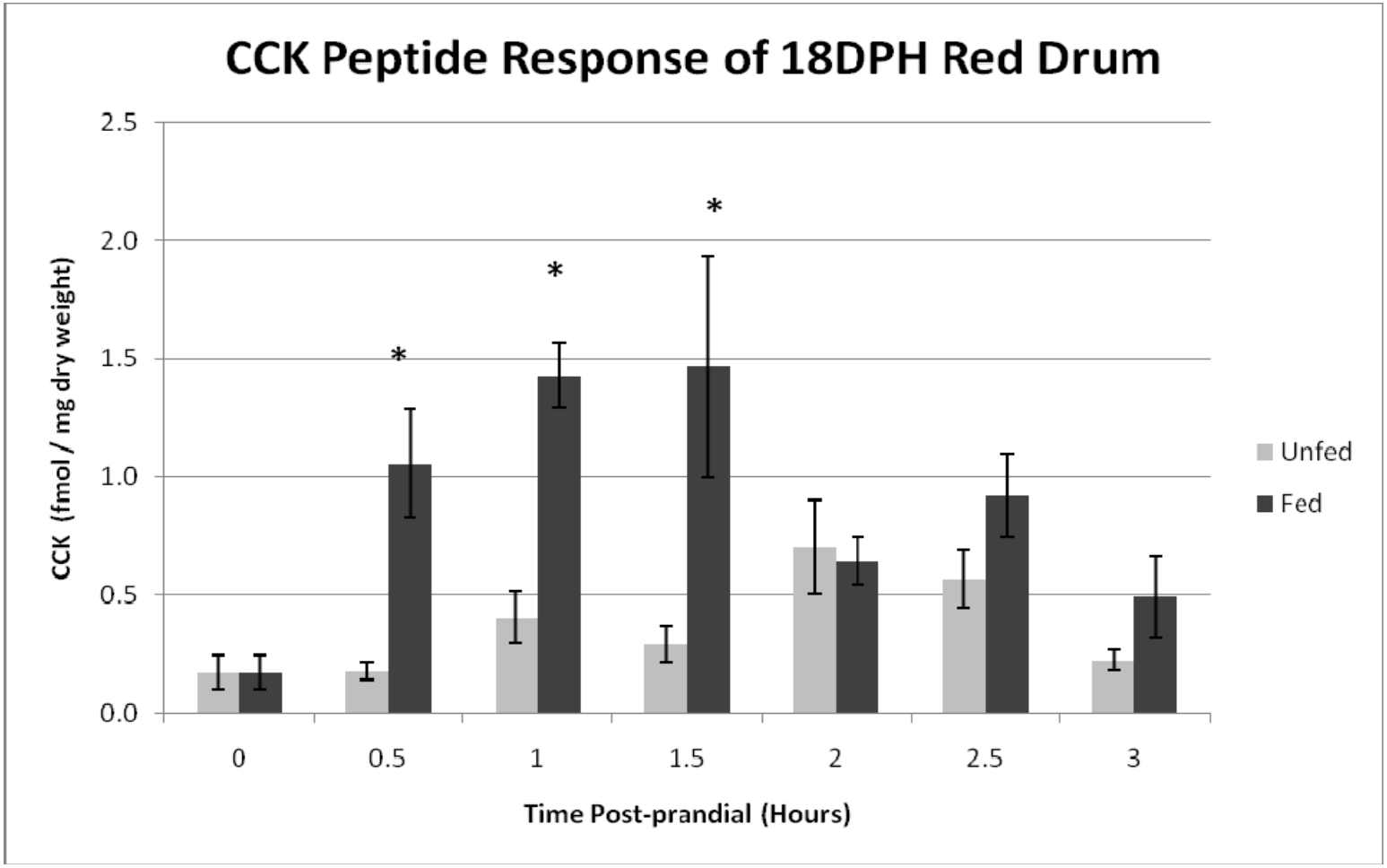

CCK Peptide Response of 18DPH Red Drum
CCK (fmol / mg dry weight)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed

Figure 3.10. Postprandial trypsin response of 18 DPH red drum larvae.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

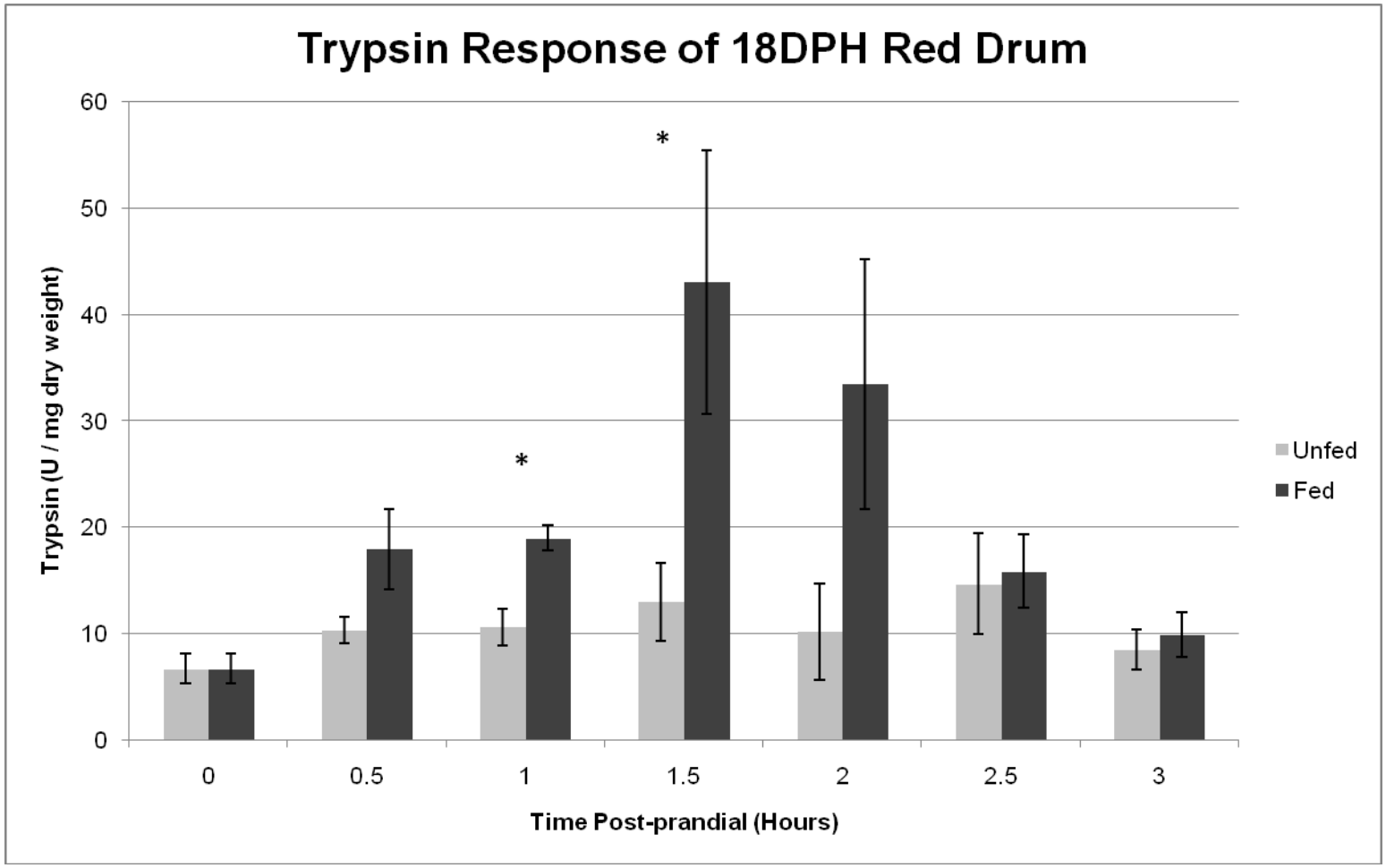

Trypsin Response of 18DPH Red Drum
Trypsin (U / mg dry weight)
60
50
40
30
20
10
0
*
*
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed

Figure 3.11. Postprandial CCK mRNA response of 18 DPH red drum larvae.

Error bars represent standard error and in periods labeled with a "*" there are significant differences between treatments at an $\alpha = 0.05$.

