Dietary Selenium in Cultured Hybrid Striped Bass

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ABSTRACT

As aquaculture continues to contribute high quality protein to a greater proportion of the world's growing population, fish producers have been pressured to increase overall production. However, associated with elevated production is greater stress due to crowding, reduced water quality, and other factors. These stressors impact the health and welfare of the farmed animal which has become of increasing concern to a more environmentally aware and health conscious consumer. New strategies must therefore be developed and adopted by the aquaculture industry to counteract negative consumer perceptions of industrial fish production while also stabilizing the industry.

Better nutrition may enhance disease resistance of farmed fish while fillet accumulations of specific health-related nutrients may simultaneously add value to the final product. This thesis summarizes research undertaken in an effort to enhance the nutritional value of fish by increasing fillet levels of selenium (Se). In addition, various biomarkers of fish health (lysozyme, ceruloplasmin and glutathione peroxidase (GSH-Px) activities) were examined to determine whether dietary Se supplementation had a positive impact upon fish immunocompetence. Moreover, the effect of vaccination was also examined using lysozyme and growth as indicators of fish performance. Hybrid striped bass (HSB), the fourth most valuable farmed fish and fifth in tonnage produced in the United States, were employed as a model animal.

Se, an essential component of the antioxidant enzyme glutathione peroxidase, with many established health benefits was supplemented to HSB diets at various concentrations but was

found to be without effect upon serum immune proteins or GSH-Px activity. This finding likely reflected the use of fishmeal within the dietary formulation, which possessed relatively high Se levels, together with sufficient storage of tissue Se within the experimental animals. Nevertheless, these studies determined that organic sources of Se were more efficiently accumulated in HSB muscle than traditional inorganic sources. A linear response occurred up to the highest dose used (3.2 mg kg^{-1}) over a 6 week study. Fillet Se accumulation (r^2=0.95) proved to be a better indicator than the liver (r^2=0.87). Se enhanced fish therefore appear to offer a route of entry for fish producers into the lucrative designer food market – especially since many hundreds of millions of people worldwide are believed to be Se-deficient. Studies undertaken with Se-deficient HSB confirmed findings from the aforementioned research and also indicated that Se-enhanced fillets might be produced using a finishing feed containing 1.5 mg Se kg^{-1} 6-8 weeks prior to harvest. Accumulation of Se using this strategy resulted in a 100g portion of HSB fillets containing between 33-109 µg Se, amounting to a dietary intake of between 25-80 µg Se; a level that would satisfy present daily intake recommendations.

Vaccination of HSB with a *Streptococcus iniae* oil-in-water vaccine was examined for its potential negative impacts upon HSB production performance. Vaccinated fish did not exhibit any significant reductions in growth but microarray studies revealed that together with many hundreds of genes, four immune-related genes were impacted by this procedure. This thesis discusses the results obtained with regard to their practical implications to the industry and welfare of cultured fish.

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Chapter 1

General introduction

Aquaculture

The percentage contribution of aquaculture production to global supplies of fish increased from $3.9^{\%}$ in 1970 to $29.9^{\%}$ in 2002 (FAO 2004). In the United States, demand for domestic aquaculture products is expected to increase in the future due to price increases in restaurant sales, expensive imported wild and cultured seafood, the establishment of a competitive domestic industry (Harvey 2006) and national demographic shifts and seafood preferences. Congressional consideration of the *National Offshore Aquaculture Bill,* may result in rapid growth of American aquaculture.

Food-borne diseases, such as bovine spongiform encephalitis (BSE) "mad cow disease" and avian influenza "bird flu" (H5N1 and its variants) may stimulate aquaculture development. United States consumers are more attuned to issues surrounding food safety and prefer to purchase American products, perceived as safer than imported products (Craig and McLean 2006). In the United States, biosecure production of aquacultured animals will undoubtedly increase as reduced availability of suitable water resources and stringent environmental regulations (EPA 2004) increase interests in the use of water recirculation systems (RAS). These systems are popular for fish rearing in the United States (Carlberg et al. 2004) and serve to heighten food safety.

Hybrid striped bass aquaculture

Commercial aquaculture of hybrid striped bass (HSB) in the United States commenced in the early 1980's (Garber & Sullivan 2006). HSB is the fourth most valuable aquacultured crop in the United States, behind channel catfish, rainbow trout, and penzeid shrimp. HSB are the fifth most important species production-wise, in a nation that cultivates more than 50 species of aquatic animals. HSB production has remained constant over the past several years at ~5 million kg. (~11 million lbs) (Carlberg et al. 2006). HSB farming is practiced in tank and pond culture, as well as cage culture (Carlberg et al. 2006). California, Texas, Mississippi and North Carolina are major producers (Bartley et al. 2001). HSB sell for about $7 per kg (~$3 per lb) for whole fish. West Coast competition from Taiwan has hindered price increases for the farmer (Carlberg et al. 2006). Limited niche markets exist for HSB in Japanese and Korean sushi restaurants (Carlberg et al. 2006). To become more competitive, production costs must be reduced by 25%, which may be achieved through selective breeding of the parental species (Hallerman 1994, Garber and Sullivan 2006). The recent identification of 9 polymorphic satellites in HSB represent a significant step in this direction, allowing for more efficient selection of broodstock (Ross et al. 2004), especially for striped bass, one of the most genotypically monomorphic of all animal species (Waldman et al. 1998).

Diseases of cultured hybrid striped bass

Infectious diseases represent a major problem for the farming of warmwater fishes (Noga 2000) especially for recirculating aquaculture systems (RAS). Approximately 10% of all cultured fish die because of disease at a global cost of billions of dollars per year. The incidence and variety of fish diseases has increased dramatically in the past 30 yrs because of increased aquaculture production and fish disease research (Noga 2000). HSB are susceptible to a broad

variety of viruses, bacteria, fungi and parasites, but perhaps the single most important disease is *Streptococcus iniae*. In America, *S. iniae* affects about 10% of larger, grow-out HSB, and across all aquaculture species, is estimated to cause disease losses greater than \$150 million (Shoemaker et al. 2001). *Streptococcus iniae* spreads very rapidly, causing meningitis, and up to 50% mortality in 48 hrs (Evans et al. 2001). Key diagnostic features of this disease include exophthalmia, hemorrhaging and serosanguinous fluid in the peritoneal cavity and intestine. Although chemical treatments have been successfully applied to control *S. iniae* in HSB, mega-supplementations of vitamins C and E failed to have any impact upon disease resistance/recovery (Sealey and Gatlin 2002). Vaccination represents an acceptable method of disease control. However, vaccination causes negative impacts upon treated fish. Most studies have been conducted with salmonids (See Table 4.1). No published evaluations report the effects of vaccines upon the performance of HSB. These must be undertaken to provide the HSB farmer with detailed information upon treatments.

Functional foods and aquaculture

Nutritional awareness of food is increasing, with 2004 the first year in which ''healthiness' competed with ''convenience' as the most important food attribute in the United States (Sloan 2004). Functional foods are fortified, enhanced or enriched with certain nutrients for the purpose of increasing their health benefits (Hasler 2002). Research into functional foods is supported by the American Dietetic Association (ADA 2004) and is more effective in health promotion and disease prevention than conventional foods (Shahidi 2004). Research into the health benefits of selenium (Se) and methods to increase it in the human diet is under investigation (Tapsell 2004).

Farmed fish retain significantly lower levels of Se, copper, zinc and vitamin E than their wild counterparts (Poppe et al. 1985, Felton et al. 1990, Maage et al. 1991, Felton et al. 1994). A Se-enhanced aquacultured product can be marketed in Se-deficient areas of the world, which has been successfully accomplished with meat, eggs, bread, and milk (Combs 2001), commanding an increased price (Cole 2000, Patterson et al. 2001). Fortified aquacultured products could provide a new source of revenue. Se deficiencies have been correlated with cancer, cardiovascular disease, viral diseases, subfertility and some of the many diseases associated with oxidative stress (Thomson 2004). Keshan Disease, which affects the heart, and Kaschin-Beck disease, which affects the joints, occurs in a mountainous belt in China with Se-deficient soils (Combs and Combs 1986).

General chemistry of Se

Se was originally discovered by the Swedish scientist M.H. Klaproth in 1817 in a copper sulfide mine in Falun, Sweden, but misidentified as tellurium (Kabata-Pendias 1998). Later it was named selenium from the Greek word, *selene* for moon by J.J. Berzelius (Kabata-Pendias 1998). Se is the 34th element in the periodic table (atomic mass=32.066) and is characterized as a trace mineral. Se has a +2 charge. In the earth's crust, Se is estimated to be present at $10\mu g/kg$, although this may vary considerably (Kabata-Pendias 1998). The element occurs inorganically as selenite, selenate or selenide, and its organic forms are predominately selenomethionine and selenocysteine. Se is an essential component of the antioxidant enzyme glutathione peroxidase, which protects cell membranes from free radical damage (Rotruck et al. 1973). It is used in the electronics industry primarily as a photoreceptor in copiers, a decolorizing agent in the glass industry, in metallurgy serves as an alloy for cast iron, copper, lead and steel, whereas its pharmaceutical use is in shampoos (George 2003).

Se geochemistry

Se tends to be depleted in central areas of continents, due to the resistance of interior rocks and sediments to weathering and of erosion facilitated migration to continental margins (Malisa 2001). Of the trace elements, Se concentration on the earth's crust appears to be the most influenced by extraterristerial deposition (Malisa 2001). European soils are typically low in Se, due to this mineral's naturally low occurrence, acid rain, and the high soil iron content; all of which impact Se presence (Aro et al. 1995). Since 1984, fertilizers in Finland have been routinely supplemented with sodium selenate, which may have contributed to the reduced heart disease rate in that country (Aro et al. 1998, Brown and Arthur 2001). Greater reliance upon European Union wheat varieties, and changes in bread making technology, has decreased the amount of Se in European diets (Macpherson et al. 1997, Rayman 1997, Rayman 2002).

Se biochemistry

Each group of three ribonucleotides, known as a codon, specifies one amino acid (Klug and Cummings 2000). To take advantage of the unique properties of Se, it has been suggested that the interpretation of the UGA codon, which codes for termination of transcription, was modified to dictate selenocysteine incorporation into proteins after the original genetic code had evolved (Hatfield and Gladyshev 2002). Sec tRNAs are the only class of tRNA molecules that specify the insertion of Se by ribosomes in the synthesis of polypeptides during translation (Hatfield and Gladyshev 2002). Selenophosphate synthetase, represents the selenium donor in biological reactions (Stadtman 1996). The weak P-Se bond in selenophosphate, enables it to easily donate Se for addition and substitution reactions (Stadtman 1996). The replacement of sulfur with Se enhances the catalytic ability of certain enzymes (glutathione peroxidases, deiodinases involved in thyroid hormone metabolism), since the enzymes become more active at

physiological pH (Birringer et al. 2002). In most selenoenzymes, Se is attached to the amino acid cysteine, known as selenocysteine, the 21st amino acid (Birringer et al. 2002). Unlike other organisms, two copies of the transfer RNA (tRNA) for selenocysteine exist in zebrafish, *Danio rerio*. This has likely occurred due to gene duplication, a common occurrence in zebrafish. However, it is not know whether the duplicated genes occur on the same or different chromosomes (Xu et al. 1999).

Se as an Antioxidant

Free radicals have detrimental impacts on the body (Harman 1956) and are especially damaging to cellular membranes, which contain many vulnerable polyunsaturated fatty acids, in a process known as lipid peroxidation, which results in the formation of hydrogen peroxide (Aaes-Jorgensen 1961, Girotti 1985). The immune system employs free radicals to kill pathogens, however excess production of free radicals during chronic infections may harm nearby cells (Secombes 1996). Antioxidants are compounds that "soak up" free radicals, rendering them harmless (Bendich 1989). Important antioxidants in the body include vitamin E, vitamin C and the Se-containing enzyme glutathione peroxidase (Lee and Dabrowski 2003, Rotruck et al. 1973). The body's antioxidant system usually provides adequate protection; however, this system may become compromised if overwhelmed by free radicals due to excess production by the immune system or as a result of dietary insufficiencies (Machlin and Bendich 1987). Through commercial feeds, livestock may be injesting large amounts of prooxidants (compounds that promote the formation of free radicals), possibly exceeding their antioxidant defenses (Miller and Madsen 1994).

Metabolism and Se

It is now understood that total Se presence is not an effective indicator of Se bioavailability. Rather, it is the proportion of individual Se-containing compounds present that determine bioavailability (Shen et al. 1997, Whanger 2003). The chemoprotective properties of Se are due to intermediates of selenium metabolism, especially methylated selenocompounds (Combs and Lu 2001). A 15 kDa selenoprotein variant is a recently-discovered selenoprotein believed to have chemoprotective capabilities (Gladyshev et al. 2001).

Selenomethionine, if not broken down into selenocysteine for selenoprotein synthesis, can be incorporated into many organs and tissues such as muscle, due to its similar structure to methionine (Jacques 2001). However, inorganic Se, when not immediately utilized by the liver for selenoprotein synthesis, is quickly eliminated (Jacques 2001). Different species may contain different proportions of selenocompounds, thereby influencing their bioavailability to humans. For example, low-molecular weight compounds, which serve as the building blocks for selenoproteins, are found in higher quantities in cod (*Gadus morhua)* and herring (*Clupea harengus)* than in plaice (*Pleuronectes platessa)* (Akesson and Srikumar 1994). However, Onning and Bergdahl (1999) found plaice to have a higher proportion of low molecular weight compounds than cod, which contained organic Se compounds in the form of selenomethionine (Crews et al. 1996). Only organic forms of selenium, selenomethionine and the trimethylselenonium ion have been found in tuna and mussels (Quijano et al. 2000). The inorganic selenium compounds, selenate, selenite and selenide are found in a variety of marine and freshwater species (Cappon and Smith 1981, Cappon and Smith 1982). The ability to differentiate selenium compounds is improving with the development of newer technology.

Livestock Se requirements

The primary sign of Se deficiency in poultry is exudative diathesis (Scott et al. 1957, Stokstad et al. 1957, Patterson et al. 1957, Schwarz et al. 1957, Noguchi et al. 1973, Bartholomew et al. 1998). Exudative diathesis is a condition in which fluids build up between the muscle and skin, especially around the breast and abdomen (Dam and Glavind 1938). This condition results due to decreased glutathione peroxidase activity, allowing free radicals to break cell membranes. Following lysis, cell contents are released into the systemic circulation (Mahan 2000). The Se requirement for poultry ranges from 0.006 mg/100g feed for laying hens to 0.2 mg/100mg for turkeys and ducks (NRC 1994). However, under the stressful conditions of commercial poultry production, this requirement increases substantially, and various studies have demonstrated that the Se requirement for immunity is much higher than requirements for growth and development (Surai 2002b). Se deficiency in poultry is implemented has a possible cause of the spread of Avian influenza "bird flu" (H5N1 and its variants) (Edens and Carver 2006). Supplementation of feeds with organic Se in breeder diets at 0.2-0.4 mg/kg may offer significant protection against free radicals in the newly-hatched chick, while also having beneficial effects upon the chick's immune response (Surai 2002b). Se in excess is toxic. In birds, symptoms of Se over-supplementation (selenosis) include decreased growth and egg production, anemia and stiffness of the leg joints, which become noticeable when dietary Se exceeds at least 5 mg/kg feed (Surai 2002a).

In swine, Se deficiency causes liver necrosis, white muscle disease, exudative diathesis, "mulberry" heart and ulcers (Ewan et al. 1969, Bengsten et al. 1978a, 1978b, Mahan and Moxon 1980). When combined with inadequate dietary vitamin E, *hepatosis diaetetica*, dietetic microangiopathy, yellow fat disease, acute circulatory failure and, during reproduction, puerperal

fever, and metritis-mastitis-agalactia complex (MMA) occur (Whitehair and Miller 1985). Prevention of the latter is attained with 0.30 mg Se/kg feed with 40 (IU/kg) vitamin E for nursery pigs (5-20 kgs); 0.20 Se (mg/kg) with 20 (IU/kg) vitamin E for grower pigs (20-60 kgs); 0.15 Se (mg/kg) with 11 (IU/kg) vitamin E for finisher pigs (60 kg to market size), and 0.30 Se (mg/kg) with 60 (IU/kg) vitamin E for gestating and lactating pigs (Mahan 2000). Weanling pigs deficient in either Se or vitamin E, or both, exhibit decreased antibody production (Peplowski et al. 1981). Pigs fed diets deficient in vitamin E and selenium have reduced lymphocyte responses when challenged with *Salmonella typhisus* (Lessard et al. 1991). In contrast, swine fed diets supplemented with 50 Se mg/kg feed and 40 mg a day of vitamin E expressed increased lymphocyte response when challenged with phytohaemagglutinin when compared to pigs fed a basal diet containing 0.05-0.04 mg/kg Se and 33 mg/kg vitamin E (Larsen and Tollersrud 1981). Se enhances production of swine IgM immunoglobulins, but not IgA or IgG (Hayek et al. 1989); this fact is important when considering fish, since teleosts only produce IgM-like immunoglobulins (Kaattari & Piganelli 1996). Signs of selenosis in pigs include decreased appetite, decreased growth and abnormal hoof formation (Miller and Schoening 1938, Mahan and Moxon 1984, Goehring et al. 1984a, Goehring et al. 1984b). Selenium in excess of 0.8 mg/kg body weight is usually toxic (Whitehair and Miller 1985).

