NUTRITIONAL CONTROL OF GENE EXPRESSION, LARVAL DEVELOPMENT AND PHYSIOLOGY IN FISH

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GENERAL ABSTRACT

During preliminary research on cobia (*Rachycentron canadum*, L.) it became increasingly clear that more in-depth information was required to provide enabling techniques for the cobia aquaculture industry to develop more rapidly. A unifying theme in many of the more important issues facing cobia aquaculture is nutrition. This led to nutritional investigations with larval and juvenile fish highlighting the impacts of dietary ingredients on animal performance. Indeed, nutrition can be viewed as a central lever of action through which many aspects of the physiology and the environmental (water) quality of the animal can be controlled.

The first project focused on studying the larval development of cobia, a fish species highly suitable for aquaculture for which the industry is nascent. I described the time-course of development of external sensory organs, gut morphology and relevant digestive enzymes under controlled conditions using electron microscopy, histology and spectrophotometric assays. The developmental sequence of larval cobia could be separated in two phases, with a transition

period between 12 and 14 days post hatch (dph). This transition is characterized by the formation of the intestinal loop, the establishment of basic cranial neuromast configuration, leading to the initiation of the onset of pancreatic enzymes and the increase of growth rate. In addition, the effects of dietary taurine supplementation and incorporation of mannan oligosaccharides (MOS) into live feeds on cobia larvae development was examined. Fish fed supplementary MOS did not grow faster but displayed higher microvilli length and density. In addition, MOS-fed fish were more resistant to salinity stress. The dietary supplementation of taurine resulted in a dramatic increase in survival, growth and development rates, and enzymatic activities.

The second project aimed at refining cobia juvenile nutrition, assessing fish meal and fish oil replacements. Novel sources, including soy protein and oil, were investigated with and without amino acid and MOS supplementations, yielding promising results. Indeed, both fish meal and fish oil were replaced completely and successfully in feeds for juvenile cobia. In addition, novel ingredients (e.g. marine algae meals and soy protein concentrate) were identified to effectively achieve such replacement.

The third and last project dealt with nutrient-gene interactions, specifically centering attention on immunostimulants for which the underlying mechanisms of action remain poorly characterized. Here, dietary MOS, nucleotides and selenomethionine (Se-met) were offered to zebrafish whose transcriptome was analyzed by microarray. The immune system, humoral or cellular, innate or adaptive, exhibited different patterns of response according to the immunostimulating nutrient used. In addition, various genes involved in cell cycle and

cytokinesis were concomitantly expressed. An intriguing observation related to the insulinomimetic effect of Se-met. In other words, Se-met impacted pathways normally regulated by insulin, such as the MAPK and PI3K pathways. Some Insulin-like Growth Factors (IGF) and IGF bindgin proteins were up-regulated. Additional research is however necessary prior to advocating for the use of these additives, in order to further investigate their respective pros and cons.

Control nutritionnel de l'expression genetique, du developpement larvaire, et de la physiologie chez les poisons

Guillaume Salze

RESUME GENERAL

Au court des études préliminaires sur le cobia (*Rachycentron canadum*, L.), il est clairement apparut que des informations approfondies permettant un développement commercial plus rapide de l'élevage de ce poisson était indispensables. Le dénominateur commun des problèmes que rencontre la culture du cobia est la nutrition, ce qui a conduit à des études nutritionnelles sur les larves et les juvéniles. Ces études ont mis en évidence le rôle prédominant des ingrédients sur les performances de l'animal. En effet, la nutrition peut être considérée comme un levier central permettant de contrôler beaucoup d'aspects physiologiques ainsi que la qualité de l'environnement (qualité de l'eau).

Le premier volet de la présente thèse porte sur le développement larvaire du cobia, une espèce particulièrement adaptée à l'élevage et pour laquelle la culture à l'échelle commerciale

est émergente. En utilisant des techniques de microscopie électronique, d'histologie et de spectrophotométrie kinétique, la séquence de développement des organes sensoriels externes a été décrite, avec celles de la morphologie gastro-intestinale et de quelques enzymes digestives importantes. Chez cette espèce, la séquence de développement peut être divisée en deux phases, avec une période de transition entre 12 et 14 jours-post-éclosion. Cette transition est caractérisée par la formation de la boucle intestinale, l'établissement de la configuration générale des neuromastes, ainsi qu'une augmentation notable du taux de croissance et de l'activité des enzymes pancréatiques. Par ailleurs, les effets de la supplémentation des proies vivantes en taurine ou mannane-oligosides (MOS) sur le développement des larves de cobia ont été examinés. Les larves recevant une supplémentation en MOS ne grossissent pas plus rapidement, mais présentent des microvillosités plus hautes et plus denses sur l'épithélium digestif. De plus, ces larves montrent une résistance accrue à un stress hypersalin. La supplémentation en taurine des proies vivantes permet une élévation considérable des taux de croissance et de développement, une nette amélioration de la survie, ainsi qu'une forte augmentation des activités enzymatiques.

Le but du second volet était d'optimiser la formulation d'un aliment pour des juvéniles de cobia, en se concentrant sur le remplacement de la farine et de l'huile de poisson. Des sources nouvelles, telles que des protéines et des huiles de soja, ont été testées avec ou sans addition de MOS ou d'acide aminés, et ont donné des résultats prometteurs. D'autre part, de nouveaux ingrédients (concentré de protéine de soja et micro-algues séchées) ont été identifiés et ont permis le remplacement total et simultané de la farine et l'huile de poisson dans un aliment pour cobia.

Le troisième et dernier volet concerne l'interaction entre nutriments et gènes, et plus particulièrement sur les ingrédients immunostimulants, dont les mécanismes d'action demeurent mal compris. Ici, des poissons-zèbres (*Danio rerio*) ont été nourris avec une addition de MOS, nucléotides, ou selenomethionine (Se-met) pendant plusieurs semaines, à l'issue desquelles leur transcriptome a été analysé avec une puce ADN. Selon le régime alimentaire testé, des profiles d'expression différents apparaissent au niveau du système immunitaire – aussi bien humoral que cellulaire, spécifique que non-spécifique. Dans le même temps, plusieurs gènes impliqués dans le mouvement et le cycle cellulaire sont exprimés. Le rôle insulino-mimétique de la Se-met est intriguant. En d'autres termes, des cascades de gènes normalement régulées par l'insuline, telles que les cascades MAPK et PI3K, se comportent différemment avec ou sans addition de Se-met. L'expression de gènes tels qu'IGF (insulin-like growth factor) et IGFbp (IGF binding protein) est aussi stimulée par la prise alimentaire de Se-met. De plus amples recherches pour mieux déterminer les avantages et inconvénients de ces suppléments alimentaires sont toutefois nécessaires avant de conseiller ou non leur utilisation régulière.

Dedication

I dedicate this dissertation to my wife, Haruka, and my parents, Philippe and Brigitte, who always have given me unconditional love and support.

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My time here at the Virginia Tech Aquaculture program has been rich in unique experiences, teachings, and surprises. I could not have completed my degree without the help of many, through good and not-so-good moments.

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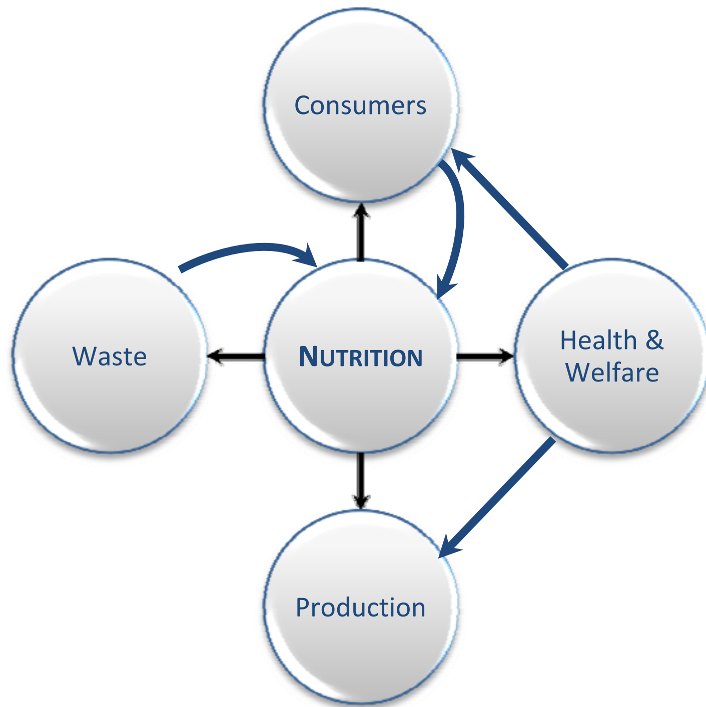
Chapter I. General introduction

In 1973, Jacques Cousteau said: “With earth’s burgeoning human population to feed we must turn to the sea with new understanding and new technology. We need to farm it as we farm the land.” With a stagnating production from fisheries (90-95 million tonnes since the 1990s), aquaculture produced 44.6% of the seafood for human consumption in 2005 (FAO, 2007a). China alone harvested 69.6% of global aquaculture production in 2006, followed by the rest of Asia (21.9%), Western Europe (3.5%) and Latin and North America (3.5%). Taking the combined growth of the world population and its demand for seafood, the FAO estimates that an additional 40 million tonnes of seafood will be necessary to maintain per capita consumption by 2030. Clearly, the aquaculture industry will become *the* essential actor in the supply of seafood needed in a hungry world.

However, the continued development of aquaculture will not be without obstacles: production, economics, public opinion, and environmental impact to name a few. All are interconnected, and sustainability is the goal for meeting these challenges. As it has for many other industries, sustainability has become a major concern for the aquaculture sector. Sustainability has been given many definitions, sometimes contradicting each other. However, I will summarize it as the way of conducting an activity to meet the needs of the present without compromising those of the future. This is a fundamental concept: it defines a philosophy of development that is increasingly adopted as mankind realizes that some practices cannot

Figure I–1: Nutrition as a central lever of action



continue as they have in the past. Thus, adopting sustainable practices goes beyond the sole environmental awareness, and must include considerations of producting and marketing new species, animal and consumer health, waste management, environmentally friendly input choices (energy, feed ingredients), improvement in the efficiency of their use and others. Considering the diversity of these issues, it is striking that one scientific discipline is able to provide answers to some of the most urgent questions: nutrition.

The direct influence of nutrition on production is obvious: the cultured organism must obtain an appropriate diet in order to develop, grow, and reproduce properly. Thus the first approach to animal nutrition is to ensure that all biological requirements are fulfilled. Often, these requirements evolve as the animal grows and matures. The priority when considering the culture of larval organisms is to ensure satisfactory development into the adult form. Because larvae are extremely small and many physiological processes are not fully functional, specific challenges arise, such as designing food particles that are attractive enough to be detected and preyed upon, small enough to be ingested, and easy enough to be digested and assimilated. For

these reasons and others, the consistent and reliable production of post-larvae is often a bottleneck to establishing commercial-scale aquaculture. For juveniles, growth to market size is clearly a priority, whereas for broodstock the goal is to produce good quality gametes and larvae. Therefore, for each stage of animal development and production, specific biological requirements must be met with unique dietary formulations.

Nutrition also impacts the organism's health and welfare. Beyond the absolute need for survival and growth, an organism must be able to find in its diet what it needs to fight pathogens and maintain its integrity. Disease is one of the most important sources of mortality in aquaculture settings and causes tremendous economic losses, not only due to direct mortality, but also because diseases result in sub-optimal performance. Additionally, diseases may cause an industrial crash, as was the case for the Ecuadorian shrimp farming industry, wiped out by Taura virus in 2000. This tragedy resulted in an estimated loss of $1 billion, and job losses for half a million people. Preventive approaches, such as HACCP (Hazard Analysis and Critical Control Points) and prophylactic procedures may be implemented to reduce the occurrence of diseases and chemical contamination. Proactive action may also complement prevention, as certainly healthy animals perform (i.e. growth, health, etc.) better. Nutrition can be employed to contribute to ensure optimum growing conditions for the cultured animal.

Consumer health also can be considered from a nutritional viewpoint. There has been in the past few years, a growing interest toward healthier foods, especially among wealthier, more educated consumers. Seafood benefits from a positive image in this regard, mainly due to the

publicity of the health advantages of n-3 fatty acids in marine organisms. Since numerous cultured aquatic species also require these fatty acids, a clear nutritional management opportunity exists to enhance the healthful benefits of the final product.

All industrial activities generate by-products of some type, and aquaculture is no exception. The culture of aquatic organisms is particular in that they live in a medium (i.e. water) that not only provides support and oxygen, but also receives wastes such as faecal matter, nitrogenous wastes, and uneaten food. These wastes must be removed from the animal's direct surroundings in order to maintain good environment quality. In an open-sea cage system, wastes are dispersed by the action of waves and currents. However, in an inland, recirculating system, wastes are collected and concentrated. They can be disposed of subsequently, or they may be utilized and transformed into new ingredients that can be incorporated into various diets again. For example, such technologies are being used to convert fish farm waste into shrimp food, thereby providing both environmental and economical benefits. Nutrition science can be employed at this level to control waste production (i.e. maximizing nutrient utilization by the cultured organism), and also to ensure that unavoidable wastes are removed from the system more efficiently, more easily concentrated, and utilizable as an additional ingredient.

Ingredient selection during dietary formulation is critical since it impacts animal performance, health, waste production and hence overall sustainability of a farm. Traditionally, fish meal has been the principal source of protein in aquafeeds. However, annual fish meal supplies remain stagnant, averaging between 6 and 7 million tonnes a year (Shepherd*, et al.*,

2005). Moreover, the aquaculture industry has been increasing at an average rate of 6.9% per year over the last 50 years, with some countries such as Turkey or Vietnam dramatically increasing their production (24.0% and 30.6% respectively between 2002 and 2004; FAO, 2007). A rising demand together with a stagnant supply has caused an increase in the retail price of fish meal, and ultimately, a heavy reliance upon this ingredient will no longer be economical: this is the "fish meal trap" (New and Wijkström, 2002). Born from a global economic perspective, it has also become an environmental issue, since the extensive use of fish meal results in net consumption of fish, as opposed to net production: in the late 1990s, approximately 2.5-3.0 kg of wild fish were necessary to produce 1 kg of Atlantic salmon (Åsgård, *et al.*, 2007). Similarly, lipid sources traditionally are derived from oily marine fishes (e.g. herring, sardine), and the "fish oil trap" is also closing. Consequently, numbers of researchers have worked on the identification and the testing of alternative protein and lipid sources, which must satisfy all the requirements that were discussed above. While the proportions of fish meal and oil in aquafeeds have been reduced significantly, more work is needed to further improve the efficient use of these finite resources.

Nutritional research can assist the aquaculture industry proactively by providing solutions to a number of problems of concern to consumers. For example, major concerns at present include issues such as negative environmental impact, animal welfare, food safety and contaminants, and zoonoses. Nutritionists, through sound dietary formulation, can ensure the health and well-being of the cultured animal. Reduced environmental impact can be attained through appropriate ingredient choices, and utilization of modern pelleting technologies.

Consumer confidence can be enhanced through emplacement of quality control and assurance programs for all ingredients to establish complete chain of custody and traceability. Moreover, the nutritional quality of the final product can be improved through judicious dietary formulation.

Approximately 300 aquatic species are cultured worldwide. However, 90.5% of the total production is concentrated in only ten species groups (FAO, 2007a). For a number of reasons, including environmental and economical, aquaculturists have examined the potential for producing new cultured species. Cobia (*Rachycentron canadum*, L.) is such a species which presents many characteristics attractive to the aquaculture industry, including extremely high growth rates and global distribution. A major problem with cultivating new species is the lack of general knowledge on their biology, with the consequence that early production levels are often very low due to high mortality rates. Thus, a high level of research and development is necessary prior to developing viable industrial production.

The following dissertation concentrates on nutritional research with cobia, a candidate species for intensive aquaculture in the United States and elsewhere. Specifically, I addressed critical gaps in our information base on cobia biology. These include:

- Larval development, emphasizing ontogeny of the gastrointestinal tract and sensory epithelia and how these systems correlate to nutritional transitions in rapidly growing animals.

- Replacement of fish meal and fish oil with alternative ingredients: new ingredients were tested, and known feedstuffs investigated and refined using novel supplementations.
- Exploration of the inner workings of nutrients on the physiology of fish using transcriptomic methods with a model species for which a microarray is readily available– the zebrafish *Danio rerio*.

Åsgård, T., Berge, G.M., Mørkøre, T., Refstie, S., 2007. Flexibility in the use of feed ingredients turns the salmon industry sustainable. Organic Aquaculture Symposium. National Organic Standards Board, Arlington, VA.

FAO, 2007. The State of World Fisheries and Aquaculture - 2006 (SOFIA). Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, pp. 180.

New, M.B., Wijkström, U.N., 2002. Use of fish meal and fish oil in aquafeeds: further thoughts on the fish meal trap. FAO Fisheries Circular No. 975.

Shepherd, C.J., Pike, I.H., Barlow, S.M., 2005. Sustainable feed resources of marine origin. Aquaculture Europe 2005. European Aquaculture Society, pp. 59-66.

Chapter II. DEVELOPMENT OF THE LARVAL COBIA GUT

Abstract

The development of a cobia aquaculture industry is constrained by the lack of a reliable supply of weaned juveniles. Together with genetic factors, nutrition plays a fundamental role in the proper development of finfish larvae. The present trial investigated the morphological and physiological ontogeny of the cobia digestive tract. Larvae were reared in a recirculated system and fed enriched rotifers in green water, followed by enriched *Artemia*, prior to being weaned on an artificial diet 25 days post-hatch (dph). The digestive tract shape was a straight tube at first feeding (3 dph), acquired a loop at 10 dph, and exhibited its final, juvenile configuration by 18 dph. During development, two valves appeared (3 dph and 18 dph) that separated fore-, mid-, and hindgut. The stomach commenced differentiation at 10 dph, and displayed gastric glands by 16 dph. Together with the observations of others, the results presented herein could be used to refine diets and rearing protocols for cobia larvae especially as this relates to the presentation of appropriate nutrient composition in respect to the development of the digestive capacity of the gut.

Keywords: *Rachycentron canadum,* pepsin, intestine, enzyme, larval nutrition, ontogeny

INTRODUCTION

Cobia (*Rachycentron canadum*) is a fast-growing (Lunger, et al., 2006), disease-resistant (McLean, et al., 2008a), globally-distributed fish which has drawn increasing attention from the aquaculture industry (McLean, et al., 2008b). In 2005, global production of cultivated cobia exceeded 20,000 metric tonnes (FAO, 2007b), representing 70% of all cobia landings. More rapid development of this industry is constrained, however, due to several bottlenecks, the most urgent being the adequate production and supply of weaned juveniles (Salze, et al., 2008). Presently, most cobia hatcheries produce 1-2 weanlings per liter regardless of the starting density, with animals being approximately 30-35mm in total length at 30 days post-hatch (dph; Holt et al, 2007). This level of production is clearly insufficient to sustain commercial hatchery operations, and significant improvements in weanling survival are needed (Benetti, et al., 2008; Holt, et al., 2007; Niu, et al., 2008a).

In fish, the gastrointestinal (GI) tract integrity is paramount to good health and performance. Indeed, it is not only involved in the digestion and absorption of nutrients, but also plays a critical role in osmoregulation, especially during larval stages when gills are absent or not fully functional (reviewed by Varsamos et al., 2005). Salts are transported against the gradient by active ion transporters through the instestinal epithelium and followed by water transfer. Fish, including larvae, regulate the amount of injested water in response to water salinity (Tytler and Blaxter, 1988). In newly hatched larvae, the drinking rate increases during

the first few days of life, and then starts to decrease until adulthood (Evans, 1993), illustrating the coordination between the GI tract and the gills.

The GI tract is characterized by its permeability and its exposition to the external environement via drinking. While this is crucial for the establishment of the gut microflora, this also constitutes an entry port for potential pathogens. Thus the GI tract is also an important site of the fish immune apparatus. All the components of the immune system are represented in the fish gut (Schley and Field, 2007; Gomez, 2008): physical barrier (e.g. gastric pH, intestinal mucus), leucocytes (e.g. macrophages and lymphocytes), and soluble factors (e.g. complement, acute-phase proteins).

During development and metamorphosis, larvae undergo numerous and dramatic changes both in external and internal morphology. In fish larvae, the GI tract is often immature at hatching, especially in marine species. Typically starting from a straight tubular form with limited histological differenciation from mouth to anus, the GI tract acquires its definitive adult conformation through a series of folding, genesis of valves, and regional specialization of the epithelium. The organogenesis speed and sequence is highly variable between species, but completion of the GI tract organogenesis is generally regarded as the completion of the individual's metamorphosis.

Currently, our knowledge of cobia larval development and organogenesis is only partial. Faulk et al. (2007a) have described some aspects of cobia gut development. In the current

study, we explore the development of the GI tract of cobia larvae held under a standard rearing protocol. An enhanced understanding of such a process might shed light onto subtle but nonetheless critical developmental steps, thereby providing the means to develop more effective feeds and feeding protocols.

MATERIAL AND METHODS

Experimental systems and fish

Seventy-two thousand 2-dph (4.4 ± 0.28mm) cobia larvae, derived from the same batch of eggs, were obtained from the University of Miami, Rosenstiel School of Marine and Atmospheric Science. Larvae were randomly stocked into one of 8 replicated 3-tank systems (Salze, et al., 2008) at a density of 10 larvae L^{-1}. Each independent unit comprised three 300 L tanks, linked to a reservoir that doubled as a fluidized KMT biofilter. Water from the reservoir was pumped to a bubble bead filter (BBF-2; Aquaculture Technologies Inc., Metaire, LA) to remove suspended solids, then through a 100 µm bag filter, a UV sterilizer (80,000 microwatts cm^{-2} sec^{-1}; Emperor Aquatics, Pottstown, PA) for water disinfection and returned to the tanks. A side-looped protein skimmer (R&B Aquatic Distribution, Waring, TX) was employed to remove small organic compounds and to decrease turbidity. Hydrodynamics of the larval tanks were maintained to provide both horizontal and vertical circulation cells to optimize prey distribution and minimize larvae-larvae interactions. Water flow was adjusted throughout the rearing trial, starting at approximately 1.5 L min^{-1}, and finishing at 12 L min^{-1} for the tanks holding the biggest fish. Initial system salinity was 35 g L^{-1}, which was reduced 1 g L^{-1} day^{-1} until a final

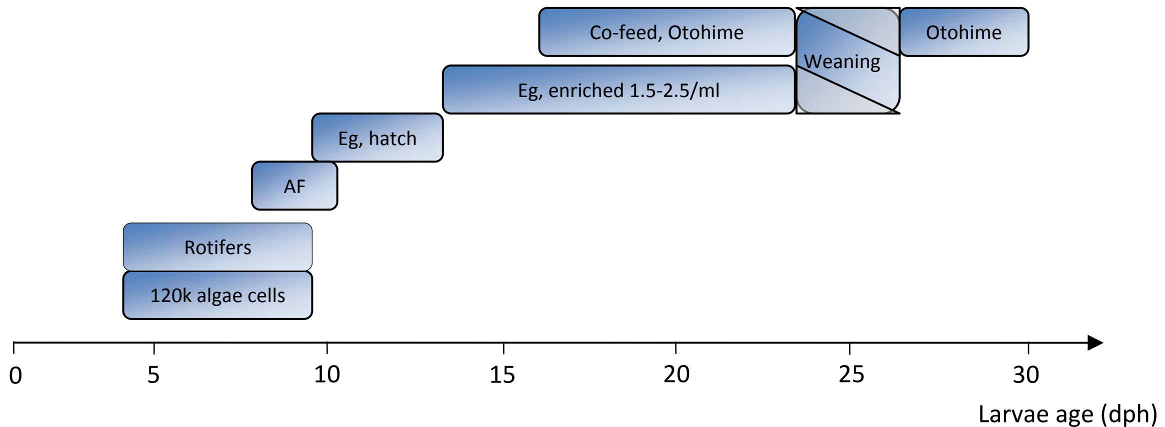
salinity of 24 g L^{-1} was achieved at 13 dph. The reduction in salinity was employed based on previous experience (Schwarz, personal communication) in cobia rearing and to reduce costs associated with the purchase of artificial sea salt. Water temperature (27.8 ± 0.6°C) was maintained using ambient air heating and the use of electronically-controlled, thermostatic titanium heaters (R&B Aquatics, Waring, TX) located in the reservoir. Bead filters were backwashed daily. Make-up water was supplied from a 38,000 L storage tank (Red Ewald Inc., Karnes City, TX). Once full, the storage water tank was treated with 20 mg L^{-1} chlorine and passed continuously through a rapid-rate sand filter in series with a diatomaceous earth filter before returning to the storage tank. Residual chlorine was neutralized with sodium thiosulfate prior to using the water. A 24-h photoperiod was maintained throughout the experimental period using fluorescent lights positioned approximately 2.5 m above the systems.

Water quality

Water quality parameters were analyzed daily, and included total ammonia nitrogen, NO_2-N and NO_3-N, which were measured spectrophotometrically (HACH DR/2400 Spectrophotometer, HACH Co., Loveland, CO); dissolved oxygen, temperature and salinity, which were monitored using a YSI model 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA); pH, measured by a HI 9024 pH meter (HANNA Instruments, Woonsocket, RI), and alkalinity ,which was determined by titration (APHA, 1998). No differences between systems in water quality were discerned throughout the trial.

Feeds and feeding

Figure II–1: Diagram of the standard feeding strategy for larval cobia



Rotifers (1.2-2.5 ml^{-1}) in "green water" (3-8 dph) were followed by smaller (AF, 1-1.5 ml^{-1}, 7-9 dph) and larger (EG, 1-1.5 ml^{-1-}, 9-11 dph) unenriched *Artemia* strains, then enriched EG *Artemia* (1-2.5 ml^{-1}, 12-21 dph). Larvae were co-fed *Artemia* and commercial dry pellets (Otohime, 15-21 dph) prior to full weaning (25 dph), at which time *Artemia* were completely replaced by artificial diet.

Green water (approx. 120,000 cells ml^{-1} *Nannocloropsis sp*. algal paste, Reed Mariculture, Campbell, CA) and L-type rotifers (*Brachionus plicatilis*, 1.5-2.5 rotifer ml^{-1}) were fed until 8 dph, at which point *Artemia* only was offered (1-2.5 nauplii ml^{-1}, see Figure II–1). The level of eicosapentaenoic acid (EPA; 20:5n-3) in the algae was 30% dry weight. Live feeds included enriched rotifers; non-enriched, small-sized (~430 µm) high highly unsaturated fatty acid (HUFA) profile AF-*Artemia* nauplii (15 mg HUFA g^{-1} dry weight); and enriched EG-*Artemia* nauplii (INVE Inc., Salt Lake City, UT). Rotifers were enriched with DC DHA Selco (INVE Inc., Salt Lake City, UT) at 0.4 g L^{-1}. Similarly, EG *Artemia* were enriched for 24 hours with DC DHA Selco at 0.6 g L^{-1}. Live prey was offered every 6 hours following complete clearing of the tank between feedings. Co-feeding of larvae with Otohime weaning feeds (Reed Mariculture, Campbell, CA)

commenced at 15 dph, with 100% artificial diets being offered from 25 dph after a 3-day weaning period (Figure II–1). The dry food was distributed every hour using automated shaking feeders (AF6 feeders, DFT3R8AC timer, Sweeney Feeders, Boerne, TX).

Sampling

Five larvae from each tank were randomly sampled every other day for histological analysis. Larvae were euthanized by an overdose of clove oil (0.3 ml L^{-1}) prior to being rinsed with distilled water in order to remove salt. Individual total lengths were recorded, and larvae were immersed in fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid, 0.05M sodium cacodylate; pH 7.4) and stored at $4^{o}C$ until processed. After completion of the weaning process, weanlings were counted, bulk-weighed and measured to determine final production, and malformation frequency as assessed by spinal deformities.

Histological processing and analyses

Depending on size, larvae were processed as whole or tailed and/or headed. Typically, 3-8 dph larvae were kept whole, 10-16 dph were tailed, and 16-27 dph larvae were both tailed and headed. Samples were immersed in 0.05M sodium cacodylate (pH adjusted to 7.4) for 15 min prior to being post-fixed in 1% osmium tetroxide (OsO_4) for 1 hour. Additional sodium cacodylate baths were performed to ensure complete removal of OsO_4. Dehydration was achieved using ethanol baths (15% to 100%, 15 min each), and completed with a propylene

oxide bath (15 min). Samples were then infiltrated and embedded in plastic (Poly/Bed 812) using standard methods (PolySciences, Inc. technical data sheet location) and positioned for longitudinal sectioning. Thick sections (1µm) were prepared, stained (toluidine blue O, counterstained with safranin O) and examined by light microscopy (Leitz Laborlux S Fixed Stage microscope equipped with a Nikon 5500 digital camera).

RESULTS

Growth and survival

Larvae grew from an average of 4.4 ± 0.1 mm at 3 dph to 33.9 ± 1.0 mm at 27 dph. Fully weaned cobia attained a weight of 0.35 ± 0.04 g. Survival was 7.09 ± 1.16% and tanks produced an average of 0.71 ± 0.12 fish L^{-1}. The rate of deformity, as assessed by the presence of spinal deformities was 2.39 ± 1.22%.

Development of the gastrointestinal tract

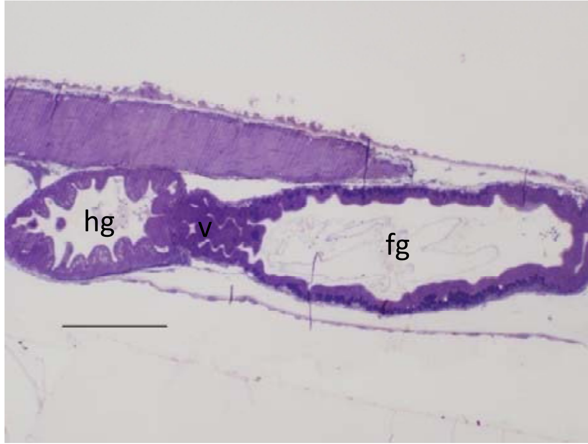
At 3 dph, the cobia gut took the form of a simple straight tube (Plate II–1A), which underwent important morphological changes in configuration throughout development. At 3 dph, the intestine was tubular with an intestinal valve demarcating the fore- and hindgut (Plate II–1A). A narrowing of the pharynx resulted in the formation of the esophagus layered by stratified squamous epithelium which, by 6 dph, exhibited a longitudinally folded, columnar

epithelium and striated muscularis (Plate II–1B). Intestinal folds were apparent, more pronounced in the caudal area. Differentiation of intestinal regions was perceptible based on staining and structural differences observed in absorptive epithelium. The absorptive epithelium of the anterior region of the intestine in 3 dph larvae contained numerous vacuoles and vesicles of varying size: larger blue-green-stained vesicles (indicative of lipid absorption) and smaller, dark blue stained vacuoles (suggestive of protein absorption) in the most anterior region. In contrast, the posterior region of the intestine was characterized by the presence of non-lipidic clear vesicles. Microvilli were distributed homogenously, and were 2 to 2.7 μm in height. At 3 dph, no goblet cells were discerned.

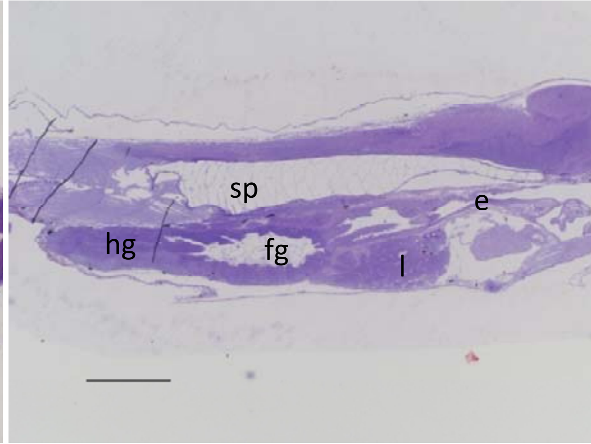
By 10 dph, the larval intestine expressed a complete loop in what ultimately formed the fore- and midgut regions. The epithelium between the esophagus and intestine had commenced differentiation into a stomach. This was expressed primarily as a thickening of the epithelium, resulting in a constricted lumen lined with longitudinal folds (Plate II–1C).

The regional functional specialization of the intestine was also apparent, especially in the anterior gut: lipid absorption took place in the midgut, after the loop. The first goblet cells were observed in the midgut of 10 dph larvae. In addition, the microvilli in the stomach region gradually shortened and disappeared (2-2.7 μm tall at 3 dph, 0.5 μm at 14 dph). In the intestine, microvilli height was reduced in all regions relative to those of 3 dph larvae, being 1.5-1.8 μm in fore- and midgut and 0.8-1.0 μm in the hindgut.

A



B



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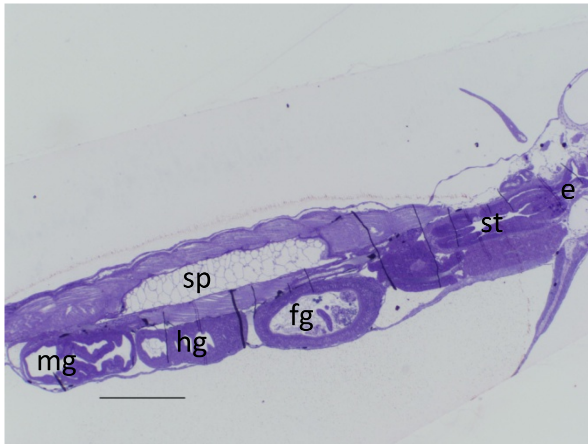

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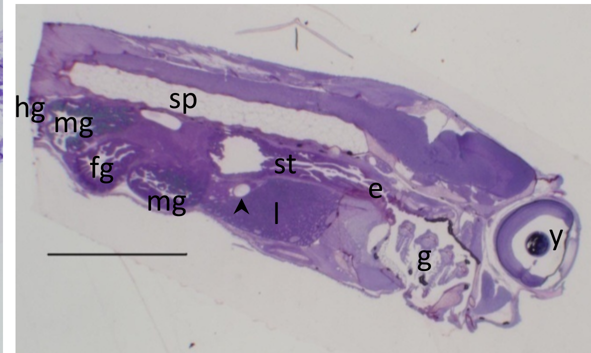


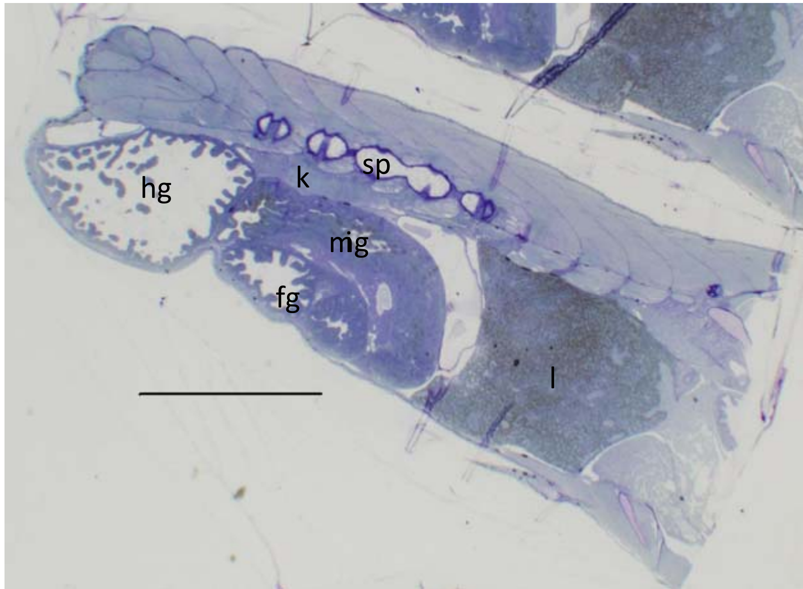
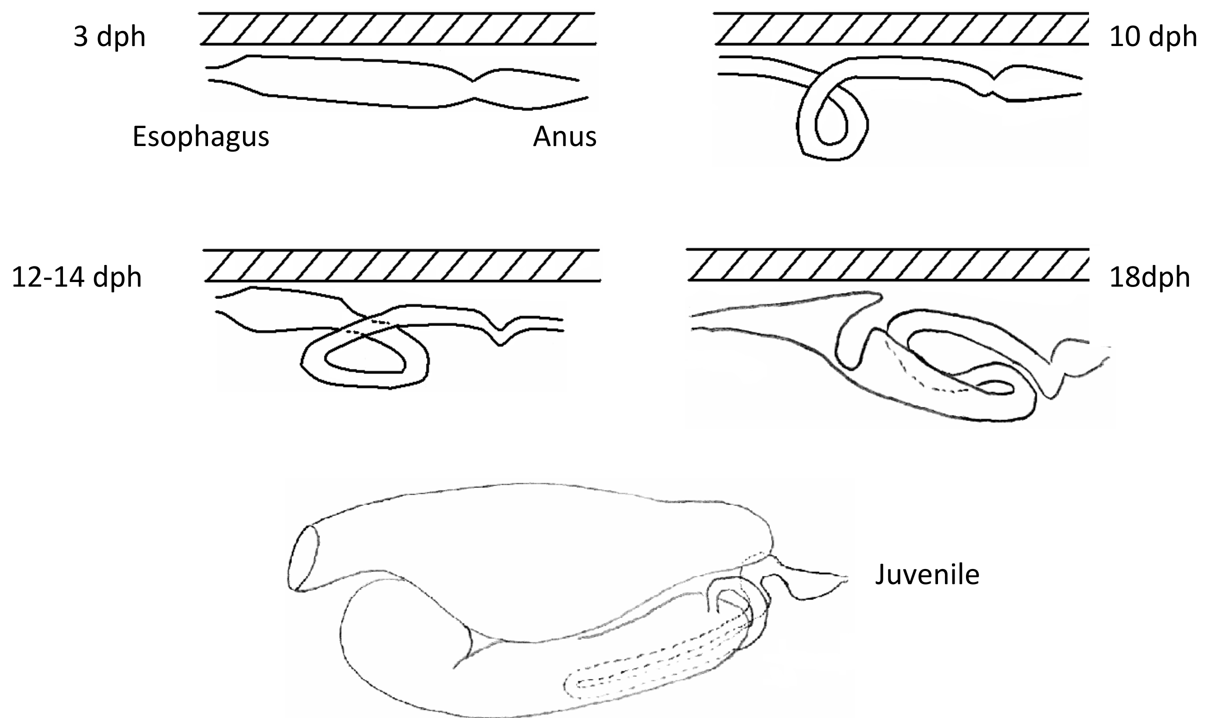


Plate II–1: Longitudinal sections of cobia larvae. Stained with toluidine blue O, counterstained with safranin O.

(A): 3 dph (bar=25µm); (B): 6 dph (bar=50µm); (C): 10 dph (bar=200µm); (D): 18 dph (bar=200µm); (E): 24 dph (bar=200µm).

e: esophagus; g: gills; k: kidney; fg: fore-gut; hg: hind-gut; l: liver; mg: mid-gut; sp: spine; st: stomach; v: valve; y: eye. Arrow head: gall bladder.

Figure II–2: Diagram describing the morphological changes of larval cobia gastrointestinal tract though development.



By 16 dph, the gastric mucosa expressed several gastric glands and by 18 dph the first sign of a fundic stomach, forming a Y shape, was observed (Plate II–1D).The intestine continued to lengthen through 18 dph, at which point the structure flattened, unlooped, elongated, and formed an additional elbow. A tubular structure, located at the posterior edge of the liver and lined by squamous epithelium was discerned and identified as the gall bladder (Plate II–1D). A second valve formed which further separated the foregut from the midgut close to the first elbow of the intestine. Throughout intestinal development, the number and structural complexity of intestinal folds increased (Plate II–1E). The goblet cells multiplied substantially, especially in the foregut, and were observed for the first time in the hindgut at 18 dph. They did not have a preferred location within an intestinal fold, and were present at fold base, side and

apex. Unlike the observations between 3 and 10 dph, microvilli height remained stable. By 24dph, the GI apparatus had achieved its metamorphosis and reflected that of a juvenile cobia (Plate II–1E). The entire process is summarized in Figure II–2.

DISCUSSION

The growth rate of cobia during the present study was comparable to that observed by Faulk et al. (2007a). However, fish from this study were approximately 40% longer than those reported by Faulk et al. (2007b) and Hitzfelder et al. (2006) and 26% longer than those reported for tank-reared cobia by Benetti et al. (2008). Although survival was low, this fell within the range of previously reported data for pond-reared larvae (Weirich, et al., 2004), and at the lower range for those reared in tanks (Faulk and Holt, 2005; Faulk, et al., 2007b; Hitzfelder, et al., 2006; Salze, et al., 2008).

Although cobia are known to have a short, active cannibalistic period during development (Salze, et al., 2008), it is unlikely that cannibalism was the main cause of mortality since the rotational and convectional water movements in the tanks was highly effective at isolating fish from one another. Deformity frequency (2.39%) was calculated for live fish at trial end and this only took account of visible spinal curvatures. The actual number of deformed weanlings therefore, was probably underestimated, since no consideration was given to jaw deformities, which are common in cobia larvae (McLean, et al., 2008a). Various deformities can appear for a range of reasons: maladapted husbandry or poor water quality (Bolla and Holmefjord, 1988;

Chatain, 1994; Rosenthal and Alderdice, 1976), poor larval and/or broodstock nutrition, particularly that of vitamins (Cobcroft, et al., 2001; Cobcroft, et al., 2004), genetics (Sindermann, 1990), and diseases (Oh, et al., 2002). In the present trial however, no signs of diseases were observed. Water quality parameters were within accepted ranges for warm marine fish larvae. Although these parameters have not been definitively determined for cobia larvae, they probably did not contribute to the observed malformations. Rather, genetic and nutritional challenges most likely exerted a more predominant role. Clearly, further research is required in broodstock management, selection, nutrition, and egg quality in order to improve survival. More specifically, I feel that protein and amino acid nutrition in larvae of such fast-growing species is critical and often overlooked.