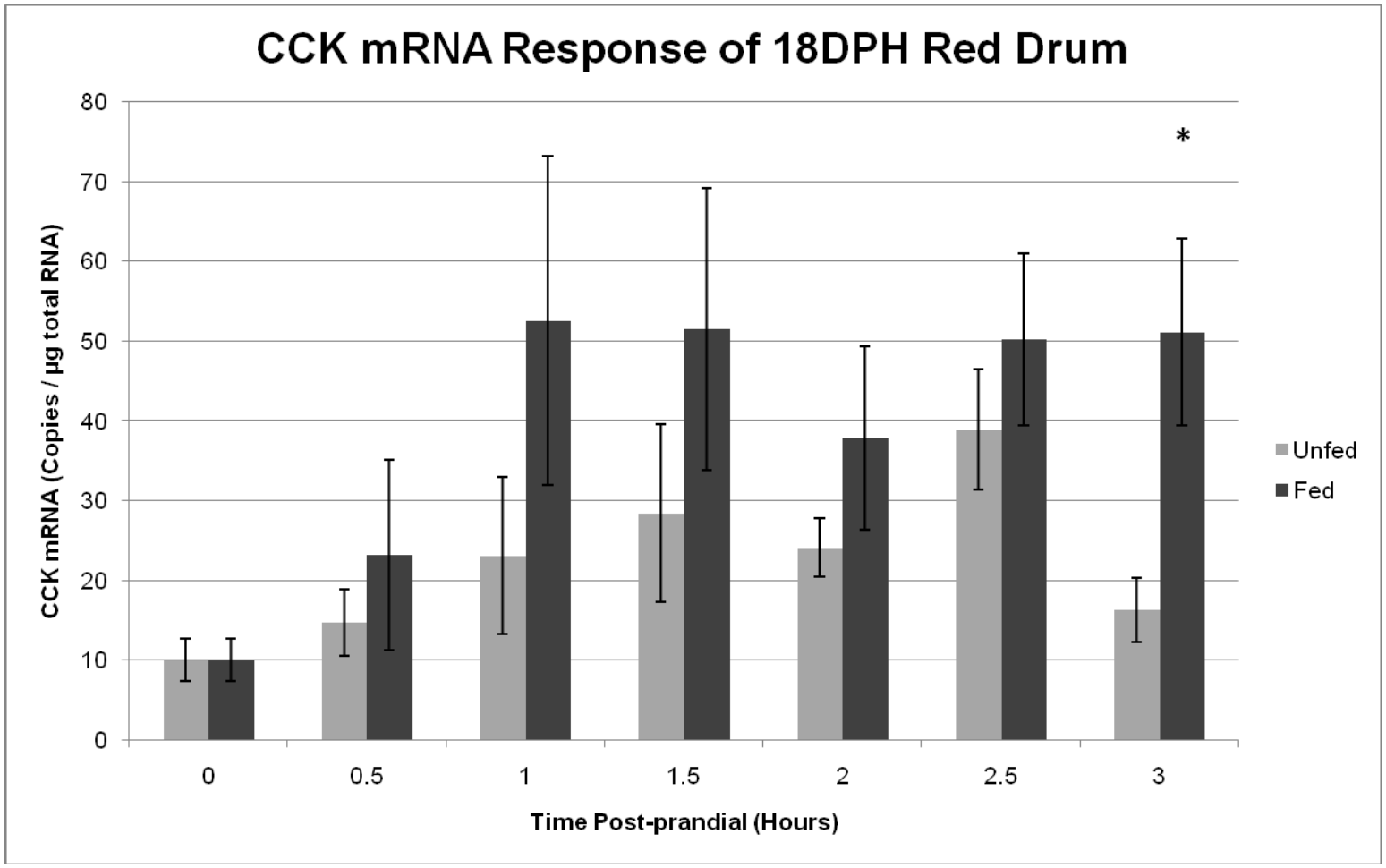
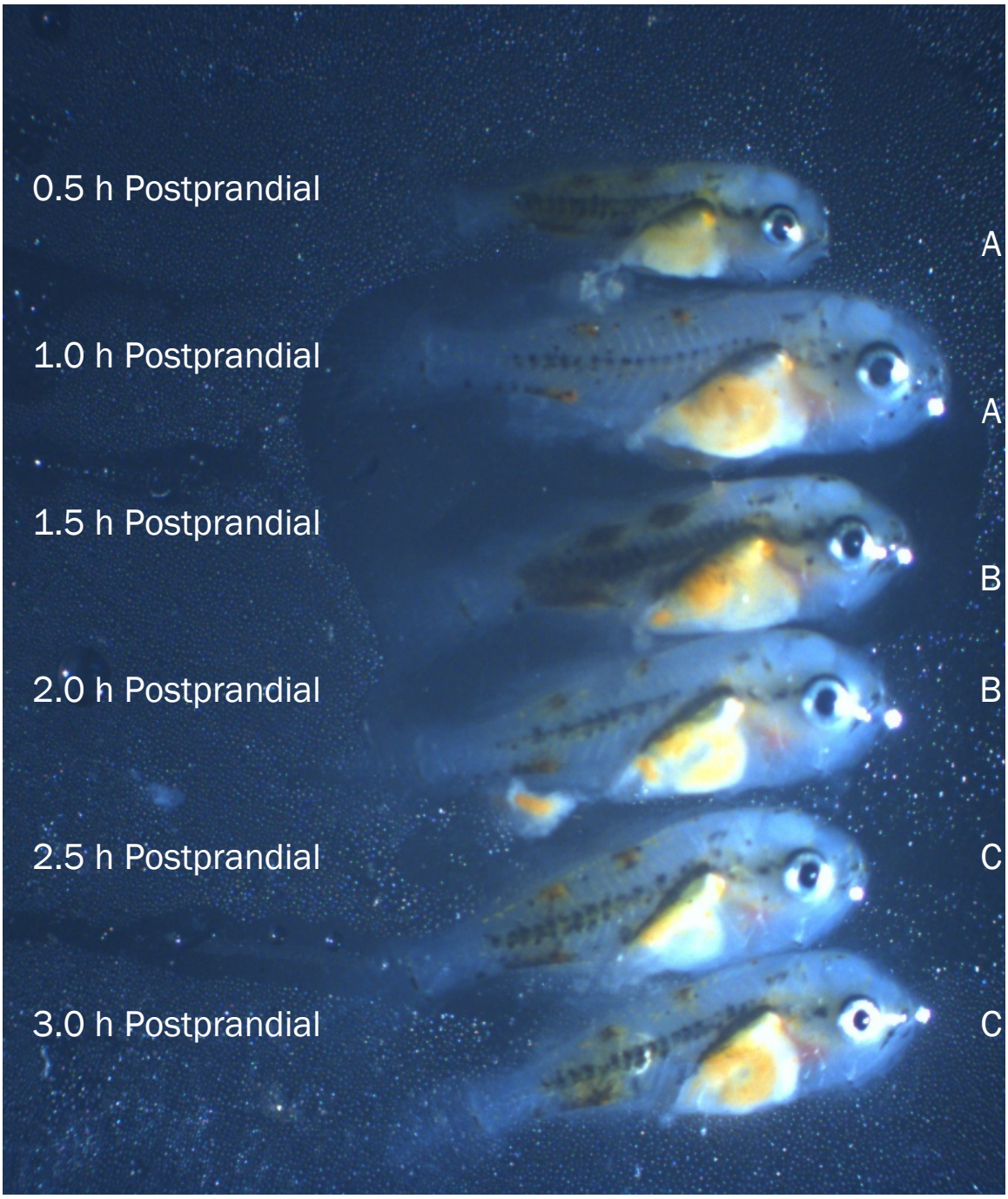

CCK mRNA Response of 18DPH Red Drum
CCK mRNA (Copies / µg total RNA)
80
70
60
50
40
30
20
10
0
*
0
0.5
1
1.5
2
2.5
3
Time Post-prandial (Hours)
Unfed
Fed

Figure 3.12. Representative larvae from each time period showing the presence of *Artemia* in the gut.

In this right-side view of the larvae, the presence of the *Artemia* in the intestine can clearly be seen. A) *Artemia* are visible throughout the gut. B) Small amounts of *Artemia* are still visible before the bend in the intestine, but the largest part of the food bolus has advanced past the bend in the anterior intestine. C) There is no longer any visible *Artemia* before the bend in the intestine though there are still large amounts to be seen after the intestine.


0.5 h Postprandial
A
1.0 h Postprandial
A
1.5 h Postprandial
B
2.0 h Postprandial
B
2.5 h Postprandial
C
3.0 h Postprandial
C

CHAPTER 4: RESPONSES OF LARVAL RED DRUM (*SCIAENOPS OCELLATUS*) TO SOLUBLE COMPONENTS OF ROTIFERS (*BRACHIONUS PLICATILIS*) AND ALGAE (*ISOCHRYSIS GALBANA*)

ABSTRACT

Regulation of pancreatic enzyme secretion by the hormone cholecystokinin (CCK) is important in pancreatic enzyme secretion and may be triggered by soluble components of larval diets. The current study investigated the CCK, trypsin, and CCK mRNA responses in larval red drum (*Sciaenops ocellatus*) at 6 and 10 DPH exposed to treatments containing the soluble fractions of rotifers (*Brachionus plicatilis*), algae (*Isochrysis galbana*), and rotifer culture water. Of these three treatments, only the rotifer treatment produced any significant response and that only in the 6 DPH larvae. At 6 DPH, larvae showed increased CCK, trypsin, and CCK mRNA after two hours of exposure to the rotifer treatment similar to what has previously been seen in postprandial experiments with 18 DPH larvae. This work demonstrates that some soluble component of the rotifers is capable of stimulating the CCK and trypsin responses in larval red drum.

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 unclear even in 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 microparticulate diets. This has proven difficult and while some success has been made (Holt, 1993; Cahu et al., 1998a; Lazo et al., 2000a; Cahu and Infante, 2001), 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 substantially,

at least some of the fish are able to feed on the microparticulate 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 (Cahu et al., 1998a). Growth of the seabass was slower, with fish fed the microparticulate diet taking 1.5 times as long to reach 3.4 mg than fish fed live prey, but the fish did manage to grow. The same type of pattern has been seen in red drum (*Sciaenops ocellatus*) larvae 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 (Lazo et al., 2000a). Both the sea bass and red drum larvae possess functional pancreatic enzymes at first feeding (Lazo et al., 2000b; Cahu et al., 2004) and these are clearly sufficient for at least some of the larvae to grow. This has lead to a investigation of the physiological basis for these differences.