Signs of Se deficiency in cattle are white muscle disease, retained placenta, abortions and stillbirths, neonatal weakness, "ill thrift" syndrome, myodegeneration in adult cattle and infertility (Maas and Koller 1985). Se requirement for most classes of dairy cattle is 0.3 mg/kg; however, for nonlactating cows in the last trimester of gestation, 1.75 mg/day is recommended, and for lactating cows producing 30 kg/day of milk, the recommended dose is 4 mg/day (NRC 2001). The requirement of beef cattle for Se is 0.1 mg Se/kg (NRC 1996). Due to the lack of

purified or semi-purified diets in cattle research because such diets are cost-prohibitive, Finch and Turner (1996) determined that no conclusive studies have been undertaken on the effect of Se upon the immune system of larger animals. The signs of selenosis are alkali disease and blind staggers that can be seen chronically when 5 to 40 mg Se/kg are fed for several weeks or months, or acutely when cows are fed 10 to 20 mg of Se/kg of body weight (NRC 2001). Selenosis was observed in cattle in the late 1800s and early 1900s in the Midwestern United States, and later attributed to the cattle grazing on high Se accumulating plants (Combs and Combs 1986) The Se level needed to induce chronic selenosis is at least 16-times greater than the Se requirement of 0.3 mg/kg (NRC 2001).

Se in fish

The recommended level of Se for growth in hybrid striped bass (HSB) is 0.3 mg/kg inorganic based on the requirements of salmonids and some warm water species (Gatlin and Wilson 1984, Webster 2002). Se is present in commercial diets at required levels; however, during disease or stress, the levels of Se may be insufficient. Se levels declined in Atlantic salmon infected with Hitra disease (*Vibrio salmonicida*) (Hjeltnes and Julshamn 1992). Se enhancements of immune parameters are well documented (Wise et al. 1993, Table 1.3). Jaramillo and Gatlin (2004) concluded that nutritional influences on immunity in HSB need further evaluation. Dietary Se toxicity studies on fish are reviewed in Table 1.1. Excess Se accumulates in the gonads of fish, reducing production of viable eggs (Sager and Cofield 1984, Baumann and Gillespie 1986, Gillespie et al. 1988). In excessive amounts, Se replaces sulfur, preventing the necessary disulfide chemical bonds needed for protein structure, causing teratogenesis in developing larvae, a diagnostic symptom of selenosis in fish (Lemly 2002b). Effects of Se deficiency, primarily brought about by reduced glutathione peroxidase activity, are

reviewed in Table 1.2. Higher waterborne Se concentrations are needed to affect fish ($\approx$40-130 *ug* /L) that naturally occurs in water (<0.1-0.4 *μg*/L) (Lemly 2002a). It is recommended that fish health should be monitored whenever Se exceeds 0.5 *μg*/L (Lemly 2002b). Differences in the rate of Se excretion between waterborne and dietary selenium by fish are thought to occur due to circulatory anatomy, with Se being absorbed by the gills entering all tissues except the liver, whilst dietary Se must pass the liver (first-pass effect; Hilton et al. 1982).

Se and humans

The Recommended Dietary Allowance (RDA) of Se for adults is 55 *μg*/day for maximum glutathione peroxidase activity (National Academy of Science 2000). The predominant natural source of Se is plants. Natural sources such of these may have varying Se conternt depending on the Se content of the soil in which they are grown (Milovac et al. 1998). Se has demonstrated to possess anticarcinogenetic properties in humans (Combs and Combs 1986). This mineral is of interest to the geomedical field because correlations between high serum Se levels and low mortality rates for cancer and cardiovascular diseases are found throughout the world (Maksimovic et al. 1998). Se deficiency in humans is the primary cause of juvenile cardiomyopathy ("Keshan Disease") and juvenile chondrodystrophy ("Kaschin-Beck Disease" or "Big Joint Disease") (Combs and Combs 1986). Keshan disease is found in the mountainous and hilly areas in a belt of Se-deficient soils in China (Combs and Combs 1986). The primary organ affected by Keshan disease is the heart (Ge et al. 1983). The most common form of Keshan disease is the subacute type (lasting 1-2 weeks) with symptoms including cardiogenic shock, facial edema, gallop heart rhythm, heart enlargement and congestive heart failure (Chen et al. 1980, Ge et al. 1983). The case-fatality of Keshan disease is estimated to have been greater than 80% in the 1940s, but due to improved health care this has declined to about 30% (Combs and

Combs 1986). Kaschin-Beck disease occurs in eastern Siberia, northern Korea and China, and affects the cartilage and growth plates of growing bones, resulting in enlarged joints, shortened fingers, toes and extremities (Combs and Combs 1986). Se deficiency may serve a role in Balkan Endemic nephropathy (BEN), a chronic kidney disease found in the Balkan countries of the former Yugoslavia (Grubor-Lajsic et al. 1998).

Symptoms of acute selenosis in humans, usually occurring through inhalation of Se in copper refineries, include tearing and burning sensations of the eyes, hoarseness, coughing, sneezing, headache and dizziness, followed by pulmonary edema (Combs and Combs 1986). Reports of chronic selenosis have occurred in the highly seleniforous soils of the northern Great Plains of the USA, parts of Venezuela and Colombia, and Enshi County in the Hubei Province of China (Combs and Combs 1986). Signs and symptoms of chronic selenosis include depression, fatigue, brittleness and loss of hair and nails, and scaly dermatitis (Combs and Combs 1986). To prevent chronic selenosis, a limit of 400 μg Se day^{-1} from food is recommended (National Academy of Science 2000). Water and air are usually negligible sources of Se (Combs and Combs 1986).

Currently, the U.S. RDA for Se is 55 μg Se day^{-1} (National Academy of Science 2000), however, 200-300 μg Se day^{-1} may be needed to exhibit Se's anticarcinogenic effects (Rayman and Clark 2000). Severe Se deficiency has been implicated only in a few diseases (Keshan and Kashin-Bech disease); however, sub-clinical signs may include cardiovascular disease, decreased immunocompetence, increased susceptibility to viral infections, subfertility, impaired thyroid function, depression and cancer (Brown and Arthur 2001, Combs & Lu 2001, Rayman 2002). Hundreds of millions of people may be Se deficient (Combs 2001).

Dietary inadequacy of Se in humans has far-reaching effects in health and disease (Foster and Sumar 1997, FAO/WHO 2002). During infections, circulating Se levels may decline rapidly, thereby placing patients into transient Se deficiency (Nichol et al. 1998, Schrauzer 2006). It is clear that a number of nutrients, including Se, must be available at the appropriate concentrations if the immune system is to operate efficiently (IOM 1999). As with other minerals that may be preferentially mined during disease and sub-clinical infections, it may be prudent especially for high-risk individuals (e.g., the chronically sick and elderly, HIV patients), to increase mineral intake above recommended daily values. This, in essence, underpins one principle of the functional food concept and a reason for examining Se accretion in aquacultured fish.

Se availability from fish

There are conflicting reports regarding Se bioavailability from fish for humans. Fox et al. (2004) found that selenium from fish is highly bioavailable. Se from fish increased plasma Se, glutathione peroxidase and selenoprotein P (a selenoprotein believed to be involved in oxidant defense and transport), but not thyroid hormones in Latvian fish consumers (Hagmar et al. 1998, Hill and Burk 2001). Consuming 150-200 g of fish per day increased plasma selenium $13^{\%}$ in Swedish subjects within three weeks (Thorngren and Akesson 1987). Human populations of northern Finland, with high fish consumption, and from areas with high reindeer consumption had higher serum Se levels along with lower blood pressure and reduced cardiovascular and cancer mortality than Finns from low Se areas (Westermarck et al. 1977, Luoma 1998). Plasma Se levels were significantly increased in Swedes consuming two fish meals per week over those who did not consume fish (Svensson et al. 1992). However, Meltzer et al. (1993) found low Se bioavailability from fish for humans and offers the possible explanations of tissue saturation, different chemical forms of Se, interactions with heavy metals such as mercury and arsenic,

environmental and health conditions of the human subjects, a high methionine content of the diet and oxidative stress due to polyunsaturated fatty acids. Extreme fish consumption did not increase plasma antioxidant levels, including selenium, in Finnish subjects (Anttolainen et al. 1996). Se from shrimp had high retention; however, there was no increase in glutathione peroxidase activity, suggesting another possible reservoir for Se accumulation (Bugel et al. 2001). Studies on the enhanced accumulation of organic sources of minerals in fish tissues are reviewed in table 1.4.

Summary

A substantial body of literature exists to indicate not only the essentiality of Se for normal health, but also that this mineral's requirements may differ significantly between populations. Moreover, during periods of stress, such as during reproduction and disease and during aging, dietary requirements for Se may increase in animals and humans. Dietary Se supplementation may enhance general immunocompetence and hence increase the ability of animals to cope with stressful situations, while also offering protection from reactive oxygen species. Moreover, because Se may be preferentially mined from the body during periods of high requirement (*e.g.*, during chronic and severe disease), supplemental Se above recommended daily levels may be beneficial. Clearly, in areas of the world where Se deficiency is common, a need exists to devise strategies for increasing Se intake. In certain instances, this has already commenced with the advent of Se-enhanced foods, as exemplified by bread, eggs, milk and meat (Milovac et al. 1998, Cole 2000, Hassler 2000, Surai and Sparks 2001, McIntosh and Royle 2002, Yaroshenko et al. 2003, Heard et al. 2004). At present, no Se-augmented seafoods are available.

Although many seafood products are already considered as "functional foods" (*i.e.*, provide significant health benefits to consumers), they also lend themselves to further manipulations. Indeed, production of value added products for aquaculture would clearly provide the industry with a new set of produce that would provide the means to develop novel business opportunities for fish farmers. Concomitantly, value added products might provide the means to stabilize and expand the industry. Se enhancement of the edible component of fish could furnish such a product. Additionally, increased dietary Se intake by fish has been reported to improve growth and feed conversion (Wang et al. 1997, Jaramillo and Gatlin 2004), and to stimulate the immune system. Accordingly, Se enhancement might provide the means to elevate the welfare of farmed fish during production.

Contemplation of applying Se enhancement to the production of farmed fish for human consumption clearly demands that basic information upon Se accumulation and tissue dynamics be determined on a species-specific basis. Differences in accumulation when different sources of Se (organic and inorganic) are used must also be appreciated. Through an enhanced understanding of Se dynamics, tactics may be sought and strategies developed for the application of specialized Se finishing diets. These areas of research have not been previously considered in depth. Consequently, in order to provide such information, the present thesis centered attention upon Se accumulation in HSB using both organic and inorganic Se. As well, since farmed animal welfare has become an issue of increasing importance to the western consumer, these studies evaluated the immune responsiveness of Se-challenged fish. As an adjunct to this welfare aspect of the present thesis, the effects of vaccination upon HSB performance was evaluated.

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Table 1.1 Studies of dietary Se toxicity in fish.

Species	Toxic level observed (mg/kg)	Se form	Lifestage	Toxic effects	Duration until onset of effects	Reference
Fathead minnow	20	Selenite Selenate Selenomethionine	59-61 days	Reduced growth, reduced appetite However offspring not affected	3 months	Ogle & Knight 1989
Bluegill	33.3	Selenomethionine	2 yrs	Reduced survival in offspring (only 7%)	P1 fed diets for 2 months days prior to spawning	Coyle et al. 1993
Chinook salmon	9.6	Selenomethionin, Se laden mosquitofish	Swim up larvae, fingerlings (70 mm)	Reduced growth, survival to seawater challenge	3 months	Hamilton et al. 1990
Rainbow trout	13	Sodium selenite	juveniles, (128 gms)	Reduced growth rate Poor FCR, mortalities, uncoordinated spiral swimming, livers discolored	1 month	Hilton et al. 1980
Coho Salmon	13.6	Sodium selenite	fingerling (4.5 gms)	↓ survival to seawater challenge	6 months	Felton et al. 1996
Bluegill	30	Selenomethionine Selenite	juveniles, (5.1 gms)	Teratogenesis in offspring Organoselenium more toxic to offspring	P1 fed diets for 9 months prior to spawning, mortalities started to occur at 4 months in the 30 ug;/g diet	Woock et al. 1987
Bluegill	13.6	Se-contaminated mayflies	3 gms	Mortality, exophthalmia, distended abdomen, edema, reduced balance, abnormal erythrocytes	6 weeks	Finley 1985
Striped bass	38.6	Se-contaminated red shiners	250 gms	Abnormal livers, kidney damage, reduced weight gain, reduced condition factor, abnormal behavior	6 weeks, by week 11 all were dead	Coughlan & Velte 1989
Bluegill	13	Selenomethionine	3 mos old, (0.2 gms)	Reduced condition factor	3 months	Cleveland et al. 1993
Rainbow trout	4.6	Selenomethionine	24 day old larvae, (0.37 gms)	Reduced growth	3 months	Vidal et al. 2005

Table 1.2 Studies of Se deficiencies in fish

Species	Lifestage Size	Se (mg/kg)	Vitamin E (mg/kg)	Defeciency Symptoms	Duration until onset of effects	Reference
Atlantic Salmon	Fry (0.10 gms)	0.1	0.5 IU/g	↑ mortality, nutritional muscular dystrophy, abnormal erythrocytes, ↓ Glutathione Peroxidase	1 month	Poston et al. 1976
Channel catfish	Fingerlings (75 gms)	0.06	30	0.06 mg resulted in ↓ growth	Examined at end of study (4 mos)	Gatlin III & Wilson 1984
Atlantic Salmon	Parr (6 gms)	0.17	40	0.17 mg resulted in ↓ growth, Abnormal erythrocytes ↓ Glutathione Peroxidase	Examined at end of study (7 mos)	Bell et al. 1987
Channel catfish	Fingerlings (10.9 gms)	0.06	11	↑ red blood cell peroxidation ↓ hepatic GPx activity ↓ superoxide anion production	4 months	Wise et al. 1993
Rainbow trout	Juveniles (27 gms)	0.025	63	↓ PCV, ↓ hepatic E, ↓ hepatic and plasma Se ↑ glutathione transferase activity	Examined at end of study (7 ½)	Bell et al. 1986.
Channel catfish	Fingerlings (80 gms)	0.06	2.5	↓ growth, anemia, myopathy, exudative diathesis, mortalities, ↓ hepatic GPx, ↑ hepatic glutathione transferase	2 ½ mos	Gatlin III et al. 1986

Table 1.3 Challenge studies conducted with fish supplemented by dietary Se.

Species	Immune Response	Reference
Chinook salmon	>0.38 mg/kg increased survival when infected with *Renibacterium salmoninarum*, but severity and occurrence of BKD was not affected	Thorarinsson et al. 1994
Channel catfish	Resistance to *Edwardsiella ictaluri* increased as Se levels increased up to 0.40 mg/kg; organic Se more effective than inorganic Se	Wang et al. 1997
Hybrid Striped Bass	0.2 mg/kg Se and glucan increased plasma lysozyme levels, but did not improve survival against bath exposure to *Streptococcus iniae*	Jaramillo & Gatlin III 2004

Table 1.4 Review of comparative studies of the bioavailability of dietary organic and inorganic chemical forms of minerals.