The general morphological development of the cobia gut followed that described by Faulk et al. (2007a), although some differences in the timing of events were evident. These differences likely reflect variations in larval rearing temperature (25.9 vs. 27.8^{o}C), but also broodstock conditioning and use of spawning induction techniques; the latter have been shown to result in differences in mRNA abundance of specific genes that impact gamete quality (Bonnet, et al., 2007) and potentially overall larval quality. The feeding protocols employed, stocking densities used and system-specific characteristics, including water quality, salinity, rearing temperature and photoperiod, may also have altered developmental rates. Noteworthy however, was the relatively late appearance of intestinal goblet cells relative to previous reports (Faulk, et al., 2007a; Schwarz, et al., 2006b), which in the present study coincided with the feeding of *Artemia*. Another observation of interest related to the changes in microvilli

height. Reduced microvilli height, without attendant increases in diameter, would result in a decreased surface area available for absorption. This incident however might be expected since as the gut increased in diameter and complexity, in terms of fold height and number and attendant number of absorptive enterocytes, there would be an overall augmentation in intestinal absorptive capacity. Support for such an explanation is supplied by studies with rats, where high energy diets resulted in a 28% reduction in microvilli length (Goda and Takase, 1994). Alternatively, reductions in microvilli height might have been driven by changes in diet, microbial flora, or salinity. In this regard, changes in microvilli height and global absortive surface area may reflect adjustments in the osmoregulatory process as the gills acquire their ion and gas exchange function.

The GI development in other marine fish larvae has been reviewed by Zambonino Infante and Cahu (2001). At 3-dph (55 degree-days; ^{o}d) European sea bass (*Dicentrarcus labrax*, L.) larvae, an absence of intestinal folds and the gradual appearance of the brush border characterized the intestinal epithelium (Vu, 1976). Walford and Lam (1993) marked the formation of the stomach between 13 and 17 dph (240-314°d) with the development of the pyloric sphincter and the decrease of gastric pH in this species. However, gastric glands were not observed before 25 dph (462°d). In contrast, differentiation of the gastric mucosa is initiated at 10 dph (290°d), and completed at 18-20 dph (522-580°d) in cobia, although the timing of appearance of gastric glands is very similar (464°d). In sole (*Solea solea*, L.), the stomach starts to differentiate on 10 dph (190°d), and the gastric glands appear at 22 dph (418°d; Bouhlic and Gabaudan, 1992). In addition, intestinal folds can be found only in the

posterior third in newly hatched sole, which is also similar to my observations in cobia. The similarities in GI ontogenetic pattern between cobia and sole may reflect comparable developmental strategies and/or natural prey items. Indeed, larvae of both species develop in estuaries and live in close contact with the benthic environment, whereas *D. labrax* is a more pelagic and oceanic species.

Taken together with the work of others, these findings could be used to refine diets and rearing protocols. The appropriate nutrient composition, as well as degree of chemical complexity of these nutrients, could be particularly important in respect to the ontogeny of digestive capacity. Further, it is likely that the development of gut-associated lymphoid tissue can be driven by initial exposure to dietary and other antigenic materials. The latter may partially explain the increasing appearance of goblet cells. Considerations of the nature of nutrients and their role in digestive ontogeny, in regards to digestion mode and capacity of the larvae, could lead to enhanced growth and survival, as well as to an earlier and more controlled weaning date in marine finfish larvae.

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Chapter III. DIETARY TAURINE ENHANCES GROWTH AND DIGESTIVE ENZYME ACTIVITIES IN LARVAL COBIA

Abstract

While present in high concentrations in natural preys of marine fish larvae (e.g. copepods), taurine is absent from rotifers and very low in Artemia, which may thus be lacking in fish larvae. Therefore, the effect of increasing taurine intake on growth, amylase, trypsin, lipase and pepsin-like activities during larval cobia development and weaning was investigated. Taurine was delivered using bioencapsulation techniques wherein rotifer and Artemia nauplii were co-enriched with 4 g taurine L-1 d-1. In control larvae, amylase activity was detectable at 3 days post-hatch (dph) and expressed a four-fold increase from trial start to weaning, with peak levels being 1.38±0.31 U fish-1 at 27 dph. Trypsin activity exhibited a similar profile to that of amylase: activity was low at 3 dph, and reached 0.08±0.00 U fish-1 at weaning. Lipase activity was not detected at 3 dph, but by 27 days achieved levels of 2.89±0.38 U individual-1. Pepsin-like activity was not detectable prior to 22 dph ($P<0.05$), denoting a lack of gastric functionality until this time point. In control fish, maximum pepsin-like activity reached 29.09 ± 1.47 UHb fish-1 at weaning. Taurine-fed larvae had a markedly increased amylase activity ($P < 0.0001$), commencing at 16 dph with maximal levels being recorded as 7.40±1.47 U fish-1. Likewise, trypsin activity attained 0.29±0.02 U individual-1 at 27 dph ($P < 0.0001$) in taurine-fed fish. As with control-fed larvae, lipase activity in taurine-enhanced fish was undetectable at 3 dph, but by 22 dph attained twice the levels observed for controls ($P < 0.0001$) at weaning. Pepsin-like activity was also first detected at 22 dph in taurine fed larvae. Maximum levels of pepsin-like activity were remarkably high at 330.95±13.39 U fish-1 at 27 dph ($P < 0.0001$). Possible underlying mechanisms of action of taurine are explored, such as mitochondrial function, protein translation, bone growth, as well as its participation in anti-oxidative processes.

Keywords: *Rachycentron canadum,* pepsin, lipase, trypsin, larval nutrition, ontogeny.

INTRODUCTION

As a species for aquaculture, Cobia (*Rachycentron canadum*, L., 1766) has garnered increasing interest due to its impressive growth performance (Lunger, et al., 2006), near-global distribution and disease resistance (McLean, et al., 2008a), desirable flesh characteristics (Duncan, et al., 2007), and ability to utilize alternative proteins (Craig, et al., 2006) and carbohydrates (Schwarz, et al., 2007). As with other new candidate species for cultivation, there currently exists a dearth of information on cobia larval development and physiology, and this may partially explain the relatively poor survival rates currently observed in many cobia hatcheries. During cobia larviculture, comparatively high mortalities are experienced at critical phases of development. This is especially so at decisive moments such as first exogenous feeding and the transition from live to formulated diets (Benetti, et al., 2008; Holt, et al., 2007; Salze, et al., 2008).

A number of explanations have been proposed to provide at least a partial rationale for poor larval survival including water quality issues, physical damage to larvae, cannibalism, various genetic factors and poor initial egg quality (Holt, et al., 2007; Salze, et al., 2008). Clearly, nutrition plays a central role in any successful aquaculture practice, especially during larviculture. Although morphological development is mainly determined genetically, critical physiological control processes can be influenced strongly by diet (Cahu and Zambonino Infante, 2001; Péres, et al., 1998). For example, inappropriate nutrition may delay and compromise larval development, resulting in impaired growth, heightened frequencies of

malformations, and ultimately, death (Yúfera and Darias, 2007). Conversely, correctly delivered premium diets with proper nutrient balance and energy serve to maximize growth, development and survival.

Rotifers (*Brachionus* sp) and brine shrimp (*Artemia salina*) are typically employed as live feeds for cultured marine fish larvae, but because they are generally considered nutritionally deficient, enrichments are necessary to enhance growth and development of fish. Research on larval fish nutrition has emphasized the fatty acid content of live feeds with comparatively little attention being given to protein nutrition. Copepods are widely recognized as nutritionally superior to *Artemia*, for they need not be enriched, and their use typically results in improved growth and survival in fish larvae (Shields, et al., 1999). The amino acid profiles of copepods differ from those of traditionally employed live feeds and they tend to be rich in taurine (van der Meeren, et al., 2008). Taurine is not an amino acid *sensus stricto* as it lacks the characteristic carboxyl group and is thus generally found in its free form and in high concentrations in many fish tissues (Sakaguchi, et al., 1988). Although considered metabolically inert, taurine functions in maintaining cellular osmolality and stabilizing cell membranes, and has a function in detoxification and antioxidation processes (Huxtable, 1992). Several studies with fishes have determined that dietary taurine has positive effects on fish growth and development (Brotons Martinez, et al., 2004; Kim, et al., 2005; Matsunari, et al., 2005; Takeuchi, 2001), while taurine supplementation permits higher fish meal replacement with alternate proteins (Gaylord et al., 2006; Lunger et al., 2007; Chatzifotis et al., 2008). However,

limited information is available on the nutritional role, if any, that taurine plays during the development of larval fishes.

Our knowledge of cobia larval digestive processes is currently only partial, and an enhanced understanding of the developmental sequence for digestive enzymes might provide the means to design more effective feeds and feeding protocols. Faulk et al. (2007a) have described the ontogeny of the pancreatic enzymes trypsin, chymotrypsin, lipase and amylase. In the current study, we extend the work of Faulk et al. (2007a) by examining the enzymatic and developmental response of cobia larvae held under different rearing protocols, while also using eggs from a dissimilar broodstock. As well, we report here, for the first time in cobia, the developmental profiles for pepsin. Knowledge of the developmental process *in toto* may be critical to adapting and adopting novel feeding strategies for larval cobia, which may in turn enhance weanling survival. Finally, because dietary taurine appears to provide growth advantage to juvenile fishes (see above), we examined the impact of increasing dietary taurine levels on larval growth and enzyme activities.

MATERIAL AND METHODS

Experimental systems and fish

Two days-post-hatch (dph, 4.4 ± 0.28mm) cobia larvae, derived from the same batch of eggs, were obtained from the University of Miami, Rosenstiel School of Marine and Atmospheric Science. Larvae were stocked randomly into one of two replicated systems (Salze,

et al., 2008) at a density of 10 larvae L^{-1}. Each independent unit comprised three 300 L tanks, linked to a reservoir that doubled as a fluidized KMT biofilter. Water from the reservoir was pumped to a bubble bead filter (BBF-2; Aquaculture Technologies Inc., Metaire, LA) to remove suspended solids, then through a 100 µm bag filter, and a UV sterilizer (80,000 microwatts cm^{-2} sec^{-1}; Emperor Aquatics, Pottstown, PA) for water disinfection and returned to the tanks. A side-looped protein skimmer (R&B Aquatic Distribution, Waring, TX) was employed to remove small organic compounds and to decrease turbidity. Hydrodynamics of the larval tanks were maintained to provide both horizontal and vertical cells to optimize larvae-prey distribution and minimize larvae-larvae interactions. Horizontal flow was controlled using the water inlet, while the vertical flow was manipulated using a circular air diffusion line placed at the base of the tank (Schwarz, et al., 2006a). Water flow was adjusted throughout the rearing trial, starting at approximately 1.5 L min^{-1}, and finishing at 12 L min^{-1} for the tanks holding the biggest fish. Initial system salinity was 35 g L^{-1}, which was reduced 1 g L^{-1} day^{-1} until a final salinity of 24 g L^{-1} was achieved at 13 dph. The reduction in salinity was employed based on previous experience (Schwarz, personal communication) in cobia rearing and as a means to reduce costs associated with the purchase of artificial sea salt. Water temperature was maintained (27.8 $\pm$ 0.6 ºC) using ambient air heating and the use of electronically-controlled, thermostatic titanium heaters (R&B Aquatics, Waring, TX) located in the reservoir. Bead filters were backwashed daily. Make-up water was supplied from a 38,000 L storage tank (Red Ewald, Inc., Karnes City, TX). Once full, the stored water was treated with 20 mg L^{-1} chlorine and passed continuously through a rapid-rate sand filter in series with a diatomaceous earth filter before returning to the tower. Residual chlorine was neutralized with sodium thiosulfate prior to using the water. A 24-h

photoperiod was maintained throughout the experimental period using fluorescent lights positioned approximately 2.5 m above the systems.

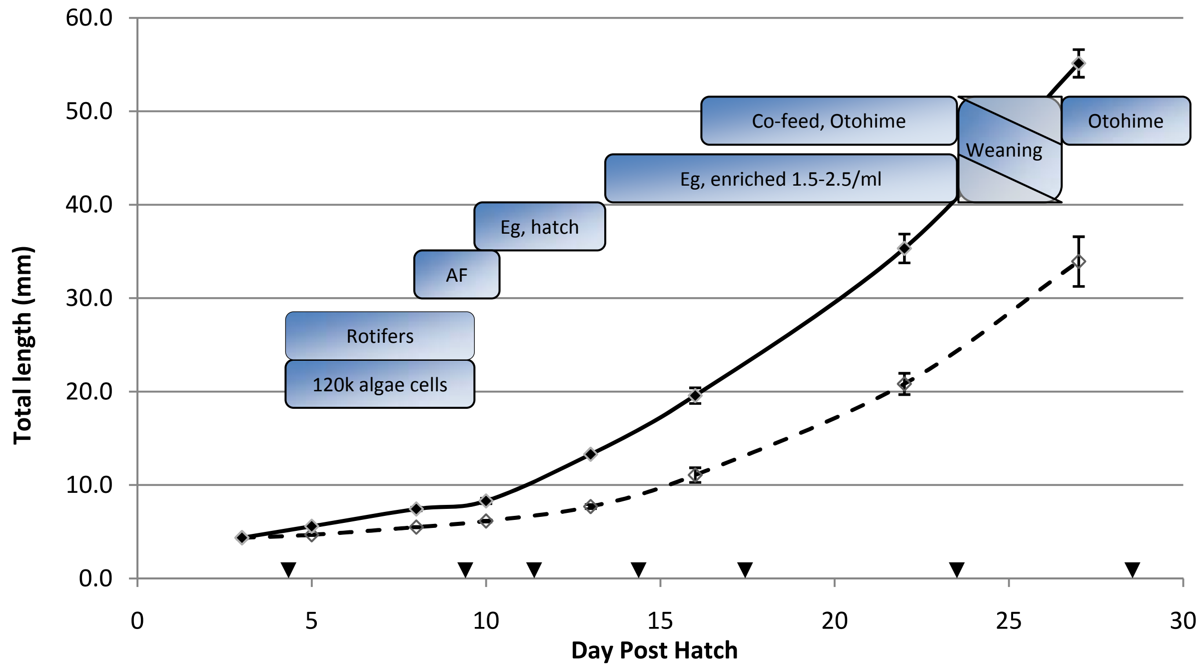
Water quality

Water quality parameters were analyzed daily and included total ammonia nitrogen (TAN), NO_2-N and NO_3-N, which were measured spectrophotometrically (HACH DR/2400 Spectrophotometer, HACH Co., Loveland, CO); dissolved oxygen, temperature and salinity, which were monitored using a YSI model 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA); pH, measured by a HI 9024 pH meter (HANNA Instruments, Woonsocket, RI); and alkalinity which was determined by titration (APHA, 1998). Averages of water quality parameters throughout the trial were as follows: temperature 27.8$\pm$0.6 ºC; salinity 27.0$\pm$3.2 mg L^{-1}; DO_2 6.2$\pm$0.3 mg L^{-1}; alkalinity 131.7$\pm$13.4 mg L^{-1}; pH 7.8$\pm$0.1; TAN 0.1$\pm$0.1 mg L^{-1}; NO_3 3.7$\pm$0.71 mg L^{-1}; NO_2 0.1+0.0 mg L^{-1}. No difference between systems in water quality was discerned throughout the trial.

Feeds and feeding

Green water (approx. 120,000 cells ml^{-1} *Nannocloropsis sp*. algal paste, Reed Mariculture, Campbell, CA) and L-type rotifers (*Brachionus plicatilis*, 1.5-2.5 rotifer ml-1) were fed until 8 dph, at which point *Artemia* only was offered (1-2.5 nauplii ml-1, Figure III–1). The level of eicosapentaenoic acid (EPA; 20:5n-3) in the algae was 30% dry weight.

Figure III–1: Growth curve of control (dashed line) and taurine-fed (solid line) cobia larvae and outline diagram summarizing feeding strategy.



Rotifers (1.2-2.5 ml^{-1}) in "green water" (3-8 dph) were followed by smaller (AF, 1-1.5 ml^{-1}, 7-9 dph) and larger (EG, 1-1.5 ml^{-1-}, 9-11 dph) unenriched *Artemia* strains, then enriched EG *Artemia* (1-2.5 ml^{-1}, 12-21 dph). Larvae were co-fed *Artemia* and commercial dry pellets (Otohime, 15-21 dph) prior to full weaning (25 dph), at which time *Artemia* were completely replaced by artificial diet. Arrows heads indicate points of dietary change and sampling day.

Live feeds included enriched rotifers, non-enriched, small-sized (~430 µm) high HUFA profile AF-*Artemia* nauplii (15 mg HUFA g^{-1} dry weight), and enriched EG-*Artemia* nauplii (INVE Inc., Salt Lake City, UT). Rotifers were enriched with DC DHA Selco (INVE Inc., Salt Lake City, UT) at 0.4 g L^{-1}. Similarly, EG *Artemia* were enriched for 24 hours with DC DHA Selco at 0.6 g L^{-1}. Live prey was offered every 6 hours following complete clearing of the tank between feedings. Co-feeding of larvae with Otohime weaning feeds (Reed Mariculture, Campbell, CA) commenced at 15 dph, with 100% artificial diets being offered from 25 dph after a 3-day weaning period (Figure III–1). The dry food was distributed every hour using automated shaking feeders (AF6

feeders, DFT3R8AC timer, Sweeney Feeders, Boerne, TX). The taurine-enriched live prey were delivered according to the same schedule and prepared in a similar manner: rotifers were enriched with 0.4 g L^{-1} day^{-1} of DHA Selco with the addition of 4 g L^{-1} day^{-1} of taurine, while *Artemia* enrichment consisted in 0.6 g L^{-1} of DHA Selco and 4 g L^{-1} of taurine per day. Taurine levels in prey items were measured using HPLC to verify the taurine enrichment.

Sampling and enzyme assays

Samples were collected after dietary change (i.e. 3, 8, 10, 13, 16, 22, and 27 dph, see Figure III–1). Larvae were randomly netted from each tank (20 larvae per tank until 16 dph, 5 larvae per tank subsequently). Larvae were rinsed with distilled water in order to remove salt, and their length was recorded prior to storage in Eppendorf tubes, which were frozen at -80°C until analyses. After completion of the weaning process, weanlings were counted, bulk-weighed and measured to determine final production.

In order to ensure easy and complete homogenization, larvae were tailed and/or headed prior to grinding. Larvae from 3-10 dph were headed, 11-16 dph larvae were headed and tailed and for 22-27 dph larvae the whole gut was excised. All dissections were carried out on an ice-chilled glass slide using a binocular dissecting microscope. Samples were homogenized using an etched-glass tissue grinder (Fisher Scientific, Pittsburgh, PA). Each larva up to 16 dph was homogenized in 50 µl of buffer (20mM Tris-HCl, 1mM EDTA, 10mM $CaCl_2$, pH=7.4). For larvae of 22 dph larvae and older, 100 µl of homogenization buffer was used. The homogenate was

centrifuged in an Eppendorf refrigerated microcentrifuge (14,000 rpm, 10 min, $3^{o}C$), and the supernatant pipetted into new tubes and frozen at $-80^{o}C$ until assay. Since enzyme activities are reduced following successive freezing/thawing cycles (Hale, et al., 2005; Jameel, et al., 1998), all analyses were conducted over 2 days, and no sample was refrozen more than once.

Enzyme kinetic assays were conducted in a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA) plate reader using spectrophotometric methods. At all time points, homogenates as well as the standard solutions were analyzed in duplicate, and each time point consisted of 3 samples (i.e. n=3 in duplicate). Lipase and amylase activities were assayed using commercial kits (Pointe Scientific, Inc., Canton, MI). Lipase and amylase plates were incubated at $30^{o}C$ for 4 min and the increase in absorbance recorded at 550 nm and 405 nm, respectively, over 10 min.

Trypsin activity was measured using N_{α}-P-tosyl-L-arginine methyl ester hydrochloride (TAME, Sigma-Aldrich, St-Louis, MO) as a substrate according to Walsh and Gertude (1970). TAME has the advantage as a substrate in being highly sensitive and selective toward trypsin (Uys and Hecht, 1987). This method was adapted to a microplate assay. Briefly, 100 µL of dH_2O, sample homogenate, or standard solution were pipetted in designated wells, and allowed to warm to $30^{o}C$. Then, 300 µL of buffered substrate solution were added to each well and the increase of absorbance recorded at 247 nm for 8 min.

Pepsin-like activity was assayed using bovine hemoglobin (Hb, Sigma-Aldrich) as a substrate according to the method described by Ryle (1984). This method was adapted for use in a microplate. After centrifugation of the reaction tubes, 250 µl of the supernatant were loaded in a 96-well plate, and the absorbance (endpoint) was read against the blank supernatant at 280 nm.

In international units, the enzyme activity (U) represents the quantity of enzyme that catalyzes the reaction of 1 µmol of substrate per minute. Enzyme activity was determined for lipase, amylase and trypsin. However, it was not possible to determine U for pepsin-like enzyme activity because of the non-specificity of tyrosine residue absorbance at 280 nm (Ryle, 1984). Thus, the pepsin activity unit is defined as $\Delta A_{280}\ min^{-1} = 0.001$ (Anson, 1938) under experimental conditions (30°C, pH=1.7, light path = 1cm). Anson's unit U_{Hb} is defined as the ΔA_{280} between sample mean and blank by reference to a standard curve using Hb.

The following formulas were used:

For lipase activity: $U = \frac{\Delta_{Abs} \times \alpha}{\beta} \times {}^{V_h}/_{L}$

For amylase activity: $U = \frac{\Delta_{Abs} \times V_a}{\varepsilon l \times V_s} \times {}^{V_h}/_{L}$

For trypsin activity: $U = \frac{\Delta_{Abs} \times V_a}{\varepsilon l \times V_s} \times {}^{V_h}/_{L}$

For pepsin-like activity: $U_{Hb} = \frac{\Delta_{Abs}}{l \times \delta \times t \times V_s} \times {}^{V_h}/_{L}$

Where Δ_{Abs} is the change in absorbance per minute at the appropriate wavelength, α is the slope of the standard curve, β is a conversion factor from L to ml (β=1000), V_s is the sample volume (ml), V_a is the assay volume (ml), ε is the extinction coefficient (M^{-1} cm^{-1}) of the product, l is the light path length (cm), δ is the Δ_{Abs} defining pepsin-like activity (δ=0.001), and t is the duration of the pepsin assay (30 min).

Statistical analyses

JMP (Version 7, SAS Institute Inc., Cary, NC, 1989-2005) was used for statistical analyses, with significance levels set at 0.05. Growth performance and enzyme activities were subjected to one-way ANOVA (age vs. treatment) and two-way ANOVA (age vs. treatment x activity) analyses, respectively.

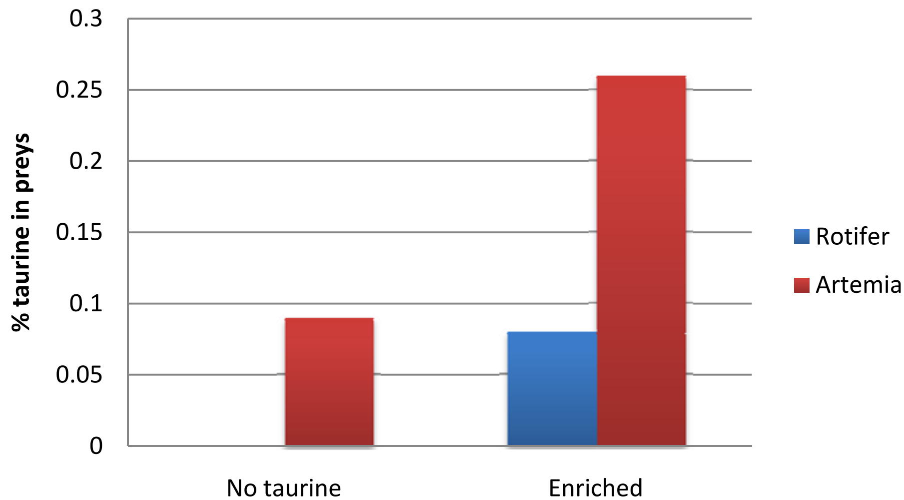
RESULTS

Growth and survival

Figure III–2 shows the enrichment in taurine of rotifers and *Artemia*: enriched rotifers contained 0.09% (wet-weight basis) taurine while unenriched rotifers were totally devoid of it. Taurine levels in *Artemia* were increased 3 folds, reaching 0.26% taurine in enriched preys, which suggest a more active uptake and/or better retention of taurine compared to rotifers. Cobia larvae receiving dietary taurine grew significantly faster than controls (P<0.0001). At 27 dph, the length, wet weight and survival of taurine-treated larvae was 55.1 ± 1.48 mm,

0.60 ± 0.09 g and 29.2 ± 0.4 %, respectively, whereas those of control cobia were 33.9 ± 1.01 mm, 0.35 ± 0.04 g, and 7.1 ± 1.16 % (Figure III–1). Additionally, taurine-fed larvae exhibited a higher degree of development than controls at the same age (opercular spikes, instestinal loop, hypural segments).

Figure III–2: Taurine enrichment of live prey items



Enzyme activities

The time-course of increase in amylase activity is presented in Figure III–3a. No variation in activity was discernable in control fish from 3 dph through to 16 dph, at which point a 4-fold increase ($P < 0.05$) in activity was measured. Thereafter, amylase activity increased until, by full weaning, highest activity levels (1.38 ± 0.31 U individual^{-1}; $P < 0.05$) were observed. Taurine addition to live feeds resulted in a much higher level of amylase activity (Figure III–3a), commencing 16 dph with concentrations being measured at 7.40 ± 1.47 U larvae^{-1} ($P<0.0001$).

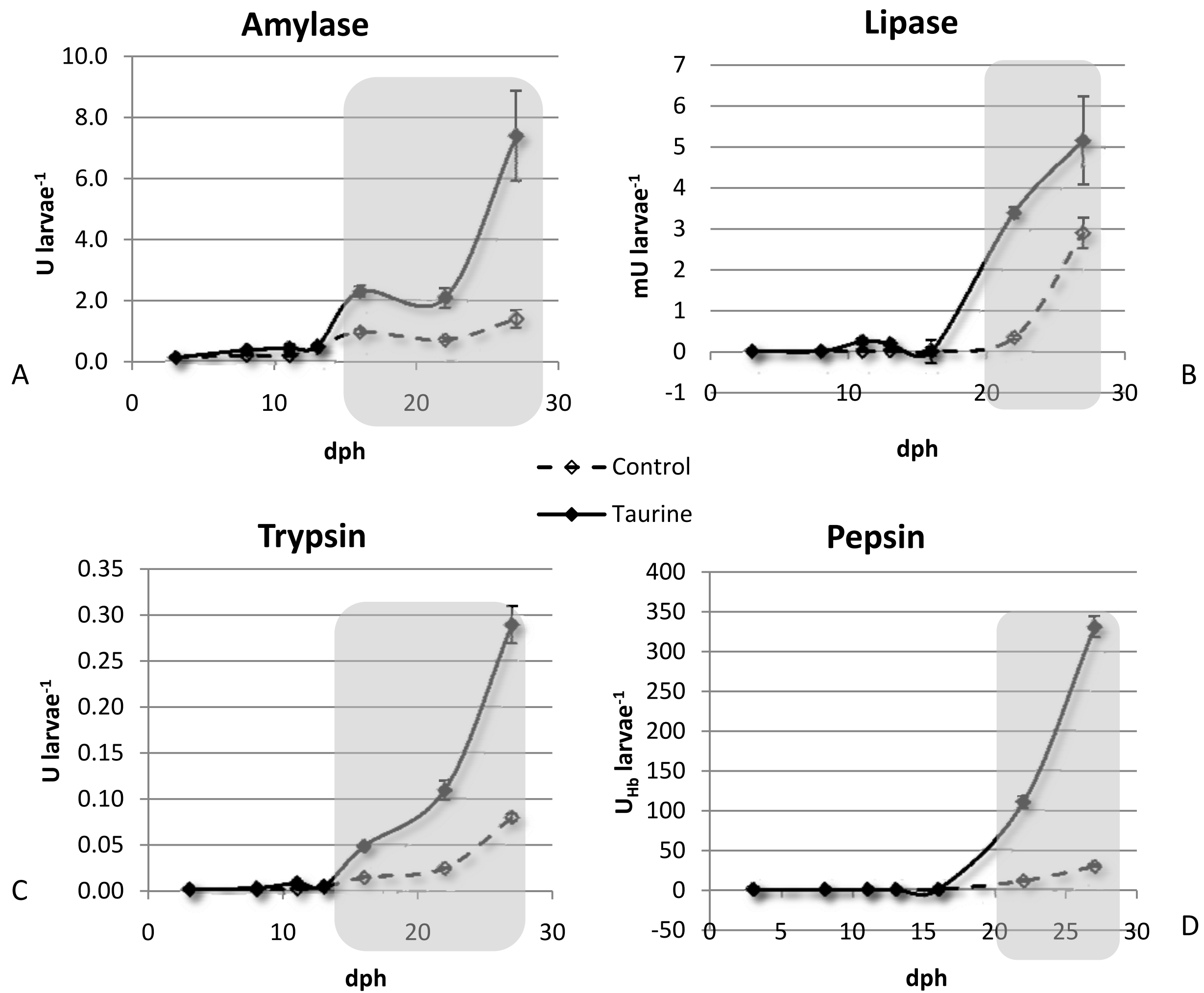
In contrast to amylase, lipase activity in control fish was not detected at 3 dph and remained undetectable until 8 dph (Figure III–3b). From 13-16 dph, lipase presence was detected but increases in activity from baseline concentrations ($P<0.05$) were not discriminated until 27 dph (Figure III–3b), at which point average activity was 2.89 ± 0.38 U $fish^{-1}$. Larval cobia fed taurine-enriched prey expressed nearly twice the lipase activity ($P < 0.0001$) when compared against controls, with the highest levels of 5.16 ± 1.07 U $fish^{-1}$ being recorded at 27 dph.

The time-course of trypsin activity in developing cobia larvae is summarized in Figure III–3c. Trypsin was immediately detectable in 3 dph control animals with activities of 0.002 U $larvae^{-1}$. Subsequently, trypsin levels remained relatively stable until 16 dph, when increased activities ($P<0.05$) were observed. At weaning, trypsin activity was quantified at 0.08 ± 0.004 U $individual^{-1}$ (Figure III–3c). In contrast to control cobia, larvae supplied with dietary taurine exhibited maximum trypsin levels (0.29 ± 0.02 U $individual^{-1}$) that were some three-fold greater ($P<0.0001$) at trial end. Moreover, trypsin activity was observed to be greater at 16 dph in taurine-treated fish ($P<0.0001$).

The developmental progress of pepsin activity is presented in Figure III–3d. Pepsin activity was not detected in 3 dph cobia larvae and remained at or below the level of assay detection until 22 dph. At weaning, pepsin activity in control fish had attained levels of 29.09 ±1.47 U_{Hb} $animal^{-1}$. Likewise, pepsin-like activity also was undetected until 22 dph in taurine-treated fish

(Figure III–3d). However, at 22 dph, pepsin levels were 11-fold higher (P<0.0001; 330.95 ± 13.39 U_{Hb} individual^{-1}) in taurine-treated fish than in controls (29.09±1.47 U_{Hb} fish^{-1}).

Figure III–3: Digestive enzyme activities in cobia larvae.



Relationship between total enzyme activity (U individual^{-1}) and age for (A) amylase, (B) lipase, (C) trypsin and (D) pepsin in cobia larvae from 3 to 27 dph. Values are mean ± SEM (n=3 per time point). Significant differences between treatments and age are indicated by shading (two-way ANOVA, α=0.05)

Discussion

The growth rate of control cobia during the present study was comparable to that observed by Faulk et al. (2007a). However, the fish from this study were approximately 40% longer than those reported by Faulk et al. (2007b) and Hitzfelder et al. (2006), and 26% longer than those reported for tank-reared cobia by Benetti et al. (2008). Although survival was low, it fell within the range of data reported previously for pond-reared larvae (Weirich, et al., 2004), and at the lower end for cobia reared in tanks (Faulk and Holt, 2005; Faulk, et al., 2007b; Hitzfelder, et al., 2006; Salze, et al., 2008). Taurine-supplemented cobia larvae on the other hand, out-performed controls significantly in terms of survival, length and weight gain such that the weanlings from this study represent the largest produced to date at 27 dph ($P<0.05$).

The precise mechanisms modulating the actions of taurine remain to be elucidated in fish. Taurine is found in many tissue types (Huxtable, 1992), and is increasingly regarded as an important, sometimes essential nutrient [e.g. in cats; (Hedberg, et al., 2007)]. Indeed, taurine is incorporated in premature human infant milk formulas (Kendler, 1989; Sturman and Chesney, 1995), to prevent retinal degeneration and cholestasis (a condition occurring when bile cannot flow to the duodenum), thereby underlining its importance during development, as also suggested for fish by the results of the present trial. As in most developing animals, the metabolic rate of fish larvae is high, denoting high demands for energy. Taurine is found in high concentrations in mitochondria, where it is involved in the buffering and stabilization of the mitochondrial matrix (Hansen, et al., 2006). Taurine modifies certain tRNA nucleotides that,

when missing, impair mitochondrial protein translation by increasing the frequency of codon misreading (Umeda, et al., 2005). Additionally, in taurine transporter knockout mice, the activity of oxidative phosphorylation enzymes is compromised (Ito, et al., 2006). Thus, taurine appears to be of critical importance to mitochondrial function and energy production, which partially could explain the poor performance of control larvae. Supplementation of taurine in diets of juvenile fish (Gaylord, et al., 2006; Lunger, et al., 2007b; Sakaguchi, et al., 1988) where better growth was observed likely occurred due to similar physiological impacts.

Marine fish larvae have an absolute requirement for long chain, highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid, ecosapentaenoic acid or arachidonic acid. These molecules must be preserved, while at the same time the developing larvae must protect themselves from oxidative stress. Taurine is known to participate in anti-oxidative mechanisms (Parvez, et al., 2008), and similar actions have been reported in fish (Sakai, et al., 1998). Consequently, the beneficial effect of taurine on cobia growth also might arise from a protective effect from oxidative stress and enhanced utilization of essential HUFA. Taurine is also of primary importance to bone growth and growth regulation, and stimulates the expression of connective tissue growth factor in human bone-forming osteoblasts (Park, et al., 2001; Yuan, et al., 2007). Moreover, taurine decreases the formation and survival of bone-degrading osteoclasts (Koide, et al., 1999) while strengthening bone structure (Jeon, et al., 2007). These mechanisms may provide a partial explanation for the advanced growth in length observed in taurine-supplemented larvae.

Despite slight differences in larval rearing protocols, including temperature, salinity, photoperiod regime and enrichment products, the ontogenic development of enzyme activities in control animals was generally similar to those reported previously (Faulk, et al., 2007a). This suggests that larval cobia development sequence is mainly driven by genetic and nutritional factors, and is relatively independent from other environmental factors (Zambonino Infante and Cahu, 2001). The timing and onset of appearance of enzyme activities however, was different, as I observed a 2 to 5-day delay for trypsin, amylase and lipase. This could have occurred due to differences in assays employed, feeding strategies used, or the effects of culture temperature. Irrespective of such considerations, when taken from a global perspective, the initial elevation in enzyme activities occurs at around 16 dph. While 4-dph cobia have been observed to ingest *Artemia* nauplii, the results of the current trial indicate that larvae may be more reliant on intracellular digestion prior to 16 dph, since pancreatic enzyme activity was very low. However, the activity of brush-border or cytosolic enzymes was not measured, which would be necessary to ascertain this hypothesis. The 16-dph time point also corresponded to the beginning of the co-feeding period, which suggests a possible role of the artificial diet in modulating enzyme synthesis and activation. In their detailed review, Zambonino Infante and Cahu (2007) considered potential influences of factors other than the enzymes themselves, including hormones, cytokines, and nutrients on the onset of enzymatic activity in fish larvae. These biomolecules could regulate enzyme synthesis at the transcriptional and translational levels. Indeed, nutrients such as protein hydrolysate stimulate the activities of cytosolic enzymes, but inhibits trypsin synthesis (Zambonino Infante and Cahu, 2007). Trypsin is also translationally regulated by hormonal mechanisms involving cholecystokinin in response to the

presence of intact proteins, and fish meal can be a poor inducer of trypsin activity (Péres, et al., 1998). Fish meal was an important ingredient in the weaning diet employed. Thus the nature of dietary protein (fish meal vs. alternate sources), as well as the ratio of intact/hydrolyzed protein, should be carefully adapted to the mode of digestion of the larvae. Just as in juvenile nutrition, not only should a proper balance of energy to protein be presented to the larvae, but it should also be delivered at the time the larva has the digestive enzymatic machinery to utilize these nutrients.

Herein I describe for the first time the ontogeny of pepsin-like activity in cobia larvae. Although some marine fish larvae are weaned before the stomach reaches full functionality (Hoehne-Reitan, et al., 2001; Yúfera, et al., 2004), ontogeny of acidic protease activity remains critical when evaluating the digestive capacity of cobia larvae. In larval cobia, acid peptidase activity remained undetectable until 22 dph. This is well after the commencement of the differentiation of the stomach mucosa and the presence of gastric glands described by Faulk et al. (2007b). Similarly, in red porgy (*Pagrus pagrus*), pepsinogen mRNA is not expressed before 30 dph, even though gastric glands are fully developed, morphologically speaking, at 26 dph; moreover, gastric pH only decreases at 35 dph in fed larvae (Darias, et al., 2005). In the present study, the *in vitro* detection of pepsin-like activity does not categorically demonstrate hydrochloric acid production such that acidic peptidase activity may not occur *in vivo* at 22 dph. Nevertheless, a remarkable feature of the present study was the 11-fold increase in pepsin-like activity in fish receiving supplemental taurine. Whether this effect provided the young fish with a digestive advantage, however, remains to be elucidated.

Dietary taurine has long been associated with bile salts and lipid digestion and assimilation in fish (Walton, et al., 1982; Yokoyama and Nakazoe, 1992), with which the lipase activity results in the present trial are in accordance. Lipid metabolism has been linked with thyroid hormone status (Shin, et al., 2006), and which is known to influence development and metamorphosis in many vertebrates, including anurans (McLean, et al., 1998) and fish (Brown and Kim, 1995). Watanabe and co-workers (2006) established a link between bile salts, energy homeostasis and metabolic energy regulation. Using microarray technology, Park *et al.* (2006) examined the impact of taurine on HepG2 human liver cells and confirmed interactions between taurine, protein translation, bone growth, signalling pathways, growth and development. Similar mechanisms of action would be likely for fish, with an abundance of taurine in a diet signifying nutrient wealth, thus accelerating growth, development, and/or cellular turnover and digestive efficiency as observed in the present trial. It is noteworthy that taurine has been demonstrated to avert pancreatic alterations caused by gestational protein malnutrition (Loizzo, et al., 2007), and it is possible that taurine had a like effect on the larval cobia, thus highlighting the importance of this nutrient in development.

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Chapter IV. MORPHOLOGICAL DEVELOPMENT OF LARVAL COBIA

Abstract

The morphological development of larval cobia *Rachycentron canadum* (L.) from 3 days-post-hatch (dph) until weaning was examined using scanning electron microscopy. Two groups of fish were studied: a control (CF) reared under standard feeding protocol, and a group in which prey items were enriched with supplemental taurine (4 g L-1 d-1; TF). TF fish grew faster (P<0.0001), attained greater size (55.1 ± 1.48 mm vs 33.9 ± 1.01 mm) and had higher survival (29.3 ± 0.4% vs. 7.1 ± 1.2 %) than CF fish. Canonical variance analysis confirmed findings with respect to differences in growth between the treatment groups, with separation being explained by two measurements. At 3 dph, larvae exhibited preopercular spikes, sensory epithelia on head and body, an open mouth exhibiting taste buds and primitive nares which took the form of simple concave depressions. The cephalic lateral line system commenced development 12-14 dph in control groups, with invagination of both supra- and infraorbital canals. At the same time, the orbital bone acquired a thorn-like or acanthoid crest. At 14 dph the mandibular and preopercular canals were observed and around 22 dph enclosure of all canals neared completion. In TF larvae, the cephalic lateral line system commenced development 4 days earlier than seen in CF cobia larvae, with enclosure commencing at 18 dph. Primordial gill arches were detected at 6 dph with arches, some of which bore filaments, appearing at 8 dph. Along the flanks of 6-dph larvae, four to five equally-spaced neuromasts delineated the future position of the trunk lateral line. As myomeres were added by the growing larvae, new neuromasts appeared such that each myomere was associated with a neuromast. Development of the trunk lateral line in terms of its enclosure was not observed in weaned CF fish (27 dph), although in the TF group, initiation of canal closure was observed. Teeth were recorded for the first time in CF fish at 20-22 dph, whereas in TF animals teeth appeared between 18-20 dph.

Keywords: ontogeny; taurine; neuromasts; lateral line; scanning electron microscopy; aquaculture;

INTRODUCTION

Larval survival during ontogenesis is dependent, among other factors, on their ability to detect, capture, handle, ingest, digest, and assimilate nutritionally complete food. In carnivorous fishes, the ability to acquire energy is perfected over time, commencing with passive encounters and ultimately evolving into active hunting. During this transformation, there is a coordinated maturation of morphological and physiological enabling mechanisms that include sensory epithelia and organs, locomotive function, oral manipulative abilities, digestive apparatus, appropriate absorptive tissues and assimilative machinery. Delay in this developmental course can lead to stunting, impaired function or even death. A good example of this is seen with the starvation and death of larvae in spite of the presence of food in the intestine (Katavic, 1986; Yúfera, et al., 1996).