The importance of pancreatic enzyme regulation in first feeding marine larvae is widely recognized, and fish like the red drum depend heavily on pancreatic secretions until the development of a functional stomach (Govoni et al., 1986; Ribeiro et al., 1999; Yufera and Darias, 2007). Research has shown that in terrestrial animals one of the important controls of pancreatic enzyme secretion is the hormone cholecystokinin (CCK), but even among fish CCK is known to cause gall bladder contraction (Rajjo et al., 1988a; Einarsson et al., 1997), regulate gut motility (Olsson and Holmgren, 2001), regulate ingestion (Gelineau and Boujard, 2001), and regulate secretion of pancreatic enzymes (Koven et al., 2002; Rojas-Garcia and

Rønnestad, 2002; Cahu et al., 2004). Due to the relative importance of pancreatic enzymes in early larvae, CCK is likely to be a major factor in their digestive physiology. In previous chapters, it has been shown that red drum begin to develop CCK-IR cells during the first few days after 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. In work with the Atlantic herring (*Clupea harengus*), Koven et al. (2002) demonstrated that when tube fed solutions containing bovine serum albumin (BSA), free amino acids (FAA), or a combination of the two, herring responded with increased trypsin and CCK compared to fish that were fed the saline carrier only. This work suggests that gut fullness alone is not sufficient to produce the CCK and trypsin responses seen following tube feeding proteins and/or amino acids. 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 (Liddle, 1997; Liddle, 2000) 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 (Koven et al., 2002). 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 (Cahu et al., 2004). 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 co-feeding rotifers and/or algae along with microparticulate diets significantly increases growth and survival (Lazo, 1999; Lazo et al., 2000a; Lazo et al., 2002). While the rotifers are actively consumed and are nutritious by themselves, the ability of algae to increase survival and growth is 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 commonly considered chemoattractants in larval feeds such as soluble polypeptides, FAA, and polyamines (Hamana and Niitsu, 2006; Srivastava et al., 2006). These compounds might be present in the water due to lysis of algal cells or the death of rotifers and therefore be available to serve as a stimulant which increases the ability of larval fish to utilize microparticulate diets better when either rotifers or algae are co-fed. Alternatively, these compounds may not be present in the culture water in high concentrations, but ingestion by the larvae may deliver concentrated amounts directly to the sites of action in the intestine. Based on earlier work, it is hypothesized that some factor in the liquid component of live prey items stimulates the digestive tract of larval red drum either directly or indirectly to produce increased amounts of CCK leading to the release of pancreatic enzymes necessary for proper digestion. The present study was therefore designed to test the hypothesis that some liquid component in live

prey or algae increases digestive function in larval red drum through stimulation of endogenous cholecystokinin (CCK) production.

MATERIALS AND METHODS

Fish and Culture Conditions

Three different spawning batches from two different broodstock tanks maintained at the Fisheries and Mariculture Laboratory of the University of Texas Marine Science Institute (FAML) were used during the course of this experiments. Two trials were run (one at 6 and the other at 10 DPH) on each batch of larvae. Eggs were hatched and the larvae reared in 400-L tanks connected to a common biofilter. Water flow from the recirculating filter was kept very low (< 20 L/h) in order to maintain the algae and feed in the tanks. Larvae were fed rotifers (*Brachionus plicatilis*) enriched with Algamac-3050 (Aquafauna Bio-Marine, Hawthorne, CA, USA) from first feeding at 3 DPH through the end of the sampling period at 10 DPH. Rotifers were enumerated four times each day and new rotifers were added to maintain the tank at 5 rotifers/mL as necessary, adjusted so that no rotifers were left in the tanks overnight. In addition, live micro-algae (*Isochrysis galbana*) were added to the tanks once per day at approximately 40,000 - 60,000 cells/ml. During periods when live *Isochrysis* was not available, another algal species (*Nannochloris oculata*) was substituted at approximately 80,000 - 100,000 cells/mL. Salinity in the

recirculating system varied somewhat during the month-long period of collection due to leaks in the recirculating system and the variable salinity of the supply water, averaging 31 ± 3 g/L. The temperature was kept at 27±1°C via ambient heating and the photoperiod was maintained at 14 h light/10 h dark via fluorescent lighting.

Treatments

Three experimental treatments and a control were used for this study. The control (SW) consisted of seawater which was filtered to 5 µm 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 µm mesh. This mesh keeps rotifers on one side of the mesh but allows excretory products of the rotifers to pass 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 µm 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 homogenized using 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.

Experimental Collections

On the day before sampling, 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, 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 raised 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 beakers for 1 h, after which one of the four treatments was added to each beaker and 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.

Sample Analysis

Triplicate samples of each of the treatments (5 mL each) were tested in duplicate for percent nitrogen (percent crude protein) using the Kjeldahl method in accordance with AOAC methods (AOAC, 2002) and for lipid as described by Faulk and Holt (2003).

Because of the small size of these larvae and the large number of fish needed for the assays, all analyses were run on whole larvae and contained both neural and gastrointestinal CCK. Also, after some preliminary tests were run it was clear that the sample of 100 fish per replicate was 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). The CCK, trypsin, and CCK mRNA analyses were performed as previously described (see Chapter 3) except that all analyses were done on fresh tissues instead of lyophilized tissues and methanol extraction was not used to separate CCK and trypsin from the same sample.

Statistical Analysis

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 ± S.E.

RESULTS

Analyses of the treatment samples were unable to detect either protein or lipid in any of the samples. There were visible differences to the eye in the case of two of the samples (a slight brownish tint to the IS and a yellowish tint to the RO), but

nothing that could be measured with the micro-kjeldahl or lipid extraction techniques used.

CCK peptide concentrations (Fig. 4.1 and Fig. 4.2) were extraordinarily low in all fish samples with an overall mean in both treatments of 0.1058 fmol/larva. In the 6 DPH fish, there were significant differences only between the SW control fish (0.085 ± 0.015 fmol/larva) and the fish in the RO treatment (0.153 ± 0.025 fmol/larva). In the 10 DPH larvae, the overall range was approximately the same at 0.067 ± 0.017 fmol/larva in the RC treatment fish to 0.126 ±0.022 fmol/larva in the RO treatment fish but no significant differences were seen between treatments.

The trypsin activity of the 6 DPH fish in the RO treatment was significantly higher than that of fish in all the other treatments. 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 ± 9.02 U/larva and the lowest activity seen in the RC treatment fish at 21.59 ± 7.10 U/larva (Fig. 4.3). 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 between groups (Fig. 4.4).

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 ± 13.93 copies/µg and the lowest number was seen in the SW control fish with 86.61 ± 10.31 copies/µg and these two treatments were the only ones found to be significantly different (Fig. 4.5). In the 10

DPH fish, CCK mRNA was highly variable resulting in no significant differences among treatments which ranged from 24.72 ± 5.82 to 91.52 ± 19.50 copies/µg in the IS and RC treatment fish, respectively (Fig. 4.6).

DISCUSSION

The present study demonstrated that some component of homogenized rotifers elicited CCK, trypsin, and CCK mRNA responses in 6 DPH red drum larvae. Only when rotifers are co-fed with microparticulate diets for at least the first five days of feeding is the growth of the larvae comparable to live prey controls (Holt et al., 1990; Holt, 1993; Lazo et al., 2000a), so it was therefore not surprising that of the three experimental treatments only the RO treatment produced a measurable response. What was surprising however was the lack of a response in 10 DPH fish to the RO treatment. 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 (Lazo, 1999). The number of CCK immunoreactive (CCK-IR) cells in the intestine (Chapter 2) also increases gradually during this time period, but there is no major expansion of CCK-IR cells. 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. What seems more likely is that the lack of significant responses seen in the 10 DPH red drum larvae is

due to a lack of power in the statistical analysis and the need for greater numbers of replicates.

The original working hypothesis for this work was that there exists some ontogenetic change between first feeding at 3 DPH and the period at which co-feeding rotifers can be discontinued (8 - 11 DPH, based on the microparticulate diet type) which allows red drum larvae to utilize microparticulate diets alone. It is possible that such a shift not only removes the need for live prey, but also reduces the stimulatory effect that adding live prey provides to earlier larvae. The results of this study suggest that 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.

Despite the unexpected results in the 10 DPH fish, the present study was able to demonstrate a CCK, trypsin, and CCK mRNA response with 6 DPH larvae in the RO treatment. This suggests that some component of the homogenized rotifer treatment was capable of stimulating a response similar to what was seen in 18 DPH larvae following feeding (Chapter 3). And while the concentration of compounds from the rotifer homogenate was likely much higher than ever experienced by larvae within the water column, the analytical techniques used to measure the nitrogen and lipid content of the treatment samples were insufficient to detect any levels of soluble compounds in the treatments used. This suggests that the larvae have highly

developed sensors for these soluble cues. While there is no direct evidence of any compounds present in the various treatments, it seems likely that the RO treatment contains at least some small amount of smaller peptides and free amino acids (FAA) which constitute a significant fraction of the soluble portion of rotifers (Srivastava et al., 2006) and that the IS treatment might contain some of the common polyamines found in *Isochrysis galbana* such as putrescine, norspermidine, and spermidine (Hamana and Niitsu, 2006). If any of these compounds are present in the RO treatment, they may be enough to elicit the response in that treatment.

In the Atlantic halibut (*Hippoglossus hippoglossus*), levels of amino acids as low as 10^-7 M have been shown to elicit an olfactory response (Yacoob and Browman, 2007). It is also possible that the RO treatment contained enough soluble compounds to directly stimulate the gut after passive ingestion during drinking by the larvae. Trypsin and CCK secretion were both stimulated in the Atlantic herring (*Clupea harengus*) by tube feeding 75 nL suspensions of lamellar liposomes containing small amounts of BSA, FAA, or both (Koven et al., 2002). These tube fed doses are quite high given that they represent roughly 2/3 the volume of the herrings gut and are likely much higher than anything obtained passively in the present study, but it demonstrates how a very small total amount could produce an effect.