Mineral	Species	Dose (mg/kg)	Duration	Chemical Form Inorganic	Chemical Form Organic	effect	Reference
Selenium	Atlantic Salmon	1-2	8 wks	Sodium selenite	selenomethionine	Selenomethione ↑ Se contents of fillets over selenite	Lorentzen et al. 1994
Selenium	Atlantic Salmon	1	4 wks	Sodium selenite	White fishmeal, Selenomethionine selenocystine	Digestibility was in the order of Se-met>selenite>se-cys>fishmeal	Bell & Cowey 1989
Selenium	Channel Catfish	0 0.02 0.06 0.2 0.4	9 wks	Sulfate Forms	Selenomethionine selenoyeast	Se-M and Se-Y had ↑ accumulation in muscle and liver	Wang & Lovell 1997
Selenium Copper Iron Manganese Zinc	Channel Catfish	0.5 10 60 5 40	6 wks	Sodium selenite Copper sulfate pentahydrate Ferrous sulphate heptahydrate Manganese sulfate monohydrate Zinc sulfate heptahydrate	Se proteinate Co proteinate Fe proteinate Mg proteinate Zn proteinate	All chelated minerals had ↑ bioavailability	Paripatananont & Lovell 1997
Zinc	Channel Catfish	0, 5, 10, 15 or 30	10 wks	Zinc sulfate heptahydrate	Zinc methionine	ZnMet more potent than ZnS for meeting zinc requirement	Paripatanont & Lovell 1995
Zinc	Channel Catfish	50-90	3 mos	Zinc sulphate (ZnSO4)	Zinc methionine (ZnMe) Zinc proteinate (ZnPr)	ZnPr not more effective than Zinc sulphate	Li & Robinson 1996
Zinc	Nile Tilapia	150	3 mos	Zinc sulphate monohydrate (ZnSO4), Zinc oxide (ZnO)	Zinc amino acid complex (Zn-AA)	Zinc sulphate had ↑ bioavailability	Carmo E Sa et al. 2005
Zinc	Atlantic Salmon	50 or 180	6 mos	Zinc sulphate (ZnSO4)	Zinc gluconate	No differencedd between forms	Maage et al. 2001
Zinc Magnesium Copper	Rainbow trout	0.05	4 $^{1/2}$ mos	Zinc magnesium copper sulfate	Zinc, magnesium copper amino acid complex (am)	am ↑ growth am ↑ bioavailability	Apines et al. 2003
Zinc Manganese copper	Rainbow trout	40 20 4	4 mos	Sulfate forms	Amino acid chelate	↑ bioavailability of chelate forms ½ dose of chelate form = full dose of inorganic form	Apines et al. 2004
Iron	Channel Catfish	20 60 180	2 mos	Ferrous sulphate heptahydrate (FeS)	Ferric methionine complex (FeM)	Both forms equally effective in preventing anemia	Lim et al. 1996

Chapter 2

The use of supplemental selenium in Hybrid striped bass diets

Abstract

One method of increasing the value of aquacultured products is to produce fillets that are fortified with minerals. Selenium (Se), a mineral that is of vital importance to normal metabolism in humans. In this study, dietary Se was fed to hybrid striped bass (HSB). Animals were fed one of nine diets supplemented with either organic (0, 0.1. 0.2, 0.4, 0.8, 1.6 or 3.2 mg kg^{-1} as SelPlex$^{®}$) or inorganic (0.2 and 0.4 mg kg^{-1} as sodium selenite) Se for a period of 6 weeks. Because basal fishmeal-based diets contained 1.22 mg Se kg^{-1}, doses of Se delivered equated to 1.22, 1.42, 1.62, 2.02 and 4.42 mg kg^{-1}. Examination of Se accumulation in HSB liver and fillet revealed a linear dose-response. Greatest fillet and hepatic accumulation of Se was observed following supplementation at the 3.2 mg Se kg^{-1} level ($P < 0.0001$). Theoretically, a 100g portion of Se-enhanced HSB fillet would contain between 33-109 μg Se, amounting to a dietary intake of between 25-80 μg Se; a level that would satisfy present daily intake recommendations. Comparison of tissue Se levels indicated that the muscle provides a better indicator of dietary Se dose-response than does liver. Dietary treatments of between 0.4 and 1.6 mg organic Se kg^{-1} reduced ($P < 0.024$) hepatic glutathione peroxidase activity. No differences were observed in ceruloplasmin, lysozyme or GSH-Px activities between organic and inorganic Se when delivered at the 0.2 mg Se kg^{-1} level. Ceruloplasmin, lysozyme and GSH-Px levels were elevated ($P \leq 0.025$) in fish fed the diet containing 0.4 mg inorganic Se kg^{-1}. Se supplementation of diets therefore may provide benefit to the cultured animal while also providing a means of adding value to farmed fish.

Key words: Hybrid striped bass, *Morone* sp., lysozyme, sodium selenite, inorganic, organic, recirculating aquaculture, ceruloplasmin, glutathione peroxidase

1. Introduction

Functional foods are fortified, enhanced, or enriched with certain nutrients for the purpose of increasing their health benefits (Mandel et al. 2005). The global functional food market has a value of in excess of $8.5 billion per year (James 1999). Fortified food products are effective for health promotion and disease prevention (Shahidi 2005). Due to their established and wide-ranging health benefits (Siddiqui et al. 2004, Ruxton et al. 2004, 2005, Peet and Stokes 2005, Nettleton and Katz 2005), unsupplemented/unfortified fish represent a complete food; especially omega-3 fatty acid-rich marine species. The recognized importance of dietary n-3 fatty acids has led to attempts to enhance their content in other meats (Rymer and Givens 2005). The nutritional value of fish can be further augmented to create a lucrative whole food market niche.

Approval of functional foods in the global marketplace has a high failure rate (Fogliano and Vitaglione 2005). For aquaculture products an obvious first step in the bionutrient selection process is to examine current functional foods. The human health benefits of dietary selenium (Se) are well established (Ryan-Harshman and Aldoori 2005), and include antioxidant properties, contributions to normal thyroid and immune function and fertility (IOM 2000), and benefits for HIV infected individuals (Rayman 2004). Methods for enhancing Se levels in eggs, milk, and meat are well documented (Hassler 2000, Surai and Sparks 2001, McIntosh and Royle 2002, Hintze et al. 2002, Yaroshenko et al. 2003, Heard et al. 2004). Populations in several areas of the world, including Europe, the Far East and Africa are known to suffer from Se deficiency (Jackson et al. 2004, Xia et al. 2005) and hundreds of millions of people are likely Se deficient (Combs 2001).

Undoubtedly, Se represents an excellent candidate bionutrient for aquaculture product enhancement. An additional advantage of employing Se is that elevated dietary levels have been reported to enhance growth, feed conversion, and immunocompetence in various fish species (Wang et al. 1997, Jaramillo and Gatlin 2004), although no positive effects have been observed in studies with fingerling channel catfish, Atlantic salmon, Nile tilapia or sub-adult Atlantic salmon (Gatlin and Wilson 1984, Julshamn et al. 1990, Lorentzen et al. 1994, Kim et al. 2003).

Selenium is an essential mineral for normal animal growth and development (Thomson 2004) and is an integral component of the enzymes thioredoxin reductase (TR) and glutathione peroxidase (GSH-Px). GSH-Px metabolizes hydroperoxides, and is thus intimately involved in cellular defense against oxidative damage (Arthur et al. 2003), whereas TR is engaged in the clearance of products of oxidative metabolism. Se is also a component of enzymes engaged in the deiodination of thyroid hormones and many of its biochemical functions are reliant upon its combined effects with vitamins C and E (FAO/WHO 2002). In the present study, supplementation of diets with Se had no effect upon serum GSH-Px activity, likely reflecting a masking effect from the relatively high endogenous Se levels in the fish meal. This could also explain the lack of clear effects upon acute phase protein responses.

The form in which Se is delivered is an important consideration. In terrestrial livestock and fish, it has been observed that organic Se is more readily absorbed, and more potent, than inorganic forms (Conrad and Moxon 1979, Bell and Cowey 1989, Paripatananont and Lovell 1997, Wang et al. 1997, Ortman and Pehrson 1999). Prior to producing a functional aquacultured product, optimal dietary levels and tissue accumulation must be established. In the present study, tissue accretion of organic and inorganic Se was evaluated to determine optimal concentrations of Se for finishing feeds. In addition, the effect of both forms of Se upon non-specific immune

proteins was examined. Hybrid striped bass (♀*Morone chrysops* x ♂*M. saxatilis*), which is the fourth most valuable crop and fifth most farmed species in American aquaculture, were used.

2. Materials and Methods

2.1 Animals and husbandry

Nine month-old juvenile hybrid striped bass (150$\pm$ g wet wt; 203$\pm$ mm length), purchased from a commercial hatchery (Keo Fish Farms, AR, USA) were PIT tagged (Biomark Inc., ID, USA) and randomly stocked into one of 18 tanks (*n*=5 tank^{-1}) of a custom designed, recirculating aquaculture system (RAS: Fig. 2.1). The RAS comprised twenty-four 120-liter glass tanks that had black-painted walls to reduce fish stress, and incorporated a bubble-bead filter (BBF-2 Aquaculture Technologies Inc., Metaire, LA, USA) to remove suspended solids, UV light sterilizer (Emperor Aquatics, Pottstown, PA, USA), a KMT fluidized bed with media (Kaldnes Inc; Providence, RI, USA) for biological filtration and a protein skimmer (R&B Aquatic Distribution, Waring, TX, USA) to remove smaller solids and decrease turbidity. A heater was placed in the biofiltration sump to maintain water temperature at 27 ^{o}C (80.6 ^{o}F). Fish were exposed to a 12:12 photophase:scotophase through fluorescent lighting positioned 3 m above the life support system. Until trial start, fish were fed a basal diet (Table 1) at 3$^{\%}$ body wt d^{-1} as two separate feedings (08.00 and 16.00 h).

2.2 Experimental treatments

Dietary crude protein and lipid levels were formulated at 40$^{\%}$ and 10$^{\%}$ respectively (table 2.1) All known nutritional requirements of hybrid striped bass (NRC 1993) were met by the experimental feeds. The basal diet was frequently analyzed to contain 1.22 mg kg^{-1} selenium (Eurofins, Des Moines, IA, USA). These diets incorporated both organic and inorganic Se supplemented at the expense of cellulose. Inorganic Se, delivered as sodium selenite (Sigma

Chemical Co., St. Louis, MO, USA) was supplemented at 0.2 and 0.4 mg kg^{-1}. Organic Se was supplemented as Selplex$^{®}$ (Alltech Inc., Nicholasville, KY) at 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg kg^{-1} for a total of nine treatments. Fish were weighed individually and measured every two weeks and group weights employed to adjust feeding rates. Diets were fed to randomly paired tanks for 6 weeks at 3$^{\%}$ body wt d^{-1} as two separate feedings (08.00 and 16.00 h).

Dietary components were mixed in a Patterson-Kelley twin shell$^{®}$ Batch V-mixer (Patterson-Kelley Co. Inc., East Stroudsburg, PA.) for 20 minutes and then homogenized into a paste by adding menhaden oil into a food mixer. The amount of distilled water required for pelleting (20-40$^{\%}$ of feed weight) was then added to the mixture and further homogenized. The paste was extruded through a Hobart D300 Floor Mixer (Hobart Co., Troy, OH) without steam using an appropriate die to provide pellets of suitable size for the fish. Duplicated samples from each feed were heated at 135°C (275°F) for 2 hrs in a gravity oven (Blue M Electric, Blue Island, IL) to determine dry matter. After air-drying overnight, feed was frozen at -10°C (14°F) until needed. Prior to use as feed, small quantities were thawed and refrigerated.

2.3 Data collection

2.3.1 Morphological and feed data

Feed conversion ratio (FCR) was calculated according to the following formula

$$FCR = (gms\ fed/gms\ gained\ in\ wgt)$$

At trial termination, all animals were weighed and measured (Fork Length), and their condition factor (CF) calculated according to the following formula:

$$CF = (weight/length^3)*1000$$

Visceral somatic indice (VSI) was calculated using the following formula:

$$VSI = (tissue\ weight\ \{g\}/wet\ weight\ \{g\}\ of\ animal)*100$$

Hepatosomatic indice (HSI) was calculating using the following formula:

$$HSI = (liver\ tissue\ weight\ \{g\}/visceral\ weight\ \{g\})*100$$

Specific growth rate (SGR) was calculated using the formula

$$SGR = ((natural\ log\ of\ final\ weight\ \{g\} - natural\ log\ of\ initial\ weight\ \{g\})*100) / t\ (days)$$

Survival was noted throughout the study

2.3.2 Serum processing

Anaesthetized (MS-222; Sigma) fish were bled from the caudal artery-vein complex using 2 ml heparinized syringes. Collected blood was transferred into Eppendorf 2 ml microcentrifuge tubes. Blood packed cell volume (hematocrit) was assessed immediately following collection. Blood was drawn into microhematocrit tubes (Fisher Scientific, Pittsburgh, PA), sealed with Cristoseal (Fisher), centrifuged at 10,000 x g for 5 min (M 24 Micro-Hematocrit Centrifuge; LW Scientific, Lawrenceville, GA) and hematocrit read using the centrifuge's combo reader. Remaining blood samples were refrigerated at 5 ^{o}C and allowed to clot overnight. Resultant serum was stored in 2 ml Eppendorf tubes at -20 ^{o}C until further analyses.

Serum samples were employed to evaluate protein levels using a hand-held VET360 temperature-compensated refractometer (Leica Optical Products Division, Buffalo, NY) and protein assay kit (Bio-Rad, Hercules, CA., Lowry et al. 1951). Glutathione peroxidase activity was quantified from liver samples using a commercial assay (Sigma-Aldrich, St. Louis, MO). Serum lysozyme activity was measured using the turbidimetric assay described by Parry et al. (1965). The assay employed 0.2 mg/ml *Micrococcus lysodeiktcus* suspended in sodium phosphate buffer (pH 7.2). 100 μl test sera were added to 100 μl phosphate buffer and 500 μl of suspended *M. lysodeiktcus* for a final volume of 700 μl. The reaction (24 ^{o}C; absorbance 540 nm)

was measured at 30, 60 120 and 180 s, and the unit of lysozyme activity defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min. Ceruloplasmin ferroxidase activity was quantified using a method based upon the catalytic oxidation of ferrous ions or ferrous complexes to the ferric state by ceruloplasmin (Anderson and Siwicki 2003, Cerón and Martínez-Subiela 2004).

2.3.3 Selenium quantification and data analyses

Tissue and water Se levels were assessed using standard methods (AOAC 1990). Five randomly-taken animals were used per evaluation with fillet samples derived from the right flank of the animal. System water samples were collected at the beginning and end of the study and frozen until analyses. Regression analyses (SAS 9.1; SAS, Cary, NC, USA) were utilized to plot muscle and liver Se accumulation versus dietary concentration. Remaining data were subjected to analysis of variance utilizing SAS 9.1. Where appropriate, data was also subjected to Duncan's multiple range test for means separation. Differences were considered significant at $\alpha < 0.05$. Comparisons between inorganic and organic forms of Se were made using PROC t-test (SAS 9.1).

3. Results

Waterborne Se levels which were < 0.1 µg l^{-1} at trial start had increased to 620 µg l^{-1} by trial termination. No mortalities were recorded throughout the period of study. Differences were recorded in weight and length growth between treatment groups. In general, little change was recorded in body condition as indicated by CF ($P > 0.05$; Table 2.2). With respect to organic Se supplementation, there were no impacts of dietary Se supplementation upon FCR, Packed Cell Volume (PCV) and plasma protein levels. However, differences ($P < 0.05$) were observed in plasma protein when comparing diets supplied with 0.2 mg Se kg^{-1} organic or inorganic Se

(Table 2.2). The lowest PCV or hematocrit value (49.4) was observed in fish fed the diet containing 0.2 mg inorganic Se kg^{-1}.

Liver Se concentrations for hybrid striped bass fed diets containing increasing concentrations of organic Se varied between 1.35 and 3.63 mg kg^{-1} DM (Table 2.3). Up to a threshold level of 0.2 mg Se kg^{-1} DM there were no differences in hepatic Se concentrations. However, differences ($P < 0.0001$) were observed in liver Se concentrations, in a dose dependent manner between 0.4 and 3.2 mg Se kg^{-1} (table 2.3). In the liver, the form in which Se was delivered (i.e., as an organic or inorganic source) was without effect upon hepatic Se concentrations ($P > 0.05$; Table 2.3). In all treatments, hepatic Se concentrations were higher than those recorded for the muscle (Table 2.3).

Examination of muscle Se levels revealed the same pattern of accumulation in terms of threshold and dose-response as that observed in the liver. Muscle Se concentrations varied between 0.30 and 1.09 mg kg^{-1} DW, with basal Se levels equaling that recorded for 0.1-0.2 mg Se kg^{-1} supplementation. The amount of Se observed in muscle was significantly ($P < 0.05$) increased over basal and 0.1-0.2 mg Se kg^{-1} diet when supplementation was $\geq$ 0.4 mg kg^{-1} (Table2.3). Differences ($P < 0.0001$) were observed in muscle Se concentrations, in a dose dependent manner between 0.4 and 3.2 mg Se kg^{-1} (table 2.3). No differences were recorded between organic and inorganic Se supplementation at the 0.2 and 0.4 mg kg^{-1} levels.

Figures 2 and 3 summarize regression analyses for the accumulation of organic selenium in liver and muscle tissues respectively. Tissue Se accumulation followed a classic dose response for muscle and liver.