Cobia (*Rachycentron canadum*, L., 1766) is monotypic of the family Rachycentridae. It has a global distribution in tropical and subtropical waters, except for the eastern Pacific (McLean, et al., 2008a). As with other candidate species for cultivation, there currently exists a dearth of information on cobia larval development and physiology and this lack may partially contribute to the relatively poor survival rates observed in hatcheries. The gross changes in external morphology of larval cobia have been described previously using wild and museum specimens (Ditty and Shaw, 1992; Finucane, et al., 1978; Hardy, 1978), but no definitive description of larval development has been provided with respect to cultured cobia. Investigations with cultured cobia larvae illustrate changes in lipid composition throughout the developmental

phase (Faulk and Holt, 2003) and diet-related effects on larval fatty acid composition (Faulk and Holt, 2005; Turner and Rooker, 2005). Moreover, diet impacts intestinal fold morphology and integrity (Salze, et al., 2008; Schwarz, et al., 2007), the rate of scoliosis and jaw deformity (McLean, et al., 2008a; Niu, et al., 2008a; Salze, et al., 2008), while also influencing hepatic enzyme activities (Niu, et al., 2008a). Two studies have examined the time-course of appearance of various intestinal digestive enzymes in larval cobia in relation to the ontogeny of the gastrointestinal (GI) tract (Faulk, et al., 2007a; Salze, 2008). These investigations determined that a fully differentiated stomach appeared between 16 and 20 days post-hatch (dph), but enzymatic functionality (i.e., pepsin-like activity) was not discerned until 22 dph (Salze, 2008).

Some of the above information has been integrated into the design of feeding protocols and diets in attempts to increase larval performance. Improved feeds have resulted in increased productivity (Benetti, et al., 2008; Faulk and Holt, 2005; Holt, et al., 2007; Niu, et al., 2008b). Notwithstanding these initial successes, reported weanling survival rates still hover around 3 larvae per liter, which is insufficient to support a more rapid growth of commercial activities. Further improvements in weanling production can be built upon increased understanding and knowledge of larval development and nutritional requirements.

Most studies with larval cobia have taken an end-point approach – that is evaluation of survival and or growth following changes to specific components of rearing protocols (e.g. temperature, salinity, feeds, density). However, more comprehensive approaches, which

examine a greater range of responses, while being less common, clearly offer superior potential in unravelling critical steps in ontogeny that may be employed to develop elite rearing strategies to enhance larval cobia survival. Accordingly, the objective of the present study was to examine the developmental sequence of cultured cobia larvae with particular regard to the ontogeny of sensory epithelia. Concomitantly, the impact of dietary modification, which here examined the effect of supplementary taurine, was explored with respect to developmental ontogeny.

MATERIALS & METHODS

Fish and husbandry

Eighteen thousand, 2 days-post-hatch (dph, 4.4 ± 0.28 mm) cobia larvae, derived from the same batch of eggs, were obtained from the University of Miami, Rosenstiel School of Marine and Atmospheric Science. Animals were stocked randomly into one of two independent, replicated recirculating aquaculture systems at a density of 10 larvae L^{-1}. The design and operating characteristics of each system were described previously by Salze, et al. (2008). Water quality parameters, analyzed daily, included total ammonia nitrogen (0.1 ± 0.1 mg L^{-1}), NO_2-N (0.1 ± 0.0 mgL^{-1}) and NO_3-N (3.7 ± 0.7 mg L^{-1}) which were measured spectrophotometrically (HACH DR/2400 Spectrophotometer, HACH Co., Loveland, CO); dissolved oxygen (6.2 ± 0.3 mg L^{-1}), temperature (27.8 ± 0.6 ºC) and salinity (27.0 ± 3.2 mg L^{-1}), which were monitored using a YSI model 85 meter (Yellow Springs Instruments., Yellow Springs,

OH); pH (7.8 ± 0.1), measured by a HI 9024 pH meter (HANNA Instruments, Woonsocket, RI) and alkalinity (131.7 ± 13.4 mg/L), which was determined by titration (APHA, 1998).

Diets

The feeding schedules employed during the present study mimicked those of Salze *et al* 2008 (see Figure III–1). Briefly, green water (approx. 120,000 cells ml^{-1} *Nannochloropsis sp*. algal paste, Reed Mariculture, Campbell, CA) and L-type rotifers (*Brachionus plicatilis*, 1.5-2.5 rotifer ml^{-1}) were fed until 8 dph, at which point *Artemia* only was offered (1-1.5 nauplii ml^{-1}). Live feeds included enriched rotifers, non-enriched, small-sized high-HUFA profile AF-*Artemia* nauplii, and enriched EG-*Artemia* nauplii. Rotifers were enriched with DC DHA Selco (INVE Inc., Salt Lake City, UT) at 0.4g L^{-1} day^{-1}. Similarly, EG *Artemia* were enriched for 24 hours with DC DHA Selco at 0.6g L^{-1}. All live-prey diets were offered every 6 hours manually. Co-feeding of larvae with Otohime weaning feeds (Reed Mariculture, Campbell, CA) commenced at 15 dph, with 100% artificial diets being offered from 25 dph after a 3-day weaning period. The dry feed was distributed every hour using automated vibrating feeders (Sweeney Feeders, Boerne, TX).

Taurine-enriched live prey were delivered according to the same schedule and prepared in a similar manner: rotifers were enriched with 0.6g L^{-1} day^{-1} of DHA Selco with the addition of 4g L^{-1} day^{-1} of taurine, while *Artemia* enrichment consisted in 0.6 g L^{-1} of DHA Selco and 4g L^{-1} of taurine. The following text will refer to control-fed fish as CF fish and larvae fed the taurine-based diet as TF fish.

Sampling and morphometric analysis

Samples (n=10 per time point) were taken every other day from 3 dph for total length measurements. A graticule was used until the fish achieved 15 mm. Samples subsequently were immersed in fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid, 0.05M sodium cacodylate; pH 7.4) and stored at 4°C until processed for scanning electron microscopy. Individual larvae were rinsed in 0.05M sodium cacodylate prior to being post-fixed in 1% osmium tetroxide and dehydrated with successive ethanol baths (15% to 100%) and critical-point-dried. Larvae were then sputter-coated with gold prior to examination using a scanning electron microscope (EVO 4.0, Zeiss, Thornwood, NY).

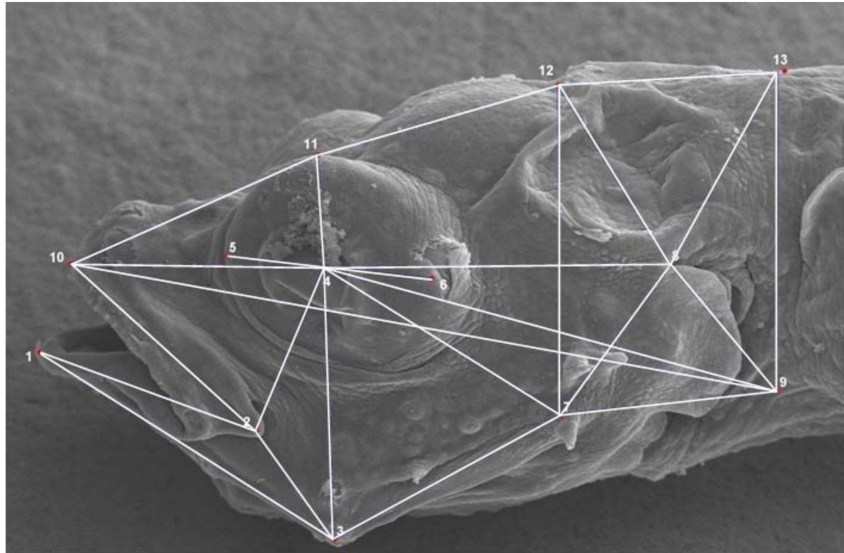
For each sample, photomicrographs were taken of the head profile for morphological analysis. Thirteen discrete points were used to examine morphological development of the head over time (Plate IV–1). These included: the tip of lower and upper jaw (premaxillary and dentary), the ventral extremity of premaxillary, the center of the eye and the two points forming its diameter along the body axis, the angular bone, the curve of the preopercle (bearing bony spikes), the insertion point of the operculum (upper side of the opercular bone), and the lower insertion point of the pectoral fin. An additional three points were added to supplement these and were located at the top of the eye (frontal bone), vertically (relative to the body axis) from the insertion of the preopercle curve spikes (supraoccipital), and vertically from the lower fin insertion point (behind the post-temporal bone). All terminology relating to skull bones employ the nomenclature of Gregory (1959) as illustrated by the overlay in Plate IV–2. During analyses, each point was plotted and extracted using the program *tpsdig*

(http://life.bio.sunysb.edu/morph/soft-dataacq.html). Subsequently, derived files were converted to comma separated files (.CSV) using the program *tpsutil* (http://life.bio.sunysb.edu/morph/soft-utility.html) for further analysis. The coordinates were then stacked and distances between selected points calculated prior to being normalized to a common scale.

Statistical analysis

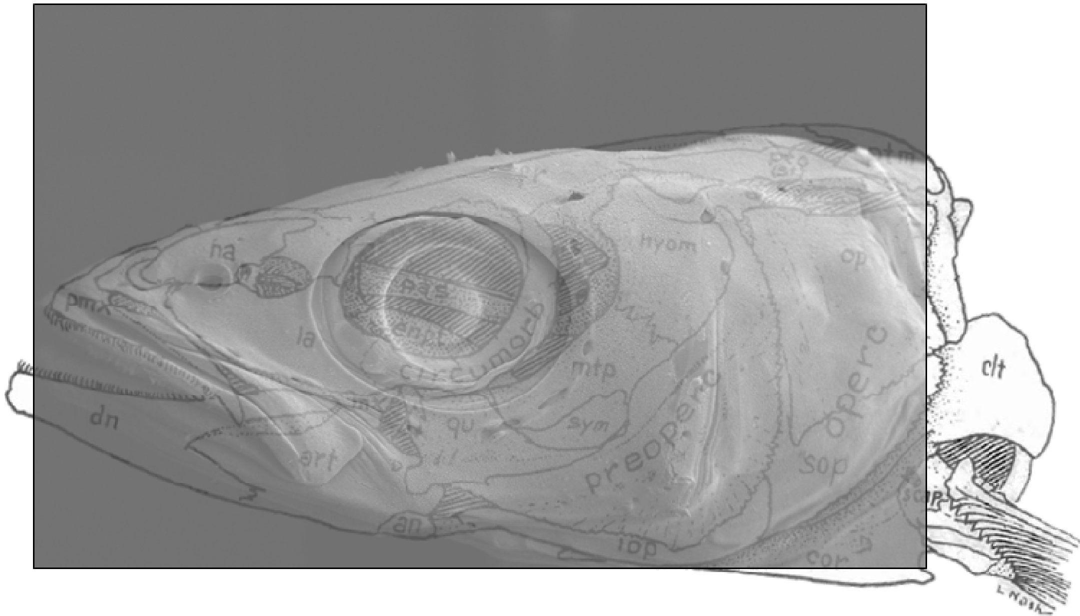
JMP (Version 7, SAS Institute Inc., Cary, NC, 1989-2005) was used to compute statistical analyses, with significance levels set at $\alpha=0.05$. Growth performance was subjected to one-way ANOVA. Image and distance analyses were performed using Canonical Variate Analysis (CVA) in SAS. Regression analysis was performed between CVA and larval age. The experiments described were undertaken in accordance with University Institutional Animal Care and Use Committee Protocol (#06-187-FiW).

Plate IV–1: Position of the points used for morphological analysis



The position of the discrete points on a on a 6 dph larvae were defined as follow: (1) the tip dentary, (2) the extremity of the premaxillary, (3) the angular bone, (4) the centre of the eye, (5 and 6) the eye diameter along body axis, (7) the preopercle curve bearing bony spikes, (8) the insertion point of the operculum (upper side of the opercular bone), (9) the lower insertion point of the pectoral fin, (10) the tip of the premaxillary, (11) apex of the eye, (12) supraoccipital bone (vertical from 7), and (13) the caudal extremity of post-temporal bone (vertical from 9).

Plate IV–2: Overlay of a 27-dph taurine-fed larvae and an adult skull.



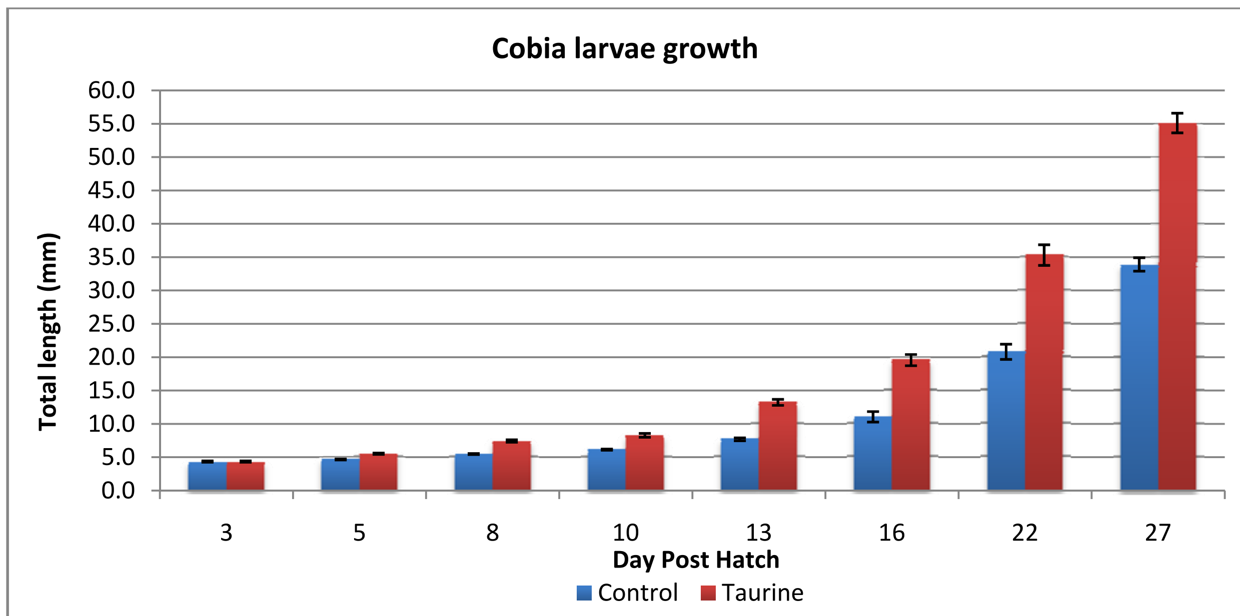
Superimposition of a scanning electron micrograph of a 27-dph taurine larvae and a diagram of an adult cobia skull by Gregory (1959) showing complete bone structure development. With permission of the American Philosophical Society.

RESULTS

Growth and survival

The growth response of experimental fish for specific time-points over the trial is summarized in Figure IV–1. TF cobia larvae grew significantly faster than CF (P<0.0001). At 27 dph, the length, wet weight and survival of TF larvae were 55.1 ± 1.48 mm, 0.60 ± 0.09 g and 29.3 ± 0.41 % respectively, compared to control larvae which were 33.9 ± 1.01 mm, 0.35 ± 0.04 g, respectively, and expressed a survival rate of 7.1 ± 1.16 %. In addition, cobia larvae fed with the dietary taurine supplement exhibited a more rapid rate of development than controls at the same age (Plate IV–3F vs. Plate IV–4F, respectively). No differences were discerned with respect to the rate of spinal deformity, which was 2.37 ± 1.22 % overall.

Figure IV–1: Histogram depicting growth of control and taurine-fed fish throughout the trial



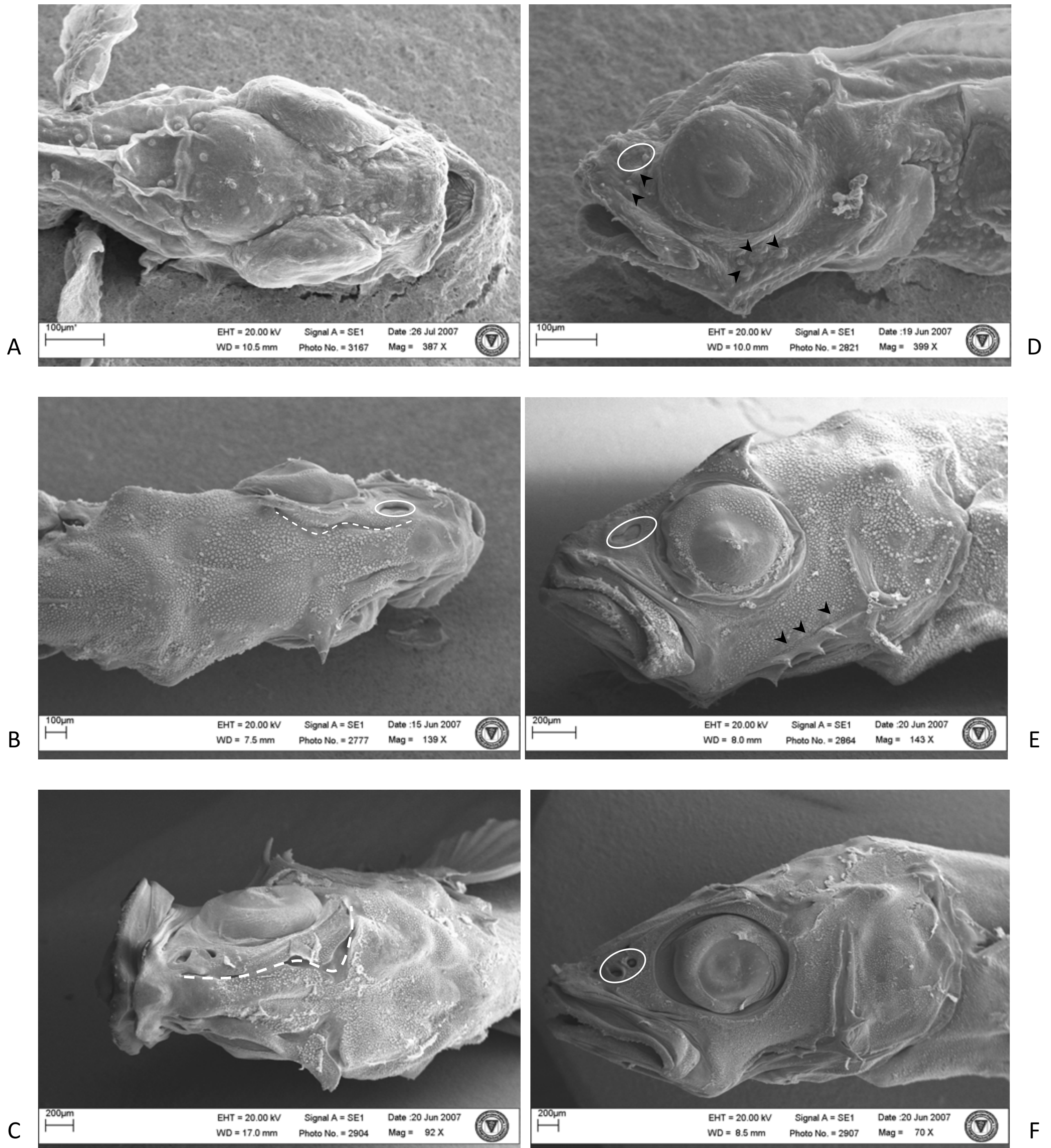
Fish presented with live feeds enriched with taurine were significantly (P<0.0001) longer than CF larvae at all points after 10 dph.

Morphometric and distance analyses

CVA of CF and TF larvae reduced the dimension for separation by forming a linear combination of the distances. An evaluation of the scores in the linear combination indicated that there was a significant difference between CF and TF cobia larvae (Wilks lambda, $P<0.0119$). Further evaluation of the differences associated with individual distances suggested a general increase in growth rate for the TF group. A normal density plot of the centroids of the canonical values of each group is presented in Figure IV–2. Although many distances suggested significant differences between the treatment and control group, a stepwise analysis revealed that most of the separation could be explained by two measurements *viz*. the half-length of the premaxilla (points 2 and 10, Plate IV–1), and the distance from the preopercular bone curve to the supraoccipital (points 7 and 12, Plate IV–1). After completion of the step-wise algorithm, these two measurements had partial F-statistics of 5.05 ($P=0.0228$) and 8.80 ($P=0.0049$) respectively, representing significant differences between the CF and the TF groups. Other distances did not significantly contribute to the separation of the treatment and control after adjusting for the separation explained by these two distances.

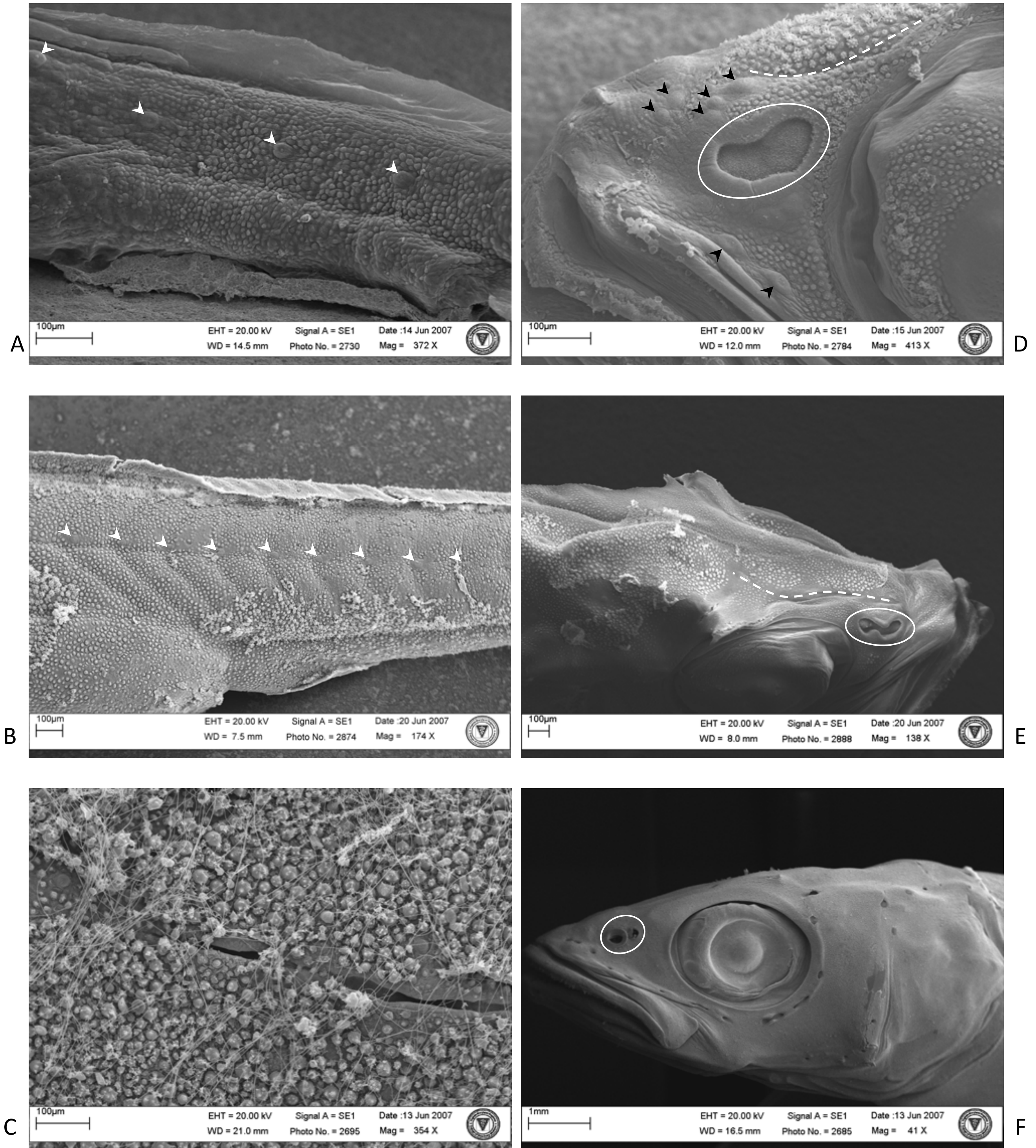
Linear regression was performed on each distance within the CF and TF groups. A positive slope of the linear model corresponds to distance growing with age. All of the linear models demonstrated sustained growth throughout development. The TF larvae exhibited consistently larger slopes which suggest more rapid growth. With the exception of two measurements (points 4-11 and points 8-13), the goodness of fit (R^2) was above 0.5 for all models.

Plate IV–3: Scanning electron micrographs of CF cobia larvae illustrating cranial development and changes in jaw and opercular structures.



A-C: top view of the head (3, 16, and 22 dph); D-F: lateral view of the head (3, 16, and 27 dph). Arrowheads, circles and dashed lines indicate neuromasts, nostrils, and cranial canals, respectively.

Plate IV–4: Scanning electron micrographs of cobia larvae illustrating morphological development.



A-B: lateral views of the CF larval trunk, showing ontogeny of the lateral line (6, 16 dph); C: invaginated and closing lateral line in a 27-dph TF larvae; D-E: detail of the head showing ontogeny of the nostrils and cranial canals in CF larvae (16 and 20 dph); F: lateral view of a 27 dph TF larvae; Arrowheads, circles and dashed lines indicate neuromasts, nostrils, and cranial canals, respectively. Y: eye.

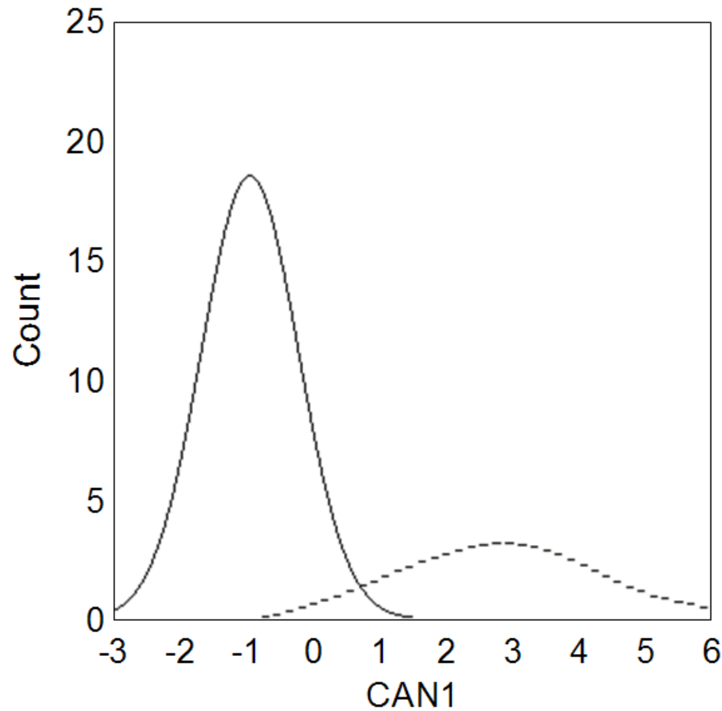


Figure IV–2: Plot of normal densities of the canonical variate calculated for morphological analysis.
The continuous line refers to CF larvae while the dashed line represents TF larvae.

Cranial development

At 3 dph, the larval mouth was open, the eyes large relative to head size (50% of head length) and sensory epithelia discernable (Plate IV–3A, D). Numerous neuromasts were evident on the cranium, being especially prominent in neurocranial and sub-orbital areas and on the lacrimal plate. Four pairs of neuromasts, which were equidistant (~56 µm) in separation, developed symmetrically to the body axis from the dermethmoid bone to the anterior base of the epiotic bone (Plate IV–3A). Additional groups of neuromasts emerged both on the nasal plate, near to the first pair of dermethmoid neuromasts, and along the premaxillary at 8 dph in CF larvae. At hatching, the nares of larval cobia appeared as shallow, open bowl-shaped structures covered by a sensory epithelium (Plate IV–3A and D). By 16 dph, these structures

became more prominent, more concave and elongated. Moreover, the nares developed a constriction which appeared in the middle of the structure's long axis (Plate IV–4D, E). This constriction merged, ultimately forming two apparently disconnected pits at 20-22 dph (Plate IV–3F). In TF larvae, the time to nares constriction occurred more rapidly (13 dph) and separation into pits was completed approximately 4 days earlier – i.e. 16-18 dph – than CF larvae.

Nascent preopercular spikes were present at 3 dph (Table IV–1), attained maximum length around 16 dph, and subsequently were resorbed at full metamorphosis. Development of the cephalic lateral line system commenced between 12 to 14 dph in CF larvae with invagination of the future supraorbital and infraorbital canals (SOC and IOC). Concomitantly, the orbital bone acquired an acanthoid crest (Plate IV–3E), the resorption of which was complete by 22 dph. The IOC was positioned over the circumorbital bone and terminated subordinately to the SOC. At 14 dph, two additional canals started to form: the mandibular and preopercular canals (Plate IV–3E). No direct connection of the latter two canals was observed. At 16 dph, all cranial canals were clearly visible and by 22 dph enclosure was near completion. In TF larvae, the SOC and IOC commenced invagination at 8 dph, with the canals enclosing at 18 dph. The paired SOCs, which adjoined over the frontal bones, connected to the trunk lateral line on their respective sides. Likewise, the development of the mandibular and preopercular canals was contracted in time relative to that of the CF group. Finally, presumptive taste-buds were discerned on the lips and palate of 3 dph larvae. In CF larvae, discrete teeth were first observed at 22 dph (Plate IV–4C).

Development of the gills, trunk and tail

At 6 dph, primordial gill arches were discerned (Plate IV–5A) and by 8 dph, of the 3 visible gill arches observed, the second and third bore filaments and developing lamellae buds (Plate IV–5B). By 13 dph, lamellae were discrete on observable filaments, which became obscured by the operculum at 16-22 dph. Similar developmental patterns for the gills were seen in fish fed taurine at 6 and 8 dph. However, in contrast to CF larvae, by 13 dph, TF larvae presented more advanced gill development, including the presence of longer lamellae.

At 3 dph, neuromasts were distinguishable along both flanks of the larvae, although cupulae were not observed on any preparations. At 6 dph, four to five neuromasts were present on either side of the trunk. These were equally spaced, occurring every 4 myomeres (~200 µm, Plate IV–4A) with an apparent anterior-to-posterior pattern of maturation. Trunk neuromasts increased in abundance over time until, at 16 dph, each myomere was associated with its own neuromast (Plate IV–4B). At this point in ontogeny however, all trunk neuromasts were naked with no canal invagination visible. Initiation of closure of the trunk lateral line was only observed in 27 dph TF larvae (Plate IV–4C), suggesting a delayed developmental sequence compared to the cranial lateral line complex.

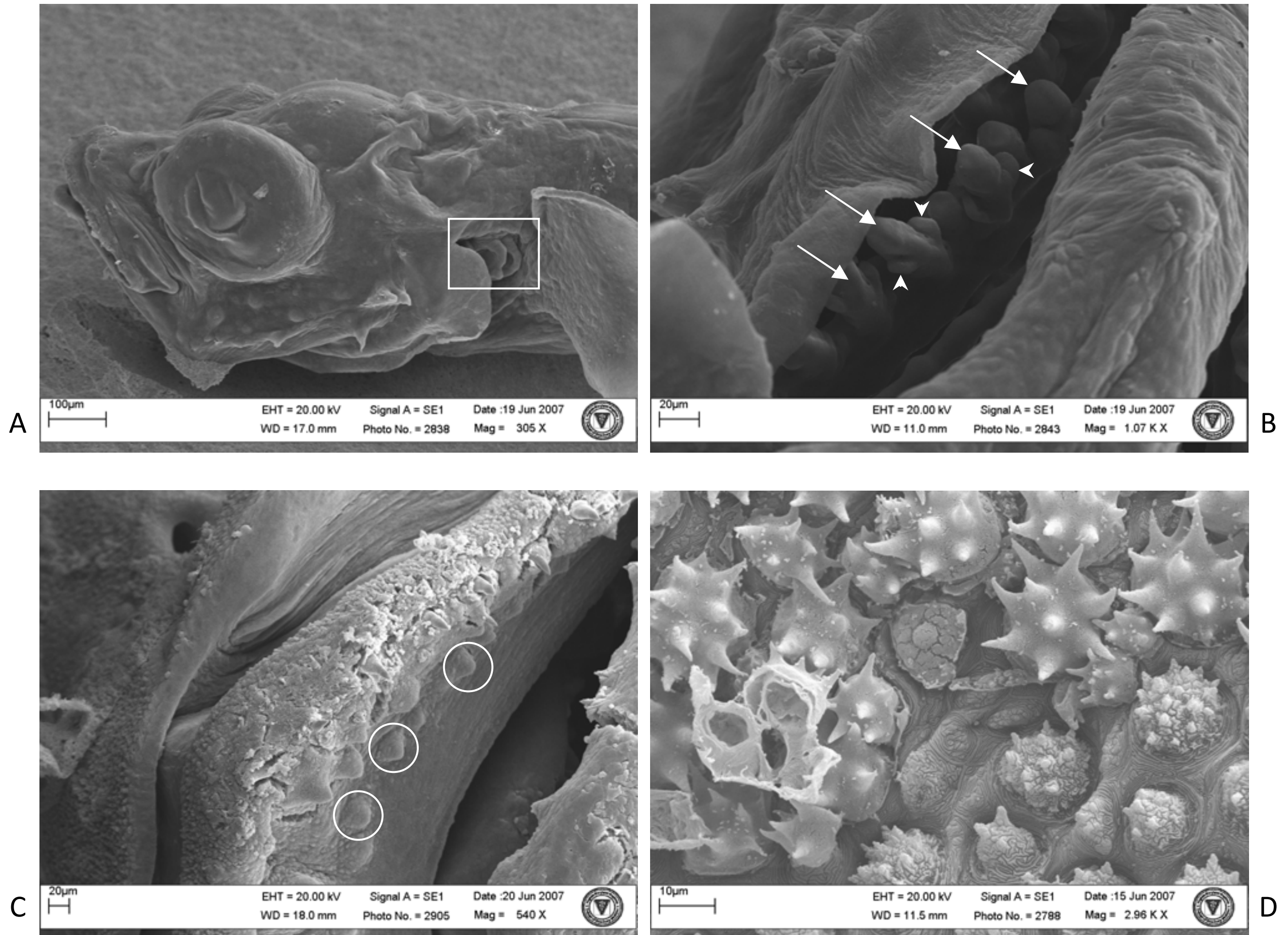
Appearance of the hypural segments was observed between 8 and 9 dph, which preceded the development of finrays and notochord flexion (10 dph; Table IV–1).

At hatching, the larval skin was scale-less with initial scaling occurring at 6 dph. Nascent scales or spicules were present over the entire body by 12 dph and displayed a characteristic crown shape (Plate IV–5D). Desquamation, indicated by detached spicules above newly-formed ones, appeared to be a continuous process from 14 dph.

DISCUSSION

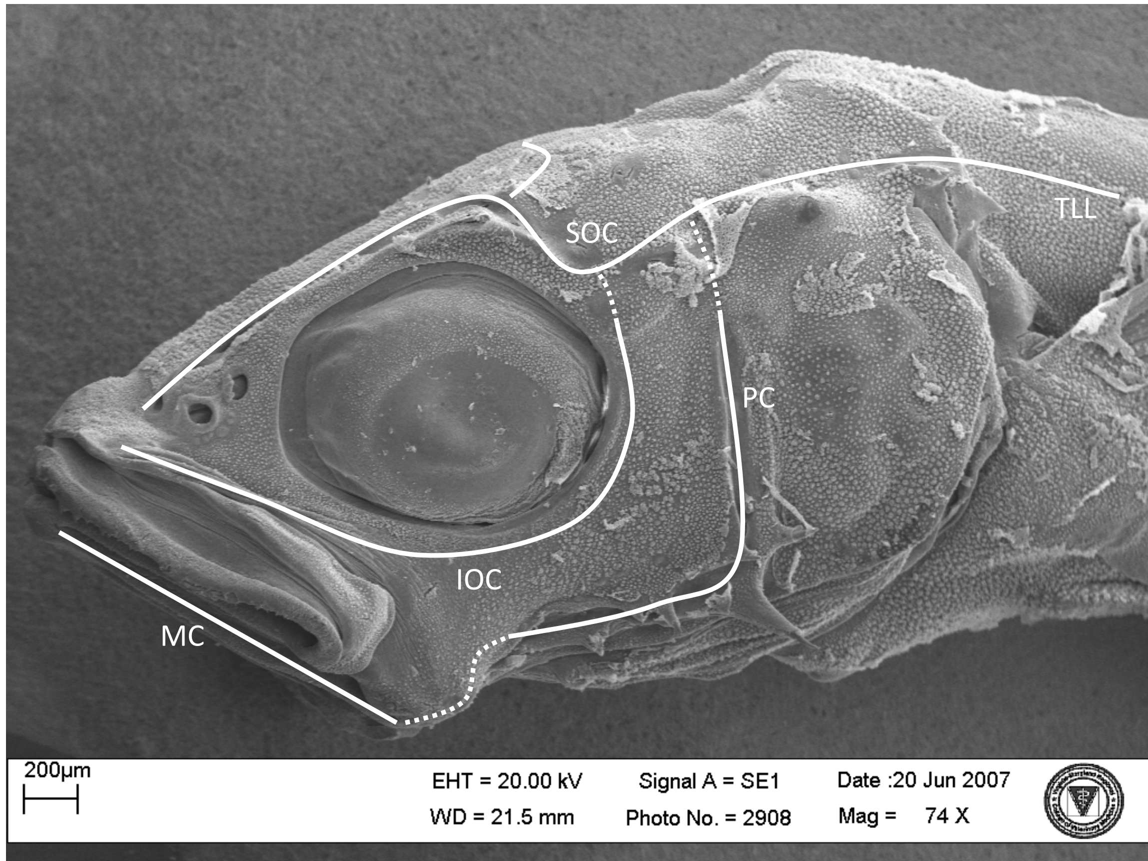
Ditty and Shaw (1992) provided a general description of wild larval cobia gross morphology, and the overall developmental features of cultured fish did not deviate fundamentally from their wild conspecifics. Specifically, Ditty and Shaw (1992) noted the appearance of notochord flexion when larvae were 5 and 6.5-8 mm respectively, which corresponded to a delay of 1 to 2 days when compared to the present study. These differences were likely the result of the lower and more variable temperatures recorded during the museum specimens capture (range 24.2-32.0 degree-days; ^{o}d). The same authors observed “minute epithelial spicules” covering the body of larval animals, which were observed in the present study even at 27 dph, indicating that transition into true scales occurs later, as indicated in Vaughn-Shaffer and Nakamura (1989).

Plate IV–5: Scanning electron micrographs of control larvae: gills, mouth, and skin



A: lateral view of a 6-dph larvae highlighting primitive gill arches (rectangle); B: ventral view of an 8 dph larvae showing gill filaments and lamellae buds (arrows and arrowheads indicate primary and secondary lamellae, respectively); C: detail of the upper lip of a 22-dph CF larvae showing emergent teeth and taste buds (circled); D: detail of the skin of a 16 dph CF larvae illustrating various stages of development of nascent and desquamated spicules.

Plate IV–6: Summary diagram of the cranial canal systems in cobia larvae



Scanning electron micrograph of a 22-dph CF larva: supraorbital canal (SOC), infraorbital canal (IOC), mandibular canal (MC), preopercular canal (PC). The two symetric supraorbital canals join together over the frontal bones, and connect to the trunk lateral line (TLL) on their respective side of trunk. Dotted lines indicate presumptive unifications of the different canals.

Compared with larvae of other marine species that hatch at similar stages of maturity, CF cobia developmental milestones occurred earlier or at a similar time in terms of ^{o}d (Table IV–1). For example, hypural segments appeared at 145°d in cobia, but at 168°d and 187°d in red drum (*Sciaenops ocellatus,* L.*)* and black porgy (*Acanthopagrus schlegelii,* Bleeker), respectively. Noticeably, preopercular spikes emerged as early as 87°d in cobia, whereas in common dentex (*Dentex dentex*, L.) , red drum, and black porgy, spikes were not recorded until later (190°d and 290°d, respectively; Table IV–1). In black sea bream (*Spondyliosoma cantharus*, L.) notochord

flexion and nostril separation transpired at 220°d and 560°d, respectively (Fukuhara, 1987), versus 203°d and 520°d for cobia. Resorption of the yolksac in cobia was completed at 132°d, which was later than that reported for red drum, black porgy and stripped trumpeter (*Latris lineata,* Forster), but similar to sea bream *(Sparus aurata*, L.) and common dentex (Table IV–1). When combined with time to mouth opening (i.e., first exogenous feeding), the earlier stages of cobia development were more similar to those reported for sea bream and common dentex.

Table IV–1: Comparison of developmental sequences between cobia and various marine species that hatch at similar states of maturity.

	Cobia[1]	**Red drum[2]**	**Black Porgy[3]**	**Sea Bream[4]**	**Common dentex[5]**	**Striped trumpeter[7]**
Mouth opening	87	72	51	36-54	83	75
Preopercular spikes	87	288-312	289		190-436	
Yolksac resorption	132	72	78	126	124	98
Hypural segments	261	168	187		160-203[6]	
Notochord flexion	290-377	168-312	221-340	432-504	457-736	268-368
Nostril separation	580-638		391-561			

Data is in degree-days (°d). [1] Present study; [2] Holt et al., 1981; [3] Fukuhara, 1987; [4] Russo et al., 2007; [5] Santamaría *et al.*, 2004; [6] Koumoundouros *et al.*, 1999; [7] Battaglene and Cobcroft, 2007.

Dietary taurine supplementation had a clear impact on growth rates, and a comparison between CF and TF groups using CVA revealed that most variation in cephalic morphological development could be explained by two distinct distances. Upon further inspection, these were

deemed almost geometrically perpendicular. Because of this relative orientation in the body axis of each cobia, they form a two-dimensional basis for cranial development. CVA illustrated that cranial growth was identical in CF and TF larvae, thereby suggesting that taurine did not affect growth patterns; rather the differences observed reflected an accelerated development rate.

Irrespective of the presence of teeth, larval cobia are commonly presented with *Artemia* at 8 dph in hatcheries, and their ability to handle such prey effectively is confirmed via direct gut content observation. Thus the lack of teeth, together with the presence of gill arches and fairly well developed operculum and large mouth, suggests that the feeding mechanism at this stage is suctorial (for discussion, see Drost, *et al.*, 1988). The larval cobia gape size, even at 3 dph indicates that larger-sized prey could be ingested than is offered normally. For example various copepods and small *Artemia* strains are smaller than 200 µm (Shields, *et al.*, 1999), and the gape size of a 6 dph cobia larvae ranges from 270 to 350 µm. However, ingestion potential does not imply digestion capabilities, and physiological and enzymatic considerations (see Chapter III) must be considered prior to adapting such feeding protocols.