Unlike the postprandial experiment with 18 DPH larvae (Chapter 3) however, the current experiment included the heads as well as the bodies of the larvae which makes interpretation of the results more difficult. Differences in CCK and CCK mRNA levels could represent either neural or gastrointestinal responses or both. The

differences in measured CCK and CCK mRNA are likely to be quite large; in Atlantic halibut larvae the level of CCK content in a whole individual was roughly 10X greater than the level in the excised gut alone (Rojas-Garcia and Rønnestad, 2002). Neural CCK might play a substantial role in the responses seen in the present study since neural stimulation (sham feeding) in dogs and humans have both been shown to cause significant rises in plasma CCK levels (Konturek et al., 2004). In goldfish (*Carassius auratus*), significant levels of CCK binding has been detected in the optic tectum which receives sensory information from both the eye and the olfactory bulb (Himick et al., 1996) which could have been stimulated by the soluble components in the present study.

While there is still much to learn about CCK in red drum, the results of the current study add significant pieces to the puzzle. It has been proposed that there exists in fish a similar secretory signalling mechanism as has been proposed in mammals (Liddle, 1994b; Schmidt et al., 1994; Liddle, 1995; Liddle, 1997; Liddle, 2000; Rønnestad, 2002). By this mechanism, a trypsin sensitive factor capable of stimulating the secretion of CCK (CCK releasing factor, CCK-RF) is secreted into the lumen of the intestine in response to some stimulus. In the absence of trypsin, this factor stimulates the secretion of CCK which then stimulates the secretion of pancreatic enzymes including trypsin. In the presence of food, the CCK-RF is protected from degradation since the food outcompetes the CCK-RF for the substrate. In humans, CCK-RF release is stimulated by nutrients such as the digestive byproducts of fats and proteins while in rats the release of CCK-RF appears

to be spontaneous (Liddle, 2000). Results from Chapter 3 and from work with the Atlantic herring (Koven et al., 2002) suggest that if red drum larvae do possess a CCK-RF, then it might also be stimulated by feeding since CCK responses were seen following feeding in both species. It is very difficult however to exclude the possibility that there may be some neural stimulus regulating the secretion of CCK in red drum, particularly in light of the present study. There is currently no way to completely separate the individual tissues and plasma in larvae (Rønnestad et al., 2007) so new methodologies must be considered if we are to understand the mechanism by which CCK secretion is induced.

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. While it was necessary to pool the samples in order to utilize the analytical methods available, these tools were still able to detect differences at 6 DPH. The exact mechanism by which the RO treatment stimulates CCK, trypsin, and CCK mRNA responses is unclear, but the fact that it was the RO treatment and not any other which produced these responses is interesting. 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.

Figure 4.1. CCK peptide response of 6 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. Results were considered significant at $\alpha \leq 0.05$. Error bars show standard error.

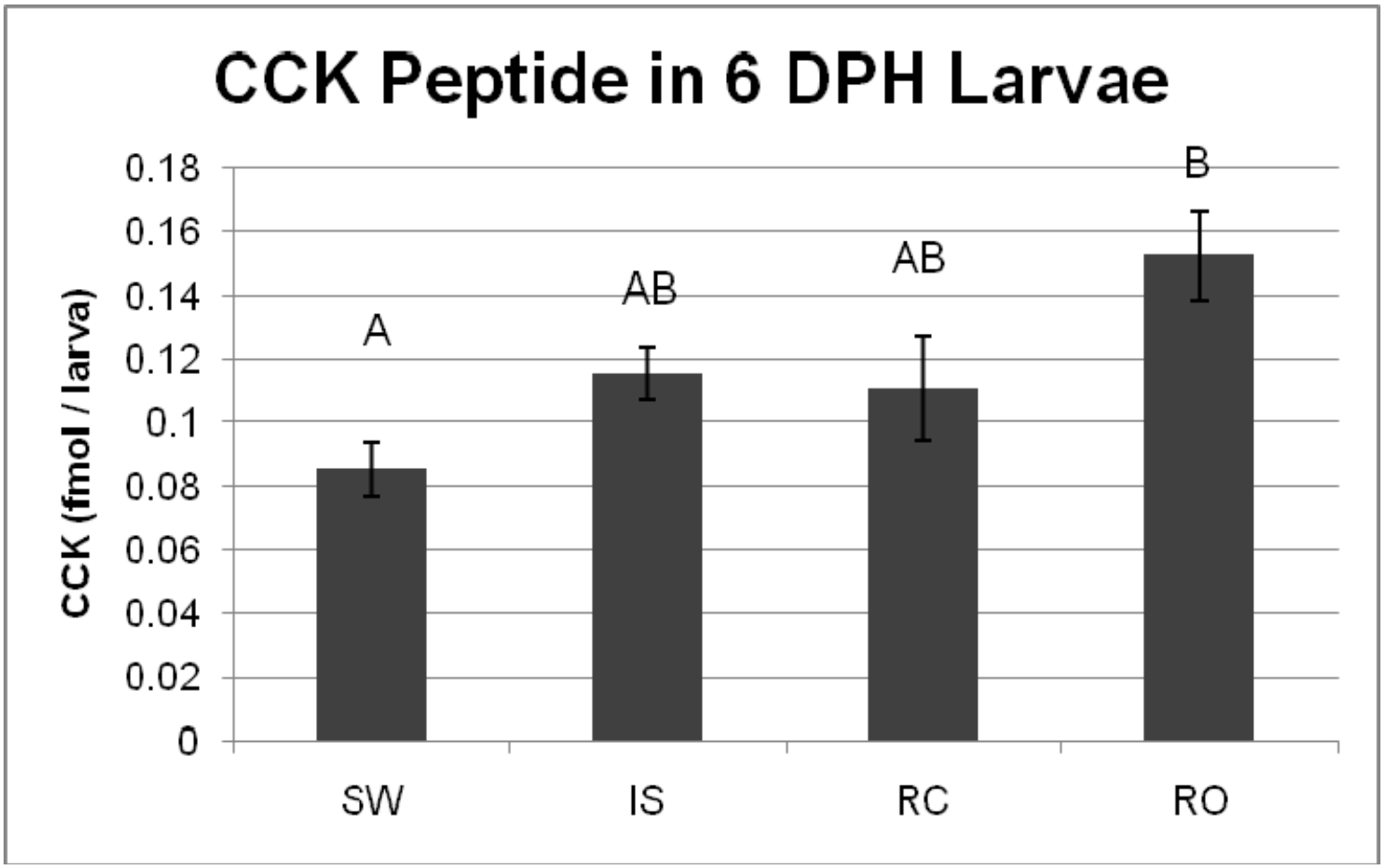

CCK Peptide in 6 DPH Larvae
CCK (fmol / larva)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
A
AB
AB
B
SW
IS
RC
RO

Figure 4.2. CCK response of 10 DPH red drum larvae.

SW = Saltwater control, IS = *Isochrysis galbana* homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. No significant differences were noted at $\alpha \leq 0.05$. Error bars show standard error.

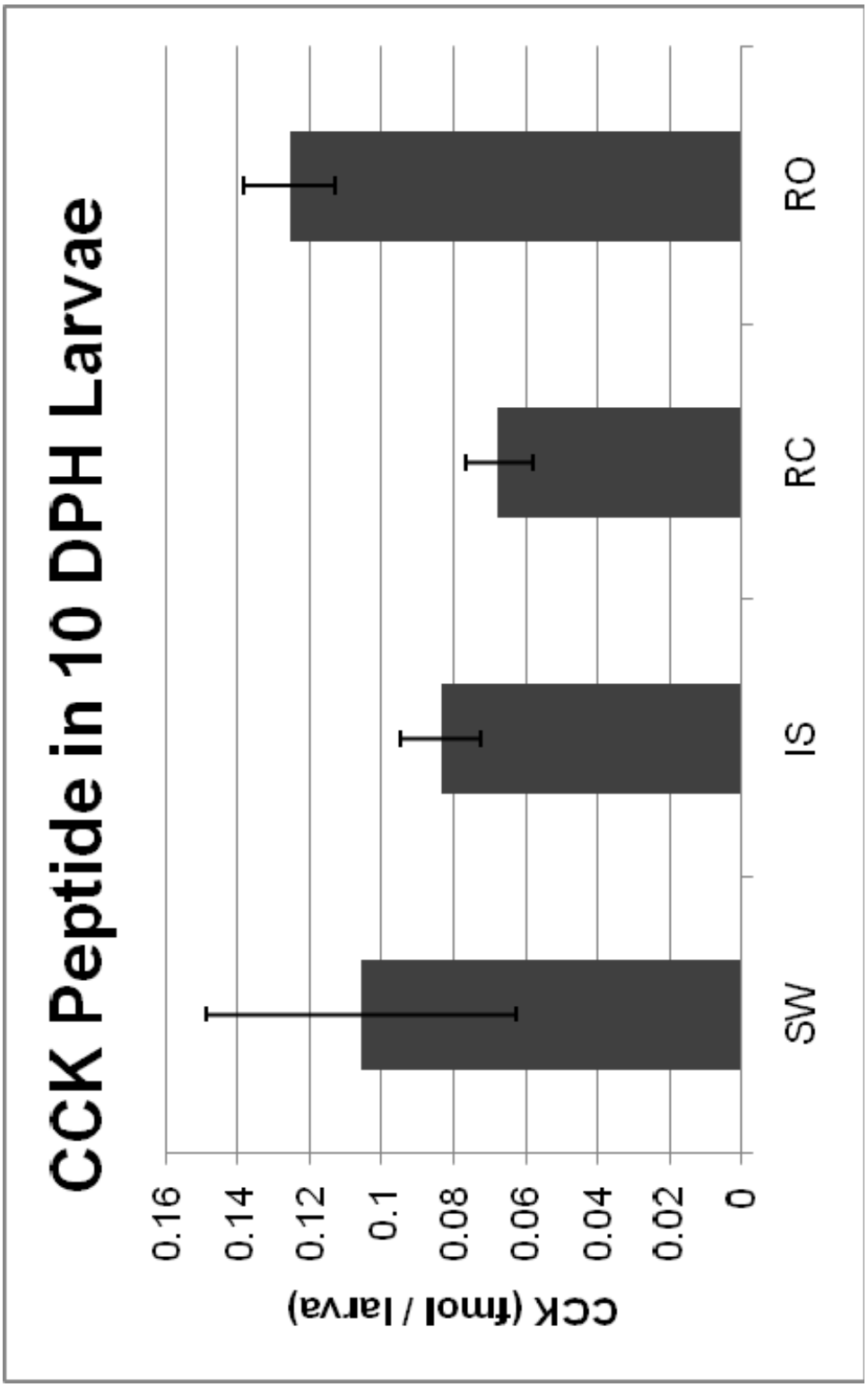

CCK Peptide in 10 DPH Larvae
CCK (fmol / larva)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
SW
IS
RC
RO

Figure 4.3. Trypsin response of 6 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. Results were considered significant at $\alpha \leq 0.05$. Error bars show standard error.