Table 2.4 summarizes treatment effects upon non-specific immune response and serum glutathione peroxidase activity. Dietary organic Se, at concentrations between 0.2 and 0.4 mg kg^{-}

[1] returned lysozyme activity that were lower than those observed when compared to fish fed the basal diet decreased ($P < 0.05$). Consideration of results for ceruloplasmin indicated a peak at 0.1-0.2 mg organic Se kg^{-1} diet. At the highest Se inclusion level, ceruloplasmin concentrations were reduced ($P < 0.004$; Table 2.4). Dietary treatments of between 0.4 and 1.6 mg organic Se kg^{-1} reduced ($P < 0.024$) glutathione peroxidase activity. No differences were observed in ceruloplasmin, lysozyme or GSH-Px activities between organic and inorganic Se at the 0.2 mg kg^{-1} level. However, all of these parameters were elevated ($P \geq 0.025$) in fish fed the diet containing 0.4 mg inorganic Se kg^{-1}.

The endogenous level of Se in the menhaden meal (1.22 mg kg^{-1}) used here fell within the recorded range for other marine and freshwater species (Meltzer et al. 1993, Fox et al. 2004, FAO/WHO 2002). With supplementation, total dietary Se intake during the present trial therefore, ranged between 1.22-4.42 mg kg^{-1}. Except where dietary requirement (Gatlin and Wilson 1984) or Se toxicity (Hilton et al. 1980) have been evaluated, previous feeding trials have not exceeded 2 mg supplemental Se kg^{-1} diet (Julshamn et al. 1990, Wise et al. 1993, Wang et al. 1997, Lovell and Wang 1997). Se at the highest dietary concentrations employed did not result in any negative production-related impact (growth, FCR) upon experimental animals. It is possible that the lack of significant effects upon measured parameters resulted due to the high endogenous Se in fish meal, which was approximately five times the requirement level of 0.3 mg/kg (Gatlin and Wilson 1984).

Discussion

In hybrid striped bass, maximum Se accumulation was observed in the liver (3.63 mg kg^{-1}) for the highest dose of supplemental Se employed. A linear dose response was observed once the threshold level of 0.2 mg Se kg^{-1} was surpassed. Similar levels of liver Se have been

reported for salmonids fed supplemental sodium selenite, DL-selenomethionine and DL-selenocysteine at concentrations of ~ 1 mg kg^{-1} diet (Bell and Cowey 1989, Karnezos 1997), although Lorentzen et al. (1994) observed greater hepatic Se accumulation (4-10 mg kg^{-1}) following 8-week feeding of Atlantic salmon with a 1-2 mg selenite or selenomethionine kg^{-1} diet. The increase in muscle Se in hybrid striped bass was linear with dose, and highest tissue concentrations (1.09 mg kg^{-1}) were measured following addition of 3.2 mg kg^{-1} diet as organic selenium. Similar values of Se build up, at like doses, have been reported for channel catfish and Atlantic salmon (Gatlin and Wilson 1984, Lorentzen et al. 1994), and the dose-response nature of dietary Se accumulation has been commented on previously (Lovell and Wang 1997, Gatlin and Wilson 1984, Karnezos 1997). Differences of Se accumulation observed in this study compared to previous studies are mostly likely due to differences in species and age, and perhaps temperature and/or culture systems.

Irrespective of these differences, however, comparison of hepatic and muscle Se levels indicated that muscle is a better indicator of dietary Se dose than does the liver. No differences were discerned with respect to tissue Se accumulation and the form delivered (i.e., organic or inorganic). This finding contrasts to earlier reports (Bell and Cowey 1989, Lorentzen et al. 1994, Paripatananont and Lovell 1997, Lovell and Wang 1997) which indicated higher tissue accretion for dietary organic Se in fish. It has been hypothesized that the enhanced bioavailability of organic Se, such as selenomethionine, occurs because it is transported across the gut in an intact form to target tissues (Ashmead and Zunino 1992). The lack of increased organic Se accumulation observed during the present study relative to inorganic Se may reflect the doses used since previous research employed lower concentrations of total dietary Se. Alternatively, other factors, including dietary ingredients, hepatic metabolism, and prevailing gut pH, may have

negatively impacted the absorption, transport, and tissue accumulation of the organic form (Meltzer et al. 1993, Fox et al. 2004, Suzuki 2005).

Fish and other livestock can accumulate dietary Se, and even though certain Se-enhanced meat products are available in the marketplace (Cole 2000), some scientists question Se bioavailability from meats (Bugel et al. 2003). Indeed, it is now understood that total Se presence does not correlate directly to bioavailability; rather, it is the proportion of individual Se-containing compounds present that determines total availability. Of the 30 or so known selenoproteins (Beckett and Arthur 2005), the physical or chemical form of each is believed to affect absorption, availability and retention. For humans, the recommended daily intake level for Se falls in the range of 0.006-0.07 mgs d^{-1} (6-70 ug d^{-1}) depending upon age and physiological status (FAO/WHO 2002, IOM 2000). This dose however, may be significantly increased, up to 0.4 mgs d^{-1} (400 μg d^{-1}), for individuals suffering from illness, gastrointestinal disorders or natural Se deficiency (IOM 2000). Se availability and absorbtion of edible fish flesh (Fox et al. 2004), is high, with an absorption and retention efficiency of 88[%] and 85[%] respectively, even following cooking or storage (Akesson and Srikumar 1994, Fox et al. 2004). Using Se accumulation levels from Table 3 and absorption and retention efficiencies listed above, a 100g portion of Se-enhanced hybrid striped bass fillets from the present study therefore, would contain between 33-109 μg Se, amounting to a dietary intake of between 25-80 μg Se; a level that would satisfy present daily intake recommendations. Production of Se-fortified fillets would require feeding of a Se-containing finishing diet for 6-8 weeks.

In summary, HSB can bioaccumulate Se in their muscle and hepatic tissues leading to the potential production of a designer food for the HSB industry. In the present study, there were no observed differences in bioaccumulation rate between organic and inorganic forms of Se. This

was mostly likely due to endogenous levels of Se in the fishmeal based diets being high. From this data, muscle accumulation appears to be a better indicator of dietary Se status, based on correlation coefficients. Additional studies must be undertaken to determine optimum dietary dose(s). Even at the highest inclusion rates, which resulted in dietary Se levels of 4.4 mg/kg, there were no detrimental impacts on production characteristics or fish health.

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Table 2.1 Composition of the basal diet used in the present study. Experimental diets All diets provided 40% crude protein, 10% lipid and supplied 3.30 kcals/g. The fish meal contained 1.2 ppm Se. The organic selenium source, Selplex[®e], was added to experimental diets at the expense of cellulose.

Ingredient (% dry matter basis)	
Fishmeal[a]	58.8
Dextrin[c]	20.0
Lipid (Menhaden Oil)[b]	4.1
Mineral (Se free)[d]	4
Vitamin[e]	3
Carboxymethyl Cellulose (CMC)[c]	1
Selplex®[f]	0
Cellulose[c]	9.1
Selenium premix[g]	0

[a] Special Select® menhaden fish meal, Omega Protein, Inc., Hammond, LA, USA. 68% crude protein (dry); 0.915 dry matter.

[b] LCP ethoxyquin-free Omega Oils, Reedville, VA, USA.

[c] US Biochemical Corporation, Cleveland, Ohio, USA.

[d] Contained (g/5kg): monocalcium phosphate 680, calcium lactate 1742, ferrous sulfate 25, magnesium sulfate $7H_2O$ 660, dipotassium phosphate 1200, monosodium phosphate 440, sodium chloride 225, aluminum chloride 0.750, potassium chloride 0.750, copper sulfate 2.5, manganous sulfate 3.5, cobalt chloride 5, zinc sulfate $7H_2O$ 15.(MP Biomedicals, Aurora, OH, USA).

[e] Contained (g/kg): ascorbic acid 50.0, dl-calcium pantothenate 5.0, choline chloride 36.2, inositol 5.0, menadione sodium bisulfite 2.0, niacin 5.0, pyridoxine HCL 1.0, riboflavin 3.0, thiamine mononitrate 0.5, dl-alpha-tocopherol acetate (250 IU/g) 8.0, vit. a palmitate (500,000 IU/g) 0.2, vitamin micro-mix 10.0, cellulose 874.1. micromix contained (g/mg) biotin 0.50, folic acid 1.8, vitamin b12 .02, cholecalciferol (40 IU/ug) 0.02, cellulose 97.66.

[f] Selplex® (Alltech Inc., Nicholasville, KY, USA)

[g] Selenium premix: 0.1 g sodium selenite (Sigma Chemical Co., St. Louis, Missouri, USA) and 499.9 g cellulose.

Table 2.2 Weight and length gain, condition factor (CF), food conversion ratios (FCR) and blood parameters of hybrid striped bass fed diets supplemented with various doses (0-3.2 mg kg^{-1}) of organic (Selplex$^{®}$) and inorganic (sodium selenite) selenium. Different superscripts in a column indicate significant differences between treatments (P $\leq$ 0.05).

Supplemental Se (ppm)	% Cum ↑ weight	Weight SGR	CF	FCR	PCV[a]	PP[b]
0.0	68.5^{abc}	1.2^{abc}	1.6^{b}	1.3	55	9.0
0.1 organic	57.1^{c}	1.1^{c}	1.7^{b}	1.9	52.7	8.9
0.2 organic	78.6^{a}	1.4^{a}	1.7^{ab}	1.4	55.0	9.5
0.4 organic	61.4^{bc}	1.1^{bc}	1.8^{a}	1.6	56.1	9.2
0.8 organic	64.5^{abc}	1.2^{abc}	1.7^{ab}	1.6	54.2	9.0
1.6 organic	72.7^{ab}	1.3^{ab}	1.7^{ab}	1.8	52.9	9.2
3.2 organic	67.4^{abc}	1.2^{abc}	1.6^{b}	1.8	52.6	9.0
Pooled SE	10.2	0.15	0.11	0.24	3.00	0.67
P < F	0.0360	0.0368	.0540	0.5379	0.5048	0.8475
0.2 organic	78.6	1.4	1.7	1.4	55.0^{a}	9.5^{a}
0.2 inorganic	72.1	1.3	1.8	1.7	49.4^{b}	7.9^{b}
T = value	1.11	1.04	-0.58	-1.94	1.98	3.26
P < F	0.2819	0.3107	0.5717	0.1917	0.0653	0.0050
0.4 organic	61.4	1.1	1.8	1.6	56.1	9.2^{a}
0.4 inorganic	57.0	1.1	1.7	2.4	56.9	8.5^{ab}
T = value	0.58	0.68	2.37	-1.32	-0.44	1.32
P < F	0.5699	0.5077	0.0296	0.3190	0.6645	0.2046

[a] PCV = packed cell volume
[b] PP = plasma protein

Table 2.3 Muscle and hepatic selenium levels, visceral (VSI) and hepatic (HIS) somatic indices of hybrid striped bass fed diets supplemented with various doses (0-3.2 mg kg^{-1}) of organic (Selplex$^®$) and inorganic (sodium selenite) selenium. Different superscripts in a column indicate significant differences between treatments ($P \leq 0.05$).

Supplemental Se (mg kg^{-1} DW)	Muscle Se (mg kg^{-1} DW)	Liver Se (mg kg^{-1} DW)	VSI	HSI
0.0	0.33^e	1.46^e	8.68^b	2.20^b
0.1 organic	0.30^e	1.35^e	9.43^{ab}	2.23^b
0.2 organic	0.32^e	1.37^e	9.93^{ab}	2.20^b
0.4 organic	0.38^d	1.46^d	9.64^{ab}	1.82^c
0.8 organic	0.45^c	2.12^c	10.71^a	2.12^b
1.6 organic	0.55^b	3.02^b	10.07^{ab}	2.14^b
3.2 organic	1.09^a	3.63^a	10.3^a	2.47^a
Pooled SE	0.07	0.12	1.12	0.19
P < F	0.0001	0.0001	0.1313	0.0002
0.2 organic	0.32^e	1.37^{bc}	9.93	2.20^b
0.2 inorganic	0.33^e	1.35^{bc}	9.79	2.10^b
T = value	-1.02	-0.04	0.45	0.98
P < F	0.3309	0.9717	0.6580	0.3403
0.4 organic	0.38^d	1.46^a	9.64	1.82^c
0.4 inorganic	0.35^{de}	1.45^a	9.73	2.25^{ab}
T = value	1.22	0.14	-0.22	0.3403
P < F	0.2496	0.8990	0.8315	0.0082

Table 2.4 Serum ceruloplasmin and lysozyme levels and glutathione peroxidase (GPx) for individual hybrid striped bass fed diets supplemented with various doses (0-3.2 mg/kg^{-1}) of organic (Selplex$^{®}$) and inorganic (sodium selenite) selenium. Different superscripts in a column indicate significant differences between treatments ($P \leq 0.05$).

Supplemental Se (mg kg^{-1} DW)	Ceruloplasmin (IU's)	Lysozyme (μg/ml)	Hepatic GSH-Px (μmol NADPH/ Min / mg protein)
0	51.4^b	363.7^b	0.39^a
0.1	89.8^a	317.2^{ab}	0.34^b
0.2	89.5^a	299.2^b	0.38^b
0.4	41.7^b	300.6^b	0.17^b
0.8	42.6^b	367.0^a	0.19^b
1.6	58.4^{ab}	309.2^{ab}	0.19^b
3.2	26.0^b	340.1^{ab}	0.27^{ab}
Pooled Se	18.9	30.2	0.12
P < F	0.0041	0.0642	0.0242
0.2 organic	89.5	299.2	0.38
0.2 inorganic	79.1	312.4	0.59
T value	0.33	-0.99	-0.95
P < F	0.7486	0.3509	0.3581
0.4 organic	41.7^b	300.6^b	0.17^b
0.4 inorganic	89.5^a	437.0^a	0.78^a
T value	-3.92	-3.48	-2.47
P < F	0.0044	0.0084	0.0249