Primordial gill arches were observed at 3 dph, whereas primitive gill lamellae were first observed at 8 dph (232°d). Similar observations with respect to mouth opening were reported by Holt *et al.* (2007), although Benetti *et al.* (2008) did not detect the formation of primary gill lamellae until 9 dph (275°d). The more rapid development of primary lamellae observed herein may have resulted due to genetic, nutritional and environmental factors. In TF fish, gill

development did not differ from that of controls through 8 dph, but thereafter a more rapid growth ensued, suggesting that from 8 dph lamellar growth and differentiation was nutritionally driven. During early life stages, fish rely on ion and respiratory gas exchange via cutaneous diffusion and later, through the circulatory apparatus (Blaxter, 1988). The diffusional respiratory mode generally predominates until gill ventilation is established and provides an adequate solution for gas exchange (Osse, 1989). The replacement of cutaneous respiration is generally considered to be a gradual process that is driven by the decreasing anatomical diffusion factor, mass-specific surface area per unit diffusion distance (Wells and Pinder, 1996). Because TF larvae grew at an accelerated pace, parallel increase in gill growth would be anticipated, as was observed. Fishes display great variation in the point at which the respiratory switch occurs (Rombough, 1988), and more definitive information on the precise timing of this process in cobia larvae will demand directed research. Because the gill is engaged in both respiration and ion regulation, its state of morphological maturity does not correlate with the decline of cutaneous respiration (Rombough, 2007).

The distribution of neuromasts and patterns of lateral line sensory system architecture are extremely diverse in fishes; even in closely related species (Bleckmann, 2007). In cobia, the distribution patterns of individual neuromasts, which were present before the formation of budding scales, coincided with the layout of the various cranial and trunk canals. Indeed, only one superficial neuromast could be observed on the head of cobia post-larvae, all other neuromasts being enclosed in the cephalic canal system. A notable feature of the development process was that as the larvae added myomeres to the trunk, new neuromasts supplemented

the populations of each flank. The neuromast series of the trunk comprised an alignment of single, as opposed to the paired neuromasts as seen for the anterior lateral line of blue tilapia *Oreochromis aureus* (Webb, 1989), or the superficial neuromasts described for the Antarctic species *Ophthalmolycus amberensis* (Lannoo and Eastman, 2006). Cranial neuromasts remain superficial in the cichlid *Archocentrus nigrofasciatus* (Tarby and Webb, 2003), whereas they are enclosed in canals in European seabass (*Dicentrarcus labrax*). In fact, the architecture observed herein for cobia is highly similar to that of the latter with respect to cranial canals (Diaz, *et al.,* 2003). This is consistent with the carnivorous behaviour of cobia, since prey are primarily detected and localized by neuromasts in the canal systems (Montgomery, *et al.,* 2002). The progressive addition of neuromasts in growing cobia resulted in different neuromast morphologies and sizes being represented on individual animals. Cobia neuromasts possessed stereocilia and several kinocilia, but all lacked a cupula. During development, neuromasts changed in morphology according to their location. Indeed, kinocilia of canal-neuromasts-to-be became shorter; the structures lost their round, papillate shape, and became flush and elongated along the lengths of presumptive canal axes. In addition, stereocilia disappeared and the neuromasts became similar to the type I form described in the freshwater eel *Anguilla japonica* by Okamura *et al.* (2002). Superficial neuromasts located on the nasal and lacrymal bones, however, maintained their papillate, type II form (Okamura, *et al*., 2002). The absence of cupula from neuromasts may represent an artifact of preparation; often, neuromasts were associated with debris which may have represented displaced cupulae.

The coincidental resorption of the acanthoid crest and preopercular spikes with the closure of cranial canals was remarkable. While these crests and spikes are generally considered to provide a defensive role, they may also serve as a calcium reserve for the completion of cranial canals. The overall developmental observations for the sensory epithelia and associated structures for cobia corroborate findings with other species of teleost (Tarby and Webb, 2003; Webb, 1989). Thus, they emulate the sequential appearance of firstly, neuromasts, secondly, canals, and ultimately scale formation. In cobia, the cranial canals formed before the trunk lateral line (TLL), but lack of histological analyses precluded determination of precise canal linkage. Nevertheless, the left and right SOC clearly connected via a bridge over the frontal bones. A common basic plan is seen in the development of the teleost lateral line system, with the SOC and IOC fusing behind the eye to unite with the TLL, and the preopercular and mandibular canals combining to likewise connect to the TLL (Cernuda-Cernuda and García-Fernández, 1996). This basic architectural arrangement appears to have been adhered to by developing cobia (Plate IV–6).

In the hatchery environment, cobia initiate striking behavior at 3-4 dph, corresponding to mouth opening and jaw movement. At this stage, it is likely that larvae are passing through a learning phase and that strikes are related more to visual acuity, as suggested by the large eye size (about 50% of the head length). From 6 dph, when endogenous reserves are exhausted, movements are more controlled and larvae more dynamic in their hunting actions, indicating positive rheotaxis. These activities become progressively more vigorous over time, suggesting that hunting is keyed not only visually but, as the cranial and TLL become more

developmentally complex, via the sensory system. The lateral line sensory system is unique to aquatic vertebrates, and experimental evidence has demonstrated that this structure is perceptive of water movement, low frequency vibrations and or movement of sound sources (Blaxter, 1987; Bleckmann, 2008; Cernuda-Cernuda and García-Fernández, 1996). The extent to which cobia rely on this sensory system for prey capture requires further examination, perhaps using pharmacological or nerve ablative blocking methods.

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Chapter V. Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia

Abstract

The potential effects of supplementing live feeds with mannan oligosaccharide (MOS; BioMos®) upon cobia *Rachycentron canadum* larval performance were examined. Characteristics of fish examined included survival to weaning, growth, ability to withstand osmotic stress and the degree of development of the brush border of the intestine. Live feeds included rotifers (*Brachionus plicatilis*) and *Artemia* which were enriched for 24 h with a commercial enrichment media alone or in combination with 0.2% (dry weight basis) MOS. Salinity challenges were performed at 6, 7, 13, and 14 days post-hatch (dph; 0 and 65 g L^{-1} for 6 dph, 0 and 55 g L^{-1} for 7+ dph) corresponding to transitions in feeding, to examine the ability of larval cobia to survive hyper- and hypohaline stress. Differences ($P<0.05$) in survival, favoring cobia receiving MOS-supplemented feeds, were discerned at 6 and 7 dph when fish were challenged at 0 g L^{-1} and at 13 dph when challenged with 55 g L^{-1} salinity water. Electron microscopy of the mid-intestine of developing larvae revealed that MOS-supplemented diets enhanced ($P<0.05$) the height of microvilli while reducing ($P<0.05$) the occurrence and size of supranuclear vacuoles. Supplementation of diets with MOS could assist cobia larvae in maintaining allostasis especially when reared at sub-optimal salinities.

Keywords: *Rachycentron canadum*; microvilli; supranuclear vacuoles; stress; feeding; intestine

INTRODUCTION

An absolute requirement for sustainable aquaculture is total life cycle closure of the cultivated species. Similarly, a critical factor during commercial operations is the timely supply of high quality, fully weaned animals. For some marine finfish species, the unreliability of weanling supply represents a crucial obstacle to more rapid expansion of the industry, especially for higher-value carnivores. Cobia are no exception to this rule, and high quality weaned juvenile supply remains a decisive bottleneck to industry expansion (Holt, et al., 2007). Although various groups have achieved successful cobia spawns (Arnold, et al., 2002; Caylor, et al., 1994; Franks, et al., 2001; Liao, et al., 2001), reported survival from egg to weanling has been low (~1 fish L^{-1}). Clearly, larval survival and weanling numbers must be increased dramatically to enable sustainable growth of cobia aquaculture (Schwarz, et al., 2007). One method of enhancing weanlings production might be through judicious manipulations of larval diets.

Mannan oligosaccharides (MOS) are complex carbohydrates derived from yeast cell walls. These compounds contain mannose as the primary carbohydrate element. MOS have a variety of beneficial effects on livestock, ranging from growth promotion in cattle to immunostimulation in swine and avian species (reviewed by Moran, 2004). In piglets, for example, dietary MOS supplementation resulted in more uniform individual weights, enhanced survival, and increased concentrations of immunoglobulin (Funderburke, 2002). The immunomodulatory (Chansue, et al., 2000; Welker, et al., 2007) and growth (Li and Gatlin,

2004; Li, et al., 2005; Li and Gatlin, 2005; Pryor, et al., 2003) benefits of dietary MOS also have been examined in fish but often with contrasting findings. Although the precise mechanism of action of dietary MOS remains to be established, it has been suggested that these compounds may beneficially influence gut bacterial colonization in poults (Juskiewicz, et al., 2006) and humans (Asano, et al., 2004), with the consequent effects of enhanced welfare, gut maturation and growth. These potential beneficial effects, alone or combined, represent significant issues when considering enhanced production of larval marine fishes. It remains appropriate, therefore, to continue the evaluation of MOS products. Herein I evaluate the effect of MOS on larval cobia growth, survival, stress resistance and gut development.

MATERIALS AND METHODS

Animals, system and husbandry

Cobia *Rachycentron canadum* were purchased from a commercial supplier (Aquaculture Center of the Florida Keys, Marathon, FL) at 2 days post-hatch (dph). A total of 54,000 larvae were stocked at 10 L^{-1} into one of 8 replicated 3-tank systems. Each independent unit comprised three 300 L tanks, linked to a reservoir that doubled as a fluidized KMT biofilter. Water from the reservoir was pumped to a bubble bead filter (BBF-2; Aquaculture Technologies Inc., Metaire, LA) to remove suspended solids, then a 100 µm bag filter, a UV sterilizer (80,000 µW cm^{-2} s^{-1}; Emperor Aquatics, Pottstown, PA) for water disinfection, and a protein skimmer (R&B Aquatic Distribution, Waring, TX) to remove particulated organic matter and decrease turbidity. Water temperature was maintained through ambient air heating and the use of

electronically controlled, thermostatic titanium heaters (R&B Aquatics, Waring, TX) located in the reservoir. Bead filters were backwashed daily. Make-up water was supplied from a 38,000 L storage tank (Red Ewald, Inc., Karnes City, TX). Once full, the water was treated with 20 mg L^{-1} chlorine and passed continuously through a rapid-rate sand filter in series with a diatomaceous earth filter before returning to the storage tank. This water then was checked for complete chlorine degassing prior to utilization. Hydrodynamics of the larval tanks was maintained to provide both horizontal as well as vertical cells to optimize larvae–prey distribution and minimize larvae–larvae interaction. Initial system salinity was 35 g L^{-1}, which was reduced 1 g L^{-1} day^{-1} until a final salinity of 24 g L^{-1} was achieved at 13 dph. At trial start, two of the eight stocked three-tank systems were randomly allocated as control and treatment systems.

Green water (approx. 120,000 cells ml^{-1} *Nannochloropsis* sp. algal paste) and L-type rotifers (*Brachionus plicatilis*) were fed to cobia larvae until 8 dph, at which point *Artemia* only was offered (1.5–3.5 individuals mL^{-1}). Algae and rotifers (2 mL^{-1}) were added to the culture tanks simultaneously every 6 h. The level of eicosapentaenoic acid (EPA; 20:5n3) in the algae was 30% dry weight. Every 6 h, non-enriched, small-sized (~430 μm) improved HUFA profile (15 mg g^{-1} dry weight) AF-*Artemia* (INVE Aquaculture, Inc., Salt Lake City, UT) nauplii were fed, following complete clearing of the tank between feedings, 6–9 dph. Densities used were 1.5 nauplii mL^{-1} 6 dph, 2.5 nauplii mL^{-1} at 7 dph and 3.5 nauplii mL^{-1} between 8 and 9 dph. Likewise, every 6 h, from 8–11 dph, 1.5–3.5 EG-*Artemia* (INVE Aquaculture, Inc.) mL^{-1} were added to the tank followed by the identical density of enriched EG-*Artemia* (INVE Aquaculture, Inc.) nauplii from 12–21 dph (see Figure III–1). EG *Artemia* were enriched for 24 h with DC DHA Selco (INVE

Aquaculture, Inc.). Experimental diets employed the identical feeding regime except that enrichments of EG *Artemia* employed MOS (0.2% dry weight; BioMos®, Alltech Inc., Nicholasville, KY) in addition to DC DHA Selco. Uptake of MOS by *Artemia* was established visually using a red food dye in pre-trial tests. Co-feeding of larvae with Otohime weaning feeds (Reed Mariculture, Campbell, CA) commenced at 18 dph. Co-feedings consisted of feed delivery 30 min and 15 min prior to *Artemia* additions with prepared diets being delivered at 2 g feeding^{-1} tank^{-1}. Dry feed only was offered every 6 h from 21 dph. Decisions regarding feed substitution during rearing were based upon daily gut content analyses. Photoperiod was maintained at 24 h light until 22 dph, at which time photoperiod was reduced to 20:4 at 260 lx.

Sampling and analyses

Throughout the study, samples of fish were measured using a microscope and attached graticule. At specific time points throughout the trial (indicated by arrows on the feeding protocol; see Figure III–1), 30 fish were randomly taken from each tank (control and MOS-enriched; N=90 treatment^{-1}) and subjected to salinity challenge. The stress tests were undertaken using eighteen 1L aerated beakers (10 fish treatment^{-1} beaker^{-1} tank^{-1} in triplicate). Salinity stressors were set 0 and 65 g L^{-1} for 6 dph, at which point the high salinity stressor was reduced to 55 g L^{-1} for the remaining challenges. The reduction in salinity was employed following preliminary salinity tests with older larvae. Animals were evaluated every 5 min to record mortality or the point at which no mortalities occurred over three consecutive time intervals. Stress was measured using the stress sensitivity index (SSI; Dhert et al., 1992), which

is the average cumulative mortality rate recorded for the replicated treatments over time. The higher the numeric value of the index, the higher the level of stress experienced.

Water quality parameters analyzed included TAN, NH_4–N, NO_2–N and NO_3–N, which were measured spectrophotometrically (HACH DR/2400 Spectrophotometer, HACH Co., Loveland, CO); dissolved oxygen, temperature and salinity, which were monitored using a YSI model 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA); pH, measured by a HI 9024 pH meter (HANNA Instruments, Woonsocket, RI), and alkalinity, which was determined by titration (APHA, 1998). Total gas pressure (N_2 and O_2) was measured inside each tank using a YSI tensionometer (Aquatic Ecosystems, Apopka, FL). Initially, water quality parameters were analyzed twice daily for each tank, which was reduced to daily measurements after 10 dph.

For microscopy, whole larvae (variable n per time point but always ≥5) were fixed (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid, 0.05 M sodium cacodylate) and subsequently post-fixed in osmium tetroxide, dehydrated with successive ethanol baths (15% to 100%) and embedded in plastic (Poly/Bed 812) using standard methods (PolySciences, Inc. technical data sheet). Samples were thick-sectioned (1 µm) stained with hematoxylin and eosin and examined by light microscopy (Leitz Laborlux S Fixed Stage microscope equipped with a Nikon 5500 digital camera) prior to preparation for transmission electron microscopy (TEM). For TEM, thin sections (60– 90 nm) were placed on copper grids and stained with uranyl acetate and lead citrate. Images were examined using a Zeiss 10CA TEM and acquired by an AMT

Advantage GR/HR-B CCD Camera System. Acquired images were examined using the University of Texas Health Sciences Center in San Antonio Image Tool.

Data were subjected to analysis of variance procedures utilizing SAS 9.1 (SAS Institute, Cary, NC, USA). Where appropriate, data also were subjected to Duncan's multiple range test for means separation. Differences were considered significant at $\alpha<0.05$. Comparisons between enterocyte morphology and salinity stress tests were made using PROC t-test (SAS 9.1).

RESULTS

At 28–29 °C across all tanks and treatments, yolk sac absorption was complete at 3 dph, correlating with first feeding. Oil globule absorption was concluded at 8 dph. Length growth of cobia was similar irrespective of treatment ($P>0.05$). At 28 dph, weanling weight averaged 0.42 g (range 0.35–0.52 g) with a survival of 24.4% (range 21.8–28.2%), resulting in an approximate production of 2.5 fish L^{-1} (range 2.18–2.82). Water quality parameters did not vary between systems: temperature ranged from 28 °C to 29 °C, salinity from 34 reduced to 24 mg L^{-1}, DO between 6 and 8 mg L^{-1}, alkalinity from 150 to 200 mg L^{-1}, Ca+ 281 ± 39 mg L^{-1}, Mg+ 753 ± 40 mg L^{-1}, pH between 7.8 and 8.0, ammonia < 0.01 mg L^{-1}, N total pressure <100, and O_2 total pressure <125.

Table V–1 summarizes the Stress Sensitivity Index (SSI) responses of larval cobia. Exposure of cobia to salinities of 0 or 55/65 g L^{-1} revealed that larvae could withstand hypersaline better

than hyposaline challenges. A tendency towards decreased stress resistance to hypersalinity was apparent with age. Fish receiving MOS-supplemented diets expressed an overall greater ability to withstand hyposaline stress ($P<0.001$). Differences ($P<0.05$), favoring MOS-treated fish, were observed in survival when subjected to 0 g L^{-1} at 6, 7 and 14 dph, while at high salinities differences were only observed in favor of MOS-fed fish at 13 dph.

Plate V–1 illustrates differences visualized in the apical region of the anterior absorptive enterocytes of the intestine of 8-dph cobia larvae. Enterocytes of fish provided with MOS supplementation expressed more uniform, densely-packed and longer microvilli than observed in the identical gut regions of larvae fed control diets (2.04 ± 0.02 µm vs. 1.18 ± 0.03 µm; $P<0.05$). Moreover, the number and size of supranuclear (SNV) vacuoles in MOS-treated cobia were lesser and smaller respectively than those observed in control samples from 8 dph throughout the length of the intestine. The width of SNV in MOS-treated fish was 0.87 ± 0.32 µm versus 1.69 ± 0.46 µm in control larvae ($P<0.01$).

Table V–1: The response of larval cobia to hyper- and hyposaline challenges

Treatment	**Salinity**	**Stress sensitivity index**			
		6dph	7dph	13dph	14dph
MOS	0 gL^{-1}	**50.6±6.8**	**60.6±6.6**	96.0±11.5	**82.3±2.4**
Control		70.0±2.5	108.0±0.0	98.0±10.5	98.6±0.7
MOS	55 gL^{-1}		23.6±3.7	**46.3±9.1**	64.0±3.6
Control			41.5±24.0	70.6±3.8	59.3±2.7
MOS	65 gL^{-1}	89.0±8.54			
Control		98.0±2.52			

The stress sensitivity index follows Dhert et al. (1992), in which lower numbers signify higher stress resistance. Numbers in bold indicate significant difference ($P<0.05$) as determined by t-tests between control and MOS-supplemented larvae.

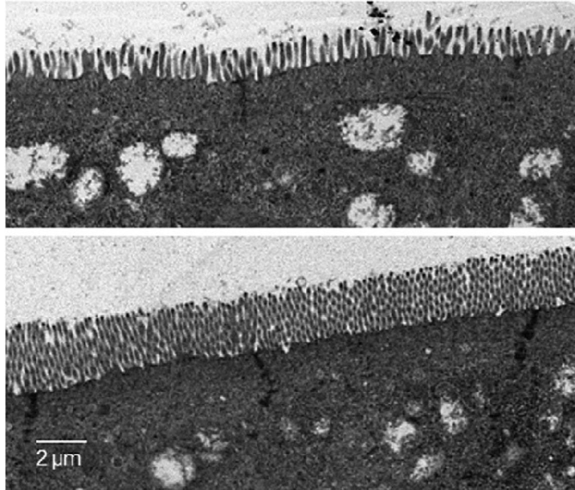
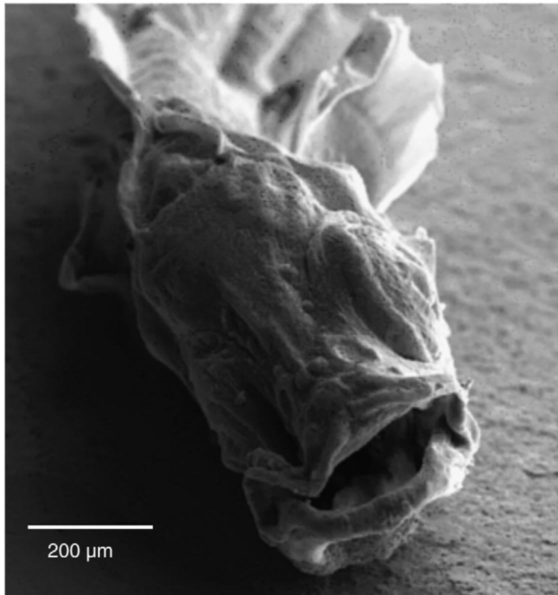


Plate V–1: Representative electron micrographs of enterocytes of the anterior intestine of 8-dph cobia larvae Upper plate: control-fed larva; lower plate: MOS-fed larva. Feed supplementation with MOS resulted in a heightening (P<0.05) of absorptive cell microvilli and a reduction in the number and size of vacuoles and vesicles in the supranuclear region of the cell.

DISCUSSION

Cobia commenced their first exogenous feeding 2–3 dph in concert with yolk sac absorption. Larval growth was similar to that reported previously (Faulk and Holt, 2006; Faulk, et al., 2007b; Hitzfelder, et al., 2006), however here I record much higher survival rates. Reduced larval mortality may have resulted from maintaining high water quality and a lowering in physical damage by convectional and rotational currents keeping larvae away from tank sides and standpipes (Rasmussen and McLean, 2004; Rasmussen, et al., 2005). Cannibalism is a factor that seriously influences larval survival in many marine species (e.g., Sawada, et al., 2005). Cannibalism was observed in all production tanks commencing around 17 dph, which coincided with the larvae's ability to orient themselves independently against currents. Grading between 14 and 16 dph could reduce these losses, and cannibalism could be decreased through manipulating tank hydrodynamics further. Since eggs were purchased from an outside supplier, no control could be exerted over the quality or method of spawning. Thus, larval mortalities due to genetic factors (Green and McCormick, 2005) and poor initial egg quality, including that

Plate V–2: Scanning electron micrograph of larval cobia at 4 dph illustrating lower jaw deformity.



Note nascent nares which ultimately develop into 6 openings and paired sensory cells between the eyes.

caused by induced spawning (Avery, et al., 2004), were inescapable. However, closer examination of newly hatched larvae revealed a propensity towards mandibular macrognathia (Plate V–2). This abnormality, characterized by extension of the lower, usually right jaw, appears to be relatively common in cobia. Initially, I believed that the condition evolved from a nutritional deficiency during first exogenous feeding. However, scanning electron microscopy (SEM) of 4-dph larvae out-ruled this hypothesis, and suggested other environmental parameters or genetic as possible causes. It is not possible to determine the importance of jaw deformation to overall larval survival although numerous individuals, exhibiting varying degrees of this syndrome, survived through the weaning process. Nevertheless, inadequate broodstock diets and conditioning as well as between-batch egg quality variations may represent elements contributing to the occurrence of these deformities and mortality.

The fact that 25% of stocked fish were fully weaned indicates that nutritional deficiency likely had negligible influence on overall survival. Nevertheless, it was conspicuous that the largest percent mortality of larvae was recorded 8–12 dph, corresponding to primary shifts in the diet, thereby suggesting digestive deficiency. The observations of Faulk et al. (2007a) on

cobia gut enzyme development might be used to argue for full gastrointestinal functionality at 12–16 dph. Nevertheless, enzymatic insufficiency may be compensated for by macromolecular absorptive mechanisms. A notable feature of absorptive enterocytes of the larval cobia intestine was the presence of supranuclear vacuoles (SNV) in the apical region of the cells. These structures may act as a supplementary mechanism for the absorption and transport of lipids and intact proteins and polypeptides in the anterior and posterior gut segments, respectively (Iwai, 1967; Iwai and Tanaka, 1968; McLean, et al., 1999; Watanabe, 1984). Control cobia had more numerous and larger SNV than MOS fed fish — a distinction which may have resulted due to the MOS driving gut development more rapidly as evidenced by the longer microvilli of the absorptive epithelia. It has been suggested that MOS acts to protect the gut by blocking bacterial adhesion, modifying gut microflora to support increased nutrient availability, reducing enterocyte cycling rates while enhancing the production of the protective mucin barrier (see Ferket, 2004). The latter may have importance in terms of ion regulation.

It is well established that marine fish larvae are able to withstand dramatic short-term changes in salinity, ranging between 0 and 65 g L^{-1} (Varsamos, et al., 2005), and cobia were not an exception to this generalization. Noteworthy was that larval cobia were more able to adjust to hypersaline challenge and that osmoregulatory capabilities appeared to improve with age. Similar observations have been reported for many other marine species (Hickman, 1959; Holliday and Blaxter, 1960; Zydlewski and McCormick, 1997), and this response likely reflects the maturation of the osmoregulatory apparatus (Alderdice, 1998). The role of the integument, gills, digestive and endocrine systems in the maintenance and control of ionic balance in fish

larvae was reviewed by Varsamos et al. (2005). For most marine teleosts, short-term tolerance to salinity stress is considered high for early stage larvae but declines dramatically during mid-larval development. Even though the first remarks concerning the role of the fish gut in maintaining hydromineral homeostasis were made 80 years ago (Smith, 1930), few studies have examined the role of the larval gut in osmoregulation and how this may be impacted by developmental stage. Whether the different abilities of MOS-treated groups to withstand salinity variations resulted due to a more rapid maturation or protection of the intestinal epithelia requires further study.

The presence and role of branchial and extrabranchial ionocytes, their potential involvement in osmoregulation and the relative importance of the gut to ionic homeostasis will demand the use of tracers to establish drinking rates as well as evaluation of variations in larval blood osmolality. An important issue with respect to the apparent protective nature of MOS against hyposaline challenge relates to the emergence of inland, tank-based cobia production. Reductions in salinity are more likely to be experienced in these commercial hatcheries than increases, and thus, fortifications of live feeds with MOS may assist the developing animal in allostasis and future performance (see Varsamos, et al., 2006).

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Chapter VI. REPLACEMENT OF FISH OIL WITH NOVEL SOURCES OF N-3 HIGHLY UNSATURATED FATTY ACIDS IN AQUAFEEDS FOR JUVENILE COBIA

Abstract

Replacement of fish ingredients in aquafeeds is a major line of research in aquaculture. However, the majority of this research has focused on alternate protein sources, despite the more pressing perspective of fish oil (FO) shortage. Two strategies may be employed to replace essential fatty acids (FA): direct supplementation, or supply of precursors to promote *de novo* biosynthesis. Both strategies were investigated in the present study through the use of two novel lipid sources: a docosahexaenoic acid (DHA)-rich algae meal (ALG) and a stearidonic acid (STA, n-3 FA precursor of EPA and DHA)-rich oil, derived from a genetically modified soybean (MSO). Starting from a control diet (100% FO), FO was gradually replaced by MSO in the first 3 experimental diets, and MSO by ALG in 3 other diets (thereby were FO-free). A 7th diet was formulated with a different algae meal produced at Virginia Tech (AVT). The response of juvenile cobia (starting individual weight: 30g) was measured in terms of growth, feed efficiency (FE), survival, body and liver composition, and biological indices. FE was high and similar across treatments (73%), while growth and hepatosomatic index were significantly impacted by dietary treatment. Proximate analysis did not reveal elevated levels of EPA or DHA levels in the muscle or liver, thus suggesting that juvenile cobia were unable to elongate STA. In contrast, juvenile cobia performed best when fed algal meals, and accumulated DHA levels in their muscle that would statisfy the USDA-recommended daily DHA intake for healthy adults. In conclusion, feeding FA precursors does not appear to be a viable strategy for FO elimination in cobia aquafeeds. FO-free diets were successfully formulated using DHA-rich algal meal in conjunction with soy oil. Essential FA must be directly supplied through the diet by an EPA- and DHA-rich source, whether naturally present or genetically engineered.

Keywords: *Rachycentron canadum*, soybean, fish oil replacement, elongation

INTRODUCTION

As the global demand for seafood products continues to rise, it is no longer feasible to rely solely upon oceanic wild fisheries, and the aquaculture industry constitutes the only alternative source for these commodities. However, aquaculture traditionally depends highly upon reduction fisheries and the resulting fish meal and fish oil, which by all accounts cannot sustain the levels of production that must be reached to fulfill this impending seafood deficit. Thus, the industry must move away from these protein and lipid sources to achieve environmental and economic sustainability, and utilize suitable alternative feedstuffs in formulated aquafeeds.

To be considered a viable alternative to fish meal or oil, a feedstuff must be widely available and competitively priced, as well as easily handled, stored and amenable to feed production (Gatlin, et al., 2007). The vast majority of research into use of alternative ingredients has focused mainly on fish meal replacement (FMR), with investigations into the use of plant proteins predominating. While other protein sources are available, their use is either discouraged (e.g. meat and bone meals due to fears of Bovine Spongiform Encephalopathy) or simply not economically feasible due to a lack of consistent and sufficient supply. As a result of these research efforts, particularly with plant-based alternatives, fish meal inclusion rates have been significantly reduced over the past decade (Gomes, et al., 1995; Lunger, et al., 2007b), although further reductions are mandatory if the aquaculture industry is to become truly sustainable.

While advances in terms of limiting the utilization of fish meal in aquafeeds have been noteworthy, depletion of fish oil resources are more pressing than that of fish meal. In fact, the FAO predicted that in 2015, global use of fish oil will exceed 145% of the historic world production levels (New and Wijkström, 2002). Fish oil replacement in aquafeeds is more problematic for several reasons. First, marine fish species require n-3 highly unsaturated fatty acids (HUFA) that are prevalent in marine fish oils. These requirements must be addressed when looking to replace fish oil, especially if fish meal, another source of these marine-derived n-3 fatty acids (FA), is also replaced or reduced in the dietary formulation. Another aspect of fish oil replacement that must be accounted for is the beneficial effects on human health that have been clearly documented. These include, but are not limited to, decreased cardiovascular disease, dementia, depression and Alzheimer's disease (Bourre, 2006; Das, 2008).

Finally, it is essential that alternate sources to fish oil are sourced through commodity crops, so they are produced consistently in sufficient quantities to support an industry. For instance, while global production of fish oil remains stagnant, the production of vegetable oils has steadily increased and reached a volume of a hundred-fold higher than that of fish oil (Bimbo, 1990). Numerous studies have successfully examined the incorporation potential of vegetable oils in fish diets in various marine carnivorous species, including European sea bass *Dicentrarcus labrax* (Montero, et al., 2005), gilthead seabream *Sparus auratus* (Izquierdo, et al., 2005), and turbot *Psetta maxima* (Regost, et al., 2003a; Regost, et al., 2003b). However, if the aquaculture industry is to utilize vegetable oils as fish oil replacements, it is imperative that the beneficial health impacts of fish oil, not only upon the targeted species, but also for the human consumer,

are retained. Undoubtedly, there is a need to further investigate alternate lipid sources that not only fulfill the biological requirements of the cultured animal, but also will provide health benefits accrued by the consumption of seafood on a regular basis.

When considering the replacement of fish oil with plant-derived lipid sources, several strategies could be employed to ensure that the n-3 FA levels are maintained for both the fish as well as the consumer. The most promising tactics relate to direct dietary supplementation with novel, alternate n-3 FA sources and/or enhancement of endogenous biosynthesis through dietary manipulation.

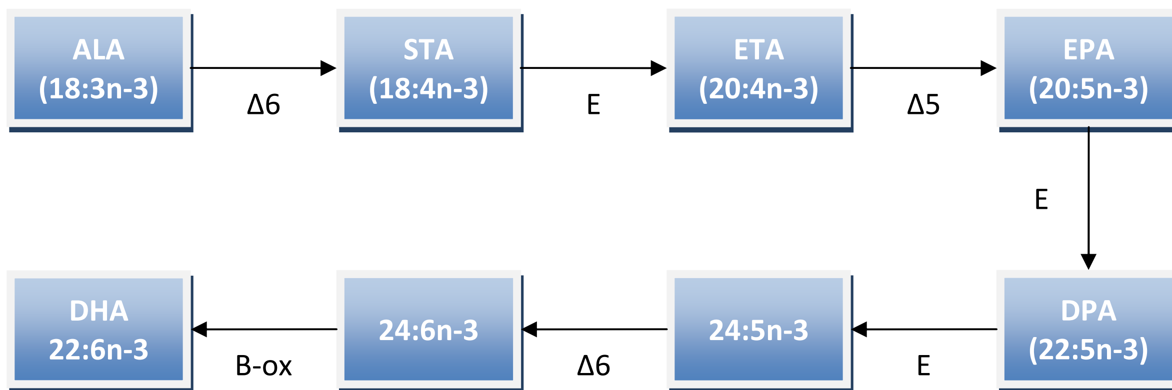
Direct supplementation

Among the required n-3 FA for marine fish species, docosahexaenoic acid (22:6n-3: DHA) is considered to be the most important. Currently, fish oils from wild marine catches constitute the major source of n-3 HUFA, although these products are hampered by market limitations that include odor issues, stability problems, and seasonal and climatic variations of fish stocks. Heavy metal and other contaminants in wild marine catches also raise concerns relating to fish oil as dietary supplements. However, oils derived from terrestrial plants are devoid of DHA and thus are unsuitable as the sole replacement for fish oil. Since microalgae are the primary synthesizers of n-3 HUFA in the natural environment, replacing fish oil with n-3 HUFA-enriched algae provides an excellent opportunity to develop alternative n-3 sources. Additionally, these micro-algae can be grown under controlled heterotrophic conditions, which allows for mass production in a sustainable manner.

Enhancement of endogenous FA biosynthesis

Freshwater fishes typically require FA of the n-6 and n-3 families, specifically linoleic acid (18:2n-6; LA), and α-linolenic acid (18:3n-3; ALA). These fishes possess the enzymatic capability to elongate and desaturate LA and ALA to produce long chain HUFA such as arachidonic acid (20:4n-6; ARA) and eicosapentaenoic acid (20:5n-3; EPA), respectively (Eckert, et al., 2006; Figure VI–1).

Figure VI–1: n-3 polyunsaturated fatty acid pathway



Adapted from Sprecher et al., 1995

In marine fishes, however, the biosynthetic rates of elongation and desaturation from ALA precursors are insufficient to produce the quantities of long-chain HUFA of the n-3 family needed for optimal health and growth; hence, they must be supplied in the diet and consequently are considered essential fatty acids (EFA). However, little work has investigated the possibility of improved production rates of long chain n-3 HUFA from intermediates in the biosynthetic pathway (Leaver, et al., 2006). A key intermediate in the biosynthesis of n-3 HUFA

is stearidonic acid (18:4n-3; STA). This FA results from the Δ^6 desaturation of ALA, and upon elongation and Δ^5 desaturation, becomes EPA (Sprecher, et al., 1995).

This study investigated the efficacy of fish oil replacement by novel algal-meal supplements. Additionally, the ability of juvenile cobia to produce endogenous n-3 HUFA from intermediate precursors in the biosynthetic pathway was estimated through the inclusion of STA-enriched oil. Finally, the impacts of these nutritional manipulations on the FA profiles of liver and muscle tissues were determined.

MATERIALS AND METHODS

Stocking and live support system

The study was conducted at the Virginia Tech Aquaculture Center (VTAC) using a recirculating life-support system composed of twenty-four, 300 L fiberglass tanks. Juvenile cobia (average individual weight 30.0 ± 0.1 g) were obtained from the Virginia Seafood Agricultural Research and Extension Center (VSAREC, Hampton, VA), and randomly stocked at 10 fish per tank. The water treatment loop consisted of a bubble bead filter (BBF-2 Aquaculture Technologies Inc., Metaire, LA) to remove suspended solids, a sump containing fluidized bed KMT media (Kaldnes Inc., Providence, RI) for biological filtration, a UV light (Emperor Aquatics, Pottstown, PA) for sterilization, a side-looped protein skimmer (R&B Aquatic Distribution, Waring, TX) to filter out smaller solids and decrease turbidity, and a thermostatically controlled heater placed in the sump to maintain water temperature at 29°C . Salinity was maintained at

20 ppt with the addition of synthetic sea salt (Marine Enterprises International, Baltimore, MD). All fish were subjected to a 12:12 light:dark cycle using a combination of fluorescent and incandescent lighting to simulate dawn and dusk.

Water quality parameters were measured every other day and included temperature and pH (HI 9024 pH meter, HANNA Instruments, Woonsocket, RI), dissolved oxygen (YSI tensionometer, Aquatic Ecosystems, 154 Apopka, FL), salinity using a refractometer (Aquatic Ecosystems, Apopka, FL), and TAN, NH4-N, NO2-N and NO3-N, which were measured spectrophotometrically (HACH DR/2400 Spectrophotometer, HACH Co., Loveland, CO). At the start of the study, ten individuals were randomly allocated to each tank (average initial weight 30g/fish).

Feeding and feed formulation

Each tank was randomly assigned to one of eight diets (n=3 tanks/diet), and the fish were hand-fed twice daily at 09:00 and 16:00 for 7 weeks. Feeding rates were determined based on the total biomass in each tank: the fish were initially fed 10% body weight, which was gradually decreased to 4% by the end of the study to maintain a level of satiation without over-feeding. Daily feeding rations were divided equally between two feedings (9am and 4pm), and uneaten feed was recorded daily. Fish from each tank were bulk-weighed weekly to monitor growth and adjust feeding rates.

Table VI–1: Formulation of the experimental diets

Ingredients	Control	50/50	25/75	0/100	50/50+	25/75+	0/100+	0/100VT
[a] Herring meal	63.8	16.0	16.0	16.0	16.0	16.0	16.0	16.0
[b] Soy concentrate		49.4	49.4	49.4	49.4	49.4	49.4	49.4
[c] Dextrin	13.0	13.0	13.0	13.0	11.0	12.6	12.4	12.4
[d] Fish oil	4.7	4.2	2.1					
[e] Stearidonic acid		4.2	6.3	8.4	4.2	6.3	5.7	5.7
[f] Algae meal								1.3
[g] DHA gold					9.3	4.6	5.5	
[h] Mineral	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
[I] Vitamin	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
[c] CMC	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
[j] CaPO4		0.8	0.8	0.8	0.8	0.8	0.8	0.8
[k] Amino acid mix		1.0	1.0	1.0	1.0	1.0	1.0	1.0
[l] BioMos®		0.3	0.3	0.3	0.3	0.3	0.3	0.3
[c] Cellufil	9.5	2.2	2.2	2.2	0.0	0.1	0.0	4.2
[m] Energy (kcal)	324.5	309.9	293.8	300.9	327.9	334.4	316.4	281.0
[n] Protein (%)	42.7	44.9	44.7	44.0	44.2	49.9	46.8	42.1
[n] Lipids (%)	11.3	8.7	7.0	8.1	11.9	9.2	8.84	7.0
[n] DHA level (%)	7.87	3.31	2.10	1.07	11.45	5.00	6.78	1.96

a: International Proteins, Minneapolis, MN; b: Nuriant, Cedar Falls, IA; c: US Biochemical Corporation, Aurora, IL; d: Omega Oils, Reedville, VA; e: Dr. Tom Clemente, University of Nebraska; f: Dr. Zhiyou Wen, Virginia Tech; g: Advanced BioNutrition Corp, Columbia MD; h: ICN Corporation, Costa Mesa CA; i: See Moon and Gatlin (1991); j: Aldrich-Sigma, St. Louis, MO; k: 30% methionine, 20% lysine, 50% taurine; l: Alltech Incorporated, Nicholasville, KY; m: calculated from other measurements; n: measured value

Diet formulations are presented in Table VI–1. The control diet was formulated with herring meal and menhaden oil as the sole protein and lipid source, respectively. In the experimental diets, proteins were supplied as a mixture of herring meal and soy protein concentrate (SPC). The alternate lipid source was obtained from a genetically modified soybean with enhanced STA content (modified soybean oil, MSO; Eckert, 2006). The lipid source was incrementally incorporated into the diets at the expense of menhaden fish oil: 50/50, 25/75, or 0/100% fish oil/MSO, respectively. The remaining experimental diets were formulated as above, but replaced the fish oil component with DHA Gold (Advanced BioNutrition Corp, Columbia MD), a

DHA-rich, algae-based meal (ALG). These diets are referred to as 50/50+, 25/75+ and 0/100+, respectively. An additional diet was designed by replacing DHA Gold with an algae meal produced on an experimental scale at Virginia Tech (AVT; Chi, et al., 2007; Pyle, et al. 2008). This meal provided 18.6% crude protein and 53.9% lipid (dry matter basis) with a DHA content of 19.5% (dry matter basis).

Sampling and measurements

At the end of the trial, 3 fish per tank were randomly sampled, and euthanized by an overdose of clove oil (Sigma-Aldrich, St Louis, MO). They then were measured, weighed, and dissected to obtain total viscera, liver and fillet masses. Viscera-somatic index (VSI; visceral mass wt * 100/body wt.), hepatosomatic index (HSI; liver wt. * 100/body wt.), and muscle ratio (MR; fillet wt. * 100/body wt.) were calculated from these measurements. Muscle and liver samples were frozen at -20°C pending proximate analysis for crude protein and lipid (AOAC, 1994).

Fatty acid analysis

Total lipids were extracted and measured according to the procedures of Folch (1957), prior to being trans-esterified according to the protocol developed by Indarti et al. (2005). The fatty acid methyl esters then were analyzed on a Shimadzu GC 2010 gas chromatograph (Shimadzu Scientific Instrument, Inc., Columbia, MD) equipped with a flame-ionization detector and a SGE

SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The fatty acids were identified by comparison of retention times with standards obtained from Sigma. Helium was used carrier gas. The temperature settings for injector, column, and detector were described previously (Chi, et al., 2007). The fatty acids were identified by comparing the retention times with those of standard fatty acids (Nu-Chek Prep, Inc., Elysian, MN) and quantified by comparing their peak area with that of the internal standard (C17:0; Chi, et al., 2007). Quantification of fatty acids was carried out by comparing their peak areas with that of the internal standard (C17:0).

Statistical analysis

Data were analyzed with one-way analysis of variance using JMP 7.0 (SAS Institute, Cary, NC). Significance level was set at α=0.05, and Tukey-Kramer HSD was used for testing means separation where appropriate.