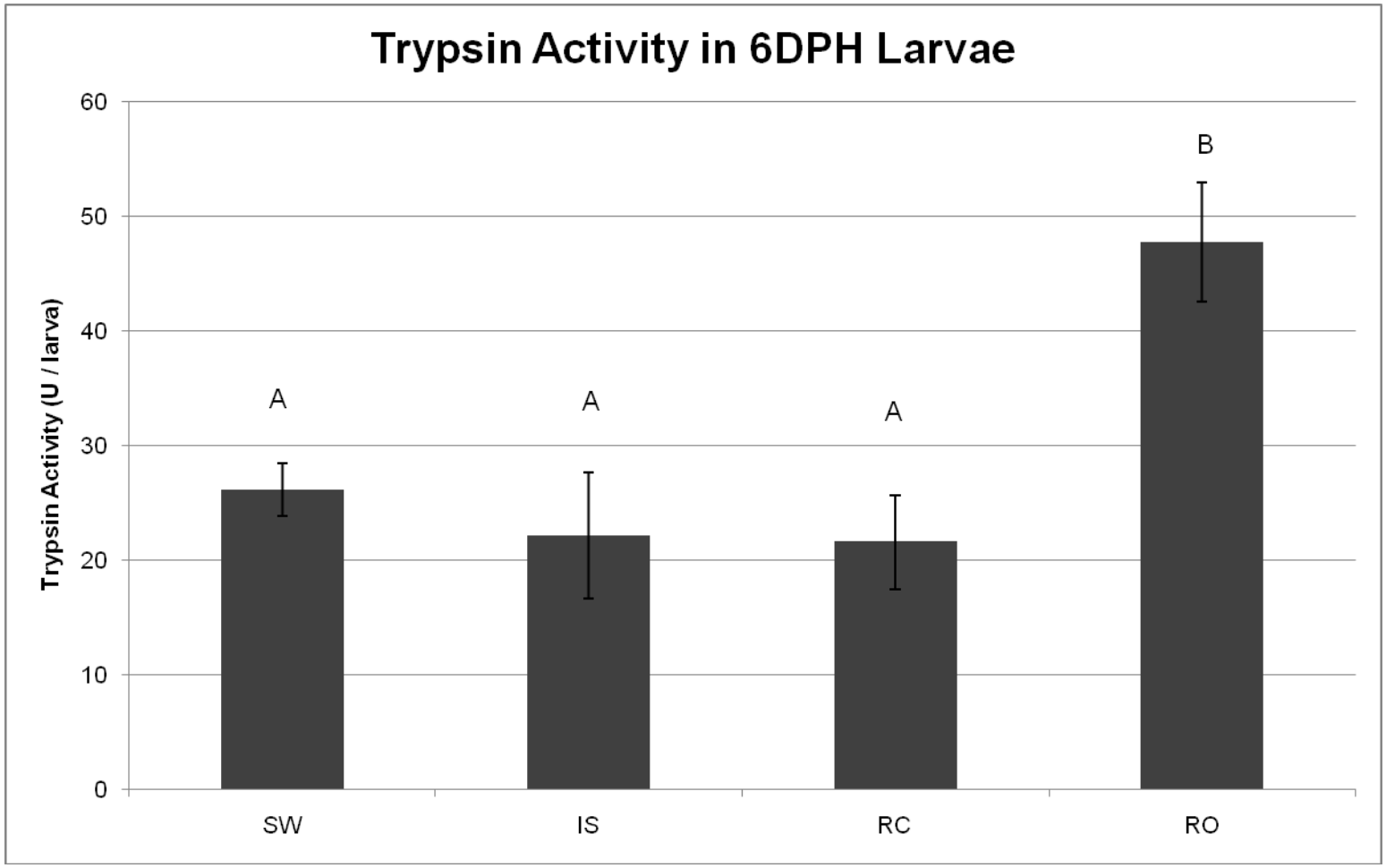

Trypsin Activity in 6DPH Larvae
Trypsin Activity (U / larva)
60
50
40
30
20
10
0
A
A
A
B
SW
IS
RC
RO

Figure 4.4. Trypsin response of 10 DPH red drum larvae.

SW = Saltwater control, IS = *Isochrysis galbana* homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. No significant differences were noted at $\alpha \leq 0.05$. Error bars show standard error.

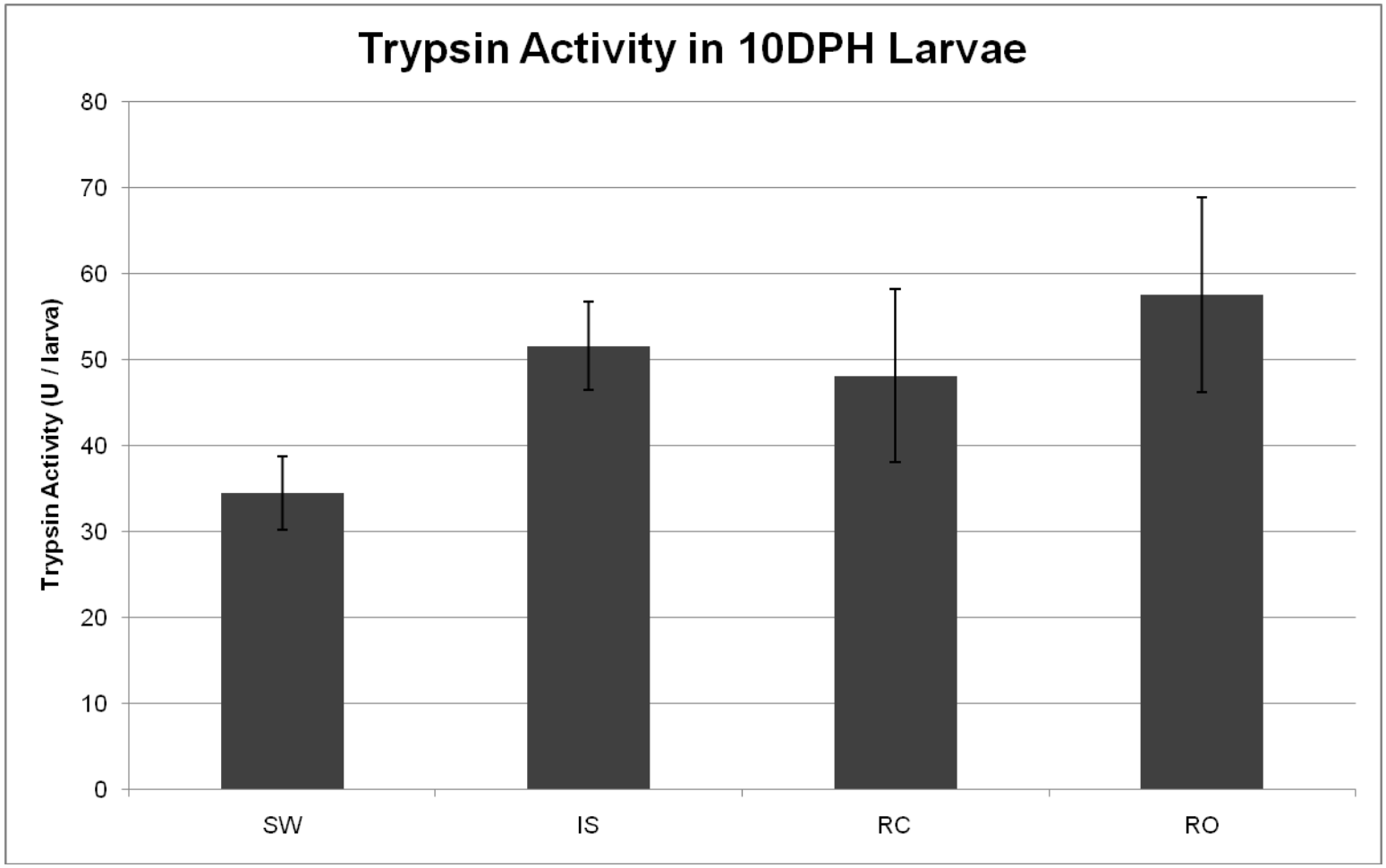

Trypsin Activity in 10DPH Larvae
Trypsin Activity (U / larva)
80
70
60
50
40
30
20
10
0
SW
IS
RC
RO

Figure 4.5. CCK mRNA response of 6 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. Results were considered significant at $\alpha \leq 0.05$. Error bars show standard error.