Figure 2.1 Schematic diagram of life support system employed during studies.
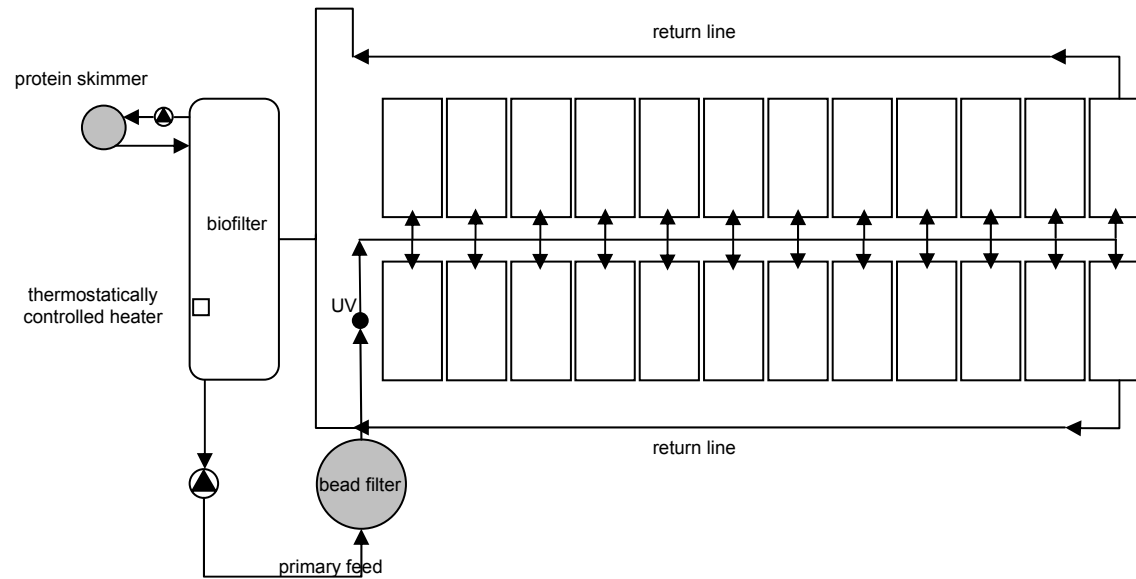
Figure 2.2 Regression of liver selenium levels of hybrid striped bass fed menhaden meal-based diets supplemented with various doses (0-3.2 mg kg^{-1}) of organic (Selplex$^{®}$) selenium. Broken line analysis indicate a breakpoint at 0.10.
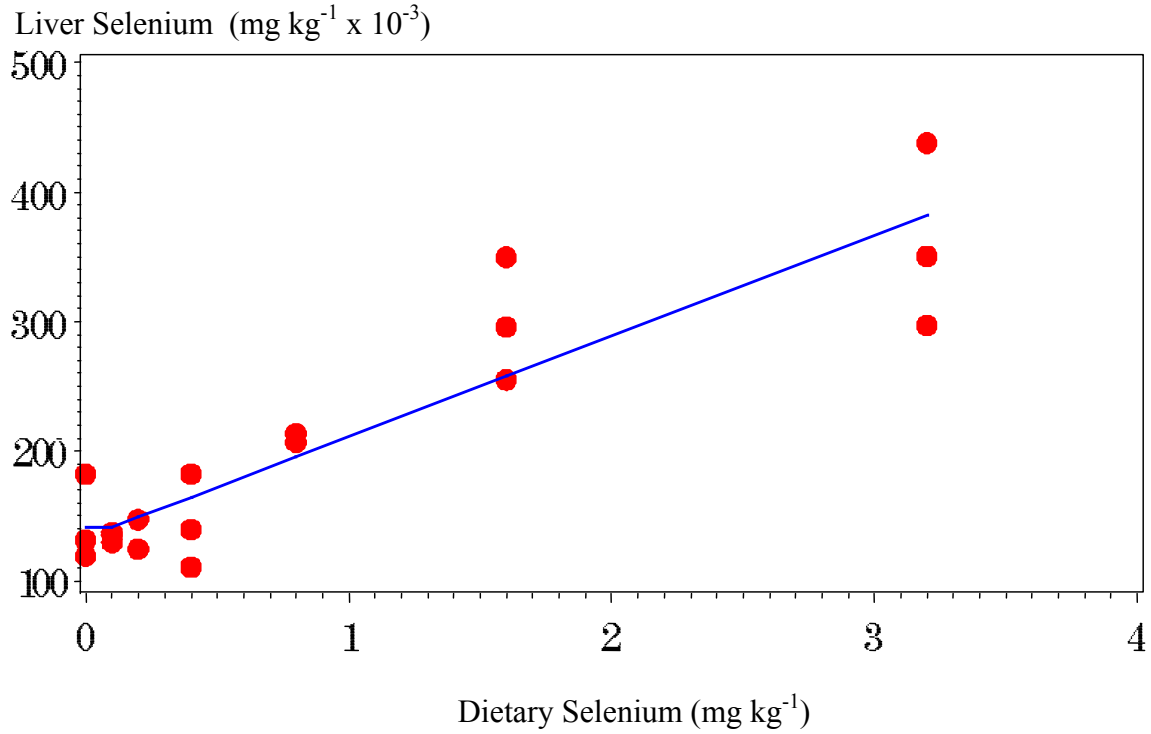
R^2=0.87, Y=1.43X+0.77, Breakpoint=0.10
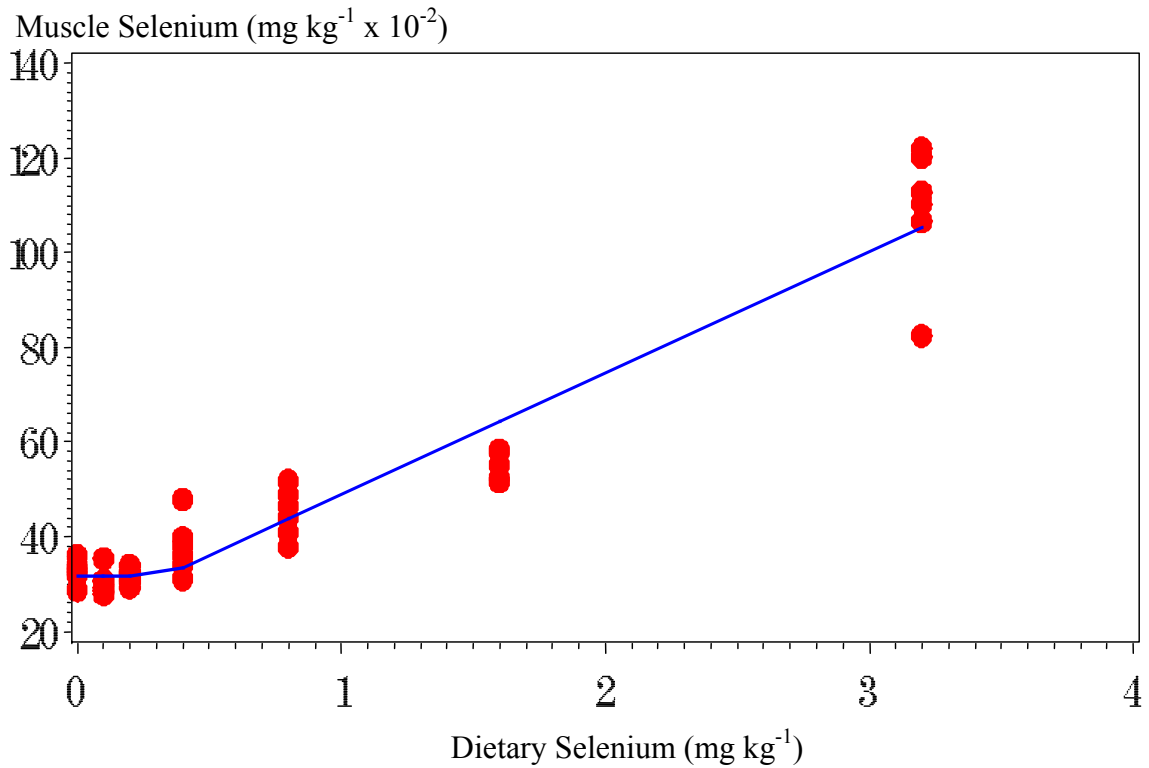
Figure 2.3 Regression of muscle selenium levels of hybrid striped bass fed menhaden meal-based diets supplemented with various doses (0-3.2 mg kg^{-1}) of organic (Selplex®) selenium. Breakpoint analysis reveals a breakpoint at 0.33 mg/kg.

Muscle Selenium (mg kg^{-1} x 10^{-2})

Dietary Selenium (mg kg^{-1})

R^2=0.95, Y=0.32X+0.26, Breakpoint=0.33

Chapter 3

Hepatic and muscular accumulation of dietary selenium in hybrid striped bass

Abstract:

Accumulation of dietary organic and inorganic selenium (1.5 mg Se kg^{-1} dry weight feed) was studied in muscle and hepatic tissues of hybrid striped bass (HSB; ♂ *Morone saxatilis* x ♀ *M. chrysops*; 58.4±19.3 g initial wet wt; 162.3±16.1 mm FL) over a 6 week period. Animals, which had been fed a Se deficient diet (0.1 mg Se kg^{-1}) for 2 months prior to study, were maintained in a recirculating system at 28±1 ^{o}C. PIT-tagged fish were randomly assigned to one of 24 120 L aquaria (n = 5 per tank) and randomly assigned to one of four diets: a fishmeal-based diet (control), soybean-casein-based feeds either supplemented with organic (SelPlex$^{®}$) or inorganic (sodium selenite) Se, or as a basal diet, purposefully deficient in Se. Se suplemented diets and control contained equal amounts of Se (1.5 mg kg^{-1}) Se Fish were fed twice daily on a 4$^{%}$ body weight basis. Samples taken at trial start (week 0), mid-way (3 weeks) and termination (6 weeks). Muscle and hepatic Se levels increased in fishmeal and Se-supplemented diets throughout the study. At trail end, greatest weight gain (P < 0.05) was observed in fish fed the control, fishmeal-based diet. Se levels accumulated at greater concentrations than the muscle. Highest levels were observed in the organic Se-fed fish (P < 0.05). Se accumulation in control and inorganic Se-fed fish was similar, whilst Se in the liver of fish fed the Se deficient diet was lowest among groups (P < 0.05). Likewise, muscle Se accumulation was found to be greatest in organic Se containing diets (P < 0.05), whereas the Se-deficient diet group exhibited lowest Se levels (P < 0.05). Plasma GP$_x$ activity was similar in HSB fed the fishmeal and organic Se diets. The fishmeal-fed group expressed higher (P < 0.05) GP$_x$ levels than those recorded for either the inorganic or basal diets. At trial end, no differences were recorded between groups for PCV or HSI, VSI or IPF. SP levels and MR were higher (P < 0.05) in fishmeal control-fed fish. FCRs were lowest in fishmeal fed-fish and highest for the Se-deficient or basal diet.

Key words: sodium selenite, organic, recirculating aquaculture, glutathione peroxidase, *Morone*

1. Introduction

As aquatic fish feed producers switch to cheaper, more sustainable plant- based sources, the need for mineral supplementation will increase (Davis and Gatlin 1996). Selenium (Se), an essential component of the antioxidant enzyme glutathione peroxidase (Rotruck et al. 1973), also plays a vital role in many key metabolic processes in animals and has many health-associated benefits (Rayman 2002, FAO/WHO 2002). This element is known to influence plant growth (Hartikainen and Xue 1999), but Se requirements for plants are below those assessed for animals. Se must be converted into selenomethionine by plants for it to be utilized by animals (IOC 2000). The Se content of soils and, hence, plants varies with geographical location. On a global basis, this has led to millions of people being in selenium deficiency (Combs 2001). Functional food products have been developed that incorporate Se at elevated levels. Organic Se additives can increase the Se content of eggs, milk, and meat (Hasler 2000, Surai and Sparks 2001, McIntosh and Royle 2002, Hintze et al. 2002, Yaroshenko et al. 2003, Heard et al. 2004). Similar mineral-enriched aquacultured products are presently unavailable although efforts to incorporate additives in fishfeed have begun (Chapter 2; Julshamn et al. 2006). Functional fish products could be marketed for niche markets to increase the range of aquacultured produce available, and help stabilize the industry.

The potential for fish fillet accumulation of Se has been established for Atlantic salmon and hybrid striped bass (Lorentzen et al. 1994, Chapter 2) and the associated market potential considered. Optimal dietary inclusion levels of organic and inorganic Se and uptake in muscle accumulation was established at 1.6 mg Se kg^{-1} dry weight of feed. However, problems were encountered with respect to the presence of Se in the fish meal component of the diet which may have been high enough to mask beneficial effects of Se to the cultured animal. Accordingly, in

the present study, diets were manufactured to be deficient in Se while fish were placed into Se deficit by feeding Se-depleted diets prior to the study's start. A commercially available selenomethionine product (Selplex[®]) derived from yeast, which contained approximately 50[%] selenomethionine, 15[%] selenocysteine and 35[%] as other seleno-amino acid forms was used as the organic form of Se (Karnezos 1997). There is evidence to suggest that organic selenium supplementation enhances the texture and taste of meat in animals (Lyons 1998, Cole 2000, Thomas and Buchanan 2006). Enhanced dietary Se may benefit health of cultured animals (see: Huntingford et al. 2006), because of the immunological benefits of dietary Se. For comparison purposes, the absorption and accretion of organic Se was judged against the accretion of sodium selenite, an inorganic Se source. Sodium selenite represents the traditional form of dietary Se supplementation in poultry and livestock, primarily due to its low cost (Newman 2004).

Usually, feeds for younger growing fish are high in protein (>500 g/kg) with moderate energy (<20 MJ/kg), whereas older fish are fed high nutrient dense (HND) diets that are lower in protein (400-450 g/kg) and higher in fat content to provide more energy (>20 MJ/kg) (Webster and Lim 2002). A fundamental constraint of HND diets is due to the limited formulation flexibility to compensate for non-nutritional material (Glencross 2005). However, a high protein (48-60%) and low carbohydrate (10-14%) and low lipid (<15%) diet fortified with vitamins and trace elements has proven suitable for initial feeds for cod diets (Lall and Nanton 2002).

As the need for alternative protein sources increases, anti-nutritional factors found in plant protein sources may hinder the absorption of nutrients (Francis et al. 2001). Organic forms of minerals have proven to be more potent due to greater digestibility than their inorganic counterparts. While chelated minerals are widely used in the livestock industries, there is little data on their use in fishes (Sarker et al. 2005, Table 1.4). Chelated (organic) compounds protect

the mineral from interactions with other minerals and compounds in the digestive tract (Ashmead 1993). Se absorption is affected by interactions with heavy metals such as mercury and arsenium, a high methionine content of the diet, levels of tissue saturation, different chemical forms of Se, environmental and health conditions of the animal, and oxidative stress from polyunsaturated fatty acids (Meltzer et al. 1993). Chelated compounds are transported intact to target tissues and are therefore more available for metabolic processes (Paripatananont and Lovell 1997) due to their higher stability (Hynes and Kelly 1995). They are also less likely to be broken down by the high acidity of the stomach (Davis and Gatlin 1996). Selenomethionine, if not converted into selenocysteine for selenoprotein synthesis, can be incorporated into many organs and tissues such as muscle, due to it's structural similarity to methionine (Jacques 2001). However, inorganic Se, when not immediately utilized by the liver for selenoprotein synthesis, is quickly eliminated (Jacques 2001). The preceding may provide partial explanation for the observed differences in muscle Se accumulations between organic and inorganic supplies observed here.

2. Materials and methods

2.1 Experimental system

Eleven month-old juvenile hybrid striped bass ($\male$ *Morone saxatilis* x $\female$ *M. chrysops*; 58.4±19.3 gms initial wet wt; 162.3±16.1 mm fork length), purchased from a commercial hatchery (Keo Fish Farms, AR, USA) were PIT-tagged (Biomark Inc., Boise, ID, USA) and randomly stocked into one of 24 tanks (n=6 tank^{-1}) of a custom designed, recirculating life support system (RALS). The 3400 L recirculation configuration (flow rate = 4 L/min) comprised twenty-four 100-L glass aquaria serviced with a 750 L KMT-based (Kaldnes Miljoteknologi, Tonsberg, Norway) fluidized bed biofilter, a bubble-bead filter (Aquaculture Technologies Inc., Metarie, LA, USA) for solids removal, a protein skimmer (R&B Aquatics, Boome, TX), and a 40-watt UV sterilizer

(Emperor Aquatics, Pottstown, PA). The fluidized bed was oxygenated using diffusion air lines connected to a 1-hp Sweetwater remote drive regenerative blower (Aquatic Ecosystems, Apopka, FL.). The aquaria had black-painted walls to reduce fish stress.

2.2 Water quality parameters

DO_2 (range: 6.9±0.3 mg l^{-1}) and pH (8.2±0.2) were monitored daily using an Y85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH), and Hanna Instrument 9024 (Aquatic Ecosystems, Apopka, FL) Total ammonia nitrogen (TAN; range: 0.08-0.70 mg l^{-1}) was monitored daily by spectrophotometric analysis (Hach, Inc., Loveland, CO, USA). Nitrite (range: 0.08-0.19 mg l^{-1}) and nitrate (range: 2.0-56 mg l^{-1}) levels were quantified once weekly. Salinity was maintained at 5 g l^{-1} and measured with a refractometer. A thermostatically controlled heater was placed in the biofiltration sump to maintain water temperature at 28±1 °C. Fish were exposed to a 12:12 photophase:scotophase through fluorescent lighting positioned 2.5 m above the RALS.

2.3 Diet preparation and feeding

Four diets: fishmeal-based control, organic Se-supplemented, inorganic Se-supplemented, and Se-deficient basal diet (table 3.1), were fed to tanks in triplicate for 6 weeks at 4% body wt d^{-1} as two separate feedings (08.00 and 16.00 h). Prior to initiation of the trial, fish were fed the Se-deficient basal diet (0.1 mg kg^{-1}) for 2 months. Crude protein and lipid levels were formulated at 40% and 10%, respectively. All known nutritional requirements of hybrid striped bass were met by the experimental feeds (NRC 1993), with the exception of the basal diet for Se. All ingredients were screened for Se content, and diets were formulated to contain 1.5 mg Se kg^{-1} total and a basal diet of 0.15 mg Se kg^{-1}, representing 50% of the Se requirement of striped bass (NRC 1993). To avoid masking effects of vitamin E upon response, all diets were formulated to

contain 24 mg vit. E kg^{-1}, just below the 28 mg vit. E kg^{-1} requirement of hybrid striped bass (Kocabas and Gatlin 1993). Vitamin E levels were determined by HPLC (Agilent Technologies 1100 Series) at Virginia Tech's College of Veterinary Medicine Toxicology Laboratory. Menhaden oil (Omega Protein Co., Reedville, VA) was used to meet the HUFA requirements of hybrid striped bass (Nematipour and Gatlin 1993). Prior to experiment start, animals were maintained for a period of 2 months on a Se-deficient diet (basal diet, Table 3.1) in order to avoid any possible masking effects of stored (tissue) Se upon animal responses.

Dietary components were mixed in a Patterson-Kelley twin shell® batch V-mixer (Patterson-Kelley Co., Inc., East Stroudsburg, PA) for 20 minutes and then homogenized into a paste by adding menhaden fish oil into a food mixer. The amount of distilled water required for pelleting (10-30% of feed weight) was then added to the mixture and further homogenized. The paste was extruded through a Hobart D300 Floor Mixer (Hobart Co., Troy, OH) without steam, using an appropriate die to provide pellets of suitable size for the fish. Duplicated samples from each feed were heated at 135 ^{o}C for 2 hrs in a gravity oven (Blue M Electric, Blue Island, IL) to determine dry matter. After air-drying overnight, feed was frozen at -10 ^{o}C until needed. Prior to use, small quantities of feed were thawed and refrigerated.