RESULTS

Growth and feed efficiency

Water quality results are presented in Table VI–2. All measured parameters were within their respective recommended ranges for juvenile cobia (Rodrigues, et al., 2007). Table VI–3 shows that over the course of the trial, fish that were fed the 50/50+ and 0/100+ achieved a heavier average final weight and experienced a higher overall weight gain (P=0.0062 and

P=0.0146, respectively). The cumulative feed efficiency did not differ statistically between treatments (73.6 ± 1.0% on average, P=0.3940, Table VI–3).

Biological indices

Overall, dietary impacts on biological indices were negligible (Table VI–4). The VSI was not significantly different among fish fed the various diets, ranging from a high of 9.2% in fish fed the control diet to a low of 8.4% in fish fed the 50/50 and 0/100 diets. Fish fed the control diet had a significantly higher HSI (1.9%; P=0.0002) than fish fed the remaining diets, in which the HSI ranged from 1.48 in fish fed the 50/50+ diet to 1.65 in fish fed the 0/100 diet. Fish fed the control diet had a significantly lower MR (32.6%; P=0.0007) compared to fish fed all other diets, which had similar MR values ranging from 36.8% in fish fed the 0/100 diet to 38.6% in fish fed the 25/75 diet.

Table VI–2: Water quality parameters

	O_2 (mg/L)	T(^oC)	Salinity (‰)	pH	TAN (mg/L)	N-NO_2 (mg/L)	N-NO_3 (mg/L)
Average	5.41	28.48	18.87	7.79	0.28	0.15	169.58
SEM	0.14	0.14	0.71	0.03	0.03	0.02	15.81

Table VI–3: Growth performance of juvenile cobia[1]

Diet	Average initial fish weight (g)	Average final fish weight (g)	Fish weight increase (%)	Cumulative FE (%)
Control	29.4	189.5^{c}	545.3^{b}	72.8
50/50	30.3	228.3^{abc}	653.3^{ab}	72.3
25/75	30.4	233.1^{abc}	666.0^{ab}	76.3
0/100	29.5	199.9^{bc}	577.6^{ab}	67.7
50/50+	30.2	249.7^{a}	726.0^{a}	76.1
25/75+	30.5	232.5^{abc}	662.0^{ab}	74.5
0/100+	29.6	245.8^{ab}	729.0^{a}	75.8
0/100VT	29.7	229.1^{abc}	672.3^{ab}	73.0
P value	0.0690	0.0062	0.0146	0.3940
Pooled SEM	0.292	9.91	33.54	2.703

[1] Values with different superscripts within a column are significantly different at α=0.05. 50/50, 25/75 and 0/100 refer to the diet with corresponding fish oil/stearidonic acid oil ratio. 50/50+, 25/75+, and 0/100+ refer to the diets with corresponding algae meal/stearidonic acid ratio. The 0/100VT diet was formulated using the algae meal produced at Virginia Tech.

Table VI–4: Biological indices at the end of the trial

Diet	VSI (%)	HSI (%)	MR (%)
Control	9.15	1.91^{a}	32.60^{b}
50/50	8.42	1.50^{b}	37.05^{a}
25/75	8.76	1.64^{b}	38.55^{a}
0/100	8.35	1.65^{ab}	36.77^{a}
50/50+	8.73	1.48^{b}	37.14^{a}
25/75+	8.74	1.59^{b}	37.23^{a}
0/100+	8.80	1.62^{b}	37.52^{a}
0/100VT	8.79	1.64^{b}	37.93^{a}
P value	0.1890	0.0002	0.0007
Pooled SEM	0.203	0.060	0.881

50/50, 25/75 and 0/100 refer to the diet with corresponding fish oil/stearidonic acid oil ratio. 50/50+, 25/75+, and 0/100+ refer to the diets with corresponding algae meal/stearidonic acid ratio. The 0/100VT diet was formulated using the algae meal produced at Virginia Tech; VSI: viscera-somatic index; HSI: hepato-somatic index; MR: muscle ratio; [1] Values with different superscripts within a column are significantly different at α=0.05.

Proximate analysis

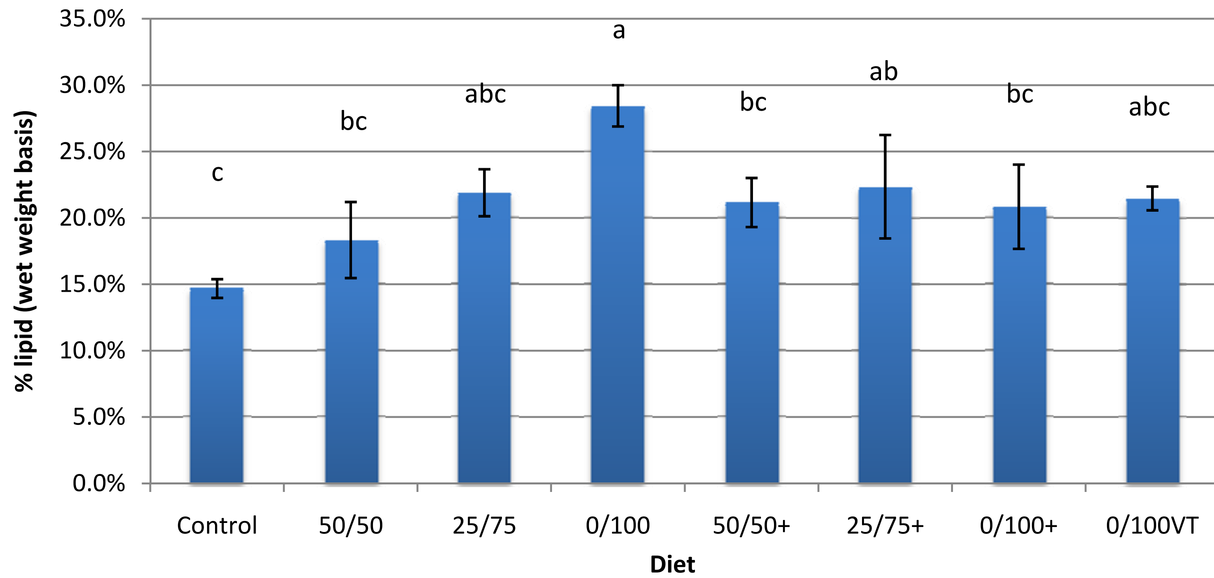
There were no significant differences in muscle lipid, protein or dry matter in cobia fed the various experimental diets (Table VI–5). Muscle lipid levels ranged from a low of 1.9% (wet wt.) in fish fed the control diet to a high of 3.9% (wet wt.) in fish fed the 50/50+ and 0/100+ diets. Muscle protein level was very stable, ranging from 18.6% (wet wt.) in fish fed the 25/75 diet to 19.3% (wet wt.) in fish fed the 50/50 diet. Muscle dry matter was also very stable, ranging from 22.9% in fish fed the control diet to 24.9% in fish fed the 25/75+ diet.

Liver lipids were significantly impacted by dietary treatment (P = 0.0122; Figure VI–1). Fish fed the control diet had the lowest liver lipid in the study (14.7%, wet wt.). There was a linear increase in liver lipid with decreasing fish oil inclusion. The diets supplemented with dietary DHA (50/50+, 25/75+, 0/100+ and 0/100VT) all had equal liver lipid values ranging from 20.8% (wet wt., 0/100+) to 22.4% (wet wt., 25/75+).

Table VI–5: Proximate analysis of muscle and liver

Diet	Muscle lipid	Muscle protein	Muscle DM
Control	1.9%	19.2%	22.9%
50/50	3.2%	19.3%	24.3%
25/75	3.2%	18.6%	23.7%
0/100	3.7%	18.9%	24.0%
50/50+	3.9%	19.2%	24.7%
25/75+	3.1%	19.5%	24.9%
0/100+	3.9%	19.1%	24.6%
0/100VT	2.7%	18.9%	23.1%
Pooled SEM	0.00450	0.36724	0.00548
P-value	0.0971	0.7729	0.1449

Figure VI–2: Percentage of total lipid in juvenile cobia livers at the end of the trial.



50/50, 25/75 and 0/100 refer to the diet with corresponding fish oil/stearidonic acid oil ratio. 50/50+, 25/75+, and 0/100+ refer to the diets with corresponding algae meal/stearidonic acid ratio. The 0/100VT diet was formulated using the algae meal produced at Virginia Tech.

FA profiles

In the muscle, dietary treatments significantly impacted all the FA investigated (Table VI–6). Most notable were levels of STA, EPA and DHA, all of which mirrored that of the diets (Table VI–7). Levels of STA in muscle tissue increased as dietary inclusion levels increased. Fish fed the control diet had significantly lower levels of STA in the muscle tissue (P = 0.0072; 1.32% of total FA), while fish fed the 0/100 diet, which replaced all the fish oil with the MSO, had the highest levels of STA at 13.32%. Similarly, EPA levels paralleled the levels of fish oil in the diet, the major contributor of EPA. Fish fed the control diet had significantly higher levels of EPA in muscle tissue (P=0.0033; 4.41% of total FA) while fish fed the 0/100+ diet had the lowest levels (0.99%). All other values for EPA ranged between 1.01 and 2.45% of total FA. DHA levels in the

muscle tissue also were significantly impacted by dietary treatment, with fish fed the 0/100 diet having the lowest levels (P = 0.0006; 1.67% of total FA) and fish fed the 50/50+ diet having the highest levels (12.53%). Fish fed the remaining diets had DHA levels in the muscle tissue ranging from 2.38 to 7.21%.

Table VI–6: Selected fatty acid profile in cobia muscle at the end of the trial

Diet	% LA	% ALA	% STA	% ARA	% EPA	% DPA	% DHA
Control	0.29^{b}	0.90^{b}	1.32^{b}	0.75^{b}	4.41^{a}	0.50^{c}	7.21^{ab}
50/50	1.51^{ab}	4.72^{ab}	5.49^{ab}	0.43^{ab}	2.45^{ab}	0.31^{c}	3.57^{bc}
25/75	1.86^{ab}	5.61^{ab}	6.85^{ab}	0.28^{ab}	1.62^{b}	0.19^{c}	2.38^{bc}
0/100	3.72^{a}	10.47^{a}	13.32^{a}	0.21^{a}	1.01^{b}	0.19^{c}	1.67^{c}
50/50+	1.94^{ab}	5.83^{ab}	6.72^{ab}	0.65^{ab}	1.27^{b}	4.57^{a}	12.53^{a}
25/75+	2.50^{ab}	7.40^{ab}	8.98^{ab}	0.47^{ab}	1.57^{b}	1.20^{bc}	4.78^{bc}
0/100+	2.17^{ab}	6.59^{ab}	8.02^{ab}	0.50^{ab}	0.99^{b}	2.14^{b}	6.10^{bc}
0/100VT	1.78^{ab}	5.11^{ab}	6.45^{ab}	0.36^{ab}	1.93^{ab}	0.28^{c}	3.41^{bc}
Pooled SEM	0.42	1.21	1.48	0.09	0.45	0.31	1.02
P-value	0.0073	0.0085	0.0072	0.0296	0.0033	<0.0001	0.0006

Values with different superscripts within a column are significantly different at α=0.05.

Table VI–7: Selected fatty acid profile in experimental diets

Diet	% LA	% ALA	% STA	% ARA	% EPA	% DPA	% DHA
Control	0.22^{d}	0.75^{d}	1.37^{d}	0.74^{a}	8.38^{a}	0.44^{cd}	7.87^{ab}
50/50	2.09^{bc}	6.34^{bc}	8.90^{bc}	0.40^{bcd}	4.14^{b}	0.23^{d}	3.31^{cde}
25/75	3.09^{ab}	8.87^{ab}	12.76^{ab}	0.26^{cd}	2.60^{c}	0.17^{d}	2.10^{de}
0/100	3.86^{a}	10.71^{a}	15.91^{a}	0.13^{d}	0.97^{d}	0.20^{d}	1.07^{e}
50/50+	1.56^{c}	4.34^{c}	6.16^{cd}	0.66^{ab}	1.17^{d}	4.29^{a}	11.45^{a}
25/75+	2.33^{bc}	6.58^{bc}	9.53^{bc}	0.25^{cd}	0.93^{d}	1.78^{bc}	5.00^{bcd}
0/100+	2.58^{bc}	7.36^{abc}	10.53^{bc}	0.43^{bc}	1.23^{d}	2.37^{b}	6.78^{bc}
0/100VT	2.98^{ab}	8.56^{ab}	12.36^{ab}	0.14^{d}	1.08^{d}	0.34^{d}	1.96^{de}
Pooled SEM	0.21	0.60	0.87	0.05	0.22	0.25	0.69
P-value	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001

Values with different superscripts within a column are significantly different at α=0.05.

Table VI–8: Selected fatty acid profile in cobia liver at the end of the trial

Diet	% LA	% ALA	% STA	% ARA	% EPA	% DPA	% DHA
Control	0.35^{b}	0.95^{b}	1.14^{b}	1.21^{ab}	6.40^{a}	0.66^{b}	9.71^{ab}
50/50	1.73^{ab}	5.03^{ab}	4.00^{ab}	0.60^{bc}	3.01^{b}	0.48^{b}	4.01^{b}
25/75	2.36^{ab}	6.36^{ab}	5.51^{ab}	0.48^{bc}	2.20^{bc}	0.17^{b}	3.08^{b}
0/100	2.35^{ab}	6.21^{ab}	4.99^{ab}	0.20^{c}	0.76^{bc}	0.16^{b}	1.35^{b}
50/50+	2.25^{ab}	6.44^{ab}	4.97^{ab}	1.56^{a}	1.48^{bc}	5.24^{a}	14.22^{a}
25/75+	2.91^{a}	7.61^{a}	6.53^{a}	1.04^{abc}	1.30^{bc}	2.86^{ab}	8.75^{ab}
0/100+	1.47^{ab}	4.14^{ab}	3.31^{ab}	0.68^{bc}	0.79^{c}	1.95^{b}	5.77^{b}
0/100VT	2.51^{ab}	6.79^{ab}	5.39^{ab}	0.56^{bc}	1.02^{bc}	1.42^{b}	4.85^{b}
Pooled SEM	0.35	0.97	0.74	0.15	0.40	0.54	1.40
P-value	0.0186	0.0277	0.0192	0.0019	<0.0001	0.0005	0.0012

Values with different superscripts within a column are significantly different at α=0.05.

The FA composition of liver tissue also was impacted significantly by dietary treatment (Table VI–8) although they did not reflect dietary FA composition as did FA composition of the muscle. Again, focusing upon STA, EPA and DHA, STA levels in the liver of fish fed the control diet were significantly lower than those observed in fish fed the 25/75+ diet (1.14 vs 6.53% respectively). Fish fed all other diets had liver STA levels that did not differ from one another or from fish fed either the control or the 25/75+ diets (Table VI–7). These values ranged from 3.31 to 5.39% of total FA. Levels of EPA in the liver tissue mirrored the results observed in muscle tissue—decreasing EPA levels with decreasing fish oil inclusion. Fish fed the control diet had the highest levels of EPA (0.0001; 6.40% of total FA), decreasing to a low of 0.76% in fish fed the 0/100 diet. Fish fed the 50/50+, 25/75+, 0/100+ and 0/100VT all had low levels of EPA compared to fish fed the control diet (1.48, 1.30, 0.79, and 1.02%, respectively, compared to 6.40%). Liver DHA levels were highest in fish fed the 50/50+ diet (P = 0.0012; 14.22) and lowest in fish fed the 0/100 diet (1.35%). Fish fed the remaining diets had intermediate values ranging from 3.08% in fish fed the 25/75 diet to 9.71 in fish fed the control diet.

DISCUSSION

This study investigated the potential ability of juvenile cobia to elongate and desaturate STA into longer-chain n-3 HUFA. As well, I examined the impacts of supplementing algae-based DHA for dietary fish oil replacement and the role of these novel alternate lipids for enhancing DHA levels in the edible flesh.

The juvenile cobia in the present study accepted the experimental diets readily, with no apparent issues regarding palatability. This is evidenced by excellent feed efficiency (FE) values, which averaged approximately 73% over the trial course (approximately 1.3 as a feed conversion ratio). Growth, as measured by percent increase from initial weight, was concordant with growth trials described previously from our laboratory and others investigating cobia (Craig, et al., 2006; Lunger, et al., 2007a; Resley, et al., 2006; Webb, et al., 2007; Zhou, et al., 2005). Levels of STA in the muscle reflected that in the diet, although it cannot be determined whether this FA was esterified into phosphatidylcholine, as observed in Atlantic salmon (Ghioni, et al., 2002), or stored as triacylglyceride. Interestingly, liver STA levels in cobia were not elevated above 5-6.5% regardless of the dietary level. This finding suggests a maximum storage concentration in hepatic tissue. The HSI was elevated significantly in fish fed the control diet compared to all other diets. Among fish fed the diets without DHA supplementation, the liver lipid content was correlated positively with dietary STA ($P=0.0009$, $R^2=0.398$), and correlated negatively with dietary n-3 HUFA ($P=0.0023$, $R^2=0.316$). This strongly suggests that the

processing function of hepatic lipids was impaired in fish fed the 0/100 diet due to a lack of long-chain n-3 HUFA.

Correspondingly, based on the liver lipid data from the present study, it appears as if juvenile cobia require a minimum of 1% DHA (of total lipid) for optimal growth and health. As elevated liver lipid is a classic symptom of EFA deficiency in many marine fishes (Castell, et al., 1972), the significantly higher liver lipid values in cobia fed the 0/100 diet suggest a sub-clinical indication of an EFA deficiency, even though weight gain was not significantly impacted. As the EFA requirement for cobia has not been determined quantitatively, this is only an estimation. However, most marine fish species require between 0.5 and 2% n-3 HUFA (as a percentage of total diet; Lochmann and Gatlin, 1993). In juvenile cobia, it appears as if an inclusion of 2% n-3 HUFA (as a percentage of dry diet) ensures optimal growth, health and hepatic function.

In this trial, the STA-rich oil utilized was derived from a genetically modified soybean strain (Eckert, et al., 2006). Additionally, this oil contained significantly higher levels of STA than those used by previous researchers (Bell, et al., 2006; Miller, et al., 2007; Tocher, et al., 2006). It is widely accepted that marine fish have limited capabilities to elongate and desaturate precursor FA such as ALA into longer-chain FA of the n-3 family (Almaida-Pagan, et al., 2007; Miller, et al., 2007; Tocher, et al., 2006). Hence, the replacement of fish oil in aquafeeds must address the EFA requirement for these species, so that growth and health of the target species is not impacted detrimentally. It is well known that in marine fishes the Δ^6 desaturation is the main rate-limiting step in the biosynthetic pathway which converts C18 precursors into STA (Miller,

et al., 2007; Tocher, et al., 1998). This enzyme is also involved in the conversion of EPA to DHA (Sprecher, et al., 1995; Yamazaki, et al., 1992; Figure 1). However, it has been shown that feeding Atlantic salmon (*Salmo salar*) diets low in ALA may stimulate some FA desaturation and elongation (Tocher, et al., 2002). Nevertheless, this bioconversion was insufficient to maintain concentrations of EPA and DHA in the tissue to the same concentrations as observed in FO-fed fish; hence the need for dietary supplementation.

While many studies have replaced a proportion of fish oil successfully in a wide variety of species (Almaida-Pagan, et al., 2007; Karapanagiotidis, et al., 2007; Mourente, et al., 2005; Peng, et al., 2008; Tocher, et al., 2003), very few have attempted to replace fish oil entirely by providing a down-stream intermediate to serve as a precursor for further elongation and desaturation reactions. Moreover, all dealt with cold water and/or salmonid species exclusively. In the present study, STA was incorporated to bypass the initial Δ^6 desaturation step and to determine whether juvenile cobia could further elongate and desaturate this precursor. In other studies involving STA incorporation in aquafeeds, Bell and coworkers (2006) investigated the total replacement of fish oil by echium oil, which contained γ-linolenic (18:3n-6; GLA) and STA, in Atlantic cod (*Gadus morhua*), and observed an accumulation of STA, GLA and dihomo-GLA (20:3n-6), along with a decrease in EPA and ARA concentrations in both flesh and liver. Tocher et al. (2006) replaced 80% of dietary fish oil with echium oil, and examined its impact in terms of elongation and desaturation into long chain n-3 HUFA in Arctic charr (*Salvelinus alpines*). Similar to the present study, these authors observed no bioconversion of these down-

stream intermediates into EPA and DHA and noted that Arctic charr could not maintain tissue n-3 HUFA or ARA levels given these precursors.

Since the present study also bypassed the initial Δ^6 desaturation step, the absence of EPA implied that either elongation or Δ^5 desaturation is more rate-limiting. However eicosatetraenoic acid (20:4n-3; ETA), which is the product of STA elongation, was virtually absent as well, thus suggesting this step might be most limiting in this biosynthetic pathway. Ghioni et al. (1999) described a similar finding *in vitro* from a cell line derived from turbot (*Psetta maxima*) in which the $C_{18\text{-}20}$ elongase activity was more rate-limiting than that of the Δ^5 desaturase. This result, however, differs from salmonids, which are capable of desaturating and elongating STA into ETA and EPA, but not DHA (Ghioni, et al., 2002). Taken together, feeding FA precursors such as STA does not appear to be a viable strategy for fish oil elimination in cobia aquafeeds. This is most likely due to the involvement of the limiting elongase and Δ^6desaturase at multiple points in the endogenous biosynthetic pathway, regardless of the carbon chain length or degree of unsaturation (Figure VI–1). However, research on genetic modification of commodity crops such as oil seeds is conducted in order to enrich these seeds in EPA and DHA directly. This would provide a safe, cheap and plentiful alternate source of long-chain HUFA for both aquaculture and human consumption (Napier and Sayanova, 2005).

Despite the requirement of a microbiological facility for their commercial exploitation, marine algae represent another promising alternative to fish oil, especially for their high n-3 HUFA content, which are of primary importance for marine carnivorous fish. They also provide

the opportunity to enhance the beneficial n-3 HUFA levels in fish that normally would not naturally accrue these FA, such as freshwater fishes. In the present trial, the algal meals (ALG and AVT) were utilized as a replacement for fish oil in the “plus” diets, i.e., the lipid component in these diets was composed of STA and algal meals only — no fish oil was present. Two types of algae meals were utilized in the present study: one that is available commercially (ALG) and the other (AVT) produced at Virginia Tech on a small, experimental scale utilizing crude glycerol as the substrate (Pyle, et al., 2008). While the inclusion rates were significantly different, the impacts upon muscle DHA levels were similar statistically. Additionally, there were no significant differences with respect to weight gain or feed efficiency in cobia fed these two algal meals. With both sources of DHA algal meals, muscle DHA contents reflected those of the respective diets and were similar statistically. As well, DHA levels in the liver tissues from fish fed both algal meals were the same (Table VI–8). This indicates comparable utilization of both forms of DHA supplementation by juvenile cobia. In terms of final product quality with respect to consumer health and DHA levels, cobia fed the diets supplemented with the algal meals contained at least 138 mg/150 g fillet compared to 205 mg/150 g fillet from fish fed the control diet containing 100% herring meal and added fish oil. These levels of DHA are relatively high, especially if one takes into account the low total lipid levels measured in the cobia fillet (1.9-3.2% wet wt.). The US Food and Drug Administration (FDA) currently recommends a daily DHA intake of approximately 150 mg DHA per day for healthy adults.

As these algae meals contained predominantly DHA, and STA is included typically in n-3 HUFA calculations in terms of EFA requirements, the diets in the present study contained

extremely high levels of n-3 HUFA. Previous work with red drum *Sciaenops ocellatus* (Lochmann and Gatlin, 1993), coho salmon *Oncorhynchus kisutch* (Yu and Sinhuber, 1979) and channel catfish *Ictalurus punctatus* (Satoh, et al., 1989) all indicated growth depression with high dietary n-3 HUFA levels . This depression was not observed herein, as cobia fed the diets containing the highest levels of n-3 HUFA (50/50+ and 0/100+) both exhibited the highest weight gain over the 7-week feeding trial. It is noteworthy that both of these diets contained no fish oil, indicating that manufacturing and administrating fish oil-free diets is a distinct possibility in cultured marine carnivorous fish. Additionally, all the experimental diets, as opposed to the control diet, contained only 16% herring meal as the fish meal source, and all numerically outperformed the 100% herring meal control diet in terms of weight gain. This is a true indication that cobia aquafeeds can be formulated with significantly lowered fish meal and fish oil inclusion rates without impacting production performance or characteristics detrimentally.

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Chapter VII. Total replacement of dietary fish meal in cobia juvenile

Abstract

Achieving true sustainability in fish farming requires the replacement of most of the fish meal and fish oil utilized as feedstuffs. The present experiment reports 2 feeding trials that resulted in the total replacement of fish meal and fish oil in juvenile cobia (*Rachycentron canadum*). The first trial was conceived as a 2x3 factorial design with three levels of fish meal replacement (FMR; 50, 75 and 100% of dietary protein) by soy protein concentrate (SPC), and two levels of mannan oligosaccharide (MOS) supplementation (0 or 0.3% of the diet). Since MOS has been reported to promote gut health and integrity, it was included in order to verify whether it would ease high levels of FMR. Lipids were supplied by menhaden oil. In the second feeding trial, fish meal was replaced by various combinations of SPC and soybean meal (SBM), again with or without MOS supplementation. In addition, some diets were supplemented with purified amino acids. Lipids were supplied by fish oil. A final diet (NOFM) was formulated using SPC, a marine worm meal, a nucleotide-rich yeast extract protein source, MOS, and a yeast source of selenomethionine. In the latter diet, lipids were supplied with a mix of soy oil and a DHA-rich algal meal. Over both feeding trials, juvenile cobia consistently exhibited excellent performance at 75% FMR and less. MOS did not have a significant effect, although a beneficial trend was observed in the first trial at 100% FMR. In the second trial, the fish fed the NOFM diet exhibited one of the best weight gains and feed efficiencies, with no mortality and no impact on muscle and liver composition. This result illustrates the crucial importance of the selection of feedstuffs for FMR and fish oil, since the NOFM diet did not receive amino acid supplementation. However, the consistent, successful replacement of 94% of the fish meal in the other diets is actually more promising to the future as they solely utilized commodities traded (soy products) as replacement sources, which is the only road to true environmental and economical sustainability for the aquaculture industry.

Keywords: *Rachycentron canadum*; sustainability; soy protein concentrate; mannan oligosaccharide; algae meal.

INTRODUCTION

Over the past several years, intense focus has been trained upon the reduction and/or elimination of fish meal protein in aquafeeds, especially those designed for high-level marine carnivores. This goal has also been driven by the desire and need for the aquaculture industry to achieve true sustainability, while attempting to fill the massive seafood deficit that must be eliminated through aquaculture (FAO, 2007; Lunger, et al., 2006). Sustainable replacements for fish meal protein are most often those of plant origin, especially the grains, pulses and oilseeds (Gatlin, et al., 2007; Gaylord, et al., 2006; Lunger, et al., 2006). Soybean meal (SBM) has been one of the most studied alternatives to fish meal, but has several limitations, including anti-nutritional factors, low levels of methionine and adverse effects on the intestinal integrity of some carnivorous species (Gatlin, et al., 2007). Additionally, SBM is relatively low in crude protein levels, especially when compared to fish meal. Hence, complete replacement of fish meal in aquafeeds designed for carnivorous species requiring higher levels of dietary protein is problematic due to these lower crude protein levels. With the recent increase in the price of fish meal, as well as the realization of the need for alternate proteins to drive the industry forward, more emphasis has been placed upon technologies that can concentrate protein content from traditionally lower-protein sources, resulting in products such as corn gluten meal and soy protein concentrate (SPC; Barrows et al., 2007). These technologies have provided new, alternative sources of protein which, in many cases, have crude protein levels similar to fish meal. As production capacities of these plant-based protein concentrates continues to increase, price and availability will make these products more cost-effective. However, their use and

optimal inclusion rates in aquafeeds designed for high-level marine carnivores must be ascertained, and many feedstuffs have similar problems in terms of inadequate amino acid profiles.

At the Virginia Tech Aquaculture Center (VTAC), recent research has concentrated upon total fish meal replacement in aquafeeds designed for cobia (*Rachycentron canadum*) utilizing a wide variety of alternate protein sources with varying levels of success (Craig and McLean, 2005; Lunger, et al., 2006; Lunger, et al., 2007a; McLean and Craig unpublished data). Over the course of these studies, diets containing 100% replacement of fish meal have been investigated using a yeast-based protein source (Lunger, et al., 2006; Lunger, et al., 2007a), with the finding that the addition of taurine in diets with high levels of fish meal replacement (FMR) significantly improved production characteristics (Lunger, et al., 2007b). Positive impacts of taurine supplementation upon weight gain also have been observed in rainbow trout (Gaylord, et al., 2006) and olive flounder (Kim, et al., 2007; Kim, et al., 2005). Unpublished results from our laboratory on feeding trials conducted with cobia indicated that supplementation of other amino acids such as methionine and lysine in addition to taurine is imperative if complete replacement of fish meal is to be achieved without detrimental impacts on production characteristics in juvenile cobia. Due to the outstanding nutritional qualities of fish meal which , include a well-balanced amino acid profile, high digestibility and palatability, and the presence of potential growth factors, it is well accepted that complete replacement will not be possible with a single alternative protein source (Craig and McLean, 2005). Drawing upon our previous findings, recent studies have investigated a blend of alternate protein sources, including yeast-

based feedstuffs, *Neried sp.* worm meals, SBM, and other organic alternative protein sources (Lunger, et al., 2007a), in combination with and without specific amino acid supplementation. This study sought to build upon these previous findings by utilizing various combinations of soy protein products and other alternative protein sources in aquafeeds that can be considered commercially feasible in terms of cost-effectiveness. Additionally, the use of mannan oligosaccharides (MOS), which have been shown to benefit larval cobia intestinal development (Salze, et al., 2008), were evaluated in feeds for juvenile cobia. The addition of MOS was investigated to determine whether this feed additive could enhance gastrointestinal (GI) tract integrity in juvenile cobia, thus aiding in the digestion of high levels of plant protein incorporated as SBM and SPC. Two separate trials were conducted, each containing at least one diet totally devoid of fish meal protein.

MATERIALS AND METHODS

Experimental system and husbandry

Both studies were undertaken using a recirculating aquaculture life support system. The 3400 L recirculation configuration (flow rate = 4 L min^{-1} per aquaria) was comprised of twenty-four, 110 L glass aquaria serviced with a 750 L (200 gal) KMT-based (Kaldnes Miljøteknologi, Tønsberg, Norway) fluidized bed biofilter, a bubble-bead filter (Aquaculture Technologies Inc., Metaire, LA) for solids removal, a protein skimmer (R&B Aquatics, Waring, TX), and a 40-watt UV sterilizer (Aquatic Ecosystems, Apopka, FL). The fluidized bed was oxygenated using diffusion air lines connected to a 1 hp Sweetwater remote drive regenerative blower (Aquatic

Ecosystems, Apopka, FL). A photoperiod using phosphorescent tubes positioned 1.8 m above the system was implemented using a 12h photophase-scotophase cycle using an automated timer with a half hour dusk/dawn period. Water quality parameters were monitored (3 times a week) during the feeding trials. Water temperature (28°C) and pH (8.4) were monitored using a Hanna Instrument 9024 pH meter (Aquatic Ecosystems, Apopka, FL). Salinity was maintained at 18 ppt using Crystal Sea synthetic sea salt (Marineland, Baltimore, MD) added to well water and monitored using a refractometer. Dissolved oxygen (7.0 ± 0.1 ppm) and total ammonia nitrogen (0.12 ± 0.01 ppm) were measured using a YSI 85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH) and by spectrophotometric analysis (Hach Inc., Loveland, CO), respectively. Nitrite (0.074 ± 0.012 ppm) and nitrate (97.7 ± 2.2 ppm) levels were quantified once a week by spectrophotometric analysis.

Juvenile cobia (*Rachycentron canadum*) were supplied by the Virginia Seafood Agricultural Research and Extension Center (VSAREC, Hampton, VA, USA). Fish were transported to the Virginia Tech Aquaculture Center (VTAC, Blacksburg, VA) and were acclimated and maintained in eight 500 L tanks for approximately 60 days. Upon commencement of the feeding trials, seven (81.7 ± 0.3 g, initial mean weight ± SEM) and five (104.0 ± 0.8 g) juvenile cobia for experiment 1 and 2, respectively, were randomly placed into each tank. Fish were hand-fed the experimental diets (three tanks per diet) twice daily, at 9h00 and 16h00 for 6 weeks, starting at 7% body weight (bw) per day, and gradually decreasing to 5% bw d^{-1}, equally divided between the two daily feedings. This maintained a level of apparent satiation without overfeeding. Fish

in tanks were group-weighed weekly to adjust the feeding rates and to monitor growth performance.

Diets

Feeding trial 1

Experimental feeds for the first feeding trial were produced as summarized in Table VII–1. All diets provided 45% crude protein and 12% total lipid (dry-matter basis) and supplied 340 kcal available energy/100 g dry diet.

Table VII–1: Formulation of diets in feeding trial 1

Ingredients	Control	50/50	50/50+	25/75	25/75+	0/100	0/100+
Herring meal	63.8	31.9	31.9	16.0	16.0	0.0	0.0
Soy concentrate	0.0	32.9	32.9	49.4	49.4	65.9	65.9
Dextrin	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Menhaden fish oil	4.7	7.2	7.2	8.4	8.4	9.6	9.6
Mineral mix	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Carboxymethyl cellulose	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Amino acid mix	0.0	0.0	1.0	0.0	1.0	0.0	1.0
BioMos	0.0	0.0	0.3	0.0	0.3	0.0	0.3
Cellufil	9.5	6.0	4.7	4.2	2.9	2.5	1.2
Available Energy (kJ/100 g diet)	340	340	340	340	340	340	340

Diets are designated in reference to the fish meal replacement ratio: the 50%, 25%, and 0% of the protein were supplied via fish meal in the 50/50, 25/75, and 0/100 diets, respectively. The + indicated MOS supplementation

The trial was designed as a 3 x 2 factorial with fish meal replacement level by SPC as one factor (50, 75 and 100% of dietary protein) and MOS inclusion as the other main factor (with and without). The diets were maintained isolipidic by increasing the levels of fish (menhaden)

oil as fish meal levels decreased, and all diets except the control were supplemented with an amino acid mix (methionine, lysine, and taurine; 20/30/50 w/w/w, respectively). The remaining diets containing MOS were formulated by adding BioMos® (Alltech Inc., Nicholasville, KY), a commercial MOS product, at a level of 0.3% of the diet at the expense of cellulose.

Feeding trial 2

In the second feeding trial, a total of six diets were prepared (Table VII–2) based on the results of the first feeding trial, to further investigate MOS and amino acid supplementation in high plant-protein inclusion level diets for juvenile cobia, as well as to attempt to completely replace fish meal and fish oil in a cobia diet. On a dry matter basis, total crude protein levels were achieved with a combination of 25.3% from fish meal, 12.6% from SPC and 32.4% from SBM. This served as the control diet. Neither the amino acid mix nor MOS was included in this diet. The next two diets (SB and SB+) were formulated to contain 12.06% fish meal, 25.3% SPC and 32.4% SBM as the dietary protein component. The SB+ diet was supplemented with MOS, but did not receive the additional amino acid supplement utilized in the first feeding trial.

Two additional diets were formulated to contain the maximum inclusion levels of soy products, in order to minimize overall protein costs. In these diets (MXSB and MXSB+), protein levels from the soybean meal were maximized, resulting in 8.5% of the diet supplied by the fish meal, 32.8% by the soy concentrate, and 39.9% by soybean meal. Both MXSB and MXSB+ diets

were supplemented with the supplemental amino acid mix, and MXSB+ also included MOS, to further investigate the impacts of MOS supplementation.

Table VII–2: Formulation of diets in feeding trial 2.

Ingredients	Control	SB	SB+	MXSB	MXSB+	NOFM
Herring meal	25.3	12.6	12.6	8.5	8.5	
Soy concentrate	12.6	25.3	25.3	23.8	23.8	25.3
Soybean meal	32.4	32.4	32.4	39.9	39.9	
Worm meal						30.0
Dextrin	10.0	10.0	10.0	8.6	8.3	9.5
Soy oil						7.3
Menhaden fish oil	8.7	10.0	10.0	10.2	10.2	
DHA Gold						1.5
Mineral mix	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix	3.0	3.0	3.0	3.0	3.0	3.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0
Amino acid mix				1.0	1.0	
BioMos™			0.3		0.3	0.3
Selplex™						0.6
NuPro™						17.5
Cellufil	3.0	1.7	1.4			
Available Energy (kJ/100 g diet)	328.0	328.0	328.0	322.4	322.4	

	Crude protein[1]	Total lipid[1]	Dry matter[1]
Herring meal	70.05	11.41	95.93
Worm meal	60.22	15.00	88.36
NuPro™	51.52	7.09	92.14
SPC	68.3	3.64	95.90

[1] Percentage on a dry matter basis. SB: soybean-based diet; MXSB: soybean-based diet in which the incorporation of soybean meal has been maximized. The + sign indicates MOS supplementation. SPC: soy protein concentrate.

A final experimental diet was designed to eliminate fish meal and fish oil entirely from the formulation. This diet formulation was based not only upon published results from our laboratory (Craig and McLean, 2005; Craig, et al., 2006; Lunger, et al., 2006; 2007a; 2007b), but

also incorporating the results of several unpublished feeding trials involving novel alternate proteinsources in aquafeeds for juvenile cobia at our laboratory. In this diet – named NOFM – proteins were supplied by 30% *Nereid* worm meal (SeaBait Industries, UK), 25.3% SPC, and 17.5% NuPro® (Alltech Inc., Nicholasville, KY), a yeast-based protein source high in nucleotides. Lipids were supplied endogenously from the worm meal as well as from soy oil and DHA-enriched algae meal (AquaGrow Gold, Advanced BioNutrition, Columbia, MD). This diet was not supplemented with amino acids, but incorporated MOS and a selenomethionine-rich yeast extract (SelPlex®, Alltech Inc., Nicholasville, KY).

Diet manufacture

In both feeding trials, all diets were manufactured at the VTAC, where the dry dietary components of the diet were first thoroughly mixed in a Patterson-Kelley twin shell® Batch V-mixer (Patterson-Kelley Co. Inc., East Stroudsburg, PA) prior to being transferred into a Hobart D300 Floor Mixer (Hobart Co., Troy, OH), where oil was added and further mixed. The amount of distilled water required for pelleting (20-40% of feed weight) then was added to the mixture and mixed until a pebble-like consistency was achieved. The mixture then was pressure pelleted using an appropriate die to provide pellets of suitable size for the fish. After air-drying, feed moisture content was approximately 15% and accurate dry matter determinations (AOAC, 1994) made so that feed quantity was based upon a dry-matter basis. Bulk diets were frozen at -20°C and smaller portions were thawed and refrigerated as needed.

Data acquisition

At the end of the trials, three (n=9) and two fish (n=6) from each tank were used for data acquisition in feeding trial 1 and 2, respectively. The fish were euthanized with an overdose of anesthetic (clove oil, 3 mg L^{-1}; Sigma-Aldrich, St. Louis, MO), prior to being measured for length and weight. Overall weight gain, specific growth rate (SGR; 100 * Ln(final weight/initial weight)/(trial duration)), and feed efficiency ratio were calculated from the latter. The fish were then dissected and the visceral mass, liver, and filets were weighed to establish the viscera-somatic index (VSI; visceral weight / total body weight * 100), hepato-somatic index (HSI; liver weight / total body weight * 100), and muscle ratio (MR; total filet weight / total body weight * 100), respectively. Proximate analyses were performed on muscle and liver, and included crude protein and lipid (AOAC, 1994).

Statistical analyses

In the first feeding trial, all data were subjected to factorial analysis of variance (ANOVA) procedures utilizing JMP 7.0 (SAS, Cary, NC, USA). In the second trial, standard ANOVA procedures were utilized. In both trials, when appropriate, Tukey-Kramer HSD was used for multiple comparisons of the means ($\alpha < 0.05$).

RESULTS

Feeding trial 1

Weight gain, FE and survival

Weight gain, feed efficiency (FE) and survival were all significantly affected by the fish meal replacement (FMR) level in the experimental diets (Table VII–3). At the end of the first feeding trial, juvenile cobia fed diets with either 50 or 75% FMR achieved equal weight gain, ranging from 199 to 205% over the six week period. Similarly, fish fed these same diets had significantly higher FE ratio values when compared to fish fed the diets containing 100% FMR. Survival was also significantly higher in fish fed the 50 and 75% FMR replacement diets, averaging 90% compared to an average survival of 50% in fish fed the 100% FMR diets. Addition of MOS had no significant effects on weight gain, FE ratio values or survival in the first feeding trial.

Table VII–3: Weight gain, feed efficiency ratio, and survival of juvenile cobia in the first feeding trial.

Dietary Treatment		Gain[1] (%)	FE[2] (%)	SGR (%)	Survival (%)
Fish meal Replacement (FMR)	MOS				
50	-	199.0 [a]	37.0 [a]	2.55 [a]	81.0 [a]
50	+	205 [a]	43.0 [a]	2.58 [a]	95.2 [a]
75	-	199 [a]	41.9 [a]	2.54 [a]	100 [a]
75	+	199 [a]	37.5 [a]	2.54 [a]	90.5 [a]
100	-	116 [b]	17.4 [b]	1.76 [b]	38.1 [b]
100	+	153 [b]	28.3 [b]	2.15 [b]	61.9 [b]
P-value					
FMR		**0.0007** ***50, 75 > 100***	**0.0015** ***50, 75 > 100***	**0.0007** ***50, 75 > 100***	**0.0042** ***50, 75 > 100***
MOS		0.7785	0.3163	0.8361	0.3965
FMR * MOS		0.3926	0.2015	0.2924	0.3597
Pooled SE		0.0352	0.0028	0.0292	0.0229

Values with different superscripts within the same column were significantly different (P < 0.05); [1] Percent increase from initial weight; [2] Feed efficiency: grams gained / grams fed; SGR: specific growth rate.