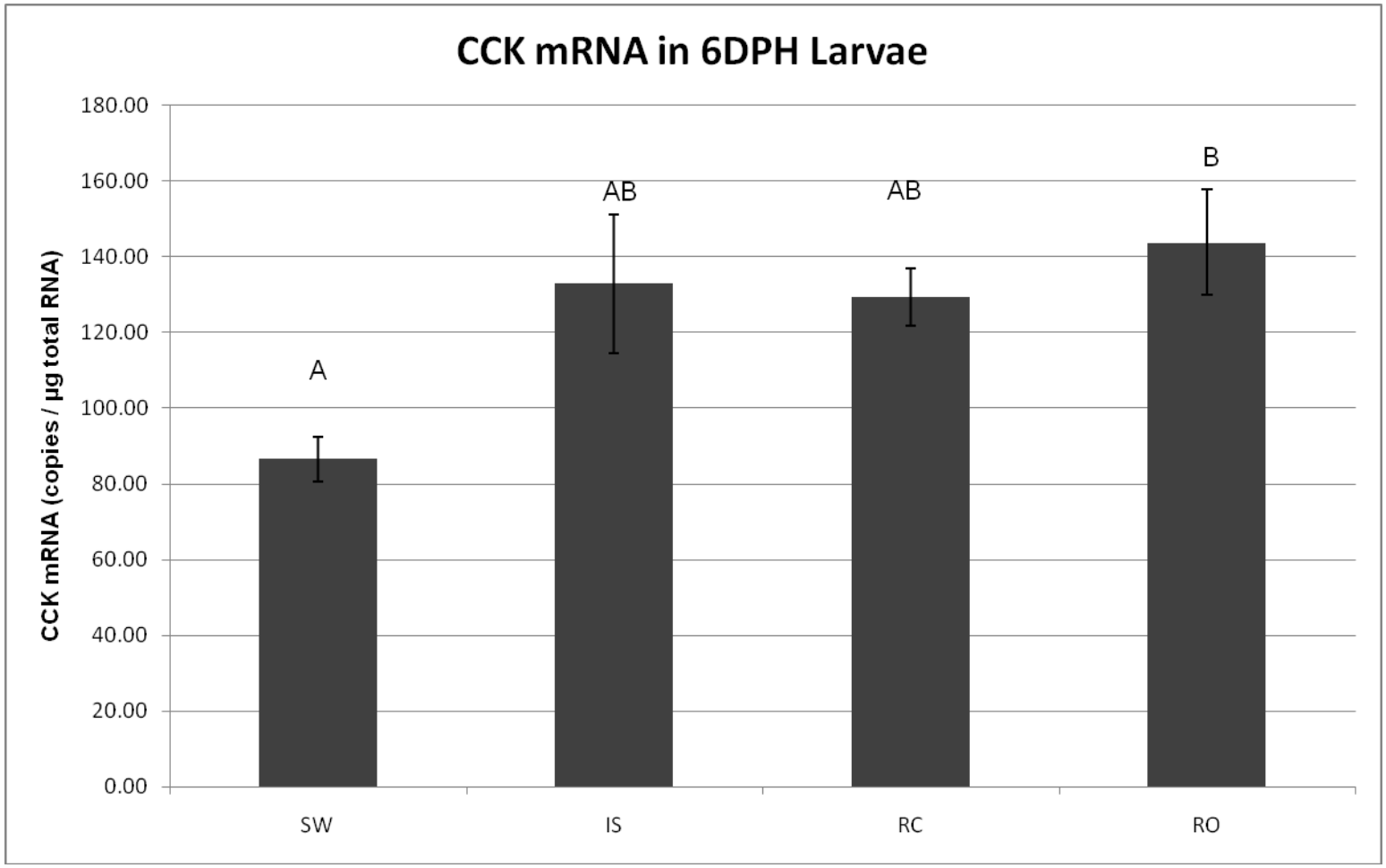
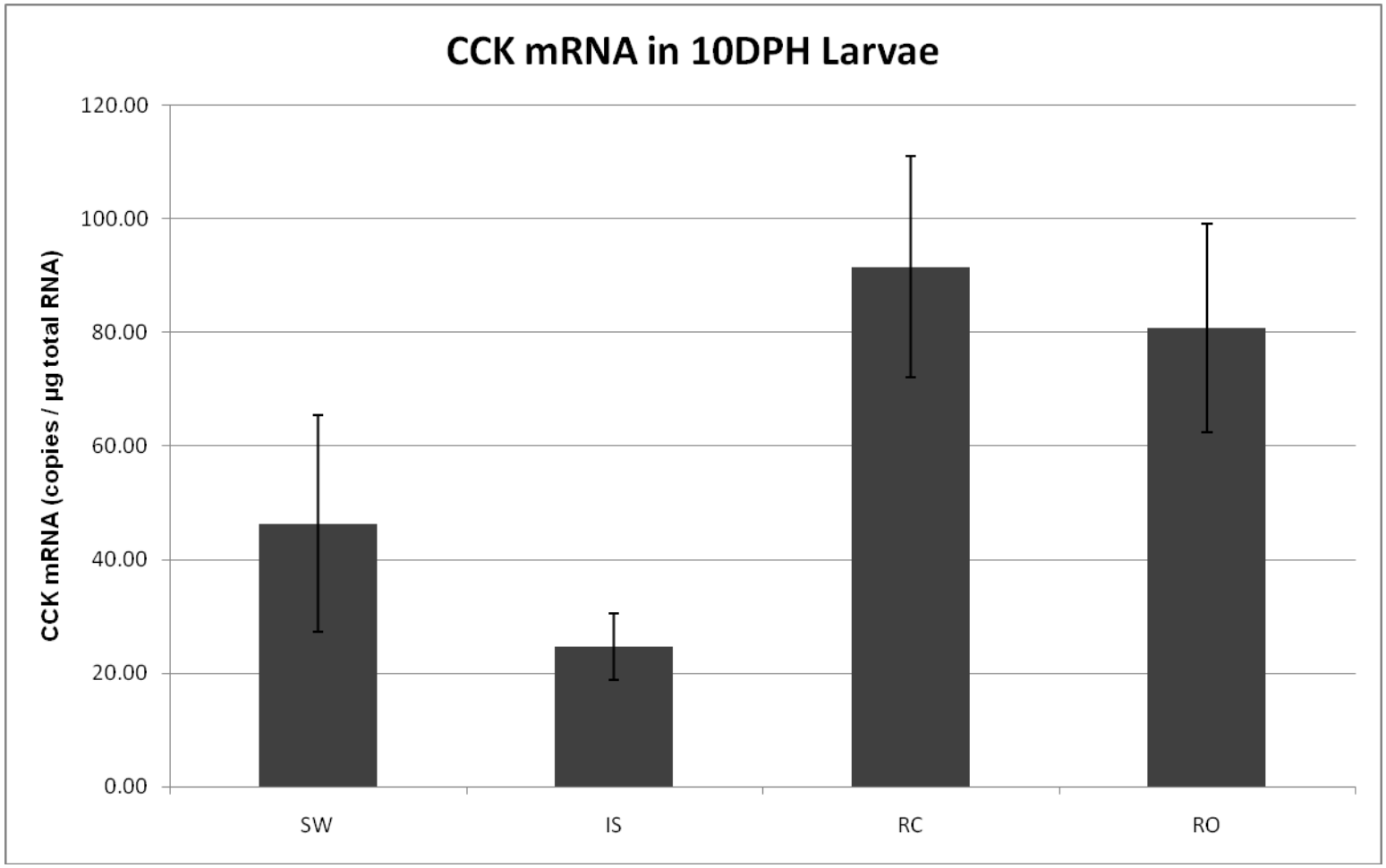

CCK mRNA in 6DPH Larvae
CCK mRNA (copies / µg total RNA)
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
A
AB
AB
B
SW
IS
RC
RO

Figure 4.6. CCK mRNA response of 10 DPH red drum larvae.

SW = Saltwater control, IS = *Isochrysis galbana* homogenate, RC = Rotifer culture water, and RO = Rotifer homogenate. No significant differences were noted at $\alpha \leq 0.05$. Error bars show standard error.


CCK mRNA in 10DPH Larvae
CCK mRNA (copies / µg total RNA)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
SW
IS
RC
RO

CHAPTER 5: GENERAL CONCLUSIONS

This work was undertaken to test the general hypothesis that some component in live prey or algae increases digestive function in larval red drum through stimulation of endogenous cholecystokinin (CCK) production. This is of interest since the inability of larval red drum (*Sciaenops ocellatus*) to be reared on microparticulate diets alone appears to be limited by the regulation of digestive function rather than by a functional insufficiency in the larvae. In order to test this hypothesis, three questions were addressed.

First, it was necessary to determine if CCK was present in larval red drum at first feeding. If CCK is capable of affecting the digestive process and modulating the ability of red drum larvae to utilize microparticulate diets, then it must be present at the time in which red drum become capable of utilizing microparticulate diets without any live prey or algae present. In order to test this, red drum CCK was first cloned and sequenced, demonstrating that red drum do possess CCK. Tissue expression of CCK mRNA was also investigated, revealing a pattern consistent with the distribution seen in other animals. This suggests that CCK possesses the same neural and gastrointestinal functions and is capable of regulating pancreatic enzyme secretions. Finally, the expression of CCK immunoreactive cells (CCK-IR) in the gut of red drum larvae was examined throughout the period of development from hatching through development of a functional stomach. The presence, however limited, of CCK-IR cells in larvae at first feeding suggests CCK is being produced at this time and may be

capable of regulating pancreatic secretions. Also, the gradual increase in CCK-IR cells during the period between first feeding at 3 days post hatch (DPH) and the time when red drum larvae can be weaned to microparticulate diets (8 - 11 DPH) indicates that the ability of CCK to regulate digestive function would develop gradually over time rather than precipitously. This matches the pattern seen in weaning experiments with red drum larvae. These results demonstrate CCK is present in all fish within three days from the onset of exogenous feeding in red drum larvae and the distribution is consistent with a role as a regulator of pancreatic enzyme secretion.