2.4 Data acquisition

Feed conversion ratio (FCR) and survival were noted throughout the study. At trial termination, all animals were weighed and measured (FL), and their condition factor (CF) calculated according to the following formula:

$$CF = (weight/length^3)*1000$$

Visceral somatic index (VSI), and muscle (MR) indices were calculated using the following formula:

$$\text{Somatic index} = (\text{tissue weight \{g\}/wet weight \{g\} of animal})*100$$

The hepatosomatic index (HSI) and intraperitoneal fat ratio (IPF) was calculated using the following formula:

$$\text{Somatic index} = (\text{tissue weight \{g\}/visceral weight \{g\} of animal})*100$$

Fish were individually weighted and measured every two weeks to adjust feeding rations. At trial start, midway (3 weeks) and final (6 weeks) fish (n=6) were euthanized by an overdose of clove oil (0.6 ml l^{-1}; Sigma Chemical Co., St. Louis, MO., USA) and dissected to remove internal organs to determine visceral somatic index (VSI), liver for determination of hepatosomatic index (HSI) and filleted for determination of muscle ratio. Samples of muscle (min. 0.5g) and liver (min. 0.25g) (n=6) were analyzed for Se by Virginia Tech's Veterinary Toxicology Laboratory by atomic absorption spectrophotometry (Varian SpectraAA 220FS) using the VGA (Vapor Generation Assembly) method.

During clove oil anesthesia, fish were bled from the caudal artery-vein complex using 2 ml heparinized syringes. Collected blood was transferred into Eppendorf 2-ml microcentrifuge tubes. Blood packed cell volume (hematocrit) was assessed immediately following collection. Blood was drawn into microhematocrit tubes (Fisher Scientific, Pittsburgh, PA), sealed with Cristoseal (Fisher), centrifuged at 10,000 x g for 5 min (M 24 Micro-Hematocrit Centrifuge; LW Scientific, Lawrenceville, GA), and hematocrit read using the centrifuge's combo reader. Remaining blood samples were transferred to a refrigerator maintained at 5 ^{o}C and allowed to clot overnight. Resultant serum was stored in 2-ml Eppendorf tubes at -20 ^{o}C until further analyses.

Serum samples were employed to evaluate protein levels using a hand-held VET360 temperature-compensated refractometer (Leica Optical Products Division, Buffalo, NY) and

protein assay kit (Bio-Rad, Hercules, CA.; Lowry et al. 1951). As a means of establishing retained biological activity of absorbed Se, glutathione peroxidase activity was quantified for serum samples using a commercial assay (Sigma-Aldrich, St. Louis, MO).

2.5 Waterborne Selenium

To examine the possibility of waterborne Se uptake through the gills, concentrations of waterborne Se also were analyzed by Virginia Tech's Veterinary College Toxicology laboratory. Initial Concentrations of Se were measured as 0.006 ppm, midway (3 weeks) at 0.002 ppm, and final (6 weeks) at 0.003 ppm.

2.6 Statistical Analyses

Liver and muscle Se concentrations, FCE, CF, weight, length, hematocrit and PP were analyzed for statistical differences at $P < 0.05$ (SAS Inc., Cary, NC, USA). Statistical analyses were conducted using Anova and Duncan's multiple range test. Levene's test for homogeneity of variance was used to test for normality.

3. Results

All fish grew both in weight and length during the experimental period (Table 2). However, differences ($P < 0.05$) in weight gain were recorded between fishmeal or control diet-fed animals (average 107% increase) and all other treatments (Table 3.2). Final weight gains in organic Se-supplemented feed, inorganic Se-supplemented feed and basal diet-fed fish were 17%, 16.3% and 9.8% above start weights, respectively. FCRs of 4.8, 5.2, 6.3 and 8.2 were observed for control, organic Se, inorganic Se and basal diet, respectively. Survival was 74%, 78%, 56% and 64% for control, organic Se, inorganic Se and basal diet, in that order. Length gain was also significantly higher ($P < 0.05$) in hybrid striped bass fed the control or fishmeal-based diets when compared against all other groups. The latter expressed equivalent length gain (Table 3.2). VSI, HSI and

IPF were similar across all treatments ($P > 0.05$). Muscle ratio (MR) was identical for all non-fishmeal fed groups with control fed fish expressing higher MRs ($P < 0.05$).

Data relating to differences in PCV, serum protein and glutathione peroxidase (GP_x) activity and muscle and hepatic Se levels are presented in Table 3.3. GP_x activity was similar for control and organic Se-based diets, and for inorganic Se-based diets similar to that recorded for fish fed the basal diet. Control GP_x activity was however greater ($P < 0.05$) than inorganic Se and basal diets while GP_x activity of the organic Se-based diet was higher ($P < 0.05$) than that observed in the basal diet. PCV did not differ across treatments but PP levels were higher ($P < 0.05$) in control than all other treatments (Table 3). FCRs were significantly higher ($P < 0.05$) in fish fed the Se deficient diet when compared to all other groups.

Feeding experimental animals upon Se deficit diets for 2 months prior to experimental start resulted in fillet and hepatic Se levels of 0.09 ± 0.012 and 0.292 ± 0.084 mg Se kg^{-1} tissue respectively. Following feeding of Se-supplemented or fishmeal-based diets, hepatic Se levels increased but continued to decline in fish maintained on the Se deficient diet (Table 3.3). By trial end, liver Se levels were of the order fishmeal = inorganic selenium > organic Se ($P < 0.05$) > basal diet ($P < 0.05$). Muscle Se also increased following feeding of fishmeal, organic and inorganic Se supplemented diets but continued to decline in hybrid striped bass maintained upon the basal, Se deficient diet. By trial end, muscle Se levels were of the order organic Se > fishmeal ($P < 0.05$) > inorganic Se ($P < 0.05$) > basal diet ($P < 0.05$) (Table 3.3).

4. Discussion

In the present study, muscle and hepatic Se levels decreased following a period of 2 months on Se-deficient diets. The recorded decrease represented a 3- and 5-fold decline when compared against previous studies (Chapter 2). Accordingly, the reaction of experimental animals to dietary manipulations can be considered tangible, especially given the reduction in dietary vitamin E. The Se deficient diet did not exhaust body Se levels since fish maintained upon the basal diet still retained measurable concentrations of Se in both the liver and muscle at trial termination. While growth retardation was noted in Se-deficient hybrid striped bass, no differences emerged with respect to weight gain when compared to fish fed Se-supplemented diets. The depressed growth observed in the fishmeal-free diets likely reflected poor performance of animals maintained on soybean meal-based feeds, an observation that has been made previously (Brown et al. 1997, Keembiyehetty and Gatlin 1997). Nevertheless, the reduced GP_x activity and deficiencies in hepatic (3-fold) and muscle (2-4-fold) Se when compared to control and Se supplemented diets suggest that these animals were Se deficient. Indeed, tissue Se levels and GP_x activity were considerably lower in hybrid striped bass than that recorded for channel catfish deprived of dietary Se for 9 weeks (Wang and Lovell 1997).

The most rapid increase in selenium concentration of hybrid striped bass fillets occurred in fish receiving diets containing organic Se, whereas for the liver, concentrations were highest in fish fed control and inorganic Se diets. These results illustrate organic dietary Se sources readily accumulates in the edible component of the fish. Organic Se supplementation is more effective in increasing the Se muscle content of channel catfish and Atlantic salmon (Lorentzen et al. 1994, Wang and Lovell 1997). Unlike previous observations (Chapter 2), organic Se accumulated at levels above that recorded for inorganic Se. This likely occurred due to the 2-

month feeding of fish on a Se-deficient diet. Accordingly, the results of the present work vindicate the inference in Chapter 2 of this thesis i.e., that the presence of Se in fish meal masked differences in Se accumulation. Hepatic accumulation of Se was strongest in the fishmeal-based diet, but significant differences between treatment groups in terms of Se presence were not recorded until trial end. This result was similar to that of a 4-week study with Atlantic salmon where no increased hepatic absorption of organic Se was found (Bell and Cowey 1989). These authors hypothesized that this resulted due to the short duration of the study. Results from the present trial would appear to corroborate this supposition. Liver Se accumulation also was delayed for a 4-week period (Karnezos 1997). Hepatic Se accumulation was found to be highest for selenite than for selenomethionine-supplemented diets in a 10-week trial with Atlantic salmon (Lorentzen et al. 1994). Our data substantiate this view but contrast to studies with channel catfish, in which liver Se saturation was achieved more rapidly with organic selenium supplementation (Wang and Lovell 1997). High FCRs and poor survival during this study were likely due to Se deficiency induced in these fish prior to study start. An indication of this possibility was provided by the apparent fragility of erythrocytes.

Organic Se diet supplementation enabled producers to accumulate Se in edible tissues such as eggs and increase fertility and hatchability in chickens (Edens 2002). Despite its prooxidant affect, sodium selenite is the traditional form of selenium supplementation of poultry and livestock due to its low cost (Newman 2004). Is there an "organic advantage" for selenium that is revelant for aquaculture? Current federal regulations limit Se supplementation to 0.3 ppm from both organic and inorganic sources for the livestock industry; however this currently does not extend to aquaculture (FDA 2004).

As global aquaculture production increases, feed waste from farming facilities will become of increasing concern (Sarker et al. 2005). Due to the greater absorption of chelated minerals, the mineral requirement of fish may be reduced by as much as $50^{\%}$, thereby reducing dietary pollution potential which may lead to eutrophication of receiving waters (Apines-Amar 2004, Talbot and Hole 1994). Feeds should not contain nutrients greater that can be successfully utilized by the animal to minimize environmental discharge (Sugiura 2000). Results with hybrid striped bass and organic Se accumulation provides an indication of the benefits of utilizing organic mineral sources for the production of possibly environmentally cleaner aquafeeds (i.e., decreased mineral release/pollution; Cho et al. 1994). Moreover, the welfare of production animals may be enhanced by using supplemental organic Se since greater immune enhancing effects than traditional inorganic forms have been reported for trout and channel catfish (Lovell and Wang 1997, Wang et al. 1997, Karnezos 1997).

Since the addition of Selplex® would only increase the price of a ton of commercial feed by only ~$1.50, it seems apparent that this feed additive would be cost effective in grow out diets during the length of the production run. Utilizing this strategy, the obvious health benefits transferred not only to the fish but also, potentially to the consumer, by organic Se supplementation would be realized. This could increase the economic viability of a HSB production facility not only through decreased losses due to disease but also through increased prices for a designer product.

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Table 3.1

Composition of four experimental diets (% on a dry matter basis) with 40$^{\%}$ Crude Protein; 10$^{\%}$ lipid; 314 kcals/g.

Ingredient	Control	Inorganic Se	Organic Se	Basal
Fishmeal[a]	69.1	0	0	0
Soybean Meal[b]	0	45.1	45.1	45.1
Casein[c]	0	17.3	17.3	17.3
Dextrin[c]	16	16	16	16
Lipid[d]	3.4	9.3	9.3	9.3
Minerals[e]	4	4	4	4
Vitamins[f]	3	3	3	3
CMC[c]	1	1	1	1
Se premix[g]	0	0.8	0	0
Selplex[®h]	0	0	0.3	0
E premix[i]	2.1	2	2	2
Cellufil[c]	1.5	1.5	2	2.3

[a] Menhaden fishmeal, Omega Protein, Inc., Houston, Texas, USA.

[b] Lipid extracted with hexane

[c] Carboxymethyl cellulose, US. Biochemical Corporation, Cleveland, Ohio, USA.

[d] Menhaden fish oil, ethoxyquin free, Omega Protein, Inc., Reedville, VA, USA.

[e] Se-free. MP Biomedicals, Aurora, OH, USA. Contained (g/5kg): monocalcium phosphate 680, calcium lactate 1742, ferrous sulfate 25, magnesium sulfate 7H$_2$0 660, dipotassium phosphate 1200, monosodium phosphate 440, sodium chloride 225, aluminum chloride 0.750, potassium chloride 0.750, copper sulfate 2.5, manganous sulfate 3.5, cobalt chloride 5, zinc sulfate 7H$_2$0 15.

[f] E-free, vitamins obtained from Sigma Chemical Co., St. Louis, MO. Formulated to contain (g/kg): ascorbic acid 50.0, dl-calcium pantothenate 5.0, choline chloride 36.2, inositol 5.0, menadione sodium bisulfite 2.0, niacin 5.0, pyridoxine HCl 1.0, riboflavin 3.0, thiamine mononitrate 0.5, dl-x-tocopherol acetate (250 IU/g) 8.0, vit. A palmitate (500,000 IU/g) 0.2, vitamin micro-mix 10.0, cellulose 874.1. micromix contained (g/mg) biotin 0.50, folic acid 1.8, vitamin B12 .02, cholecalciferol (40 IU/ug) 0.02, cellulose 97.66.

[g] Sodium selenite, Sigma Chemical Co., St. Louis, MO. (0.1g/499.9 g cellufil, 200ppm)

[h] Alltech Inc. (600 ppm, Nicholasville, KY)

[i] x-tocopherol, Sigma Chemical Co., St.Louis, MO. (0.5 e/499.5 g Cellufil, 1000ppm)

Table 3.2

Weight and length gain and morphological characteristics of experimental groups fed diets varying in selenium content and type: at study start (baseline), following 2 months feeding on a Se-deficient diet, 3 weeks post-trial initiation, and at trial end (6 weeks). Data that differed significantly are indicated by superscript column-wise for specific time points. ND = not detectable; $n \geq 6$ per data point.

Treatment	Week	Weight	Length	CF	VSI	HSI	IPF	MR
Baseline	0	58.4±19.3	162.3±16.1	1.32±0.12	11.0±1.1	14.5±2.6	ND	13.9±3.4
Fishmeal		82.0±17.7[a]	178.9±14.8	1.42±0.16[a]	10.8±1.0	22.7±4.9	39.5±6.7	15.6±5.1
SelPlex®	3	75.9±19.9[ab]	178.4±13.5	1.31±0.14[ab]	10.7±1.5	17.4±2.1	42.1±7.7	13.1±4.2
Inorganic		77.5±25.1[ab]	185.3±36.8	1.25±0.25[b]	10.8±0.8	19.7±4.1	39.1±7.0	16.1±6.5
Soybean		60.2±25.6[b]	167.4±24.7	1.20±0.09[b]	11.0±1.2	15.4±3.7	37.3±8.5	16.4±5.1
Fishmeal		121.0±29.5[a]	198.1±13.0[a]	1.53±0.14[a]	9.9±0.6	16.6±3.3	35.6±5.1	13.0±2.5[a]
SelPlex®	6	79.9±24.0[b]	181.9±13.4[b]	1.29±0.11[b]	9.8±1.2	13.5±3.2	33.6±9.5	11.0±2.1[b]
Inorganic		67.9±31.8[b]	173.1±21.6[b]	1.23±0.10[b]	10.1±2.3	15.4±6.3	31.7±10.1	9.8±2.1[b]
Soybean		64.1±13.6[b]	174.1±11.7[b]	1.20±0.07[b]	10.3±2.0	14.1±2.5	29.3±7.6	10.7±1.9[b]

Table 3.3

Blood parameters, glutathione peroxidase (GP_x) activity (nmol mg^{-1} protein min^{-1}) and muscle and hepatic Se accumulation (µg g^{-1} tissue) of experimental groups fed diets varying in selenium content and type: at study start (baseline) following 2 months feeding on a Se deficient diet, 3 weeks post-trial initiation and at trial end (6 weeks). Data that differed significantly are indicated by superscript column-wise for specific time points. NR = not recorded; $n \geq 6$ per data point.