Biological indices

Fish meal replacement also had significant impacts on hepatosomatic index (HSI) and muscle ratio (MR), but not viscerosomatic index (VSI; Table VII–4). Fish fed the 50% FMR diets had significantly larger HSI (1.25%) compared with fish fed the 100% FMR diets (1.05%). Fish fed the diet containing 75% FMR had intermediate HSI values of 1.2%. In terms of MR, a similar trend was observed to that just described, with fish fed the 50% FMR diets having significantly higher MR values (32.6%) compared to fish fed the 100% FMR diets (29.2%), with those fed the 75% FMR diets having intermediate values (31.4%). Visceral somatic indices were not impacted by FMR, with means ranging from 8.3% in fish fed the 75% FMR diet to 9.0% in fish fed the 100% FMR diet, and with intermediate values recorded for fish fed the 50% FMR diet (8.6%). Inclusion of MOS had no significant effects on VSI, HSI, or MR.

Table VII–4: Biological indices of juvenile cobia in the first feeding trial

Dietary Treatment		VSI[1]	HSI[2]	MR[3]
Fish meal Replacement (FMR)	**MOS**			
50	-	8.4	1.2 [a]	33.5 [a]
50	+	8.8	1.3 [a]	30.6 [a]
75	-	8.7	1.3 [ab]	31.4 [ab]
75	+	8.1	1.1 [ab]	31.4 [ab]
100	-	9.0	1.0 [b]	28.8 [b]
100	+	9.0	1.1 [b]	29.6 [b]
P-value				
FMR		0.2753	**0.0050**	**0.0440**
MOS		0.2211	0.4590	0.0898
FMR*MOS		0.1337	0.1024	0.4317
Pooled SE		2.59×10^{-5}	7.7×10^{-7}	4.77×10^{-4}

Values with different superscripts within the same column were significantly different ($P < 0.05$); VSI: viscera-somatic index; HSI: hepato-somatic index; MR: muscle ratio.

Feeding trial 2

Weight gain, FE and survival

In the second feeding trial, diet impacted weight gain significantly, with fish fed the SB and SB+ diets, without amino acid supplementation, displaying the lowest overall weight gain of 99 and 121% increase from initial weight, while fish fed the MXSB and MXSB+ diets, as well as the NOFM diet, all returned significantly greater weight gains (Table VII–5). Juvenile cobia fed all the latter diets had similar weight gains, ranging from 218% in fish fed the NOFM diet to 242 % in fish fed the MXSB diet. Fish fed the control diet exhibited an intermediate weight gain of 187%.

Table VII–5: Weight gain, feed efficiency ratio and specific growth rate for juvenile cobia in feeding trial 2

	Wt Gain (%)	FE (%)	SGR (% d^{-1})	Survival (%)
Control	187 ^{ab}	51 ^{a}	2.50 ^{ab}	93
SB	99 ^{c}	34 ^{b}	1.64 ^{c}	100
SB+	121 ^{bc}	40 ^{b}	1.88 ^{bc}	100
MXSB	242 ^{a}	59 ^{a}	2.91 ^{a}	100
MXSB+	232 ^{a}	59 ^{a}	2.86 ^{a}	100
NOFM	218 ^{a}	56 ^{a}	2.75 ^{a}	100
Pooled SE	16.41	2.3	0.142	2.72
P<F	0.0002	<0.0001	<0.0001	0.4582

Values with different superscripts within the same column were significantly different (P < 0.05).

Specific growth rates (SGR) were significantly impacted by diets, mirroring the results observed with respect to weight gain, ranging from 1.64 to 1.88% in fish fed the SB and SB+ diets, respectively, to 2.75 to 2.91% in fish fed the NOFM and MXSB diets, respectively. Fish fed the control diets presented an intermediate SGR of 2.50%

FE ratio values were impacted significantly by diet, following a trend similar to that observed with regards to weight gain (Table VII–5). Fish fed the MXSB (59%), MXSB+ (59%), NOFM (56%) and control diets (51%) had statistically higher FE ratio values than fish fed the SB and SB+ diets (34% and 40%, respectively).

Biological Indices and tissues analyses

Biological indices were all significantly impacted by dietary treatments (Table VII–6). With respect to VSI, fish fed the SB and SB+ (10.62 and 10.78%, respectively) diets had significantly higher VSI ratios than fish fed the MXSB diet (9.07%). Fish fed the remaining diets had intermediate responses ranging from 9.33 (MXSB+) to 9.67% (control).

Hepatosomatic indices were also significantly affected by dietary treatments: MXSB and MXSB+ -fed fish presented the lowest HSI levels (1.68 and 1.75%, respectively), compared to that observed in fish fed the SB+ diet (2.55%). Fish fed the remaining diets had intermediate responses ranging from 2.02 (control) to 2.30% (SB).

Finally, muscle ratio was significantly decreased in fish fed the SB and SB+ diets (30.06 and 31.63, respectively). Fish fed the remaining diets exhibited significantly higher MR values, ranging from 36.87 (MXSB+) to 37.02% (control).

Table VII–6: Biological indices and proximate analyses in experiment 2

	VSI	HSI	MR	Muscle lipid	Liver lipid
Control	9.67 ^{ab}	2.02 ^{ab}	37.02 ^{a}	4.21	29.13
SB	10.62 ^{a}	2.30 ^{ab}	30.06 ^{b}	3.98	16.58
SB+	10.78 ^{a}	2.55 ^{a}	31.63 ^{b}	4.81	18.59
MXSB	9.07 ^{b}	1.68 ^{b}	36.98 ^{a}	3.49	16.68
MXSB+	9.33 ^{ab}	1.75 ^{b}	36.87 ^{a}	4.29	17.66
NOFM	9.43 ^{ab}	2.17 ^{ab}	36.88 ^{a}	2.68	24.14
P<F	0.0052	0.0022	<0.0001	0.4164	0.4401
Pooled SE	0.339	0.162	1.065	0.710	4.999

Values with different superscripts within the same column were significantly different (P < 0.05); VSI: viscera-somatic index; HSI: hepato-somatic index; MR: muscle ratio.

Tissue composition in cobia from feeding trial 2 was not significantly impacted by dietary treatment (Table VII–6). Muscle lipid concentrations ranged from 2.68% (wet weight) in fish fed the NOFM diet to 4.81% (wet weight) in fish fed the SB+ diet. Liver lipid was more variable, but also was not significantly affected by dietary treatment. Fish fed the control diet had the highest liver lipid levels (29.13% wet weight), while fish fed the SB diet had the lowest recorded liver lipid levels (16.58% wet weight).

DISCUSSION

This represents the fourth time that high fish meal replacement (≥ 75%) levels have been conducted without negative impact on production characteristics at the VATC. Such fish meal replacement levels have been achieved by including supplemental amino acids, particularly taurine, methionine and lysine. In addition to underlying amino acid requirements, combining various alternate protein sources (e.g. yeast-based protein, SBM, SPC) reinforces previous

hypotheses that a single alternate protein source cannot effectively replace fish meal (Craig and McLean, 2005).

In the first feeding trial, juvenile cobia responded well to FMR levels of 50 and 75%, showing equal weight gain and >91% survival. Fish fed an internal control diet consisting of 100% herring meal as the protein source performed statistically well, but numerically lower than fish fed the FMR 50 and 75% diets (167% increase from initial weight, data not presented). Conversely, when 100% of the fish meal was replaced with a single ingredient (SPC), even with amino acid supplementation, production performances declined significantly in all evaluated areas (e.g. weight gain, SGR, survival, FE, Table VII–3).

The use of dietary MOS has been investigated in fish with ambiguous results: improvements in growth and health status in rainbow trout (Yilmaz, et al., 2007) and European sea bass (Torrecillas, et al., 2007) were observed, while no significant effects were discerned in trials with Nile tilapia (*Oreochromis niloticus*; Craig and McLean, 2003), Gulf of Mexico sturgeon (*Acipenser oxyrinchus desotoi*; Pryor, et al. 2003) and European sea bass (Sweetman and Davies, 2006). In the present experiment, juvenile cobia did not benefit from dietary MOS supplementation in either feeding trial. However, when replacing 100% of the fish meal, a beneficial trend was observed: fish fed the 100+ diet experienced a 24% increase in weight gain and a 38% increase in survival on average, when compared to the non-MOS supplemented 100% FMR diet. While these numbers were not significantly different, they may still be relevant from a commercial production stand-point when using high FMR levels. Based on these data, as

well as those observed by Salze et al. (2008) where beneficial effects were noted in larval cobia, producers should be practical in their choices regarding dietary MOS supplementation.

In the second feeding trial, fish meal provided approximately 9% of the dietary protein in the SB diets, while only 6% in the MXSB diets. Despite this reduction in fish meal, a doubling of weight gain was achieved when the supplemental amino acid mix was included in the MXSB diets. This, again, emphasizes the critical importance of supplementation of limiting amino acids when high replacement levels (>75%) of dietary fish meal are employed. The amino acid mix was developed at VTAC based upon novel findings with respect to taurine supplementation in feeds for juvenile cobia (Lunger, et al., 2007b). During the course of many other unpublished cobia studies, experimental diets were analyzed for amino acid composition which aided in the refinement of the present amino acid supplement regime.

One of the most important and far-reaching aspects of this experiment was the successful total elimination of fish meal concomitantly with fish oil in cobia aquafeeds. In the second feeding trial, this was achieved with a unique combination of alternate protein and lipid sources as well as dietary additives (SelPlex® and MOS). The dietary protein components of the NOFM were comprised of a *Nereid* sp. worm meal, SPC, and NuPro®, each supplying complementary amino acid and nutrient profiles. Nereid worms are marine in origin (Dall, et al., 1991) and thus their lipid component contains high levels of long chain highly unsaturated fatty acids (HUFA) of the n-3 family required by marine fishes. The SPC, containing 71% of crude protein, provided the majority of the dietary protein. Finally, NuPro®, a yeast-based protein source,

complemented the protein supply while also added valuable nutrients such as nucleotides, di- and tri-peptides, and other potential nutrients from the yeast cytoplasm. Noteworthy are the overall performances of fish fed this diet, given that it was not supplemented with the amino acid mix. These findings highlight the importance of selecting alternate protein sources based upon appropriate dietary amino acid profiles for the considered species.

In addition, NOFM was also devoid of any fish oil, but utilized two alternate lipid sources, namely soy oil and a concentrated marine algal meal. Importantly, the latter contained the n-3 HUFA, primarily docosahexaenoic acid (DHA). The combination of the algal meal and the marine lipids from the worm meal satisfied the requirements of cobia for n-3 HUFAs without using fish oil.

These data are especially germane to the ongoing concerns regarding sustainable production of marine carnivores – often species with higher market values. The complete elimination of both fish meal and fish oil has now been successfully achieved in aquafeeds for cobia juveniles. However, the ground-breaking NOFM formulation relied on novel and sometimes unique alternative feedstuffs, and therefore may hinder the economical feasibility and sustainability of such diets. Hence, the consistent, successful replacement of up to 94% of the fish meal protein achieved at VTAC is actually more promising to the future of the aquaculture industry and the surrounding issues of true sustainability. Indeed, the use of commodity traded plant protein sources as alternatives to fish meal is the only avenue to true environmental and economical sustainability in the global aquaculture industry.

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Chapter VIII. Expression of Immune-Relevant Genes in a Cyprinid Following Feeding with a Mannan Oligosaccharide

Abstract

Among the various available dietary immunostimulants, yeast-derived mannan oligosaccharides (MOS) are commonly used in fish and animal feeds. Considering the inconsistencies reported on the effects of dietary MOS, I explored its impacts on the zebrafish transcriptome. Fish were fed a MOS-free control diet or a MOS-enriched diet for 9 weeks prior to extracting mRNA from the whole fish and comparing the expression profiles using a microarray technologies. Several immune-related genes were affected by dietary MOS, including *Kruppel-like factor2*, *Complement 3 Component*, *Major Histocompatibility Complex (MHC) class I uea*, *MHC I uad* and *MCH I ufa*. In addition, some genes were involved in cell-cycling (e.g. *SKP2*), and cytokinesis and vesicle trafficking (e.g. *COPB2, DDX41*). Noticeably, no up- or down-regulation of mucin genes was observed, which may be to the result of the up-regulation of the mucus regulator *IL-13 receptor α2.* This lack of altered mucin expression may be due to dose, timing, species and previous physiological state. This study highlighted a mechanism of action for dietary MOS, explaining previous observations, while also suggesting possible reasons for the previously reported inconsistencies. More research is required to explain the conditional benefits of dietary MOS.

Keywords: *Danio rerio*, microarray, nutrigenomics, immunostimulant, probiotic, transcriptome.

INTRODUCTION

The vertebrate gastrointestinal (GI) tract is a complex ecosystem that hosts a vast number of microbes along its length. In humans, at least 500-1000 different Bacteria, Eukarya and Archaea species inhabit the gut (Xu and Gordon, 2003). Moreover, microbial abundance and diversity varies along the intestine's length (Magalhaes, et al., 2007) in many vertebrate species, including teleosts. In fishes, changes in microbial loading and species composition follow shifts in culture conditions, with pathogen challenge, and after dietary modification (Moffitt and Mobin, 2006; Ringø, et al., 1995). These GI mutualists have significant effects on a range of host functions, including development of gut-associated lymphoid tissue and hence immunocompetence; epithelial cell proliferation and thus gut barrier management; angiogenesis and development of the enteric nervous system thereby impacting peristaltic mechanisms; as well as playing a role in nutrient processing (Hooper and Gordon, 2001). The manner in which mutualistic bacteria influence host gut functions is diverse and includes modulation of the expression of host genes (Hooper, et al., 2001). Therefore, perturbating the normal gut flora may impact host gene expression.

A number of studies indicate that dietary pre-, pro- and synbiotics (see: Schrezenmeir and de Vrese, 2001 for definitions) each exert beneficial effects on various immunological functions in aquacultured animals (Chen and Ainsworth, 1992; Gildberg and Mikkelsen, 1998; Villamil, et al., 2003). Similar claims have been made also for mannan oligosaccharides (MOS). Oligosaccharides occur naturally in many feed ingredients. A number of oligosaccharides are

produced commercially either by disaccharide isomerization through enzymatic hydrolysis, or by extraction from microbial cell walls (Perry, 1995). However, MOS derived from yeast cell walls, are utilized most commonly in animal feedstuffs (Monsan and Paul, 1995). In fishes, dietary MOS supplementation reportedly confers enhanced resistance to mycobacteriosis and vibriosis challenges in hybrid striped bass (*Morone saxatilis* x *M. saxatilis*; Li and Gatlin, 2005). In European sea bass (*Dicentrarchus labrax*), MOS reportedly increases the phagocytic index (Torrecillas, et al., 2007) whereas Staykov et al. (2005; 2007) observed augmented serum lysozyme and complement activity in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) fed diets that included MOS at 0.2%. A few studies also have commented on improved gut structure of fish fed MOS-containing diets (Salze, et al., 2008; Sweetman and Davies, 2006; Yilmaz, et al., 2007).

Irrespective of reported benefits of MOS supplementation, several studies conducted on a diverse species range reported a lack of such an effect. These include trials with Nile tilapia (*Oreochromis niloticus*; Craig and McLean, 2003), Gulf of Mexico sturgeon (*Acipenser oxyrinchus desotoi*; Pryor, et al. 2003) and European sea bass (Sweetman and Davies, 2006). In channel catfish (*Ictalurus punctatus*) fed rations containing various, commercially-available MOS preparations, no differences were seen in plasma lysozyme, bactericidal, or spontaneous hemolytic complement activities. Moreover, MOS did not impact respiratory burst activities of phagocytes or survival of catfish when immersion-challenged with *Edwardsiella ictaluri* (Welker, et al., 2007). Other studies also have provided inconclusive results with MOS. These inconsistencies may reflect inappropriate dosage or indicate species-specific differences in

responsiveness. Clearly, before advocating a widespread use of MOS in aquafeeds, it is necessary to establish more precisely a categorical beneficial effect of this ingredient while taking account of potential negative impacts of such treatments on the welfare of the cultured animal. Accordingly, I examined the effect of feeding MOS to a model cyprinid, the zebrafish (*Danio rerio*). Commercially available genechip microarrays (Affimetrix Inc., Santa Clara, CA) were employed in order to evaluate this ingredient's impact on whole-body gene expression profiles with particular attention to immune-related genes.

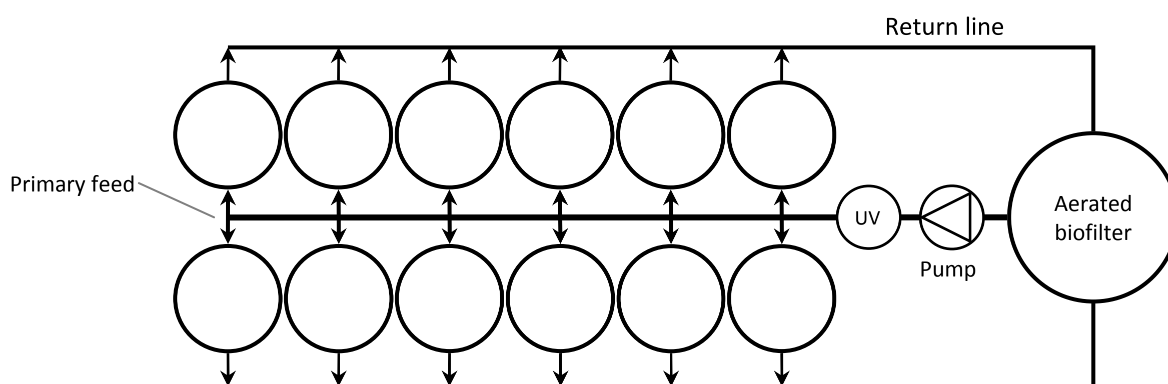
MATERIALS AND METHODS

Animals and holding

Zebrafish were obtained from a local supplier (Zooquatics, Blacksburg, VA, USA), transferred to the Virginia Tech Aquaculture Center (VTAC), and stocked in a custom-made recirculating life support system (RLS; Figure VIII–1). The RLS comprised twelve circular 10-L plastic aquaria for animal holding, a biological filtration unit that retained 0.1 m^3 of plastic media (Kaldnes North America, Inc., Alexandria, VA), 1/6-hp water pump and a 150-watt UV sterilizer (Emperor Aquatics, Inc., Pottstown, PA). Oxygenation was provided by a 1-hp remote-drive regenerative blower (Sweetwater, Aquatic Ecosystems, Apopka, FL) which diffused air directly into the biofilter, and water flow was sufficient to ensure oxygenation of the tanks. Photoperiod was maintained at a 12h photophase-scotophase using an automated timer, with light provided by banks of commercial phosphorescent tubes positioned 8 m above the experimental system. Water quality parameters – including total ammonia nitrogen (TAN, range 0.1 ± 0.1 mg L^{-1}),

nitrite (0.03 ± 0.02 mg L^{-1}) and nitrate (44.6 ± 12.4 mg L^{-1}), pH (7.6 ± 0.3), dissolved oxygen (7.8 ± 0.3 mg L^{-1}) and temperature (23.5 ± 0.6°C) were monitored daily using a pH meter (Hanna Instruments, Woonsocket, RI), spectrophotometric analyses (DR2010; Hach Company, Loveland, CO) and a combined temperature-oxygen meter (YSI Inc., Yellow Springs, OH).

Figure VIII–1: Diagram of the zebrafish recirculating life support system



Each tank was of 10 L volume. Water was returned to the biofilter-sump via gravity feed. The biological filtration unit comprised a 280 L sump containing 140 L of water and 0.1 m3 of KMT media, vigorously aerated via air stones. Water from the biofilter-sump was pumped back to tanks using a 1/6 hp pump (A.O. Smith, Tipp City, Ohio) via a 150-watt UV sterilizer.

Diet formulation and husbandry

Ten zebrafish (initial weight 0.3 ± 0.04 g) were randomly stocked into 6 tanks and each tank then arbitrarily assigned one of two diets. Experimental diets (Table VIII–1) were formulated on a dry-weight basis and prepared at the VTAC. The control diet (CD) was devoid of MOS, whereas the experimental diet (MD) contained 0.5% MOS at the expense of cellulose. Lipids were provided in both diets by menhaden oil, and were formulated to be isonitrogenous (40%

crude protein), isolipidic (10% lipid) and isoenergetic (270 kcal g^{-1}; Table VIII–1). Diets were pelleted in an industrial mixer, crumbled to the desired size, and air dried prior to being stored at -20°C until feeding. Fish were hand fed at 3% body weight d^{-1} split in two feedings (09.00 and 16.00 h) for a period of 9 weeks.

Table VIII–1: Composition of experimental diets (g/100 g on a dry matter basis) formulated to provide 40% crude protein and 10% lipid (dry matter basis).

Ingredient	Control	MOS
Herring meal[1]	53.2	53.2
Dextrin[2]	5.4	5.4
Lipid[3]	5.3	5.3
Mineral mix[4]	4	4
Vitamin mix[5]	3	3
Carboxymethyl cellulose[2]	1	1
Cellufil[2]	28.1	27.6
MOS[6]	0	0.5
Total	100	100
Energy[7]	272	272

[1] Herring meal, International Proteins, Minneapolis, MN; [2] US Biochemical Corporation, Cleveland, OH, USA; [3] Menhaden oil Omega Oils, Reedville, VA, USA; [4] See Cotter et al. (2008); [5] ICN Corporation, Costa Mesa, CA; [6] BioMos, Alltech Inc., Nicholasville, KY; [7] Calculated (kcal/100 g diet).

Sample collection and analyses

At the end of the trial, three fish were randomly sampled per tank. The target tissue (whole fish) was immediately placed into a mortar containing liquid N_2, and crushed with a pestle that

was pre-cooled in liquid N_2. The samples then were pooled and utilized for total RNA isolation and microarray assays (i.e., three fish per chip, three chips per treatments).

RNA isolation and microarray preparation

Total RNA samples were isolated from the whole fish using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Each sample was precipitated with ethanol to concentrate the total RNA, and the resultant pellet was brought up to volume in RNase-free distilled water. RNA quality, quantity and DNA contamination were determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The quality-checked total RNA was used for synthesizing biotin-labeled cRNA. Briefly, 10 μg of total RNA was used to generate first-strand cDNA with a T7-linked oligo(dT) primer. After second-strand cDNA synthesis, *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY), resulting in approximately 100-fold amplification of the cRNA population. The cRNA was fragmented (15 μg per sample), spiked with internal controls (Affymetrix Inc., Santa Clara, CA) and hybridized overnight to the Affymetrix *D. rerio* (DsRed) zebrafish gene chips (n = 6 chips total). The genechips were washed and stained with streptavidin-phycoerythrin, before being scanned on the GeneChip scanner (Affymetrix, Inc.).

Data preprocessing

After import into the Bioconductor package (version 1.5) in R (version 1.9), the CEL files (containing the probe-level data, 22 gene spots per gene) were preprocessed using the Robust Multi-chip Average (RMA) method, which normalizes across a set of hybridizations at the probe level to perform within- and between-chip (quantile) normalizations and to adjust the background. Preprocessing of the data with RMA is more sensitive and specific, and provides a more robust dataset than the standard Affymetrix MAS 5.0 scaling or dCHIP techniques. The normalization process reduced unwanted technical variation, as verified by examining the individual gene chips for reproducibility within the given conditions using two-dimensional scatter plots and hierarchical clustering with GeneSpring (version 7.2, Agilent Technologies, Inc., Santa Clara, CA). Assessing the replication performance among genechips within a treatment group provides reasonable statistical confidence in the expression values (Saviozzi and Calogero, 2003).

Filtering and data analyses

Uninformative genes were eliminated from the dataset after implementing the preprocessing steps. These included genes with signals very near background, those that were considered absent by the Affymetrix scanner and genes that did not change expression values appreciably across conditions. A review of the scatter plots showed that this excluded gene set was highly variable at the lowest end of the expression scale (below 150 units of intensity).

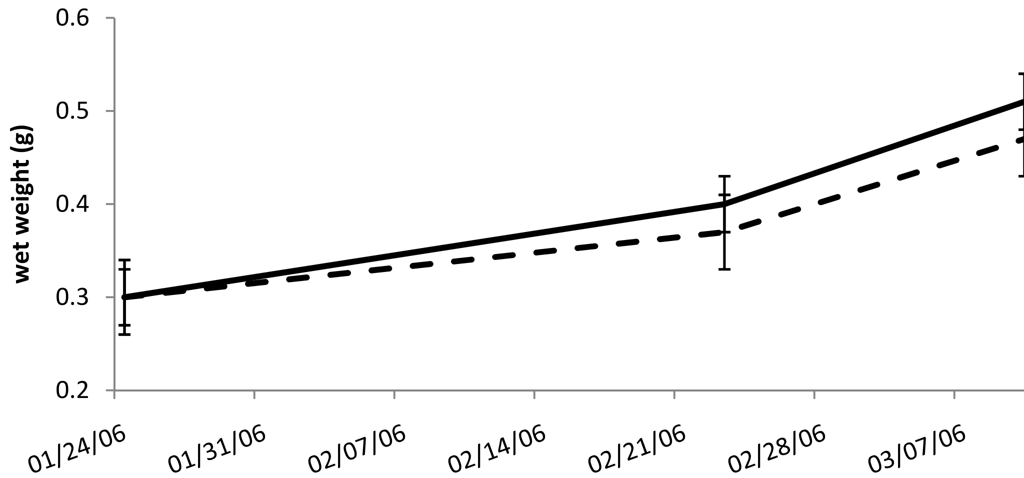
Since I was primarily interested in discovering genes with robust expression levels, eliminating the genes at the low end of the expression scale should not curtail this discovery. Filtering was performed using GeneSpring. On this quality-checked dataset, statistical analyses and fold-change assessments were performed using GeneSpring as follows: 1) executed *t*-tests on the gene values between the control and MOS-treated samples, 2) examined the fold-change differences of the genes with significant effects ($P < 0.05$).

Results

No mortalities were recorded during the experimental period in either control or MOS groups. Both groups grew equivalently over the trial's duration (Figure VIII–2), although each registered significant growth increment between trial start and end ($P < 0.05$).

The final working dataset included 811 genes whose expression was significantly affected by treatments. In the present study, MOS significantly stimulated the transcription of 363 genes while down-regulated 448 other genes ($P < 0.05$). In addition, 29 and 6 genes were up- and down-regulated, respectively, more than 2-fold, although inter-chip variation prevented finding of statistical significance. Table VIII–2 and Table VIII–3 present a selection of up- and down-regulated genes of interest, respectively. Gene up-regulation varied between 2.0-8.4 folds with 5 genes, or 17%, being up-regulated at levels above 3.0. A range of 2.0-4.9 folds was recorded for down-regulated genes, with 28% of these being up-regulated above 3.0. 34% and 23% of the up- and down-regulated genes, respectively, were yet uncharacterized in terms of function.

Figure VIII–2: Growth of control- and mannan oligosaccharide-fed zebrafish



Dashed and solid lines represent the growth of control-fed and mannan oligosaccharide-fed zebrafish, respectively, over the 9-week trial. No differences in growth were detected between groups at any individual time point, but significant (P > 0.05) growth increment was detected for both groups between time zero and trial end.

Table VIII–2: Selected genes up-regulated in mannan oligosaccharide-fed zebrafish relative to controls.

Gene	Full name	GenBank No	Fold change
COPB2	Golgi coatomer protein complex subunit beta 2	BM080957	8.4
Try2	Trypsinogen precursor	BQ264039	4.9
CYP51	Cytochrome P_{450}, family 51	BI879986	3.8
IL13Rα2	Interleukin 13 receptor, alpha 2	BM102667	2.8
MiAgP	Microtubular aggregate protein	AW019242	2.6
gbgt1l4	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1, like 4	BM529391	2.6
MHC I uad	Major histocompatability complex class I UAD	AF182155	2.5
MHC I uea	Major histocompatability complex class I UEA	BC053140	2.4
CYP881	Cytochrome P_{450} 881		2.3
KLF2	Krűppel-like factor 2	NM_131856	2.2
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (hypothetical protein)	BQ616029	2.0
MYD118	Negative growth-regulatory protein	BG739102	2.0
	Angiopoietin-like factor		2.0
C3C	Complement component C3C	AF047415	2.0

Table VIII–3: Selected genes down-regulated in mannan olgisaccharide-fed zebrafish.

Gene	Full name	GeneBank No	Fold change
BIRC6	Baculoviral IAP repeat-containing	BE605449	4.9
hNRP A0	Heterogeneous nuclear ribonucleoprotein A0 (histone cluster 1, H2aj)	BM776600	3.3
ETIF1A	Eukaryotic translation initiation factor 1A	BM037130	2.4
Latrophilin	Latrophilin		2.3
SKP2	Ubiquitin ligase S-phase kinase-associated protein 2		2.3
MHC 1 ufa	Mhc 1 ufa antigen-presenting and processing		2.3
α2mg1	α-2-macroglobulin-1	BG304084	2.2
SCAMP2	Secretory carrier membrane protein 2		2.1
RhaOLL	rhamnose-binding lectin OLL	BM531135	2.3

DISCUSSION

No differences in growth were detected between the experimental groups, thereby suggesting no negative impact of dietary MOS on overall fish performance. However, differential expression levels of various genes between the groups suggest subtle differences in growth and metabolic processes.

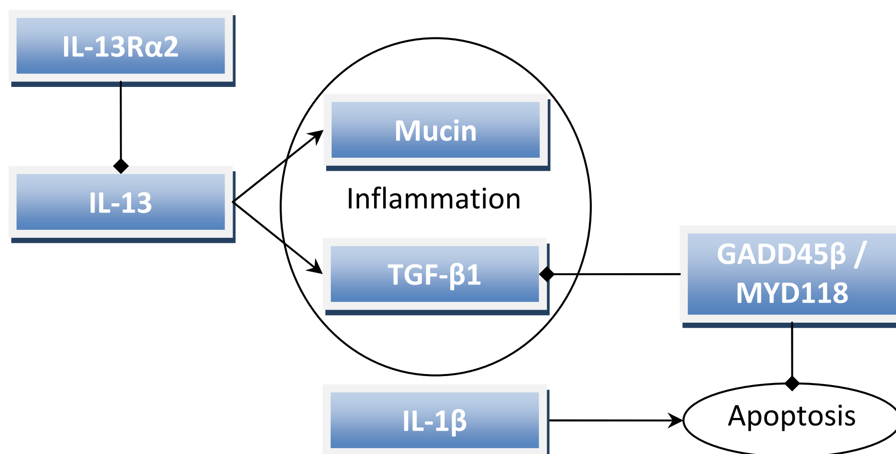
In control zebrafish, higher levels of expression for baculoviral IAP repeat-containing 6 (*BIRC6*, also called *BRUCE* or *Apollon*) and Secretory Carrier Membrane Protein 2 (*SCAMP2*) were observed. These genes play key roles in conserving the trans-Golgi network, regulate delivery of membrane vesicles during cell division, facilitate degradation of apoptotic proteins

through ubiquitination and are engaged in cytokinesis, cell growth and cellular transformation (Hao, et al., 2004; Hauser, et al., 1998; Liao, et al., 2007; Pohl and Jentsch, 2008).

Various studies with fish illustrate that diet and nutritional condition can have profound impacts on gene expression profiles for specific tissues (McLean and Craig, 2006; Panserat, et al., 2008; Rise, et al., 2006; Salem, et al., 2007). In general, microarray studies have assisted to explain the biological basis for the measured biological responses of animals to various manipulations. For example, starvation of rainbow trout down-regulates many hundreds of genes involved in protein synthesis, lipid metabolism and transport, glucose metabolism and immune function (Salem, et al., 2007) – reactions that could have been predicted based on known whole-animal responses (Loughna and Goldspink, 1984; Morata, et al., 1982; Sumpter, et al., 1991). Likewise, the feeding of probiotics to trout resulted in up-regulation of genes engaged in immunity (Panigrahi, et al., 2007), thereby confirming previously measured whole-animal biological responses (Gomez and Balcazar, 2008). Based on recorded reactions of various fishes to dietary MOS, similar findings might have been anticipated for the present study. However, little information exists regarding the precise action mechanisms of dietary MOS, and observations made here provide a distinctive process that has hitherto not been considered.

Surprisingly, mucin genes, such as *MUC5B* or *MUC2*, were not up-regulated significantly in the MOS-fed fish. This may be due to a dilution of the transcripts, since mRNA was extracted from the whole fish, and mucin genes may not be up-regulated in all mucosal tissues. However,

Figure VIII–3: Relationship between IL-13α2, inflammation, and apoptosis



IL-13Rα2, which is involved in regulating mucus dynamics, was up-regulated by dietary MOS. *IL-13Rα2* may act as a feed-back mechanism to reduce mucus production. By counteracting IL-13, IL-13Rα2 is also a suppressor of inflammatory response (Zheng, et al., 2008, Figure VIII–3), suggesting that the fish were not under pathological stress. This is also indicated by the down-regulation of *rhamnose-binding lectin OLL*, which is involved in the recognition of bacterial pathogens (Tateno, et al., 2002).

Increased mucus production and secretion obviously dictates enhanced cellular activity in terms of membrane trafficking. The role of the Golgi complex in mucin production has been recognized since 1914 (Cajal, 1914), and a more than 8-fold up-regulation of Golgi-associated particle *102K* was recorded in MOS-fed zebrafish. Golgi coatomer protein complex subunit beta 2 (*COPB2*) has been implicated in the exocytic pathway and especially in membrane traffic regulation within the cell (Lippincott-Schwartz, et al., 2000; Schekman and Orci, 1996). The DEAD box protein *DDX41*, through interactions with *nexin-2*, is likewise engaged in membrane

trafficking (Abdul-Ghani, et al., 2005) and transcription levels for this gene were 2-fold higher in MOS-fed zebrafish. These transcriptomic outcomes of dietary MOS provide strong evidence for the hypersecretion of mucus and gut barrier enhancement, and may represent one mechanism explaining the increased resistance of some MOS-fed fish species to artificial bacterial challenges. Nevertheless, further augmentation of other host defense mechanisms would likely be necessary to explain all the reported benefits of dietary MOS. This might be explained by the 2.3 to 2.6-fold up-regulation of zebrafish major histocompatibility complex class I UAD/UEA genes (*mhc 1 uda*/*uea*). For an immune response to be triggered, MHC molecules must process and present antigens to T-cells and Kruppel-like factor 2a (*KLF2*), a zinc finger transcription factor, plays a critical role in T-cell cycling (Carlson, et al., 2006). When over-expressed, and for fish in receipt of MOS, a 2.2-fold up-regulation was recorded; *KLF2* inhibits cell-cycle progression and ensures against premature activation and death of mature T cells (Buckley, et al., 2001; Kuo, et al., 1997; Sebzda, et al., 2008). *KLF2*, which activates T-cells by its induction of *IL-2* (Wu and Lingrel, 2005), may serve to bolster T-cell populations.

Several studies indicate enhanced development and maturation of the intestine in various species following dietary MOS supplementation. For example, addition of 2% MOS to turkey diets increased ileal villus height, surface area, crypt depth and goblet cell density (de los Santos, et al., 2007) and height of ileal villi increased in rabbits fed MOS at 0.1-0.2% (Mourao, et al., 2006). Fishes do not possess villi. Rather, the surface area of the intestine is increased through complex folding and or by appendages such as caeca (Harder, 1975). Nevertheless, the microscopic integrity of the gut of Senegal sole (*Solea senegalensis*), rainbow trout and larval

white sea bream (*Diplodus sargus*) and cobia (*Rachycentron canadum*) was apparently improved due to MOS supplementation (Sweetman and Davies, 2006; Yilmaz, et al., 2007; Salze, et al., 2008, respectively). In larval cobia, a beneficial effect of MOS was also recorded with regard to salinity stress resistance, possibly related to the accelerated maturation of the gut (Salze, et al., 2008).

Given the histological findings of MOS impact on gut growth, and hence the presumptive enhancement in intestinal barrier function, it might be anticipated that supplemental MOS also impacts genes involved in cellular cycling. This effect was seen in the ubiquitin ligase S-phase kinase-associated protein 2 (F-box protein *SKP2*), a major cell cycle regulation protagonist (Nakayama and Nakayama, 2006). In control zebrafish, the expression level for the *SKP2* gene was over twice that recorded for MOS-fed fish. *SKP2* specifically targets cyclin-dependent kinase inhibitor *p27* for degradation (Tsvetkov, et al., 1999), thus promoting cell cycle progression (Nakayama and Nakayama, 2005). The lower expression levels for *SKP2* and a concomitant decrease in *p27* may have decreased apoptosis in MOS-fed fish, which could thereby provide a partial explanation for the changes observed in gut architecture in MOS-fed animals (Nakayama and Nakayama, 2005). One MOS mechanism may be its ability to augment intestinal populations of beneficial mutualists while preventing pathogens establishment. Boosting the symbiotic and commensal gut flora also might drive changes in the gut architecture. However, hybrid tilapia (*O. niloticus* x *O. aureus*; Genc, et al., 2007) and rainbow trout (Yilmaz, et al., 2007) fed MOS at levels above 1.5% of diet did not display changes in gut structure. These contradictory responses are difficult to explain, but may be related to dose,

species, age and/or previous nutritional status. While MOS is already used in aquaculture, further research is needed to determine whether changes in mucin composition occur, and whether increased mucus production is associated with enhanced presence of antimicrobial compounds. Another factor that will require further research relates to establishing optimal dietary doses of MOS and cost-benefit analysis. Due to its apparent beneficial protective actions in terms of conferring resistance to pathogens, MOS might act to decrease industry reliance on antibiotics. It is possible that dietary MOS supplementation might only be necessary during high disease risk periods.

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Chapter IX. GENE EXPRESSION PROFILING OF ZEBRAFISH FED DIETS CONTAINING HIGH LEVELS OF NUCLEOTIDES

Abstract

Zebrafish (*Danio rerio*) were fed either a control or nucleotide-enhanced diet for 9 weeks. At the trial termination, fish were assayed for transcriptional alterations by dietary free nucleotide supplementation. As determined by microarray–mediated assay of gene expression, nucleotide-supplementation impacted 681 genes significantly ($P < 0.05$). The nucleotide diet stimulated the transcription of 368 genes while down-regulating 313 others ($P < 0.05$). Categorization of the up-regulated genes revealed that 22% were engaged in transport and signaling, 20% were implicated in transcription and translation, and 5% each in immunity and cell cycling. Sixteen percent of the significantly up-regulated genes could not be categorized while the remainders were uncharacterized. Since dietary nucleotides are efficiently catabolized in the intestinal epithelium, such result could be explained by a signaling role of nucleotides, starting in the absorptive cells. Indeed, the expression of numerous genes involved in signaling cascades, such as *FAST* (Fas-activated serine/threonine kinase), were impacted by dietaty nucleotides.

Keywords**:** transcriptomics, nutrigenomics, genomics, nucleotide, immunity, microarray

INTRODUCTION

Apart from constituting the basic components of nucleic acids (DNA or RNA), nucleotides can remain unincorporated as 'free' molecules of significant dietary importance. Nucleotides are low molecular weight intracellular compounds of three components: a nitrogenous base, a sugar molecule and one or more phosphate groups. Whether as free molecules or nucleic acids, nucleotides play key roles in a wide variety of metabolic processes and are present in all foods (Gil, 2002). While not considered essential, nucleotides positively influence lipid metabolism, immunity, as well as tissue growth, development and repair (Aggett, et al., 2003; Gutiérrez-Castrellón, et al., 2007). Because rapidly proliferating tissues, including those comprising the immune system and intestine, are unable to meet the cellular nucleotide requirements by *de novo* synthesis, it has been suggested that dietary nucleotides might be required conditionally (Van Buren and Rudolph, 1997). Studies on animals, as well as humans, support this possibility, since during disease states, dietary nucleotides assist in the repair of insulted tissues (Carver, 1999).

In fish, aquafeed nucleotide supplementations of 0.5-5.0% appear to enhance active components of the non-specific immune system, elicit elevations in leukocyte count and respiratory burst activities, and increase survival to bacterial and viral challenges (Burrells and Williams, 2006; Jha, et al., 2007; Li, et al., 2004; Russo, et al., 2006; Sakai, et al., 2001). Furthermore, dietary nucleotides have been shown to lower the incidence of salmon louse (*Lepeophtheirus salmonis*) infestations, (Burrells, et al., 2001a) and to exert beneficial effects on

intestinal structure, osmoregulatory capacity (Burrells, et al., 2001b; Burrells and Williams, 2006), and response to vaccination (Ramadan, et al., 1994). Although the potential production benefits conferred by dietary nucleotides are wide-ranging, the mechanism(s) of action of these compounds nevertheless remain poorly characterized. A chief goal is the determination of optimal protocols for nucleotide administration including dose, frequency and timing of use. Moreover, selection of the appropriate nucleotide source and the form in which these are delivered to provide the most advantageous response remains fertile ground for investigation. In the present study, I examined the response of the cyprinid zebrafish (*Danio rerio,* Hamilton, 1822) to dietary nucleotide supplementation. Gene expression profiling using microarray technologies was employed with particular reference to genes engaged in the immune system.

MATERIALS AND METHODS

Animals and holding

The zebrafish were obtained from a local supplier (Zooquatics, Blacksburg, VA, USA), transferred to the Virginia Tech Aquaculture Center (VTAC), and stocked in a custom-made recirculating life support system (RLS). The RLS comprised twelve circular 10 L plastic aquaria for animal holding, a biological filtration unit that retained 0.1 m^3 of plastic media (Kaldnes North America, Inc., Alexandria, VA), and a 150-watt UV sterilizer (Emperor Aquatics, Inc., Pottstown, PA). Oxygenation was provided through diffusion air lines connected to a 1-hp remote-drive regenerative blower (Sweetwater, Aquatic Ecosystems, Apopka, FL). Photoperiod was maintained at a 12-h photophase-scotophase using an automated timer, with light

provided by banks of commercial phosphorescent tubes positioned 8 m above the experimental system. Water quality parameters – including total ammonia nitrogen (TAN, range 0.1±0.1 mg l^{-1}), nitrite (0.03±0.02 mg l^{-1}) and nitrate (44.6±12.4 mg l^{-1}), pH (7.6±0.3), dissolved oxygen (7.8±0.3 mg l^{-1}) and temperature (23.5±0.6°C) were monitored daily using a pH meter (Hanna Instruments, Woonsocket, RI), a spectrophotometer (DR2010; Hach Company, Loveland, CO) and a combined temperature-oxygen meter (YSI Inc., Yellow Springs, OH).