Second, it was necessary to determine if CCK in red drum was capable of being induced following a feeding event. If endogenous CCK is responsible for the up-regulation of pancreatic secretion, then a meal should increase both CCK and pancreatic enzyme secretion in the larvae. In order to test this, juveniles and larvae were allowed to feed for fifteen minutes and then sampled over the next three hours postprandial. In addition to CCK levels, trypsin was measured as an indicator of pancreatic enzyme secretion and CCK mRNA was measured as an indicator of CCK gene expression. Results of this study showed that feeding is capable of inducing a CCK and trypsin response in both juveniles and larvae within the first hour postprandial. What is more, gene expression of CCK was also seen to be increased in both larvae and juveniles within two and a half hours following this feeding event. These results demonstrate that endogenous CCK is capable of being induced by a

feeding event and that this CCK increase is correlated with an increase in pancreatic enzyme secretions.

Thirdly, it was necessary to determine if some component of rotifers or algae is capable of inducing an endogenous CCK response independent of feeding. If rotifers or algae are able to up-regulate the ability of larvae to utilize microparticulate diets through endogenous CCK rather than simply providing a nutritional crutch until development reaches a point at which the fish can utilize the diet, then some component of these treatments must be capable of inducing CCK secretion. In order to test this, the fractions of homogenized rotifers and *Isochrysis* capable of passing a 0.45 µm membrane filter were introduced to unfed larvae. After two hours, the CCK and trypsin responses of the larvae were measured. Results of this study show that the filtrate of rotifers was capable of inducing CCK and trypsin secretion in larval red drum without ingestion. In addition, the rotifer treatment was also shown to up-regulate CCK gene expression. These results demonstrate that some component of rotifers is capable of inducing endogenous CCK although the pathway of this induction is not yet known.

The results of these three investigations demonstrate that some component of rotifers is capable of inducing endogenous CCK and that this does lead to increased pancreatic enzyme secretion. This supports the original hypothesis and suggests that co-feeding with live prey provides a stimulus necessary for proper digestion which may not be present in microparticulate diets. While the work presented here does not conclusively establish that it is possible to rear red drum

larvae on microparticulate diets alone if some soluble stimulus is provided along with the diet, this work does establish that CCK is present in red drum larvae and is responsive to components of live prey.

APPENDIX

ANOVA TABLES

Chapter 3

Juveniles:

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	988.235[a]	1	988.235	2.090	.186
Intercept	6428.760	1	6428.760	13.598	.006
Treat	988.235	1	988.235	2.090	.186
Error	3782.287	8	472.786		
Total	11199.282	10			
Corrected Total	4770.522	9			

a. R Squared = .207 (Adjusted R Squared = .108)

b. Time Period = .00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	753398.194[a]	1	753398.194	13.988	.006
Intercept	872475.721	1	872475.721	16.199	.004
Treat	753398.194	1	753398.194	13.988	.006
Error	430872.814	8	53859.102		
Total	2056746.729	10			
Corrected Total	1184271.008	9			

a. R Squared = .636 (Adjusted R Squared = .591)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	35608.669[a]	1	35608.669	18.599	.003
Intercept	53212.648	1	53212.648	27.793	.001
Treat	35608.669	1	35608.669	18.599	.003
Error	15316.787	8	1914.598		
Total	104138.104	10			
Corrected Total	50925.456	9			

a. R Squared = .699 (Adjusted R Squared = .662)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	543192.942[a]	1	543192.942	16.608	.004
Intercept	649877.753	1	649877.753	19.870	.002
Treat	543192.942	1	543192.942	16.608	.004
Error	261650.737	8	32706.342		
Total	1454721.433	10			
Corrected Total	804843.679	9			

a. R Squared = .675 (Adjusted R Squared = .634)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	20355.437[a]	1	20355.437	27.584	.001
Intercept	37845.874	1	37845.874	51.286	.000
Treat	20355.437	1	20355.437	27.584	.001
Error	5903.474	8	737.934		
Total	64104.784	10			
Corrected Total	26258.911	9			

a. R Squared = .775 (Adjusted R Squared = .747)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	441436.908[a]	1	441436.908	2.525	.151
Intercept	505665.169	1	505665.169	2.892	.127
Treat	441436.908	1	441436.908	2.525	.151
Error	1398641.974	8	174830.247		
Total	2345744.051	10			
Corrected Total	1840078.882	9			

a. R Squared = .240 (Adjusted R Squared = .145)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	52617.614[a]	1	52617.614	8.662	.019
Intercept	119644.219	1	119644.219	19.697	.002
Treat	52617.614	1	52617.614	8.662	.019
Error	48593.744	8	6074.218		
Total	220855.578	10			
Corrected Total	101211.358	9			

a. R Squared = .520 (Adjusted R Squared = .460)

b. Time Period = 3.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	765.625[a]	1	765.625	4.705	.062
Intercept	4695.022	1	4695.022	28.853	.001
Treat	765.625	1	765.625	4.705	.062
Error	1301.774	8	162.722		
Total	6762.421	10			
Corrected Total	2067.399	9			

a. R Squared = .370 (Adjusted R Squared = .292)

b. Time Period = .00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2054.062[a]	1	2054.062	4.571	.065
Intercept	14412.653	1	14412.653	32.071	.000
Treat	2054.062	1	2054.062	4.571	.065
Error	3595.141	8	449.393		
Total	20061.856	10			
Corrected Total	5649.203	9			

a. R Squared = .364 (Adjusted R Squared = .284)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17022.226[a]	1	17022.226	11.196	.010
Intercept	49465.902	1	49465.902	32.535	.000
Treat	17022.226	1	17022.226	11.196	.010
Error	12163.161	8	1520.395		
Total	78651.289	10			
Corrected Total	29185.386	9			

a. R Squared = .583 (Adjusted R Squared = .531)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4185.707[a]	1	4185.707	5.308	.050
Intercept	22360.604	1	22360.604	28.355	.001
Treat	4185.707	1	4185.707	5.308	.050
Error	6308.797	8	788.600		
Total	32855.108	10			
Corrected Total	10494.504	9			

a. R Squared = .399 (Adjusted R Squared = .324)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	25973.293[a]	1	25973.293	6.753	.032
Intercept	43943.641	1	43943.641	11.426	.010
Treat	25973.293	1	25973.293	6.753	.032
Error	30767.502	8	3845.938		
Total	100684.436	10			
Corrected Total	56740.795	9			

a. R Squared = .458 (Adjusted R Squared = .390)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2678.460[a]	1	2678.460	12.497	.008
Intercept	6791.236	1	6791.236	31.686	.000
Treat	2678.460	1	2678.460	12.497	.008
Error	1714.624	8	214.328		
Total	11184.319	10			
Corrected Total	4393.083	9			

a. R Squared = .610 (Adjusted R Squared = .561)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6070.310[a]	1	6070.310	9.208	.016
Intercept	12574.116	1	12574.116	19.074	.002
Treat	6070.310	1	6070.310	9.208	.016
Error	5273.788	8	659.223		
Total	23918.214	10			
Corrected Total	11344.098	9			

a. R Squared = .535 (Adjusted R Squared = .477)

b. Time Period = 3.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	527363.667[a]	1	527363.667	5.495	.047
Intercept	1907837.569	1	1907837.569	19.880	.002
Treat	527363.667	1	527363.667	5.495	.047
Error	767727.106	8	95965.888		
Total	3202928.343	10			
Corrected Total	1295090.773	9			

a. R Squared = .407 (Adjusted R Squared = .333)

b. Time Period = .00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	283915.760[a]	1	283915.760	4.293	.072
Intercept	2005803.710	1	2005803.710	30.329	.001
Treat	283915.760	1	283915.760	4.293	.072
Error	529078.497	8	66134.812		
Total	2818797.967	10			
Corrected Total	812994.257	9			

a. R Squared = .349 (Adjusted R Squared = .268)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7356.029[a]	1	7356.029	.044	.839
Intercept	2695354.122	1	2695354.122	16.084	.004
Treat	7356.029	1	7356.029	.044	.839
Error	1340633.806	8	167579.226		
Total	4043343.957	10			
Corrected Total	1347989.834	9			

a. R Squared = .005 (Adjusted R Squared = -.119)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	960764.414[a]	1	960764.414	.207	.661
Intercept	2.333E7	1	2.333E7	5.036	.055
Treat	960764.414	1	960764.414	.207	.661
Error	3.706E7	8	4632438.117		
Total	6.135E7	10			
Corrected Total	3.802E7	9			

a. R Squared = .025 (Adjusted R Squared = -.097)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	59606.120[a]	1	59606.120	1.457	.262
Intercept	1081074.672	1	1081074.672	26.426	.001
Treat	59606.120	1	59606.120	1.457	.262
Error	327276.345	8	40909.543		
Total	1467957.137	10			
Corrected Total	386882.465	9			