Treatment	Week	PCV	PP	GP_x	Muscle Se	Hepatic Se
Baseline	0	32.1±5.5	6.0±0.8	0.03±0.03	0.09±0.012	0.29±0.08
Fishmeal		36.2±6.7[ab]	7.4±1.0[a]		0.14±0.05[c]	0.75±0.16[b]
SelPlex®	3	31.7±4.2[b]	6.5±0.9[ab]	NR	0.18±0.04[b]	0.71±0.21[b]
Inorganic		35.2±7.3[ab]	6.7±0.9[ab]		0.10±0.01[a]	0.82±0.11[b]
Basal		37.5±6.9[a]	6.2±1.4[b]		0.09±0.01[a]	0.42±0.07[a]
Fishmeal		38.5±7.5	7.3±1.1[a]	0.35±0.2[a]	0.19±0.02[a]	1.37±0.26[b]
SelPlex®	6	32.7±5.9	5.8±0.7[b]	0.29±0.37[ab]	0.24±0.05[b]	1.05±0.30[c]
Inorganic		28.7±5.8	5.8±0.8[b]	0.12±0.12[bc]	0.10±0.01[c]	1.33±0.49[b]
Basal		27.8±10.3	5.0±1.4[b]	0.09±0.18[c]	0.07±0.01[d]	0.47±0.14[a]

Chapter 4

Vaccination of hybrid striped bass: growth, immune reaction and gene expression

Abstract

Hybrid striped bass (42.6±4.9 gms initial wet wt; 139.3±6.1 mm length), were randomly stocked into one of 6 tanks (n=6 tank^{-1}) of a custom designed recirculating aquaculture system (RAS). Tanks were randomly paired and fish either left untreated, vaccinated, or placebo injected. The vaccine employed was an experimental formalin-killed *Streptococcus iniae* oil-in-water adjuvanted bacterin. Livers were prepared for microarray evaluation using standard techniques using a *Danio rerio* gene chip to assess global changes in gene expression. No differences were observed of final weights, lengths, CF, FCR, or HSI, although differences ($P < 0.001$) were determined for VSI, which was higher in control animals. Packed cell volume and serum protein levels were similar across groups ($P > 0.05$). Time-course of changes in serum lysozyme activity exhibited an initial reduction in lysozyme activity, followed by a reboundiwhich peaked 25 days post-treatments. Evaluation of lysozyme activity among time-points revealed differences ($P < 0.05$) between 11 and 25 days post-vaccination. Examination of the hepatic microarray datasets revealed only four immune-discrete genes that were impacted 53-days following vaccination when compared against control fish. These included the up-regulated TCIRG1, or T-cell immune regulator 1 ($P < 0.0467$) and IL20RA, or interleukin 20 receptor alpha ($P < 0.0433$), and down-regulated cytokine inducible kinase, plk3 ($P < 0.01$) and mouse immune responsive protein, IRG1 ($P < 0.01$). Vaccination therefore, may provide greater benefit than simply elevating the protective response to a single pathogen.

Key words: lysozyme, transcriptomics, microarray, *Morone*, *Streptococcus iniae*

1. Introduction

Vaccination has become increasingly important to aquaculture as a means of minimizing losses from and preventing the spread of disease. Indeed, it has been suggested that almost every farmed salmon is vaccinated against several key pathogens (Engelstad 2005). Such strategies improve not only the welfare (see Huntingford et al. 2006) of the cultured animal but also its plate safety (i.e., residue-free status). Other than providing protection against specific pathogens, vaccination appears to enhance nonspecific immunity in general, thereby increasing the fish's overall resistance to infections (Adams et al. 1988). Vaccination also potentially provides protection for fish that have not been injected, or which have not received the full dose of vaccine, due to "herd immunity"; that is, animals may be protected indirectly due to a reduced incidence of disease in fully vaccinated fish (see Anderson and May 1985 a, b, Garnett 2005). A further benefit of vaccination has been a reduction in use of antibiotics and concomitant diminution of risks associated with the development of antibiotic resistant strains of pathogens (Alderman and Hastings 1998, Lorenzen and LaPatra 2005). Vaccination also can result in savings for the producer by decreasing costs associated with the purchase of medications in the event of disease. These savings could be reinvested to improve husbandry and productivity (Horne and Robertson 1987).

Along with the many benefits connected with vaccination, however, there are some negatives (Table 4.1). For example, intra-abdominal lesions caused by vaccination may persist throughout a production cycle and result in downgrading of fish at harvest by as much as 50% (Midtyling 1996, Midtyling et al. 1996). The stress experienced by fish during the vaccination process may also have an overall negative effect, albeit short-term, upon the immune system (Dunn et al. 1990, Press and Lillehaug 1995), growth performance (Lillehaug et al. 1992,

Espersen et al. 1999) and appetite (Midtlyng et al. 1996; Table 4.1). Because vaccination increases the value of fish, if mortality does occur post-treatment, greater economic loss is suffered (Rønsholdt and McLean 1999). Other problems relating to vaccines include accidental self injection, which may cause side effects in humans (see Culora et al. 1996, Leira and Baalsrud 1997). This is of major concern especially where a "per fish vaccinated" fee is employed since workers are generally more hurried and less careful during the vaccination procedure.

Irrespective of the problems associated with vaccination, its use will clearly increase. In some instances, vaccines represent the only viable method for the control of specific diseases. Such is the case for *Streptococcus iniae*, which is recognized as one of the most problematic diseases for hybrid striped bass farmers (Shoemaker et al. 2001). In the United States no FDA-approved drug is available to treat the disease and indeed, therapeutics are generally ineffective against this pathogen (Bercovier et al. 1997), possibly due to its intramacrophage existence (Zlotkin et al. 2003). *S. iniae*, which has been recorded in over 30 wild and cultured marine and freshwater species (Buchanan et al. 2005), gains access to the animal via the snout. It causes a systemic disease that spreads rapidly and can result in $50^{\%}$ mortality within 48 h (Evans et al. 2001). The lack of effective treatments has led to several groups examining the efficacy of various drugs and experimental vaccines (Evans et al. 2004, Li et al. 2004, Abutbul et al. 2005, Whittington et al. 2005, Buchanan et al. 2005). These include successful studies with a killed-toxoid enriched *S. iniae* vaccine and a live-attenuated vaccine. However, while traditional evaluations of vaccine effectiveness (dose, efficacy, survival, etc.) have been undertaken, no studies have examined the impact of *S. iniae* vaccination upon other biological and production-related characteristics in fish.

Hybrid striped bass presently represent the fourth most valuable crop and fifth most farmed species in American aquaculture (Harvey 2006). The species generally is farmed in ponds, with some harvested from cage operations. Since hybrids are accommodating to intensive cultivation, an increasing tonnage has been derived from recirculating life support systems (RLSS). A major feature of RLSS is that they permit greater control over environmental and water quality parameters and thereby allow increased stocking density. Occasionally, however, hybrid striped bass, like other species, and especially those reared at higher temperatures and densities, become more susceptible to disease outbreaks. For cultured hybrid striped bass, *S. iniae* has become an increasingly important pathogen, with estimates of losses to the industry exceeding $100 million annually (Shoemaker et al. 2001). Clearly, vaccination against S*treptococcus* infections represents the preferred method of crop protection. However, prior to anticipating the use of approved commercial vaccine formulations against *S. iniae*, farmers must be informed of the possible negative consequences of vaccination upon the performance characteristics of reared fish. Accordingly, the present study examined the effect of an experimental *S. iniae* vaccine upon growth, immunity, and gene expression in hybrid striped bass. Availability of such information would provide the aquaculturist with a more complete portfolio of knowledge with which decision-making (i.e.., to vaccinate or not) could be more easily made.

2. Materials and Methods

2.1 System and animal husbandry

Juvenile hybrid striped bass (♂*Morone saxatilis* x ♀*M. chrysops*; 42.6±4.9 g initial wet wt; 139.3±6.1 mm length), purchased from a commercial hatchery (Keo Fish Farms, Keo, AR, USA) were PIT tagged (Biomark Inc., ID, USA) and randomly stocked into one of 24 tanks (*n*=6

tank^{-1}) of a custom designed RLSS. The RLSS comprised twentfour 120-liter glass tanks that had black-painted walls to reduce fish stress, and incorporated a bubble-bead filter (BBF-2 Aquaculture Technologies, Inc., Metaire, LA, USA) to remove suspended solids, UV light sterilizer (Emperor Aquatics, Pottstown, PA, USA), a KMT fluidized bed with media (Kaldnes Inc; Providence, RI, USA) for biological filtration, and a protein skimmer (R&B Aquatic Distribution, Boerne, TX, USA) to remove smaller solids and decrease turbidity.

DO$_2$ (6.5±0.6 mg l^{-1}) and pH (7.7±0.5) were monitored daily using an Y85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH), and a Hanna Instrument 9024 (Aquatic Ecosystems, Apopka, FL.). Total ammonia nitrogen (TAN; range: 0.06-1.31 mg l^{-1}) was monitored daily by spectrophotometric analysis (Hach Inc., Loveland, CO). Nitrite (range: 0.06-0.60 mg l^{-1}) and nitrate (range: 2.0-32.1 mg l^{-1}) levels were quantified once weekly. Salinity was maintained at 5 ppt and measured with a refractometer. A heater was placed in the biofiltration sump to maintain water temperature at 28 ^{o}C. Fish were exposed to a 12:12 photophase:scotophase through fluorescent lighting positioned 2.5 m above the life support system. A 30 min. dusk-dawn dimming of lights was employed.

Throughout the trial period, a fishmeal-based diet (Table 4.2) was fed at 4$^{\%}$ body wt d^{-1} as two separate feedings (08.00 and 16.00 h). Crude protein and lipid levels were formulated at 40$^{\%}$ and 10$^{\%}$, respectively. All known nutritional requirements of hybrid striped bass were met by the experimental feeds (NRC, 1993).

2.2 Experimental treatments

Three experimental treatments were given: fish were either left untreated, vaccinated, or sham injected. The vaccine employed was an experimental formalin-killed *Streptococcus iniae* oil-in-water adjuvanted bacterin (Kent Sea Tech Corporation, Mecca, CA). Injected fish received

either vaccine (4 x 10^5 of cells/ml) or Courtland saline as a 100 µl volume. Fish were weighed and measured semi-monthly for 8 wks, with group weights employed to adjust feeding rates.

2.3 Data collection

2.3.1 Morphological and feed data

Feed conversion ratio (FCR) was calculated according to the following formula

$$FCR = (\{g\} \text{ fed } / \{g\} \text{ weight gained})$$

At trial termination, all animals were weighed and measured (Fork Length), and their condition factor (CF) calculated according to the following formula:

$$CF = (weight/length^3)*1000$$

Visceral somatic (VSI), hepatosomatic (HSI) and muscle (MR) indices were calculated using the following formula:

$$VSI = (visceral \text{ wet weight } \{g\} / \text{ wet weight } \{g\} \text{ of animal})*100$$

$$HSI = (liver \text{ wet weight } \{g\} / \text{ visceral wet weight } \{g\})*100$$

$$MR = (muscle \text{ wet weight } \{g\} / \text{ wet weight } \{g\} \text{ of animal})*100$$

Fish were individually weighted and measured every two weeks to adjust feeding rations. Survival was noted throughout the study.

2.3.2 Serum processing

Anaesthetized (MS-222; Sigma) fish were bled from the caudal artery-vein complex using 2 ml heparinized syringes. Collected blood was transferred into Eppendorf 2 ml microcentrifuge tubes. Blood packed cell volume (hematocrit) was immediately assessed following collection. Blood was drawn into microhematocrit tubes (Fisher Scientific, Pittsburgh, PA), sealed with Cristoseal (Fisher), centrifuged at 10,000 x *g* for 5 min (M 24 Micro-Hematocrit Centrifuge; LW Scientific, Lawrenceville, GA) and hematocrit was read using the

centrifuge's combo reader. Remaining blood samples were transferred to a refrigerator maintained at 5 ^{o}C and allowed to clot overnight. Resultant serum was stored in 2 ml Eppendorf tubes at -20 ^{o}C until further analyses.

Serum samples were employed to evaluate protein levels using a hand-held VET360 temperature-compensated refractometer (Leica Optical Products Division, Buffalo, NY) and protein assay kit (Bio-Rad, Hercules, CA; Lowry et al. 1951). Serum lysozyme activity was measured using the turbidimetric assay (Parry et al. 1965). The assay employed 0.2 mg/ml *Micrococcus lysodeiktcus* suspended in sodium phosphate buffer (pH 7.2). 100 μl test sera were added to 100 μl phosphate buffer and 500 μl of suspended *M. lysodeiktcus* for a final volume of 700 μl. The reaction (24 ^{o}C; absorbance 540 nm) was measured at 30, 60 120 and 180 s. The unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per min. Ceruloplasmin ferroxidase activity was quantified based upon the catalytic oxidation of ferrous ions or ferrous complexes to the ferric state by ceruloplasmin (Cerón and Martínez-Subiela 2004).

2.4 RNA isolation and microarray preparation

Total RNA was isolated from collected livers 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 brought up to volume in RNase-free distilled water. The quality and the amount of starting mRNA was confirmed with a bioanalyser/agarose gel system (Agilent Technologies, Palo Alto, CA). The quality-checked total RNA was used for synthesizing biotin-labeled cRNA. Briefly, we used 10 μg of total RNA to genereate 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 cRNA. The cRNA was fragmented (15 μg per sample), spiked with internal controls (Affymetrix Inc., Santa Clara, CA) and hybridized overnight to the zebrafish gene chips ($n = 9$ chips total; i.e. three chips per treatment). The chips were washed and stained with streptavidin-phycoerythrin, before being scanned on the GeneChip scanner (Affymetrix, Inc.).

2.5 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 RMA method to adjust the background, and to perform within- and between-chip (quantile) normalizations. Preprocessing of the data with RMA was more sensitive and specific and thus provided a more robust dataset than the standard Affymetrix MAS 5.0 scaling or dCHIP techniques. The normalization process reduced unwanted technical variation. For an explanation and comparison of these methodologies, as well as their importance in microarray data analysis, the reader is referred to Saviozzi and Calogero (2003).

2.5.1 Filtering

Uninformative genes were eliminated from the dataset after implementing the preprocessing steps. Thus, genes with signals very near background, those that were considered absent by the Affymetrix scanner, and those that did not change expression values appreciably across conditions were excluded. Since we were primarily interested in discovering genes with robust expression, eliminating the genes at the low end of the expression scale should not curtail this discovery. Filtering was performed in GeneSpring (version 7.2, Silicongenetics, Redwood City, CA).

2.5.2 Data analyses

After arriving at our quality-checked dataset (number of genes = 1,210), the individual gene chips were examined for reproducibility within the given conditions using two-dimensional scatter plots and hierarchical clustering in GeneSpring (Silicongenetics, Redwood City, CA).

2.6 Data analyses

All data were subjected to analysis of variance. Means were compared by Duncan's Multiple Range Test with differences considered significant at the $P \leq 0.05$ level (SAS Inc., Cary, North Carolina).

3. Results

No mortalities were recorded during the present trial. Irrespective of control, placebo or vaccination treatments, hybrid striped bass grew at the same rate over the 8 wk of observation. Treatment, therefore, had no effect upon weight, length or condition factor. Final weights of fish from the control, placebo and vaccine groups were 150.4±30.4g, 149.4±21.1g and 157.1±35.8g respectively (Table 4.3), representing a tripling in animal weight over initial values. Final fish lengths were 205±12.1 mm, 204.9±9.0 mm 207.1±14.1 mm, and conditionf factors were 1.72±0.08, 1.73±0.06 and 1.74±0.05 for the control, placebo and vaccinated hybrid striped bass respectively (Table 4.3). Feed conversion ratios (FCR) throughout the trial averaged 2.33. Data relating to hepatosomatic and visceral indices, IPF and muscle ratios are presented in Table 4.3. Hepatosomatic indices ranged between 1.6±0.1 and 1.7±0.2 whereas those for IPF ranged between 5.0±0.8 and 5.2±0.7, and muscle ratios ranged between 26.0±4.3 and 28.9±5.5, respectively. Differences ($P < 0.001$) were observed between treatments with regard to VSI, which were higher in control fish (Table 4.3).

Blood-related parameters are summarized in Table 4.4. Packed cell volume and serum protein levels were similar across groups ($P > 0.05$). Time-course of changes in serum lysozyme activity of control animals and those receiving vaccination or placebo injection are presented in Fig. 4.1. Following vaccination, which incorporated a handling stressor, serum lysozyme activity decreased but subsequently rebounded. While no differences were detected between treatments at the same time points examined, evaluation of lysozyme activity between time-points revealed differences ($P < 0.05$) between 14 and 28 days post-vaccination (Fig. 4.1). Examination of the hepatic microarray datasets revealed only four immune related genes that were impacted 56-days following vaccination when compared against control fish. These included the up-regulated *TCIRG1*, or T-cell immune regulator 1 ($P < 0.0467$) (Genbank# BG303543) and *IL20RA*, or interleukin 20 receptor alpha ($P < 0.0433$) (Genbank# BI843576), and down-regulated cytokine inducible kinase, *plk3* ($P < 0.01$) (Genbank# AI957812) and mouse immune responsive protein, *IRG1* ($P < 0.01$) (Genbank# AW567349), as discussed below.