Fish husbandry and diet formulation

Eight zebrafish (initial weight 0.3 ± 0.03 g) were randomly stocked into 6 tanks, and each tank was arbitrarily assigned one of two diets. The experimental diets were formulated on a dry- weight basis and prepared at the VTAC. The control diet (CD) was formulated with herring meal as the sole protein source. In the experimental nucleotide-enriched diet (ND), fish meal was completely replaced by a yeast cytoplasm extract (NuPro®, Alltech Inc., Lexington, KY) which contained a minimum of 5% nucleotides (on dry-matter basis). Lipids were provided in both diets by menhaden oil, and both diets were formulated to be isonitrogenous (40% crude protein), isolipidic (10% lipid) and isoenergetic (270 kcal g^{-1}; Table IX–1). The diets were pelleted in an industrial mixer, crumbled to the desired size, and air-dried prior to storage at -20°C until feeding. The fish were hand-fed at 3% body weight d^{-1} split into two feedings (at 09.00 and 16.00 h) for a period of nine weeks.

Sample collection and analyses

At the end of the trial, three fish were randomly sampled per tank. The whole fish were immediately placed into a mortar containing liquid N_2, and crushed with a pestle that was pre-cooled in liquid N_2. The samples then were pooled and utilized for total RNA isolation and microarray assays (i.e., three fish per chip, three chips per each of two treatments).

Table IX–1: Formulation of the experimental diets with nucleotide supplementation

	Control	ND
Herring meal	53.2	0
NuPro	0	78
Dextrin	5.4	5.4
Lipid	5.3	8.6
Mineral	4	4
Vitamin	3	3
Carboximethyl cellulose	1	1
Cellufil	28.1	0
Total	100	100
Crude proteins	40%	40%
Crude lipids	10%	10%
Energy (kcal)	272	272

ND: nucleotide diet. Numbers represent the inclusion percentage on a dry matter basis. Crude proteins, crude lipids, and energy content values are calculated.

RNA isolation and microarray preparation

Total RNA samples were isolated from the whole fish using the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Each sample was precipitated with ethanol to concentrate the total RNA, and the resultant pellet was dissolved in RNase-free distilled water. RNA quality, quantity and DNA contamination were assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies,

Palo Alto, CA). The quality-checked total RNA was used for synthesizing biotin-labeled cDNA. Briefly, I used 10 μg of total RNA to generate first-strand cDNA with a T7-linked oligo(dT) primer. After second-strand cDNA synthesis, *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY), resulting in approximately 100-fold amplification of the cRNA population. The cRNA was fragmented (15 μg per sample), spiked with internal controls (Affymetrix Inc., Santa Clara, CA) and hybridized overnight to the Affymetrix *D. rerio* (DsRed) zebrafish gene chips (n = 6 chips total). The genechips were washed and stained with streptavidin-phycoerythrin, before being scanned on the GeneChip scanner (Affymetrix, Inc.).

Data preprocessing

After import into the Bioconductor package (version 1.5) in R (version 1.9), the CEL files (containing the probe-level data, 22 gene spots per gene) were preprocessed using the Robust Multi-chip Average (RMA) method, which normalizes across a set of hybridizations at the probe level to perform within- and between-chip (quantile) normalizations and to adjust the background. Preprocessing of the data with RMA is more sensitive and specific, and provides a more robust dataset than the standard Affymetrix MAS 5.0 scaling or dCHIP techniques. The normalization process reduced unwanted technical variation, as verified by examining the individual gene chips for reproducibility within the given conditions using two-dimensional scatter plots and hierarchical clustering with GeneSpring (version 7.2, Silicongenetics, Redwood City, CA). Assessing the replication performance among gene chips within a treatment group

provides reasonable statistical confidence in the expression values (Saviozzi and Calogero, 2003).

Filtering and data analyses

Uninformative genes were eliminated from the dataset after implementing the preprocessing steps. These included genes with signals very near background, those that were considered absent by the Affymetrix scanner and genes that did not change expression values appreciably across conditions. A review of the scatter plots showed that this excluded gene set was highly variable at the lowest end of the expression scale (below 150 units of intensity). Since I was primarily interested in discovering genes with robust expression levels, eliminating the genes at the low end of the expression scale should not curtail this discovery. Filtering was performed with GeneSpring. On this quality-checked dataset, I performed statistical analyses and fold-change assessments using GeneSpring as follows: 1) executed *t*-tests on the gene values between the control and nucleotide-treated samples, 2) examined the fold change differences of the significant genes ($P < 0.05$).

RESULTS AND DISCUSSION

The final working dataset included 681 genes whose expression differed significantly among treatments. In the present study, ND stimulated the transcription of 368 genes, while down-

regulating that of 313 other genes significantly ($P < 0.05$). Further, ND significantly up-regulated two genes by greater than 2-fold, and down-regulated a further 19 by 2-fold or greater.

Table IX–2: Summary of the immune-related genes impacted by dietary nucleotides

Gene	Full name	GenBank #	Fold-change
ITGβ5	Integrin β5	NM_001082836	1.3
FAST	*Fas*-activated serine/threonine kinase	BM861730	1.3
PI3K	phosphoinositide-3-kinase	BG728812	1.1
RhoGEF	Rho guanine nucleotide exchange factor	BM186508	2.6
RasGEF	Ras guanine nucleotide exchange factor	AW115682	1.4
PIP5K	phosphatidylinositol-4-phosphate 5-kinase	BI845641	1.1
Bcl-2i	*Bcl-2* inhibitor of transcription	BM035043	1.4
NFAT	Interleukin enhancer binding factor 3 isoform c – Nuclear factor of activated T-cells	AI721516	1.1
NFκBr	NFkB-repressing factor	AI957777	1.3
LYN	*v-yes-1* Yamaguchi sarcoma viral oncogene homolog 1 – Protein tyrosine kinase	AF164753	1.2
MHC I UBA	Major Histocompatibility Complex I UBA	NM_131471.1	3.1
SA3	serum amyloid A 3	BI883568	2.5
Cathepsin Ba	Cathepsin B, a	BC044517.1	3.6
SWI/SNFe1	*SWI/SNF* related, subfamily e, member 1	BC044363.1	1.2
CC1	Complement component 1	CD014253	5.0
CC3	Complement component 3	BI878414	2.8
CC7-1	Complement component 7-1	AA497156	2.3

In addition, 35 and 34 genes were up- and down-regulated, respectively, by more than 2-fold, although no statistical differences in transcription were observed ($P>0.05$). When

categorizing the up-regulated genes according to function, 22% were involved in transport and signaling, and 20% were implicated in transcription and translation. Cell cycle- and immune-related genes each represented 5% of up-regulated genes. Finally, 16% of the significantly up-regulated genes could not be categorized, and a further 32% remained uncharacterized. Table IX–2 presents a summary of the most relevant genes up-regulated in ND-fed fish.

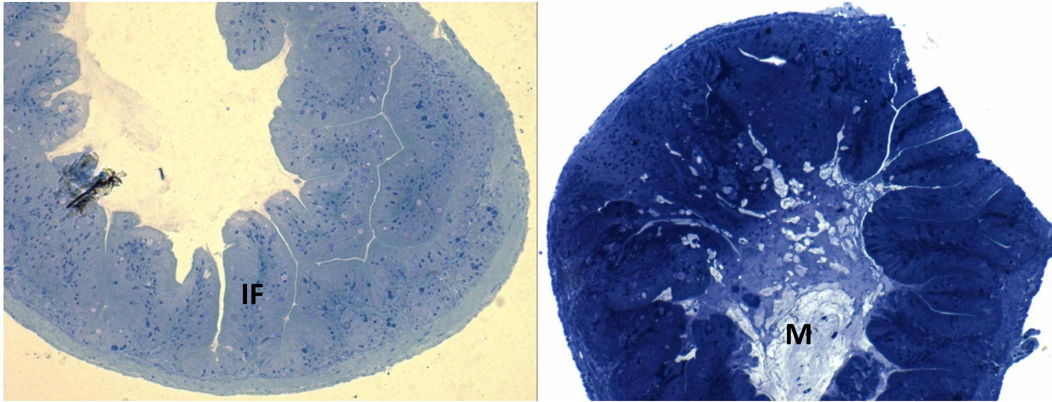
While 5% of the up-regulated genes are reportedly involved directly in immune system function, numerous other genes also were engaged indirectly in immune response via signaling or transcription/translation regulation. However, upon further inspection, certain genes that stimulate or repress lymphocyte activation were expressed concomitantly. For example, the ND-fed fish expressed higher levels of *Lyn*, a membrane-associated tyrosine kinase that participates in antigen-mediated signal transduction in B-cells (Yamanashi, et al., 1991). Nuclear Factor of Activated T-cells (*NFAT*), which participates in the activation of both T- and B-cells (Kneitz, et al., 2002), was also up-regulated in ND-fed fish. In contrast, a predicted *Fas*-activated serine/threonine kinase (*FAST*) was also up-regulated (Table IX–2). *Fas* is one of the most potent triggers for lymphocyte apoptosis (Tian, et al., 1995). In addition, the inhibitor factor of nuclear factor κ light polypeptide gene enhancer in B-cells 1 (*NFκB*), which is involved in regulating inflammation and transcription from the RNApol II promoter (Meyer, et al., 1991), apoptosis (Cahir-McFarland, et al., 2000) and ubiquitin-mediated proteolysis, was up-regulated in the ND-treated fish.

Interestingly, expressions of immunoglobulin (Ig)-coding genes were not increased in the ND-fed fish, a finding that is inconsistent with previous reports in mammals (Jyonouchi, et al., 1994; Navarro and Maldonado, 1999; Sudo, et al., 2000). In tilapia (Ramadan, et al., 1994), rainbow trout (Leonardi, et al., 2003), and hybrid striped bass (Li, et al., 2004), nucleotide-enriched diets have also been reported to increase Ig. However, unlike previous studies with fish, experimental animals used herein were not exposed to pathogen challenge, which may explain the lack of an Ig effect. It is also possible that the transcription of Igs was masked between treatments because the mRNA was extracted from whole fish. Indeed, Low et al. (2003) described an up-regulation of IgM expression in fish gills and spleen, and a down-regulation in the kidney. Nonetheless, the up-regulation of a *MHC I UBA* gene by over 3-fold in the present study indicates that cell-mediated immunity remained unsuppressed. Overall, these results diverge slightly from previous findings in which lymphocyte-mediated responses were stimulated, but rather suggest an intensification of lymphocyte signal transduction, and turnover of the lymphocyte pool.

Fish immunity relies primarily upon innate processes, such as those involving phagocytes and the complement cascade (Ellis, 2001). In the present study, several genes related to the innate immune system (humoral and cellular components) were up-regulated between 2- and 5-fold. Although individual variation likely prevented statistical separation, this may remain biologically relevent. These genes included *RasGEF* domain 1Ba, *RhoGEF*, *PI3K*, *PIP5K*, *complement components 1*, *3* and *7*, *serum amyloid A3* (which is engaged in the acute phase response), and *cathepsin B*, a lysozomal proteinase. Similarly, the dietary nucleotides increased

lysozyme expression in turbot *Psetta maxima* (Low, et al., 2003), as well as serum complement and macrophage activity in common carp (Sakai, et al., 2001). According to the results from the present study, the enhanced phagocytic activity could be explained by a stimulation of vesicle trafficking and focal adhesion. Focal adhesion, large protein complexes interacting with the cytoskeleton, is critical for processes such as cell cycling and cell motility. The latter is particularly relevant in cell-mediated immunity, whereby macrophages penetrate an infection site (Niedergang and Chavrier, 2005). Rho GTPases, which belong to the Ras superfamily of GTPases, are known to regulate processes such as cell motility and vesicle trafficking (Boguski and McCormick, 1993; Ridley, 2006); they are activated by guanine nucleotide exchange factors (GEFs). Upon activation, Rho GTPases stimulate the reorganization of the actin cytoskeleton (Hall, 1998; 2005). In addition, a *SWI/SNF-like actin- dependent factor* was also up-regulated, which suggests an epigenetic mechanism involving chromatin remodeling to enable transcription regulation of targeted gene activity regarding the cytoskeleton. Noteworthy was the observation that ND did not stimulate two other well-described Rho proteins, namely *Cdc42* and *Rac1*, which are involved in the formation of filopodia and lamellipodia, respectively (Niedergang and Chavrier, 2005). This may reflect mRNA dilution. Alternately, since RhoGEF itself activates *Cdc42* and *Rac1*, this finding could indicate a more general activation of cellular actin filaments. Precisely directed research is necessary to further clarify the influence of dietary nucleotides on these genes.

Plate X–1: Mid-intestine cross sections of control and nucleotide-fed zebrafish



Note the increased mucus production in the ND-fed fish (right) compared to the CD-fed fish (left). IF: intestinal fold; M: mucus. Stained with toluidine blue O and counterstained with safranin O.

In fishes, numerous studies have converged upon a potential mechanism for dietary nucleotides enhancing resistance to bacterial and viral pathogens (Li and Gatlin, 2006); Burrels et al. (2001a) also observed a positive effect of dietary nucleotides on sea lice infestation of salmonids, and the authors suggested that nucleotides reduced immunosuppressive factors such as cortisol and prostaglandin E_2. While we did not observe these effects at the transcriptional level, histological sections of the zebrafish intestine revealed a significant increase in mucus production in ND-fed fish compared to CD-fed fish ($P<0.05$; Plate IX–1). Accordingly, the mucin-encoding gene *Muc5b* was up-regulated in ND-fed fish (2.23-fold; Table IX–2). The observed enhanced mucus production could also partly explain the multipotent immunostimulation by reinforcing first barriers (e.g. intestinal epithelium and skin) and shedding of infesting parasites. This is consistent with previous studies with human infants, in which diarrhea occurrence is reduced and the intestinal microflora composition is improved with dietary nucleotide supplementation (Singhal, et al., 2008). In fish, the gastrointestinal (GI) epithelium is an extremely important interface for digestion, immune function and

osmoregulation. Therefore, the effects of dietary nucleotides on GI function and integrity could have significant implications for growth, development and health. However, further research is required to validate this hypothesis.

Most studies with fish – including the present one – have reported immunostimulating properties for dietary nucleotides (Jha, et al., 2007; Li and Gatlin, 2006), but the effect of dietary nucleotides on growth remains unclear. Oliva-Teles et al. (2006) reported a significant growth response to different nucleotides (brewer's yeast or yeast-extracted RNA) in gilthead sea bream (*Sparus aurata*) juveniles (12-46g). Nucleotide-fed fish were 12-19% heavier than the control fish after 10 weeks. Conversely, Li et al. (2007) observed a transient positive effect of dietary nucleotide supplementation in juvenile red drum (*Sciaenops ocellatus*, 10-30g); namely, the growth and feed efficiency response was only significant during the first week of the trial. In the latter study, nucleotides incorporated into experimental diets were either via a purified mix or a commercial product, and both sources impacted fish growth similarly. Previously, Rumsey and coworkers (1992) observed a growth depression in rainbow trout fed free purines. Fournier (2002) observed no nitrogen-sparing effect of nucleotides in rainbow trout and turbot, along with a 2-fold increase in urea-nitrogen excretion rate. In the present study, ND had no significant impact on individual fish growth. However, adult fish were used, which likely limited overall growth potential. Other authors also have speculated that different growth responses result from different nucleotide mixes (Li and Gatlin, 2006). Preliminary studies suggested that mammals respond differently to nutritional immunostimulation depending on their genotype and antioxidant backgrounds (Grimble, 2001). Since dietary nucleotides are efficiently

catabolized in the intestinal epithelium, such broad impact on gene expression could be explained by a signaling role of nucleotides, starting in the absorptive cells. I observed the up-regulation of guanine-based second messenger pathways (Ras GTPases). Cyclic AMP (adenosine-based) is another important second messenger involved in different functions than guanine-based messengers. Thus the ratios between the different nucleic bases as well as their phosphorylation status may be relevant in terms of nutritional effects. Additional research, combining transcriptomics and more traditional assay methods, is necessary to further investigate this hypothesis.

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Chapter X. MICROARRAY EVALUATION OF THE RESPONSE OF ZEBRAFISH TO DIETARY SELENOMETHIONINE

Abstract

The effect of supplementing feed with organic selenium (selenomethionine; Se-met) on gene expression was examined in zebrafish. Two diets were used: a control and one that incorporated Se-met at 0.06% of the diet on a dry-matter basis. Feeds were fed to triplicate groups of fish (n = 8 fish per tank) twice per day to satiation for 9 weeks. At trial termination, three animals per tank were sacrificed and pooled. The tissues were immediately prepared for total RNA isolation, quality checked and subsequently hybridized overnight to an Affymetrix zebrafish gene chip (n = 3 per treatment). Se-met addition to the diet resulted in up-regulation ($p<0.05$) of 6112 genes. Of these, 1279 were up-regulated more than 2-fold, 158 more than 3-fold, and 5 at over 6-fold when compared to control groups. A narrower examination showed that Se up-regulated a number of genes involved in leukocyte activation and differentiation and regulation of the complement cascade. A number of non-immune-specific genes that nonetheless have significant relevance to body defense were also up-regulated, including those involved in mucin production, endocytosis and exocytosis. A noteworthy effect of dietary Se-met treatment was seen on a range of genes associated with the insulin signaling cascade, suggesting that Se may be insulinomimetic in fish. Significant differences also were observed in various gene classes involved in the maturation process, with the Se-met treatment apparently suppressing gamete development. The present study indicates that Se may play a more wide-ranging role in vertebrate metabolism than currently appreciated.

Keywords: transcriptomics, nutrigenomics, insulinomimetic, immunity, selenium, mucin

INTRODUCTION

The last two decades have witnessed fundamental changes in nutritional research, with traditional physiological and epidemiological studies being supplemented by molecular and genetic analyses (Milner, 2004). The ascendancy of the latter methodologies reflects not only acquisition of new technologies and knowledge, but also the recognition that nutrients: 1) modify gene expression, 2) may alter normal metabolism, and 3) affect normal health (Corthésy-Theulaz, et al., 2005). This has stimulated the scientific community to engage molecular techniques to more rigorously define inter-relationships between diet, health and disease (Kaput and Rodriguez, 2004). These approaches herald a new era for the pharmaceutical and feed ingredient industries in particular, since both will be able to develop, refine and modify bioactive and natural compounds that target specific diseases and/or provide health benefits. Fundamental differences exist, however, between clinical and animal production settings and in how these emerging technologies may be applied at the practical level.

In humans, it is well established that many medications express individual variability in efficacy and toxicity. This may result due to environmental factors or genetic variation. Indeed, many genes have small sequence differences or polymorphisms, and these distinctions may affect protein-protein or protein-substrate interactions. Inconsistencies in some epidemiological studies may partially be explained by polymorphisms, as exemplified by those upon folate metabolism and altered risk to colon cancer and responsiveness to hypolipemiant

drugs (Davis and Hord, 2005; Ruano, et al., 2005). These recognized deviations from the “norm” in human subjects has led to the development of the concept of “personalized medicine”, wherein gene expression profiling (GEP) is employed to provide tailored treatment regimens based upon molecular classification of gene subtypes (Chin, et al., 2004; Jain, 2004). The use of GEP, in which transcriptomic or microarray methods are employed, has recently been applied to adjust dietary intake of humans to provide health benefit - the so-called “nutrigenomics” approach (Castle and Ries, 2007; Lau, et al., 2008). In the animal nutrition field, far from adapting nutrition to each individual, these same technologies may be harnessed to assist in the formulation of élite animal feeds.

The recognition that certain nutrients interact beneficially with the immune system of aquatic animals has led several feed manufacturers to develop diets that focus upon enhancing health through nutrient manipulation. These specialty feeds originally commenced with simple vitamin, and especially ascorbic acid, additions, but subsequently have evolved into more complex formulations that incorporate various minerals, vitamins, β-glucans, nucleotides, and other immunostimulating ingredients (McLean, et al., 2008). The cynic might suggest that today’s “enhanced” feeds simply reflect a deeper understanding of fish nutritional requirements and that even greater refinement to aquafeeds is possible. Gene expression profiling represents one means by which nutrient-gene interactions can be evaluated and information on key nutrients rapidly accumulated with respect to their overall impact potential on animal production performance. In addition, GEP could be applied to examine the negative impacts of stressors and latent infections on nutritional status. Such information may be of

significance where micronutrients are preferentially mined, leading to a transient or sustained imbalance which may have significant consequences to the operation of the immune system (Institute of Medicine, 1999).

A resurgence of interest in selenium (Se) utilization in vertebrates has emphasized the essentiality of this trace element, while highlighting a greater potential role of this mineral in a wider range of biochemical pathways than previously imagined (Surai, 2006). In fish, Se is present in the form of selenocysteine, and it has been suggested that the Se requirement for fish in general may be higher than for other vertebrates (Kryukov and Gladyshev, 2000). In higher vertebrates, Se compounds are known to be involved in a variety of biological processes, but are especially important as antioxidants and perhaps as transport proteins. Se represents one mineral that may become rapidly depleted during stress events (Gill and Walker, 2008), such that dietary over-formulation (i.e. above established requirement levels) for this mineral may be a rational option, especially during periods in the production cycle where the risk of stress events is high. A prerequisite to recommending over-formulation for any nutrient, however, is a detailed picture of not only biological response, but also a mechanistic understanding of observed effect. The latter is important since nutrient excess can have negative as well as positive impacts on a range of physiological control processes.

Microarray technologies permit an exquisite way in which the effects of dietary over-formulation of one ingredient can be examined on a genome-wide basis. Accordingly, in order to gain greater insight of the biological effects of supplemental dietary Se on fish, the impact of

supplementing feeds with selenomethionine was examined using zebrafish *Danio rerio*. The response of Se-fed fish was compared against a control treatment in which Se was fed at requirement levels only. Zebrafish were chosen as a model due to the commercial availability of zebrafish gene chips.

MATERIALS AND METHODS

Animals and holding

Zebrafish were obtained from a local supplier (Zooquatics, Blacksburg, VA, USA) and transferred to the Virginia Tech Aquaculture Center (VTAC), and stocked in a custom-made recirculating life support system (RLS). The RLS comprised twelve circular 10-L plastic aquaria for animal holding, a biological filtration unit that retained 0.1 m^3 of plastic media (Kaldnes North America, Inc., Alexandria, VA), and a 150-watt UV sterilizer (Emperor Aquatics, Inc., Pottstown, PA). Oxygenation was provided through diffusion air lines connected to a 1-hp remote-drive regenerative blower (Sweetwater, Aquatic Ecosystems, Apopka, FL). Photoperiod was maintained at a 12-h photophase-scotophase using an automated timer, with light provided by banks of commercial phosphorescent tubes positioned 8 m above the experimental system. Water quality parameters, including total ammonia nitrogen (range 0.1 ± 0.1 mg l^{-1}), nitrite (0.03 ± 0.02 mg l^{-1}) and nitrate (44.6 ± 12.4 mg l^{-1}), pH (7.6 ± 0.3), dissolved oxygen (7.8 ± 0.3 mg l^{-1}) and temperature (23.5 ± 0.6°C), were monitored daily using a pH meter (Hanna Instruments, Woonsocket, RI), spectrophotometer (DR2010; Hach Company, Loveland, CO) and a combined temperature-oxygen meter (YSI Inc., Yellow Springs, OH).

Fish husbandry and diet formulation

Eight zebrafish (initial weight 0.3 ± 0.02 g) were stocked randomly into 6 tanks, and each tank was arbitrarily assigned one of two diets. The experimental diets were formulated on a dry-weight basis and prepared at the VTAC. The control diet (CD) was formulated with herring meal as the sole protein source. In the experimental Se-enriched diet (SD), I incorporated Se (SelPlex, Alltech Inc., Lexington, KY) at the expense of cellufil. Lipids were provided in both diets by menhaden oil, and these preparations were formulated to be isoproteic (40% crude protein), isolipidic (10% lipid) and isoenergetic (270 kcal g^{-1}; Table X–1). The diets were pelleted in an industrial mixer, crumbled to the desired size, and air-dried prior to storage at -20°C until feeding. The fish were hand-fed to satiation twice per day for two feedings (at 09.00 and 16.00 h) for a period of nine weeks.

Table X–1: Dietary formulations employed during the present investigations.

Ingredient	Control diet	Se-met diet
Herring meal[1]	53.2	**53.2**
Dextrin[2]	5.4	**5.4**
Lipid[3]	5.3	**5.3**
Mineral mix[4]	4	**4**
Vitamin mix[4]	3	**3**
Carobimethyl cellulose[2,5]	1	**1**
Cellufil[2]	28.1	**28.04**
SelPlex[6]	0	**0.06**
Total	100	**100**
Available energy[7]	**272**	**272**

[1]International Protein, Minneapolis, MN, USA; [2] US Biochemical Corporation, Columbus OH, USA; [3] 1:1 mixture of corn oil and menhaden oil (Omega Oils, Reedville, VA); [4] See Cotter et al. (2008a); [5] Carboxymethyl cellulose; [6]Alltech Inc., Nicholasville, KY, USA; [7]Calculated

Sample collection and analyses

At the end of the trial, I sampled three fish per tank randomly. Samples were immediately placed into a mortar containing liquid N_2, and crushed with a pestle that was pre-cooled in liquid N_2. The samples then were pooled and utilized for total RNA isolation and microarray assays (i.e. three fish per chip, three chips per treatment).

RNA isolation and microarray preparation

The total RNA samples were isolated from the tissue samples with the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Each sample was precipitated with ethanol to concentrate the total RNA, and the resultant pellet was dissolved in RNase-free distilled water. RNA quality, quantity and DNA contamination were determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The quality-checked total RNA was used for synthesizing biotin-labeled cRNA. Briefly, I used 10 µg of total RNA to generate first-strand cDNA with a T7-linked oligo(dT) primer. After second-strand cDNA synthesis, *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY), resulting in approximately 100-fold amplification of the cRNA population. The cRNA was fragmented (15 µg per sample), spiked with internal controls (Affymetrix Inc., Santa Clara, CA) and hybridized overnight to the Affymetrix *D. rerio* (DsRed) gene chips (n = 6 chips total). The gene chips were washed and stained with streptavidin-phycoerythrin, before being scanned on the GeneChip scanner (Affymetrix, Inc.).

Data preprocessing

After import into the Bioconductor package (version 1.5) in R (version 1.9), the CEL files (containing the probe-level data, 22 gene spots per gene) were preprocessed using the Robust Multi-chip Average (RMA) method, which normalizes across a set of hybridizations at the probe level to perform within- and between-chip (quantile) normalizations and to adjust the background. Preprocessing of the data with RMA is more sensitive and specific, and provides a more robust dataset than the standard Affymetrix MAS 5.0 scaling or dCHIP techniques. The normalization process reduced unwanted technical variation, as verified by examining the individual gene chips for reproducibility within the given conditions using two-dimensional scatter plots and hierarchical clustering in GeneSpring (version 7.2, Silicongenetics, Redwood City, CA). Assessing the replication performance among gene chips within a treatment group provides reasonable statistical confidence in the expression values (Saviozzi and Calogero, 2003).

Filtering and data analyses

Uninformative genes were eliminated from the dataset after implementing the preprocessing steps. These included genes with signals very near background, those that were considered absent by the Affymetrix scanner and genes that did not change expression values appreciably across conditions. A review of the scatter plots showed that this excluded gene set was highly variable at the lowest end of the expression scale (below 150 units of intensity).

Since I was primarily interested in discovering genes with robust expression levels, eliminating the genes at the low end of the expression scale should not curtail this discovery. Filtering was performed using GeneSpring. On this quality-checked dataset, I performed statistical analyses and fold-change assessments utilizing GeneSpring as follows: 1) executed *t*-tests on the gene values between the control and Se-met-treated samples, 2) examined the fold-change differences of the genes showing statistically significant differences in expression between treatments ($P < 0.05$).

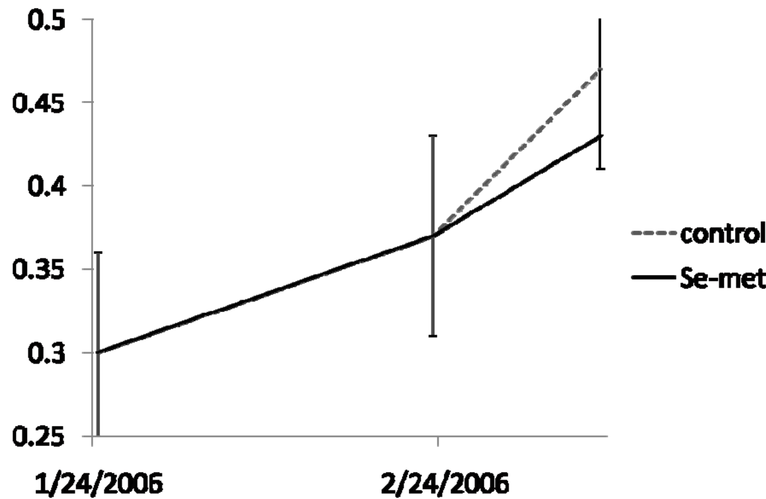
RESULTS

No mortalities were recorded throughout the trial, and fish in both control and treatment groups grew well over the 9-week period (Figure X–1). The final working dataset included 9210 genes whose expression differed significantly between treatments. This represented over 60% of the entire zebrafish genome. Of these genes, 6112 expressed significantly higher levels of transcription in Se-fed fish, while 3098 had greater levels of transcription in control zebrafish. For animals maintained on the Se-supplemented diet, 1279 genes were up-regulated greater than 2-fold, while 1427 genes exhibited 2-fold or greater levels of expression in control groups.

The most highly up-regulated transcript observed for Se-fed fish, at 8.3-fold, was a hypothetical protein of unknown function. As summarized in Table X–2, microarray analysis of Se-fed zebrafish revealed significant up-regulation of various genes involved in the immune system, including lymphocyte secreted C-type lectin ($P < 0.008$), IL-16 ($P < 0.008$), stromal cell-

derived factor 1 (P < 0.0005), *TLH29* protein precursor (P < 0.007), *CD59* antigen p18-20 (P < 0.02), and *ETS1* protein (P < 0.01). A number of genes engaged in membrane trafficking, mucus dynamics and growth also were impacted significantly (Table X–2).

Figure X–1: Mean growth of control and Se-fed zebrafish over the 9-week trial



No significant differences were recorded in growth (wet-weight basis) over time between treatments.

In the control group, the most highly up-regulated gene, at 110-fold, was a transcribed sequence also of unknown function. However, a number of identified genes expressed 50-fold+ up-regulation in the control groups (Table X–3). Most of these genes were reproduction-related and included zona pellucida glycoprotein 2.2 (*Zp2.2*) which expressed a 103-fold increase in transcription when compared against Se-fed fish, zona pellucida glycoprotein 2.3 with a 91-fold higher level of transcription, *zygote arrest 1* with a 59-fold increase and zona pellucida sperm-binding protein 3 precursor (*ZP3*) which expressed a 51-fold increase in transcription when compared against Se-fed zebrafish (Table X–3).

Table X–2: Selection of genes significantly up-regulated in Se-fed zebrafish.

Short name	Full name	GenBank #	Fold-change	P-value
Sepw1	Selenoprotein W, 1	NM_178287.2	2.0	0.0013
Sepw2b	selenoprotein W, 2b	AW232459	3.8	0.0008
Sepp1a	Selenoprotein P, plasma, 1a	BE605475	2.0	0.0337
Sept2	Selenoprotein T, 2	AW077198	1.7	0.0102
GPX4b	glutathione peroxidase 4b	BM036392	1.5	0.0396
CLEC11A	lymphocyte secreted C-type lectin	D39203	1.8	0.0076
Cxcl12a	stromal cell-derived factor 1	BM184127	3.2	0.0005
TLH29	TLH29 protein precursor	BI702655	2.3	0.0071
CD59	CD59 antigen p18-20	BM185911	2.8	0.0187
ETS1	ETS-1 protein	BG985481	1.4	0.0105
C3	Complement C3 precursor	AW116315	2.2	0.0027
C4A	complement component 4A preproprotein	BI672168	2.3	0.0199
CFAH	Complement factor H precursor	AI397473	2.3	0.0108
C8β	complement component C8β chain	AW116668	2.5	0.0427
C9	Complement component C9	BQ284848	2.7	0.0386
HSD11β2	hydroxysteroid 11-beta dehydrogenase 2	BG799163	4.2	0.0154
MUC2	mucin 2 precursor, intestinal	BI673162	1.9	0.0224
Egfl6	EGF-like-domain, multiple 6	BQ260226	2.0	0.0273
CTGF	connective tissue growth factor	BE693178	3.1	0.0003
crfb4	Cytokine receptor family member b4	BM082475	1.7	0.0007
CCL-C5a	Chemokine CCL-C5a	BQ479755	1.6	0.0162

Table X–2, continued

Short name	Full name	GenBank #	Fold-change	P-value
IL-16	Interleukin 16			
RAB36	RAB36, member RAS oncogene family	BM342307	2.7	0.0005
RAB25	RAB25, member RAS oncogene family	AL717027	2.2	0.0023
RAB12	RAB12, member RAS oncogene family	BM036926	2.1	0.0055
mknk2	MAP kinase-interacting serine/threonine kinase 2	BQ450321	3.1	0.0018
IGFbp1	insulin-like growth factor binding protein 1	AL910822	1.5	0.0167
IGFbp2	insulin-like growth factor binding protein 2	NM_131458.1	1.4	0.0030
IGFbp3	insulin-like growth factor binding protein 3	CA474205	1.4	0.0085
glrx3	Glutaredoxin thioredoxin-like 2	AI544790	1.7	0.0122
HNF-4α	Hepatocyte nuclear factor 4, alpha	AF473824.1	1.9	0.0025
foxp2	Forkhead box P2	BQ783717	2.4	0.0017
AKT3	similar to AKT3 protein kinase	BQ132705	1.3	0.0263
adcyap1b	Adenylate cyclase activating polypeptide 1b – GH-releasing hormone	NM_214715	1.6	0.0297
ctgf	connective tissue growth factor	BE693178	3.1	0.0003

Table X–3: Selection of genes expressing significant up-regulation in control zebrafish when compared against Se-fed zebrafish.

Short name	Full name	GenBank #	Fold-change	P-value
Zp2.2	Zona pellucida glycoprotein 2.2	NM_131827	102.9	0.00004
zp2l1	Zona pellucida glycoprotein 2, like 1	BQ262819	78.2	0.00004
ZP3p	ZP sperm-binding protein 3 precursor	BQ078419	50.8	0.0011
ZP3	ZP sperm-binding protein 3	NM_131331.1	108.0	0.00001
ZP2.3	zona pellucida glycoprotein 2.3	BM859647	90.8	0.00001
Zar1	Zygote arrest 1	AY283178.1	59.0	0.0000
TESK2	Testis-specific protein kinase 1	BQ132833	2.3	0.0150
brd2	Bromodomain-containing 2a	BI891861	1.6	0.0075
PBP	progesterone binding protein	BQ284702	2.0	0.0107

DISCUSSION

In order to maintain and replenish endogenous supplies of Se, all vertebrates must ingest this mineral on a regular basis (Daniels, 1995). Absorption of Se from the gut is dependent on the form in which it is consumed; the methionine derivative of Se, as used herein, is transported via the methionine carrier system (Wolffram et al., 1989). In fish, dietary selenomethionine is readily absorbed and accumulates both in muscle and hepatic tissues in a dose-dependent and rapid manner (Cotter et al., 2008a,b). As might be expected, dietary Se supplements increase tissue levels of Se-containing or Se-dependent proteins, including selenoproteins P, T, W (Yeh, et al., 1995; Burk and Hill, 2005; Shrimali, et al., 2008), and the activity and concentrations of glutathione peroxidase (GPx), the first selenoprotein to be

identified (Rotruck, et al., 1973; Flohé, et al., 1973). GPx catalyses the reduction of hydrogen peroxide and lipid peroxides, thereby protecting organisms from oxidative damage. During Se deficiency, GPx activity decreases (Lei, et al., 1998) whereas Se supplementation increases GPx activities. In the present trial, the *GPX4B* glutathione peroxidase gene was up-regulated together with the selenoproteins P, T and W. More surprising was that a dietary level of 0.06% supplemental Se exerted major changes in transcription levels of many genes not normally associated with Se, thus suggesting an influence of Se on their expression.

The close relationship between diet and fish health, especially in consideration of the micronutrients, has accelerated research and discovery in this area. A number of vitamins and minerals have been linked to disease and immunity in cultured fish, and although the mode of action of these nutrients varies, many are believed to act via the enhancement of innate immunity and especially by stimulating leukocyte activity. In several trials with laboratory and farm animals, Se deficiency has been linked to increased virulence of viral, bacterial and protozoan pathogens (Boyne and Arthur, 1986; Beck, et al., 1994, 2001; Nelson, et al., 2001; Gomez, et al., 2002). Conversely, Se supplementation confers beneficial effects during infections (Davis, et al., 1998; de Souza, et al., 2003), perhaps mediated through its action on a variety of immune mechanisms (Boyne and Arthur, 1979; Gynag, et al., 1984; Mckenzie, et al., 1998). In fish, a recurrent observation during pathogen exposure, both in field and laboratory environments, has been the probable participation of Se in the disease process. Thus, Atlantic salmon infected with *Vibrio* exhibit significantly reduced hepatic Se levels (Hjeltnes and Julshamn, 1992). Supplemental dietary Se has been reported to enhance antibody production

and survival in channel catfish challenged with *Edwardsiella* (Wang, et al., 1997). Beneficial impacts on innate immunity in *Edwardsiella*-infected hybrid striped bass (Jaramillo and Gatlin, 2004) and elevated glutathione peroxidase activity in *Renibacterium*-infected Chinook salmon also have been reported (Thorarinsson, et al., 1994). Taken together, these observations suggest that immune-related advantages may incur when Se is supplemented into aquafeeds.

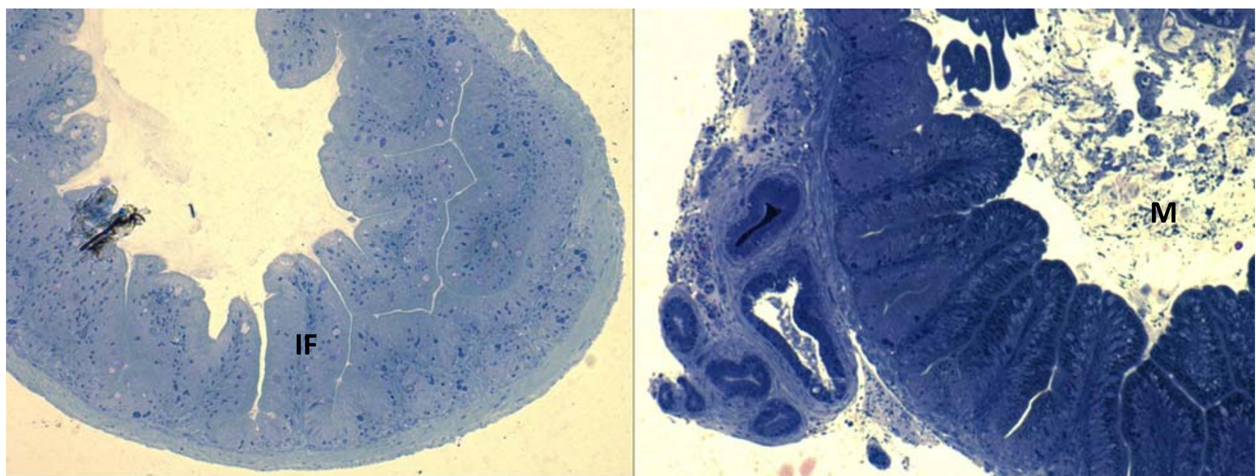
In the present study, whether by direct or indirect action, supplemental dietary Se up-regulated a number of immune-specific genes in zebrafish by 1.4-2.8-fold. These included lymphocyte-secreted C-type lectin, *IL-16*, stromal cell-derived factor 1 and 2, *TLH29* protein precursor, CD59 antigen p18-20, and *ETS1* protein. These genes are involved in leukocyte activation and differentiation and regulation of the complement cascade. In addition, genes involved in the humoral component of the innate immune system were also up-regulated (2.3- to 2.8-fold), such as the complement component *C3*, *C4*, complement factor H, membrane attack complex (*MAC*) components *C8β* and *C9*. The concomitant up-regulation of the *CD59* antigen is noteworthy since CD59 blocks the final step of MAC formation, thus inhibiting its functionality (Farkas, et al., 2002). Supplemental dietary Se thereby may act to maintain the functionality of the MAC while controlling ultimate formation through a CD59 feedback. This enhanced potential may improve both the speed and amplitude of the immune response during infection. Further research is necessary to investigate this hypothesis. A number of non-immune-specific genes, that nonetheless have significant relevance to innate immunity, were also up-regulated, including those involved in exocytosis, endocytosis and vesicle transport. In fish, the innate immune system (phagocytic cells, natural killer cells, inflammatory cells,

cytokines, complement, and acute phase proteins [APP]), plays a significant role in protection against pathogens. Integral to the APP response (APPR) are changes in nutrient utilization brought about by the action of a number of cytokines superseding the normal hypothalamic-somatotropic control of nutrient utilization. During the APPR, concentrations of specific circulating nutrients are preferentially mined (Louw, et al., 1992), presumably to assist in the hepatic synthesis of APP. When such nutrients are inadequate, the APPR may be compromised (Grimble, 2001), leading to increased susceptibility to disease. Se may represent such a nutrient and if this were the case, then benefit may arise by Se over-formulation of fish diets. Noteworthy in these trials was the upregulation of the hydroxysteroid 11-β dehydrogenase 2 (*11β-HSD2*), which controls occupancy of non-selective mineralcorticoid and glucocorticoid receptors by glucocorticoids (Albiston, et al., 1994; Baker, 2004). In fish, glucocorticoids decrease phagocytic activity, antibody production, complement activity, lymphocyte number and disease resistance (Maule, et al., 1989; Ortuno, et al., 2002). Logically, amplified expression of the *11β-HSD2* gene would thus be expected, as was the case. Taken together with those relating to growth, these results suggest the existence of a nutrition-neuroendocrine-immune syntax which acts synergistically to ensure optimal physiological functionality of the organism.