a. R Squared = .154 (Adjusted R Squared = .048)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.964E6[a]	1	1963845.814	5.678	.044
Intercept	6052326.531	1	6052326.531	17.500	.003
Treat	1963845.814	1	1963845.814	5.678	.044
Error	2766755.042	8	345844.380		
Total	1.078E7	10			
Corrected Total	4730600.856	9			

a. R Squared = .415 (Adjusted R Squared = .342)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.635E7[a]	1	6.635E7	9.929	.014
Intercept	8.764E7	1	8.764E7	13.114	.007
Treat	6.635E7	1	6.635E7	9.929	.014
Error	5.346E7	8	6682816.991		
Total	2.075E8	10			
Corrected Total	1.198E8	9			

a. R Squared = .554 (Adjusted R Squared = .498)

b. Time Period = 3.00

Larvae

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.932[a]	1	1.932	14.229	.005
Intercept	3.811	1	3.811	28.071	.001
Treat	1.932	1	1.932	14.229	.005
Error	1.086	8	.136		
Total	6.828	10			
Corrected Total	3.018	9			

a. R Squared = .640 (Adjusted R Squared = .595)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.628[a]	1	2.628	34.438	.000
Intercept	8.416	1	8.416	110.306	.000
Treat	2.628	1	2.628	34.438	.000
Error	.610	8	.076		
Total	11.654	10			
Corrected Total	3.238	9			

a. R Squared = .811 (Adjusted R Squared = .788)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.449[a]	1	3.449	6.176	.038
Intercept	7.746	1	7.746	13.869	.006
Treat	3.449	1	3.449	6.176	.038
Error	4.468	8	.559		
Total	15.663	10			
Corrected Total	7.917	9			

a. R Squared = .436 (Adjusted R Squared = .365)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.009[a]	1	.009	.071	.797
Intercept	4.544	1	4.544	36.914	.000
Treat	.009	1	.009	.071	.797
Error	.985	8	.123		
Total	5.538	10			
Corrected Total	.994	9			

a. R Squared = .009 (Adjusted R Squared = -.115)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.316[a]	1	.316	2.738	.137
Intercept	5.553	1	5.553	48.096	.000
Treat	.316	1	.316	2.738	.137
Error	.924	8	.115		
Total	6.793	10			
Corrected Total	1.240	9			

a. R Squared = .255 (Adjusted R Squared = .162)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK Peptide

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.178[a]	1	.178	2.286	.169
Intercept	1.299	1	1.299	16.687	.004
Treat	.178	1	.178	2.286	.169
Error	.623	8	.078		
Total	2.100	10			
Corrected Total	.801	9			

a. R Squared = .222 (Adjusted R Squared = .125)

b. Time Period = 3.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	145.237[a]	1	145.237	3.658	.092
Intercept	2005.906	1	2005.906	50.520	.000
Treat	145.237	1	145.237	3.658	.092
Error	317.642	8	39.705		
Total	2468.785	10			
Corrected Total	462.879	9			

a. R Squared = .314 (Adjusted R Squared = .228)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	175.645[a]	1	175.645	15.622	.004
Intercept	2193.065	1	2193.065	195.052	.000
Treat	175.645	1	175.645	15.622	.004
Error	89.948	8	11.244		
Total	2458.658	10			
Corrected Total	265.593	9			

a. R Squared = .661 (Adjusted R Squared = .619)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2260.813[a]	1	2260.813	5.446	.048
Intercept	7856.809	1	7856.809	18.927	.002
Treat	2260.813	1	2260.813	5.446	.048
Error	3320.846	8	415.106		
Total	13438.468	10			
Corrected Total	5581.659	9			

a. R Squared = .405 (Adjusted R Squared = .331)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1358.157[a]	1	1358.157	3.424	.101
Intercept	4770.730	1	4770.730	12.027	.008
Treat	1358.157	1	1358.157	3.424	.101
Error	3173.412	8	396.676		
Total	9302.299	10			
Corrected Total	4531.569	9			

a. R Squared = .300 (Adjusted R Squared = .212)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.411[a]	1	3.411	.039	.848
Intercept	2336.618	1	2336.618	26.989	.001
Treat	3.411	1	3.411	.039	.848
Error	692.614	8	86.577		
Total	3032.642	10			
Corrected Total	696.024	9			

a. R Squared = .005 (Adjusted R Squared = -.119)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.570[a]	1	4.570	.230	.644
Intercept	851.929	1	851.929	42.922	.000
Treat	4.570	1	4.570	.230	.644
Error	158.787	8	19.848		
Total	1015.286	10			
Corrected Total	163.357	9			

a. R Squared = .028 (Adjusted R Squared = -.094)

b. Time Period = 3.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	181.135[a]	1	181.135	.456	.518
Intercept	3613.801	1	3613.801	9.100	.017
Treat	181.135	1	181.135	.456	.518
Error	3176.974	8	397.122		
Total	6971.910	10			
Corrected Total	3358.109	9			

a. R Squared = .054 (Adjusted R Squared = -.064)

b. Time Period = .50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2165.607[a]	1	2165.607	1.656	.234
Intercept	14338.340	1	14338.340	10.963	.011
Treat	2165.607	1	2165.607	1.656	.234
Error	10462.698	8	1307.837		
Total	26966.644	10			
Corrected Total	12628.305	9			

a. R Squared = .171 (Adjusted R Squared = .068)

b. Time Period = 1.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1330.332[a]	1	1330.332	1.219	.302
Intercept	15993.601	1	15993.601	14.649	.005
Treat	1330.332	1	1330.332	1.219	.302
Error	8734.035	8	1091.754		
Total	26057.968	10			
Corrected Total	10064.367	9			

a. R Squared = .132 (Adjusted R Squared = .024)

b. Time Period = 1.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	471.145[a]	1	471.145	1.295	.288
Intercept	9623.645	1	9623.645	26.449	.001
Treat	471.145	1	471.145	1.295	.288
Error	2910.804	8	363.850		
Total	13005.593	10			
Corrected Total	3381.949	9			

a. R Squared = .139 (Adjusted R Squared = .032)

b. Time Period = 2.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	317.983[a]	1	317.983	.738	.415
Intercept	19897.844	1	19897.844	46.187	.000
Treat	317.983	1	317.983	.738	.415
Error	3446.460	8	430.808		
Total	23662.288	10			
Corrected Total	3764.443	9			

a. R Squared = .084 (Adjusted R Squared = -.030)

b. Time Period = 2.50

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA copies

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3028.296[a]	1	3028.296	7.890	.023
Intercept	11374.431	1	11374.431	29.635	.001
Treat	3028.296	1	3028.296	7.890	.023
Error	3070.510	8	383.814		
Total	17473.237	10			
Corrected Total	6098.806	9			

a. R Squared = .497 (Adjusted R Squared = .434)

b. Time Period = 3.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.007[a]	3	.002	5.166	.028
Intercept	.161	1	.161	358.979	.000
Treat	.007	3	.002	5.166	.028
Error	.004	8	.000		
Total	.172	12			
Corrected Total	.011	11			

a. R Squared = .660 (Adjusted R Squared = .532)

b. DPH = 6.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.006[a]	3	.002	1.172	.379
Intercept	.110	1	.110	65.446	.000
Treat	.006	3	.002	1.172	.379
Error	.013	8	.002		
Total	.129	12			
Corrected Total	.019	11			

a. R Squared = .305 (Adjusted R Squared = .045)

b. DPH = 10.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1379.859[a]	3	459.953	7.754	.009
Intercept	10374.726	1	10374.726	174.894	.000
Treat	1379.859	3	459.953	7.754	.009
Error	474.561	8	59.320		
Total	12229.145	12			
Corrected Total	1854.420	11			

a. R Squared = .744 (Adjusted R Squared = .648)

b. DPH = 6.00

Tests of Between-Subjects Effects[b]

Dependent Variable:Trypsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	869.813[a]	3	289.938	1.414	.308
Intercept	27661.092	1	27661.092	134.863	.000
Treat	869.813	3	289.938	1.414	.308
Error	1640.840	8	205.105		
Total	30171.746	12			
Corrected Total	2510.653	11			

a. R Squared = .346 (Adjusted R Squared = .101)

b. DPH = 10.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5676.216[a]	3	1892.072	4.070	.050
Intercept	181939.944	1	181939.944	391.380	.000
Treat	5676.216	3	1892.072	4.070	.050
Error	3718.947	8	464.868		
Total	191335.107	12			
Corrected Total	9395.163	11			

a. R Squared = .604 (Adjusted R Squared = .456)

b. Age = 6.00

Tests of Between-Subjects Effects[b]

Dependent Variable:CCK mRNA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8566.763[a]	3	2855.588	3.419	.073
Intercept	44434.448	1	44434.448	53.202	.000
Treat	8566.763	3	2855.588	3.419	.073
Error	6681.642	8	835.205		
Total	59682.854	12			
Corrected Total	15248.406	11			

a. R Squared = .562 (Adjusted R Squared = .397)

b. Age = 10.00

GLOSSARY

BSA	Bovine Serum Albumin
CCK	Cholecystokinin
CCK-8S	Sulfated CCK octapeptide
CCK-IR	CCK Immunoreactive (cells)
CCK-RF	CCK Releasing Factor
cDNA	Complementary Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
DPH	Days Post Hatch
ELISA	Enzyme-Linked ImmunoSorbent Assay
FAML	The Fisheries and Mariculture Laboratory of the University of Texas at Austin's Marine Science Insitute
mRNA	Messenger RNA
MS-222	Tricaine Methanesulfonate
PBS	Phosphate Buffered Saline.
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
qRT-PCR	Quantitative Reverse Transcriptase PCR
RT^-	Reverse Transcription Negative control

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