A particularly interesting outcome of Se supplementation of zebrafish diets was that several genes involved in regulating mucus dynamics were up-regulated. Mucous glycoproteins, or mucins, provide the structural basis for the formation of gels that lubricate and protect epithelial surfaces. Mucus is a potent barrier to bacterial colonization and is able to entrap and remove pathogens via increased flow and turnover. In order to avoid being washed away,

pathogens must adhere to mucus and grow rapidly. This is a complex process, mediated by adhesins (Ascencio et al 1998; Collado et al. 2008), and bacterial strains that are more adherent are often more pathogenic (van der Marel et al. 2008). An increase in mucus production at exposed surfaces (gut, gill, eye, fins, body), therefore, may aid the host in purging pathogens. Several studies have demonstrated secretagogue roles for cytokines and growth factors, including epidermal growth factors (Cohan, et al., 1991; Borchers, et al., 1999; Shekels and Ho, 2003), and in the present study, elevated transcription levels were recorded for a number of genes with secretagogue potential. Increased mucus production and secretion indicates enhanced cellular activity in terms of membrane trafficking, and numerous genes engaged in the regulation of membrane traffic within the cell were significantly up-regulated. The preceding transcriptomic outcomes of dietary Se provide strong evidence for the hypersecretion of mucus which was further evidenced through histological examination of the intestine (Plate XI–1).

Plate XI–1: Representative mid-intestine cross section of control-fed and Selenomethionine-fed zebrafish



Note the increased mucus production in the selenomethionine-fed fish (right) compared to the controls (left). IF: intestinal fold; M: mucus. Stained with toluidine blue O and counterstained with safranin O.

Accordingly, hypersecretion of mucus may represent a further mechanism through which Se enhances resistance of fish to pathogen challenges (i.e., augmented barrier functions). In cultivated fishes, a building body of evidence suggests that certain pathogens, including the causative agents of columnaris disease, *Flavobacterium columnare*, vibirosis *Vibrio anguillarum*, and cold-water vibriosis in Atlantic salmon (*Salmo salar*) modify their growth (Denkin and Nelson, 1999) and gene expression in response to elevated mucus presence. These reactions include increased extracellular protease activity (Staroscik and Nelson, 2008), increased motility, and up-regulation of genes involved in oxidative stress responses (Raeder, et al. 2006). Accordingly, even given enhancement of barrier function that Se-driven increased mucus production might confer, further augmentation of host defense mechanisms would likely be necessary in the production environment.

In higher vertebrates, an intriguing new physiological role for dietary Se has been proposed - that it possesses insulinomimetic properties. Evidence in support of a function for Se in the insulin signal cascade includes its stimulation of glucose transport and translocation of glucose transporters in rat adipocytes *in vitro* (Ezaki, 1990), normalization of blood glucose levels in diabetic rats (McNeill, et al., 1991), and regulation of some insulin-mediated metabolic processes (Berg, et al., 1995; Becker, et al., 1996). Moreover, Se alleviates or prevents the adverse effects of diabetes on cardiac, renal and platelet function (Battell, et al., 1998; Douillet, et al., 1996a,b). Evidence to suggest that Se may also exert a similar insulinomimetic effect in teleosts is provided by the present study. A number of MAP and tyrosine kinases, which are involved in the distal signaling of the insulin signaling cascade (Kusari, et al., 1997; Stapleton,

2000), were significantly ($P \leq 0.03$) up-regulated in zebrafish receiving Se-supplemented diets. Transcription levels increased for thioredoxin-like and glutathione peroxidase (see: Holmgren, 1979; Jung and Thomas, 1996), *HNF-4a* (Fajans, et al., 2001), forkhead box protein (Walter, et al., 2008) and *akt3*, which is known to be a regulator of cell signaling processes in response to insulin and other growth factors (Cichy, et al., 1998). Insulin drives the hepatic expression of insulin-like growth factors (IGF) and associated binding proteins (IGFbp), and in zebrafish fed Se-supplemented feeds significant ($P \leq 0.04$) up-regulation of numerous IGF-related genes was observed (Table X–2). Insulin-like peptides and their binding proteins modulate somatic growth and metabolism while also influencing a number of other developmental processes (Nelson and Sheridan, 2006). Se-supplemented diets also increased transcription levels of a number of growth-related hormones and receptors, including growth hormone-releasing hormone, connective, stem-cell and epidermal growth factors and thyroid receptors (Table X–2). The results of the present trial therefore, may provide a molecular explanation for the positive growth effects observed for fish fed Se-enhanced diets (Abdel-Tawwab, et al., 2007; Wang, et al., 2007). Se also may act as an appetite stimulant, as inferred by the observations of Cotter, et al. (2008) with hybrid striped bass, and demonstrated in Se-deficient chicks (Bunk and Combs, 1980). Although the present research provides strong circumstantial evidence for an insulinomimetic effect in fish, confirmation of such a role will nevertheless require more detailed study. Such experiments might include evaluation of circulating glucose levels and hormone profiles, more detailed evaluation of changes in gluconeogenic enzyme activities, as well as more thorough examination of protein synthesis.

Development and maturation of testes and ovaries demands the carefully orchestrated expression of various gene classes that ultimately produce viable gametes (von Schalburg, et al., 2005). A conspicuous feature of control groups was higher levels of transcription for reproduction-related genes, some of which were 100-fold higher than those observed in Se-fed zebrafish. Examples of prominently over-expressed genes and their fold-change included: zona pellucida glycoprotein 2.2 (100-fold), zona pellucida B protein (78-fold), sperm-binding glycoprotein *ZP3* (65-fold), *zygote arrest 1* (60-fold), zona pellucida glycoprotein 2.4 (58-fold) and oocyte maturation factor (24-fold). Other significantly up-regulated genes of reproductive importance are exemplified by testis-specific protein kinase 1 (2.3-fold), bromodomain-containing 2 (2-fold) and progesterone binding protein (2-fold). The zona pellucida provides communication between oocytes and follicle cells during oogenesis and protects oocytes, eggs and ultimately embryos during development. The zona pellucida also normalizes interactions between ovulated eggs and free-swimming sperm during and following fertilization (Wassarman, et al., 1999). The oocyte-specific gene *zygote arrest 1* functions during the oocyte-to-embryo transition (Wu, et al., 2003), while bromodomain-containing 2 expression appears to correlate with stages of oocyte maturation (Trousdale and Wolgemuth, 2004). Testis-specific protein kinase 1 expression increases in testicular germ cells at the stages of late pachytene spermatocytes (Toshima, et al., 1998), whereas sperm-binding glycoprotein *ZP3* facilitates the binding of sperm to egg (Rodeheffer and Shur, 2004). These data indicate that experimental animals in the control group were of both sexes and that, unlike the case for Se-fed fish, control animals were maturing. The slight predominance of oocyte-related genes compared to sperm-related genes could be due to a skewed sex-ratio, or may indicate a higher sensitivity of female

zebrafish to dietary Se-met. One of the most important aspects of Se toxicity in fish is its negative impacts on reproduction. Se bioaccumulation has been associated with reduced growth (Hamilton, et al. 1990), embryonic deformity and complete reproductive failure of fish inhabiting Se-contaminated aquatic ecosystems (Lemly, 1993b; Maier and Knight, 1994). Clearly the distinct differences in gene expression, with regard to reproduction, between groups signify a significant concern since it is unlikely that the results represent a chance occurrence. Accordingly, future studies with supplemental Se should examine this potentially negative impact more thoroughly.

The present experiment demonstrates nutrient-gene interactions in fish. Moreover, the results indicate that Se as an essential mineral probably plays a more wide-ranging role in vertebrate metabolism than currently is appreciated. Although clear production-related benefits may accrue by supplementing diets with organic Se, there nevertheless exist significant questions that must be answered prior to advocating the general use of Se-enriched diets by the aquaculture industry.

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Chapter XI. General Conclusions

This dissertation represents two-and-a-half years of research, directed by three major themes: larval development, alternative sources of dietary proteins and lipids, and gene expression in response to nutrition.

Larval development

The larval development of cobia was extensively studied in the first chapters, from external and internal, morphological and physiological, digestive and sensory points of view. During fish larval development, the following steps are critical to any cultured species: the transition from endogenous to exogenous diet and from live prey to inert diet. Proper development ensures low mortality rates during these critical transitions. In cobia, resorption of endogenous reserves (yolk-sac and oil globule) occurs around 145-174°d (5-6 dph). Histological investigation revealed that the gastrointestinal tract undergoes important changes during this period. However, these changes are more dramatic between 290 and 464°d (10-16 dph), including re-arrangement of gut configuration with loops and valves. Concomitant to morphological changes, a dramatic increase was observed in all studied digestive enzymes, except pepsin, as well as the formation of external sensory organs, such as nostrils and the cranial canal system. Clearly, this point in

ontogenetic development constitutes a major onset in digestive capacity. It is also noteworthy that no dietary manipulation explored in the present work hastened or delayed this transition, which occurred at 12-14 dph (348-406°d), even in taurine-fed larvae. This suggests a strong genetic influence at this level. However, as demonstrated by taurine supplementation studies, dietary manipulations at optimum temperature may prepare the organism for an easier and more efficient transition, subsequently resulting in higher survival, and faster development and growth thereafter.

This has numerous and essential consequences from the farmer's perspective. Live prey, as well as being less reliable in terms of their nutritional adequacy, is more difficult and expensive to produce than formulated diets. During larviculture, most of the mortalities occur prior to and during weaning. Thus, larval rearing may be viewed as a race to weaning. In my studies, cobia larvae were weaned between 696 and 783°d (24-27 dph), and based on the results from the taurine-fed larvae, there is little doubt that the weaning process can be compressed and with better survival. However, more research is necessary to achieve this goal. Especially, studies of brush border enzymes would provide valuable information regarding the transition between intra- to extra-cellular digestions. In addition, there is a general imbalance in knowledge and understanding between lipid and protein nutrition in fish larvae, which is surprising in the context of rapidly growing and developing organisms. Undoubtedly, more research in this area would benefit larval rearing for various cultured species.

ALTERNATIVE PROTEIN AND LIPID SOURCES

The sixth and seventh chapters of this dissertation were dedicated to the refinement of cobia juvenile nutrition through the replacement of fish meal and fish oil by various alternative ingredients. Several main results arose from this series of experiments. First of all, cobia was shown to grow efficiently on a wide variety of feedstuffs, which was somewhat surprising for a marine carnivore. This “omnivorous tendency” adds to the long list of desirable traits and characteristics of cobia, because it allows nutritionists to work with a greater range of ingredients, which in turn facilitates choices that would combine biological performance, cost, and respect for the environment (see Chapter I).

Second, it is unlikely that a single, natural ingredient could solely replace 100% of its fish counterpart (i.e. meal or oil) without any detrimental impacts, and considering the economical aspect as well as the volumes necessary to supply the global aquaculture industry. Rather, a combination of several feedstuffs, selected not only for their intrinsic value, but also for their complementarity, would be more realistic. Numerous factors must be taken into account when attempting such replacements, and satisfactory results were herein attained by mixing multiple feedstuffs. In fact, performance on experimental diets often surpassed that on fish meal-based control diet in experiments with cobia. No contaminants or degradation of the fish meal utilized in the formulations were detected (data not shown), which suggests that the diversity of ingredients may be beneficial for the fish. Indeed, the successful formulation of a fish meal- and

fish oil-free diet that sustain cobia's growth potential is, as shown in Chapter VII, technically feasible.

Third, total replacement of fish meal and oil has been previously reported in other species, including tilapia, rainbow trout and Atlantic cod. However, in marine carnivorous species, 100% replacement often is reported with reduced growth and health issues (e.g. enteritis-like conditions). Hence, the results of the present studies are of primary importance, as I did not observe any detrimental effects from the experimental diets. This may, upon further investigation, assist in the replacement of both fish meal and fish oil for other carnivorous species, which would contribute to alleviate the aquaculture-related pressure on corresponding fisheries (e.g. herring and menhaden).

However, the economic feasibility of these experimental formulations was not estimated, since complete economic assessment was beyond the scope of this dissertation. Nonetheless, I quickly explore this important aspect. Most of the alternative ingredients used here were derived from soy, which is commonly used for this purpose. I discussed the advantage that alternative ingredients are chosen among commodities. However, this implies that such commodities are already traded on other markets, with which the aquaculture industry will inevitably compete, and their price will fluctuate under the influence of the laws of supply and demand. Consequently, we may witness shifts in the competitiveness of these ingredients. From a solely environmental perspective, there is no need for an absolute and complete replacement of fish meal and oil in diets of farmed aquatic species. Rather, aquaculturists must

achieve high-enough replacement rates to produce seafood while respecting the capture capacity of natural stocks and taking into account economic dynamics.

Nutritional gene expression profiling

The last chapters of this dissertation diverged from the applied science as it is traditionally conducted in aquaculture to explore realms that were until recently dedicated to mammalian and avian research, as well as other more basic scientific research. In aquaculture, diseases constitute a major concern (see Chapter I), which drives an intense search for diets with immuno-stimulating properties. A few promising ingredients of particular interest have been discovered so far. However, only a phenotypic response is usually measured (e.g. mortality during a challenge, serum immunoglobulin levels, lysozyme activity, etc.), and the underlying mechanisms remain poorly defined. When considering a function as complex as immunity, it is important to understand these mechanisms in order to utilize these ingredients in the most appropriate situations. By exploring the response of an organism at the gene level, results from microarrays provide pieces of this complex puzzle and give us an insight into potential mechanisms.

As previously reported, dietary mannan oligosaccharides (MOS) altered the expression of several immune-related genes, including MHC or complement component C3. However, against expectations, transcription levels of mucin genes were not increased, neither did MOS influence gut structure. This study is not the first to report inconsistencies in the effects of dietary MOS in

fish, which may elicit different responses according to the species, age, as well as past and current health and nutritional status. It may be that the incorporation of MOS in formulations is advantageous only under certain conditions, which, however, remain to be determined. In spite of this, MOS already are utilized in aquaculture, and additional research investigating observed inconsistencies would allow refinement in the use of this feed additive.

One aspect of the zebrafish response to dietary nucleotide enrichment was the stimulation of immune function, and more particularly innate immunity. In general accord with previous reports, I observed an increase in transcription levels of immune genes like *MHC I UBA*, *RasGEF*, complement *C7* and lysozyme components. However, a number of these genes are involved in the signal transduction between leukocytes. In fact, most of the transcriptional responses to dietary nucleotides involved signalling molecules, guanine-based, cyclic AMP and other secondary messengers. Such routes of action would be in accordance with some of the paradoxes when it comes to the nutritional role of nucleotides: the *de novo* and salvage pathway are sufficient for ensuring anabolic needs, and supplemental presence of nucleotides then may associate with a variety of receptors, even after being processed by the intestinal epithelium and/or liver. This may be of particular relevance for developing animals such as larvae, since proper signalling is paramount to well canalized ontogenesis. However, more directed research is necessary to establish which nucleotide family, or combination of families, would best promote larval development, as well as determine a precise protocol (dose, frequency, etc.) to this end.

The first striking aspect of the response of zebrafish to selenomethionine (Se-met) was its amplitude and expansion across the genome: almost two-third of the 14,900 scanned transcripts were significantly impacted by the mere addition of 0.06% of Se-met in the diet, which constitutes by far the most important overall response observed in the present research. This clearly demonstrates the far-reaching roles of selenium in fish. Indeed, in addition to the known selenium-containing proteins such as GPX, the regulation of a vast number of genes was unforeseen since their relation to selenium was never expected. These include genes involved in mucus production, gonad maturation, as well as insulin-related pathways. This has direct implications for the aquaculture industry: if dietary supplementation of Se-met may benefit fish health, metabolism and growth during larval and juvenile stages, it might hinder oocyte and sperm production in broodstock. However, the extent of selenium impacts on fish physiology raises numerous questions. Additional investigations on the complex effects of dietary selenium and their inter-relation must be conducted prior to recommending – or otherwise – the use of this micronutrient in teleosts.

In conclusion, this series of gene expression studies not only confirmed the close relationship between diet and gene expression, but also highlighted its relevance to applied research. Indeed, the investigation of practical feedstuffs using transcriptomic tools led to a better understanding of their roles and actions, hence allowing us to utilize them more appropriately and efficiently. Nevertheless, transcriptomics only reveals a snapshot of the gene expression profile at a specific time-point. Time-series experiments could provide valuable insights on the sequence of mechanisms in place. In addition, proteomics would complete this

molecular approach by following the gene products to their respective biologically active forms. Thus, there is no doubt that future investigations combining "omics" with traditional tools will yield a more complete picture of the molecular mechanisms involved.

Chapter XII. APPENDIX

Detailed Laboratory Protocols

HISTOLOGY AND MICROSCOPY

PREPARATION OF POLY/BED 812

PROCEDURE

1. Verify proper performance of balance by checking with standard weights. Record weights on balance log. (see SOP code #41-3M2)
2. Place a plastic tri-pour beaker inside a large weigh boat on balance and tare.
3. Slowly pour 51.13g of Poly/bed 812 into beaker and tare. Record weight in balance record book. Pour slowly. (May decant down a wooden applicator stick and add drop wise to prevent adding too much.)
4. Slowly pour 27.02g of DDSA into beaker and tare again. Record weight in balance record book.
5. Slowly pour 21.85g NMA into beaker and record weight in balance record book.
6. Place beaker on magnetic stir plate under fume hood and stir resin components slowly for 5 minutes.
7. Resin may be stirred by hand using two wooden applicator sticks. Avoid whipping air bubbles into mixture and stir a full 5 minutes before adding DMP-30 and 5 minutes again after.
8. Using a sterile 1.0cc syringe and working under a fume hood, draw up 2.0ml of DMP-30 and add to resin mixture.
9. Allow mixture to stir for 15 minutes.
10. Place lid on beaker to prevent hydration by exposure to air.
11. Make fresh before each use, and keep tightly sealed if you are not using mixture immediately.
12. Pour extra resin in waste resin bottle under hood, or cure in oven before discarding.

13. Adjust quantity needed by dividing or multiplying weights proportionally.

EQUIPMENT REQUIREMENTS:

1. Balance and standard weights
2. Plastic tri-pour beaker
3. Magnetic stir plate and stir bar
4. Plastic 1cc syringe
5. Disposable plastic gloves
6. Large weigh boat to protect balance
7. Disposable gloves

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Poly/Bed 812
2. DDSA
3. NMA
4. DMP-30

THICK SECTIONING USING THE REICHERT JUNG ULTRACUT E MICROTOME

PROCEDURES:

1. Place a trimmed block (see code# 41-6.16) into the specimen holder as close to the center as possible. Tighten block holding plate into place using one of the two wrenches stored on the microtome stage.
2. Place specimen-holder into the advancing arm with adjusting knobs on either side pointing horizontally.
3. Tighten the holding screw on right side of advancing arm while holding the specimen holder to prevent any rotation.
4. Back up the coarse feed by turning the large center-front knob counter-clockwise, and reset the ultra/micro feed by turning the knob at the right rear of the microtome clockwise.
5. Obtain a freshly-broken glass knife (code# 41-3.1). Do not touch the cutting edge or the front surface, handling only by the sides.
6. Place the knife in the knife holder with the front edge pushed forward against the front plate of the knife holder, then tighten holding screw securing knife.
7. Remove trimming stage and position knife stage onto microtome.
8. Slide knife/holder assembly into the knife stage, adjust the angle of the knife to 4o and tighten into place.
9. Check to be sure that all securing screws are tight: block holder in advance arm, specimen in holder, knife in holder, knife holder in stage.
10. Manually move the knife stage very slowly toward the block while looking through the binoculars, until the knife edge is about 1mm away from the block face. Be VERY CAREFUL not to touch the block face with the knife edge at this point. Lock stage into place by moving the lever at the lower right front to the right. Check to be sure it is secure.
11. Place the handwheel lever into the top locked position and lock the advancing arm. The top of the block face should be approximately 1mm above the knife edge. If it is not, rotate the hand wheel until it is.
12. The length of the cutting stroke can also be adjusted on the hand wheel (0.5-15mm).

13. Adjust the lateral position of the knife so that the trimmed section is a little to the right of center.
14. Place the hand wheel lever in the middle manual operation position.
15. Using small increments, advance the knife using the coarse feed (center front) knob. After each advance, slowly crank the hand wheel once to make sure the knife is not too close.
16. Adjust angle of knife with respect to the trimmed block face.

- The rotation of the specimen in its mount is adjusted by using the Rotation knob (thinner one on the specimen-holder). This should initially be set at 90o, but may need to be adjusted to accomodate for trimming imperfections. As the knife approaches the block, adjust rotation so that the knife edge and lower edge of trimmed face are parallel on the vertical plane. Do not cut at this point.
- The angle of the knife with respect to the trimmed block face is adjusted by turning the long knob on the left side of the knife stage. Adjust so that the knife edge is parallel with the lower edge of the face on the horizontal plane.
- The thicker knob on the specimen holder (tilt angle knob) adjusts the tilt of the block face so that block face and knife edge are parallel at all points from top to bottom. Using the shadow from the knife edge on the face of the block, determine the need for this adjustment by manually moving the block up and down and watching for a consistently even shadow width from top to bottom.
- Slowly approach the block face, using manual control, until sections are coming off onto knife. Continue to advance 0.5 to 2.0 um at a time using the fine feed knob (large knob on the left of the microtome) until complete face is being sectioned. Adjust knife angle if only one side of block is being cut, or specimen tilt if only top or bottom are being sectioned, always backing up knife and re-advancing following any change in adjustments.

17. Back the knife out and move it laterally to the right, lining the left center edge of the knife up with the faced-up block.

18. Re-approach the block as described in steps 15 and 16, adjusting knife angle if necessary to due to variations in edge.
19. Check the electronic control panel to see that the automatic feed is set on micro-feed. It is the bottom display marked "semi". Set the reading on 98 by pressing the appropriate + or – buttons.
20. Begin cutting 1.0um sections by slowly and evenly turning the hand wheel on right of microtome manually. Sections should come off shiny and intact. Check for visible knife marks. Move the knife to a new position or replace if necessary.
21. Clear knife edge of section debris using compressed air.
22. Wet a small paint brush with distilled water and glide it close to, but not touching, the knife edge until a small pool of water beads up against the cutting surface. You will see section debris floating in this pool. Without touching the knife edge, pick up debris by rolling it onto the brush.
23. Rinse brush thoroughly.
24. Obtain a clean microscope slide and wipe any dust or debris off with a kimwipe. Then place a few drops of water on the slide, wipe again with the kimwipe and dry. Label the slide according to sample.
25. Place several drops of distilled H2O on the slide, labeled side up.
26. Set speed between 1.0 - 1.5 mm/sec.
27. Move the handwheel lever to the lowest position to begin automatic micro-feeding. While peering through the binoculars, slowly bring water from the paintbrush up to the knife edge. As the section comes off the block, gently roll it onto the brush.
28. Gently roll the section off of the brush and onto the pool of water on the slide.
29. Rinse brush and repeat steps 27-28 four to six times, discarding any sections that appear to wrinkle.
30. Move hand wheel lever to middle or manual operation position and place slide with sections onto hot plate for staining procedure (code# 41-6.13; 41-6.15).

EQUIPMENT REQUIREMENTS:

1. Reichert-Jug Ultracut E Ultramicrotome
2. Glass knives
3. Small paint brush
4. Microscope slide
5. Trimmed TEM block
6. Kimwipes
7. Compressed air
8. Pipettes and bulbs
9. Hot plate

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Distilled water

STAINING THICK SECTIONS WITH TOLUIDINE BLUE AND SAFRANIN O

PROCEDURES:

1. Place slide on hot plate set on "LO" or approximately 70o for at least five minutes to fix sections onto slide.
2. Place 2-3 drops of toluidine blue on slide.
3. Spread with toothpick to cover all sections.
4. Place slide back on hot plate for 7-10 seconds or until metallic appearing ring forms around edge of stain. Avoid allowing stains to dry onto slide before rinsing.
5. Remove slide and thoroughly rinse excess stain into waste stain beaker with a distilled water wash bottle.
6. Using a kimwipe, remove any excess water from the underside of the slide.
7. Return slide to hot plate and leave on until excess water disappears.
8. Remove slide from hot plate and place 1-2 drops safranin stain on slide.
9. Repeat step 3 using a different toothpick.
10. Place slide back on hot plate for 3 seconds.
11. Repeat steps 5-7.
12. Remove slide from hot plate and check under light microscope for stain quality.
13. Pour waste stain from beaker into 1 gallon waste container labelled stains and record amount.
14. Avoid skin contact with stains if at all possible. Can remove fairly well with "eradistain" cream.

EQUIPMENT REQUIREMENTS:

1. Hot plate
2. Slide with sections
3. 5.0ml plastic syringes with disk filters to hold stain
4. Toothpicks
5. Kimwipes
6. Waste containers for stain (beaker and gallon jug)

7. Wash bottle

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Safranin O stain, 0.5% (see SOP code #41-5C3)
2. Toluidine blue stain, 1% (see SOP code #41-5C6)
3. Distilled water

CUTTING AND COLLECTING THIN SECTIONS FOR TEM

PROCEDURES:

1. Once the block is trimmed properly for thin sectioning (code# 41-6.19), mount specimen holder with block in the arm of the microtome.
2. Place a fresh glass knife in the knife holder of the microtome and adjust the tilt angle of knife to 3-4o.
3. Align block face with knife edge and adjust knife cutting angle and tilt of block face as needed to achieve a knife approach parallel to the full face of the block.
4. Take a few sections with the glass knife to assure proper alignment. Make sure you are getting complete sections of the cutting surface.
5. Back off the knife without changing the alignment established.
6. Remove glass knife and replace it with the diamond knife, (see code# for use and cleaning of the diamond).
7. Fill the trough of the diamond knife with distilled water.
8. Adjust the water level so that the meniscus is concave and a silver reflection is apparent on the surface, but water level is still even with the edge of the blade.
9. Make sure that the adjustments on the microtome advance are set for thin sections.
10. Carefully approach block face, advancing 1/2 micron at a time until you see the first indication of a section come off the knife onto the water surface. DO NOT advance further after this point but allow microtome to advance automatically as set for thin sections.
11. Section should be complete and even in color.
12. If you are only getting partial sections, back off diamond, adjust angle by 1/4 to 1/2o as appropriate, and re-approach as described in step 10.
13. If alignment is correct and block face is trimmed properly (top and bottom edges parallel), sections should float into boat in a straight ribbon.
14. Check the thickness of the sections by observing the interference colors of light reflected from the sections. Sections should appear silver to light gold in color.

15. Stop the microtome with the arm in the lower position when you have enough good sections in the knife boat.
16. Ribbon may have to be broken into sections or sections moved into accessible position for picking up. This can be done with a single eyelash or dog hair attached to an applicator stick (hair may be cleaned in 10% nitric acid solution if it appears dirty or adheres to sections).
17. Sections can be picked up onto grids by several acceptable methods, including the following:

- Pick up new, dry grid with forceps and dip in 10% nitric acid.
- Rinse with distilled water and blot dry with triangle of filter paper.
- Position dull side over sections to be picked up and lower grid to touch surface.
- Lift grid, now containing sections, and blot dry from back (shiny) side.
- Store grids in 100% ethanol. Remove a few and air dry on filter paper before use.
- Pick up grid with forceps and carefully lower, at an angle of 30 to 45°, beneath the surface of the water in front of sections.
- Move the grid under the sections.
- Slowly raise the grid, still at an angle, and pick up the sections.
- An eyelash or the side of the trough may be help to keep the sections centered on the grid.

18. Take two to three grids of each block.
19. Hold grids in a petri dish until they are stained.

EQUIPMENT REQUIREMENTS:

1. Microtome (see codes# 41-2.15, 41-2.16)
2. Glass knives (see code# 41-3.1)
3. Diamond knife (see code# 41-6.20)
4. Block trimmed as described in code# 41-6.16.
5. Filter paper, cut into triangles
6. Clean new 200 mesh grids (or size required)

7. Disposable pipette with bulb or dust-off
8. Forceps
9. Eyelash
10. Interference color index and thickness scale card
11. Beaker
12. Petri dish

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Distilled water
2. 100% ethanol
3. Nitric acid, 10%

STAINING THIN SECTIONS WITH 2% URANYL ACETATE AND LEAD CITRATE

PROCEDURES:

1. Procedure for 4 or fewer grids
2. Pipette small amount (about 5ml) of each stain (uranyl acetate and lead citrate) into screw top centrifuge tubes, and cap loosely.
3. Spin for 5 minutes at speed #6 in IEC Clinical centrifuge.
4. Place square of dental wax in petri dish.
5. With pipette, place a small drop (approx. 5-8mm in diameter) of uranyl acetate on dental wax for each grid to be stained.
6. Float 1 grid on each drop, section side (dull side) down, place lid on petri dish and time for 12 minutes.
7. Rinse each grid either by holding in forceps and decanting distilled water down forceps and over grid, or by moving each grid through a series of 4 or 5 drops of fresh distilled water. Rinse thoroughly to prevent contamination.
8. Dry grids, using filter paper cut into triangles and place back into holder.
9. Place uranyl acetate drops into waste container and wash dental wax with distilled water.
10. Wipe dental wax dry with kimwipes.
11. Place sodium hydroxide pellets in petri dish and the place dental wax on top of them.
12. Minimize exposure of lead citrate to the air in order to prevent precipitation of lead crystals on sections. (Lead precipitate can be removed by exposing grids to 10% acetic acid for 1 minute).
13. Place drops of lead citrate on dental wax as in step 4, then float each grid as before, section side, place cover on dish and time for 5 minutes.
14. Rinse quickly and thoroughly with dist. water (step 6).
15. Dry grids and place in grid box, being careful to record grid identity on grid box formaldehyde.
16.
17. Procedure for more than 4 grids, using the Hiraoka Staining Kit (including plastic grid holder, mounting block and staining tray) for multiple grid staining

18. Place plastic grid holder onto curved mounting block in order to open slots for receiving grids.
19. Carefully place grids into holding slots, being careful not to bend them.
20. With great care, lift plastic grid holder out of one end of block and bend back to a flat position, thus closing slots and allowing grids to be grasped securely.
21. Place Staining tray into petri dish and fill to near overflowing with distilled water.
22. Invert grid holder with grids and place on staining tray, submersing grids into distilled water in well. Let stand for about 2 minutes to wet surface of grids.
23. Remove grid holder, pour off water and fill well with uranyl acetate.
24. Replace grid holder, submersing grids into stain for 12 minutes. Pour used stain into waste container for uranyl acetate and lead citrate.
25. Rinse grids with several changes (3 - 5) of distilled water for 1 to 2 minutes each.
26. Place stain tray into petri dish, surrounded by NaOH pellets.
27. Fill well with lead citrate stain, replace grid holder, then petri dish cover and allow staining for 5 minutes.
28. Pour used stain into waste container and rinse grids as in step 8.
29. Pour rinse water in waste container.
30. Dry grids and place in grid box, being careful to record identity of each on grid box record sheet.
31. Freshly cut grids offer the best staining properties.

EQUIPMENT REQUIREMENTS:

1. Petri dish
2. Dental wax (for procedure A)
3. IEC Clinical centrifuge
4. Pipettes and bulbs
5. Timer
6. Kimwipes and filter paper triangles
7. forceps

8. Waste container for used stains
9. Hiraoka Staining Kit (for procedure B)
10. Waste container for sodium hydroxide pellets
11. Grids with sections
12. Screw top centrifuge tubes
13. Test tube rack

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Uranyl Acetate
2. Lead Citrate
3. NaOH pellets, Sodium hydroxide
4. Distilled water in squirt bottle

INSTRUCTIONS FOR USE OF LADD CRITICAL POINT DRYER

PROCEDURES:

1. Turn power switch on about 10 minutes before use.
2. Remove three knurled nuts from chamber door, remove door and check O ring to be sure it is clean and seated correctly.
3. Replace chamber cover and tighten down the knurled nuts by tightening each one only ¼ turn at a time until all three are tight. All three must be tightened gradually at the same time to distribute pressure evenly.
4. Close all valves on chamber (vent, fill, drain and drain rate). Do not place undue force on vent rate valve as copper threads may easily strip.
5. Open main CO2 tank valve fully. CAUTION….NEVER STAND IN FRONT OF THE CONNECTION NOZZLE.
6. Open fill valve and vent valve, watching for CO2 level to rise on the glass front to approximately 3/4 full. (Observe by standing to the side; do not stand directly in front).
7. Set timer for 5 minutes.
8. After 5 minutes, drain and vent chamber completely.
9. Close drain and vent valves and then repeat steps 6-8 several times (5 to 8) until temperature gage has dropped to between 10o and 15oC.
10. The CPD is now ready for samples (dehydrated in ethanol to 100%).
11. Close the main valve on the CO2 tank completely and open drain and vent valves to empty chamber.
12. Specimens should be placed in appropriate holders or baskets while resting in a petri dish filled with absolute ETOH to prevent them from drying out during this process.
13. Prepared sample baskets should now be first blotted briefly but thoroughly on a paper towel to remove excess alcohol (without allowing samples to dry out), and then placed onto the specimen carrier in the CPD chamber.
14. Place samples into chamber, check O ring and replace chamber cover as in step 3.

15. Fill and Vent/drain as in steps 6-9 for 4 to 8 times depending on sample number and size. Avoid draining completely as samples should remain covered with CO2 at all times. Record number of fills on card attached to top of CPD. Allow samples to stand 5 minutes after each fill.
16. Repeat procedure until "fog" has subsided in chamber during drain process, indicating alcohol is gone.
17. When alcohol is gone, fill the chamber to 3/4 full and close all valves on chamber.
18. Close the main valve on the CO2 tank.
19. Turn the power switch to heat and begin to monitor the rise in temperature and pressure.
20. When the pressure reaches 1300-1350, begin careful venting to maintain pressure in that range until temperature reaches 42o at which point it will turn off automatically.
21. Set timer for 15 min and start timing at this point.
22. Begin to slowly drain off pressure with careful adjustments of the vent and vent rate valves. Bleed off pressure at about 100 psi/minute, using the timer as a guide.
23. When pressure gage reaches 0, crack the knurled nuts on the chamber cover. If difficult to loosen, wait a few minutes for all pressure to subside.
24. Turn power off and leave vent and drain valves open and chamber door loosely secured after use.
25. Remove specimens to desiccator or mount on stubs (see SOP code# 41-6D2, 41-6D3).
26. Record date, investigator and acct# on card attached to top of CPD.
27. Make sure the main CO2 valve is completely closed when finished.

EQUIPMENT REQUIREMENTS:

1. Ladd Critical Point Dryer
2. Timer
3. Paper towels
4. Petri dish
5. Sample holders

6. Samples

REAGENTS, SOLUTIONS AND CHEMICALS:

1. Syphon CO2
2. Absolute ethyl alcohol

INSTRUCTIONS FOR USING THE SPI-MODULE SPUTTER COATER

PROCEDURE:

1. Carefully remove the glass work chamber and metal top plate assembly.
2. Place mounted samples (see SOP code #41-6D2, 41-6D3 to be coated on the base plate.
3. Check O-rings for debris and replace glass chamber and metal top plate assembly.
4. Turn "power" switch of the Central Scientific Co. mechanical pump to "ON".
5. Switch "ON" power to SPI-Module Control Unit.
6. Switch "ON" power to SPI-Module Sputter Coater. After 10 to 15 seconds the fall in pressure within the work chamber will register on the vacuum gage.
7. Watch for the "Ready" light and "Power" light to illuminate when the pressure falls below 0.2mbar, indicating sufficient vacuum for the high voltage power to be applied.
8. When a vacuum of 0.04 - 0.02mbar is reached, set timer to desired setting.
9. Open the gas leak valve very slightly until the chamber pressure just begins to rise (about 0.06mbar).
10. Depress the "test" button and notice plasma current level.
11. Adjust current level up or down as required by making slight changes in the gas leak valve until an 18mA plasma current is obtained.
12. Depress the "START" button to deposit gold on sample. Re-depressing the "START" button will repeat the process to double the time as set.
13. After coating is complete, turn OFF power switches to the SPI-Module control and Sputter Coater units, and turn off the vacuum pump. The gas leak valve should also be closed.
14. "Vent" the work chamber by lifting the "vent" valve on top of the metal top plate of the chamber.
15. Lift and set aside the work chamber, then remove the samples.
16. Samples should be viewed immediately or placed in holder and stored in desiccator.
17. Replace glass chamber and metal top plate assembly on baseplate.

EQUIPMENT REQUIREMENTS:

1. SPI-Module Sputter Coater
2. Forceps
3. Desiccator
4. Sample holder
5. Critical point dried or air dried specimens

ENZYME ASSAYS

HOMOGENIZATION

PROCEDURE

1. Take the tubes containing frozen larvae out of the freezer to thaw;
2. Meanwhile, prepare dissecting microscope, scalpel, twizers. Chill two glass microscope slides on ice. Prepare a shallow cup with ice under the dissecting microscope;
3. When larvae are thawed, place them on a chilled microscope slide under the microscope. Dissect while keeping the slide on the ice. 3-10 dph larvae are headed, 11-16 dph larvae are headed and tailed, 22-27 dph larvae have their whole gut carefully removed;
4. Place pooled larvae in a etched-glass tissue grinder for homogenization with homogenization buffer (HB; 20mM Tris-HCL, 1mM EDTA, 10mM CaCl2, pH=7.4): 50 µl per larva until 16 dph, 100 µl per larva for older larvae;
5. Grind the tissue carefully until a homogenous solution is obtained and no big debris is visible;
6. Transfer to centrifuge eppendorf tube, and repeat the procedure for all samples. Alternate microscope slide in order to keep them chilled;
7. Centrifuge samples in a refrigerated micro-centrifuge (14000 rpm, 15 min, 3oC) to separated cellular debris, and transfer supernatant (avoid possible top fat layer) to another set of eppendorf tubes;
8. Analyze immediately or freeze at -80oC.

EQUIPMENT AND REAGENTS:

1. Dissecting microscope
2. Dissection kit: scalpel, twizers
3. Refrigerated micro-centrifuge
4. Tris-HCl pH=8 (Sigma-Aldrich #93378)
5. EDTA (Sigma-Aldrich #E5134)
6. CaCl2 (Sigma-Aldrich #C1016)

TRYPSIN ASSAY

PROCEDURE

1. Prepare trypsin assay buffer (TAB): 0.01 M CaCl2, 0.04 M Tris-buffer, pH=8. Mix 0.222 g CaCl2 and 1.134 g of Tris-buffer in 200 ml distilled water. Store at 4oC for 3 months;
2. Prepare substrate solution (TSAS): 1.5x10-3 M TAME. Mix 29.3 mg of TAME in 50 ml TAB. Store TAME at -20oC, and TSAS at -80oC;
3. If frozen homogenates are used, thaw at room-temperature. Re-centrifuge if necessary (14000 rpm, 2 min, 3oC) and dilute the 22 and 27 dph samples to 25% of full strength with homogenization buffer (HB);
4. Prepare standard series: prepare mother solution by dissolving 15 mg purified trypsin in 15 ml TAB. Dilute 100 times to obtain a 10 µg ml-1 solution. Do 5 serial dilutions to obtain the standard series: 10, 5, 2.5, 1.25, and 0.625 µg ml-1;
5. Turn on the spectrophotometer, and set temperature to 30oC;
6. In a UV-transparent 96-well plate, pipette 100 µl of distilled water (blank), standard, or sample in designated well. Run all samples in duplicate. Pre-incubate at 30oC for 3 min;
7. Using a channel pipette, add 200 µl TSAS in each well as quickly and cleanly as possible. Avoid splashing and touching the plate with pipette tips;
8. Plate the plate in spectrophotometer and read the increase of absorbance at 247 nm for 8 min

EQUIPMENT AND REAGENTS:

1. UV capable spectrophotometer
2. UV-transparent 96-well plates (Fisher Scientific #EK25801)
3. Refrigerated micro-centrifuge
4. Micro-pipettes
5. $CaCl_2$ (Sigma-Aldrich #C1016)
6. Tris-HCl pH=8 (Sigma-Aldrich #93378)
7. Nα-P-Tosyl-L-Arginine methyl ester Hydrochloride, TAME (Fisher Scientific #AC13920)
8. Purified trypsin from bovine pancreas (Sigma-Aldrich #T1426)

PEPSIN ASSAY (adapted from Ryle, 1984)

PROCEDURE

1. Turn on the water-bath and set at 30°C;
2. Prepare the trichloroacetic acid (TCA) solution by mixing 20 g TCA into 500 ml of distilled water;
3. Prepare the neutral haemoglobin (Hb) solution by mixing 2.5 g of purified bovine haemoglobin into 90 ml of distilled water in a beacher with vigorous mixing. Store at 4oC for up to two weeks;
4. Prepare acid Hb solution with a 0.3 M HCl solution 4:1 (v:v). This solution must be used within 24h.
5. Prepare standard series: dissolve 10.4 µg of purified porcine pepsin in 10 ml distilled water buffered at pH=4.1 to create a 30 µM solution. Create the standards by serial dilutions: 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.234 µM. Standard solution may be frozen at -80oC for the next analysis;
6. In 15 ml tubes, pipett 15 µl of distilled water, standard, or sample;
7. Pipett 1 ml of acidified Hb solution;
8. Incubate at 30oC for 30 min exactly;
9. Stop the reaction by introducing 5 ml TCA solution in each tube. Shake;
10. Centrifuge the tubes at 1000 G for 10 min;
11. Transfer ~1 ml of the supernatants into UV-transparent cuvettes;
12. Read the absorbance (endpoint) at 280 nm.

EQUIPMENT AND REAGENTS:

1. UV capable spectrophotometer
2. UV-transparent cuvettes semi-micro (Fisher Scientific # 13-688-74)
3. Centrifuge
4. Stirring plate
5. Micro-pipettes

6. 0.3 M HCl solution
7. trichloroacetic acid (Sigma-Aldrich #T9159)
8. Haemoglobin (Sigma-Aldrigh #H2625)
9. Purified pepsin (Sigma-Aldrich #P7012)