4. Discussion

Previous challenge-based studies with hybrid striped bass and the vaccine used herein displayed a high level of protection over extended periods, as long as 4 months (Buchanan et al., 2005). Fish in the present experiment were not challenged, since the primary objective of this study was to determine whether the experimental *S. iniae* vaccine imposed negative effects upon animal production characteristics. No differences were discerned between treatment groups for FCR, or weight and length growth indicating that treatment had no impacts upon animal performance. This finding contrasts with the observations of others who have employed oil-based vaccine preparations. In general, the use of oil, aluminum, and other types of adjuvant has been reported to negatively impact fish growth and appetite (Table 4.1). However, until

relatively recently, side-effect-based investigations were exclusively undertaken with salmonids ((and thus at < 17 ºC (63ºF); Table 4.1)). Nevertheless, studies with other species also indicate that vaccination has varying negative impacts upon farmed fish. While not affecting growth, vaccination caused injection site lesioning in coldwater marine species such as Atlantic cod *Gadus morhua* (Mikkelsen et al. 2004) and turbot *Scophthalmus maximus* (Björnsdóttir et al. 2004). In sea bass, *Dicentrachus labrax* (Afonso et al. 1998), an apparent reduction in appetite was noted, although this indicator was not explicitly monitored. Lesions caused by injection in this study was not noticed. In Arctic charr *Salvelinus alpinus*, Pylkko et al. (2000) observed negative growth responses immediately following vaccination, but over the entire study no negative growth impact was recorded. Studies with rainbow trout *Oncorhynchus mykiss* (Mulvey et al. 1995) and common whitefish *Coregonus lavaretus* (Lonnstrom et al. 2001) likewise record no effects of vaccination upon growth.

It has been hypothesized that the adjuvant component is not responsible for the observed growth reductions in vaccinated fish. Rather, it is the antigen or antigen x adjuvant interaction that is causative (Rønsholdt and McLean 1999), a suggestion that appears to be supported by the findings of Melingen and Wergeland (2002). Poppe and Breck (1997) speculated that growth reduction and loss of appetite following vaccination results from irritation of the gut or intrusion upon normal swim bladder function. Mutoloki et al. (2004) discerned a correlation between the magnitude of the reaction of vaccinated fish and the release rate of antigen from the adjuvant. Further, it has been proposed that reduced growth might be anticipated due to the increased energetic costs of a stimulated immune system (Ackerman et al. 2000). Indeed, results with respect to visceral somatic index in hybrid striped bass may support this supposition, since comparison between vaccinated and unhandled control animals revealed greater lipid deposition

in the latter. Conceivably, reduced intraperitoneal fat depots may have resulted from the increased metabolic costs associated with the heightening of the immune state. But growth and FCRs were not influenced during the present study, which would argue against this suggestion, as too would the apparent reduction in lysozyme activity immediately following vaccine injection.

Reduction of serum lysozyme activity in vaccinated hybrid striped bass contrasts with the reported actions of vaccines in a number of salmonids where plasma lysozyme increased (Table 4.1). Lysozyme activity remained unchanged in goldfish *Carassius auratus* vaccinated against *Aeromonas salmonicida* (Robertson et al. 2005) and Nile tilapia *Oreochromis niloticus* vaccinated against *S. iniae* (Whittington et al. 2005). In Nile tilapia immunized against *Mycobacterium* extracellular products, significantly higher plasma lysozyme levels were recorded 4 days post-vaccination (Chen et al. 1998). Similar responses have been reported in other species, including whitefish (Koskela et al. 2004), bester – beluga sturgeon (*Huso huso)* x sterlet sturgeon (*Acipenser ruthenus)* (Kolman et al. 1999) and olive flounder *Paralichthys olivaceus* (Li et al. 2005). The magnitude of change and indeed presence of lysozyme recorded in the present study was in general much lower than those observed with other species. Indeed, the only other reports for lysozyme activity in hybrid striped bass are an order of magnitude higher (Li et al. 2004). This difference may reflect technique, husbandry methods employed (water temperature, quality etc.), fish source, size and age, and immunological maturity and status (i.e., previous challenges), since each of these parameters are known to impact lysozyme activity (Schrock et al. 2001, Langston et al. 2002). Moreover, adjuvant and vaccine type (preparation and pathogen) have likewise been shown to affect lysozyme activity (Ackerman et al. 2000). This apparent broad variability in lysozyme response following (a)biotic challenges

might thus be used to question the scientific value of assessing this apparently almost indifferent parameter as an indicator of immune response. Clearly, more sensitive markers of cause and effect might be gained by examining the impact of vaccination upon gene expression.

The use of heterologous microarrays requires rigorous validations, although excellent correlations have been observed from human gene chips hybridized with distantly-related mammalian species (bovine, porcine and canine), from the *Arabidopsis* chip for cross-plant species studies, and from a cichlid chip for research with various cichlid species, a salmonid, poeciliid and cyprinid fishes (Becher et al. 2004, Ji et al. 2004, Renn et al. 2004). In the present study, a zebrafish *Danio rerio* microarray was employed to examine gene expression profiles 53 days post-vaccination. While a variety of genes exhibited up-, and down-regulation, only four, following rigorous data pre-processing and filtering, were found to exhibit direct significance to the immune response of fish. These included T-cell immune regulator 1, a V-ATPase. All cells must regulate their pH within very narrow limits, and the V-ATPases, a family of ATP-driven proton pumps, act to regulate and maintain a reservoir of ions within various cellular organelles to enable this. V-ATPases are important constituents of macrophages because these cells often function in regions where pH may become restrictive (Brisseau 1996), as exemplified by areas of inflammation which occur following vaccination of fish (Afonso et al. 1998, Mutoloki et al. 2006). Likewise, up-regulation of the interleukin-20 receptor alpha suggests a response to inflammation (Xu 2004) but reports also indicate a role for *IL20RA*-stimulated monocyte expression of IL-6, TNF-α and reactive oxygen species in T-cells (Wang et al. 2003). Polo-like kinase 3 (*plk3*) is intimately involved in cell cycle regulation and appears to be activated when cells are exposed to H_2O_2. Down-regulation of this enzyme may be considered beneficial since apoptosis would be arrested (Xie et al. 2001, Wang et al. 2002). *IRG1* promotes the interferon

response to viral infection, and these genes may be down-regulated to enable focus upon non-viral defenses which may be anticipated in response to vaccination for bacterial diseases. Hence, the vaccine induced expression of at least a few genes involved in immune response.

Clearly, injection of hybrid striped bass with the experimental *S. iniae* formulation had limited impact upon production performance. Such findings have economic significance to aquaculture, since feed costs represent the largest operating variable during the commercial fish farming, and losses (feed and growth) would not be incurred with use of this vaccine. Indeed, appetite, as indicated by FCRs, remained unaffected, which contrasts to the situation for salmonids (Table 4.1). The application of "aquanomic" (McLean and Craig 2006) technology to pinpoint specific genes affected by vaccination represents an obvious target for future research delineating biomarkers for more thorough investigations of the consequences of vaccination upon whole animal-response to (re)vaccination.

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Table 4.1. Reported impacts of vaccination upon performance characteristics of salmonid species.

Adjuvant	Antigen	Vaccine	Dose (*ul*)	Species	Temp C°	Duration	Effects	Reference
Aluminum sulphate	*Vibrio Anguillarum*	Experimental	50, 100 or 200	Rainbow Trout	13-15	6 wks	↓ growth No differences between dose levels	Horne et al. 1994
	A. salmonicida	Experimental	100	Atlantic Salmon	12-15	3 mos	Growth not affected	Mulvey et al. 1995
Aluminium hydroxide	*A. salmonicida*	Aquavac Furovac IJ® Aquaculture Vaccines Ltd, Saffron Walden, England	100	Atlantic Salmon	7-9	1-2 yrs	↓ weight 4.1%	Lillehaug et al. 1992
	A. salmonicida	Aquavac Furovac IJ® Aquaculture Vaccines Ltd, Saffron Walden, England	100	Atlantic Salmon	2.8-7.2	1.5 yrs	↓ appetite 2-4 wks post vac.	Midtlyng 1996
	A. salmonicida	Experimental Aquaculture Vaccines Ltd, Saffron Walden, England	400	Atlantic Salmon	5-7.5	1.5 yrs	↓ appetite 2-4 wks post vac.	Midtlyng 1996
Aluminium phosphate	*A. salmonicida*	Furogen® Aqua Health Ltd, Charlottetown, Canada	100	Atlantic Salmon	7-9	1-2 yrs	↓ weight 4.1%	Lillehaug et al. 1992
	A. salmonicida	Furogen® Aqua Health Ltd, Charlottetown, Canada	100	Atlantic Salmon	2.8-8.5	1.5 yrs	↓ appetite 2-4 wks post vac.	Midtlyng 1996
	A. salmonicida	Experimental Aqua Health Ltd, Charlottetown, Canada	100	Atlantic Salmon	5-7.5	1.5 yrs	↓ appetite 2-4 wks post vac.	Midtlyng 1996
Mineral Oil	*A. salmonicida* *V. anguillarum*	Apoject 1800® Alpharma Inc. Dirdal, Norway	200	European Whitefish	15	7 wks	↑ lysozyme activity ↓ growth	Koskela et al. 2004
	A. salmonicida *V. anguillarum*	Lipogen® duo Aqua Health Ltd, Charlottetown, Canada	100	European Whitefish	15	7 wks	↑ lysozyme activity ↓ growth	Koskela et al. 2004
	V. salmonicida *V. anguillarum* *A. salmonicida* IPN	Lipogen Quattro® Aqua Health Ltd, Charlottetown, Canada	200	Atlantic Salmon	8.6-14	3 wks	↓ growth	Melingen & Wergeland 2002
	A. salmonicida	Experimental vaccines Alpharma Inc., Dirdal, Norway	100 or 200	Atlantic Salmon	8	1 yr	↓ weight & ↓ Gsp of vac groups at 3 mos No differences at 6 and 12 months Antigens are inflammatory and magnitudeof response is regulated by the adjuvant	Mutoloki et al. 2004
	A. salmonicida	Apoject 1-Fural® Alpharma Inc. Dirdal, Norway	200	Rainbow Trout	6.4-10.6	7 wks	↓ weight 8%	Ronsholdt & McLean 1999
	A. salmonicida	name not reported	Not	Arctic	10.3,	10 wks	No negative effect on growth at	Pylkko 2000

		Alpharma Inc. Dirdal, Norway	reported	Charr	14.1 or 18.1		temp between 10.3 and 14.1	
	A. salmonicida	Biojec 1500® Biomed Inc. Bellevue, USA	200	Atlantic Salmon	2.8-8.5	1.5 yrs	↓ appetite 2-4 wks post vaccination	Midtlyng 1996
	A. salmonicida	Biojec 1500® Biomed Inc. Bellevue, USA	200	Atlantic Salmon	7.2	3 mos	↓ growth	Midtlyng & Lillehaug 1998
	V. anguillarum *V. salmonicida* *V. viscosus* *A. salmonicida* IPNV	Lipogen® Pentium Aqua Health Ltd, Charlottetown, Canada	200	Atlantic Salmon	12	4.5 wks	↓ growth 20% ↓ feed intake ↑ FCR's ↓ Gsp	Sorum & Damsgard 2004
	A. salmonicida *V. anguillarum* *V. salmonicida*	Biojec 1900® Biomed Inc. Bellevue, USA	200	Atlantic Salmon	4.9-6.3	3 mos	↓ growth	Midtlyng & Lillehaug 1998
Squalene Oil	*A. salmonicida*	Apoject 1-fural® Alpharma Inc., Dirdal, Norway	200	Atlantic Salmon	7.2	3 mos	↓ growth	Midtlyng & Lillehaug 1998
	A. salmonicida *V. anguillarum* *V. salmonicida*	Apoject 3-fural® Alpharma Inc., Dirdal, Norway	200	Atlantic Salmon	4.9-6.3	3 mos	↓ growth	Midtlyng & Lillehaug 1998
Glucan	*A. salmonicida*	Norvax FUR® Norbio AS Norway	100	Atlantic Salmon	5-7.5	1.5 yrs	↓ appetite 2 weeks post vac	Midtlyng 1996
	A. salmonicida	Norvax FUR® Intervet Norbio Bergen, Norway	200	Atlantic Salmon	7.2	3 mos	↓ growth	Midtlyng & Lillehaug 1998
	A. salmonicida *V. anguillarum* *V. salmonicida*	Norvax triple® Intervet Norbio Bergen, Norway	200	Atlantic Salmon	4.9-6.3	3 mos	↓ growth	Midtlyng & Lillehaug 1998
Levamisole	*A. salmonicida*	Experimental Lovens Kemiske Fabrik Ballerup, Denmark	100	Atlantic Salmon	5-7.5	1.5 yrs	↓ appetite 2-4 wks post vac	Midtlyng 1996
V. anguillarum Lipopoly-saccharide	*A. salmonicida*	Experimental Microtek Ltd., Saanichton, Canada	100	Rainbow Trout	8-17	5, 12 & 24 wks	↑ Gsp ↑ O_2 consumption at 3 wks No change in white blood cell counts, FCE's	Ackerman et al. 2000

Table 4.2 Composition of the basal diet used in the present study. All diets provided 40 % crude protein, 10% lipid and supplied 3.30 kcals/g.

Ingredient (% dry matter basis)	
Fishmeal[a]	58.8
Dextrin[c]	20.0
Lipid (menhaden oil)[b]	4.1
Mineral (Se-free)[d]	4
Vitamin[e]	3
Carboxymethyl cellulose (CMC)[c]	1
Cellulose[c]	9.1
Selenium premix[g]	0

[a] Special Select® menhaden fish meal, Omega Protein, Inc., Hammond, LA, USA. 68% crude protein (dry); 0.915 dry matter.

[b] LCP ethoxyquin-free Omega Oils, Reedville, VA, USA.

[c] US Biochemical Corporation, Cleveland, Ohio, USA.

[d] Contained (g/5kg): monocalcium phosphate 680, calcium lactate 1742, ferrous sulfate 25, magnesium sulfate $7H_2O$ 660, dipotassium phosphate 1200, monosodium phosphate 440, sodium chloride 225, aluminum chloride 0.750, potassium chloride 0.750, copper sulfate 2.5, manganous sulfate 3.5, cobalt chloride 5, zinc sulfate $7H_2O$ 15.(MP Biomedicals, Aurora, OH, USA).

[e] Contained (g/kg): ascorbic acid 50.0, dl-calcium pantothenate 5.0, choline chloride 36.2, inositol 5.0, menadione sodium bisulfite 2.0, niacin 5.0, pyridoxine HCL 1.0, riboflavin 3.0, thiamine mononitrate 0.5, dl-alpha-tocopherol acetate (250 IU/g) 8.0, vit. A palmitate (500,000 IU/g) 0.2, vitamin micro-mix 10.0, cellulose 874.1. micromix contained (g/mg) biotin 0.50, folic acid 1.8, vitamin B12 .02, cholecalciferol (40 IU/ug) 0.02, cellulose 97.66.

[f] Selplex® (Alltech Inc., Nicholasville, KY, USA)

[g] Selenium premix: 0.1 g sodium selenite (Sigma Chemical Co., St. Louis, Missouri, USA) and 499.9 g cellulose.

Table 4.3 Morphological indices of hybrid striped bass 8 weeks following vaccination, placebo injection, or no treatment. Data with different superscripts in a column were different (P < 0.05).

Treatment	Weight (g)	Length (mm)	CF[1]	IPF[2]	HSI[3]	VSI[4]
Control	150.4±30.4	205.0±12.1	1.72±0.18	5.0±0.8	1.7±0.2	13.1±2.0[a]
Placebo	149.4±21.1	204.9±9.0	1.73±0.06	5.2±0.7	1.6±0.3	10.2±1.2[b]
Vaccinated	157.1±35.8	207.1±14.4	1.74±0.05	5.1±0.8	1.6±0.1	10.2±0.8[b]

[1]CF = condition factor.

[2]IPF = intraperitoneal fat ratio.

[3]HSI = hepatosomatic index.

[4]VSI = visceral somatic index.

Table 4.4 Hybrid striped bass packed cell volume (PCV), plasma protein (PP) and serum lysozyme activity (units ml^{-1}) 8 weeks following vaccination, placebo injection, or no treatment.

Treatment	PCV	PP	Lysozyme
Control	41.4±6.3	8.1±0.9	97.2±27.9
Placebo	40.4±7.1	7.8±0.9	71.5±30.7
Vaccinated	42.7±7.7	7.6±0.8	126.5±34.6

Figure 4.1 Time-course of serum lysozyme response in hybrid striped bass following vaccination (△), placebo injection (□) or remaining untouched (◇) for a 56-day period. Vaccinated fish received a single dose (100μl) of an experimental oil-in-water *Streptococcus iniae* vaccine, whereas placebo injections comprised an identical volume of Courtland saline. No differences were discernable at specific time points for serum lysozyme activity, although significant elevations ($P < 0.05$; *) in activity were observed between days 11 and 25 post-vaccination (n = 5 per time and treatment).
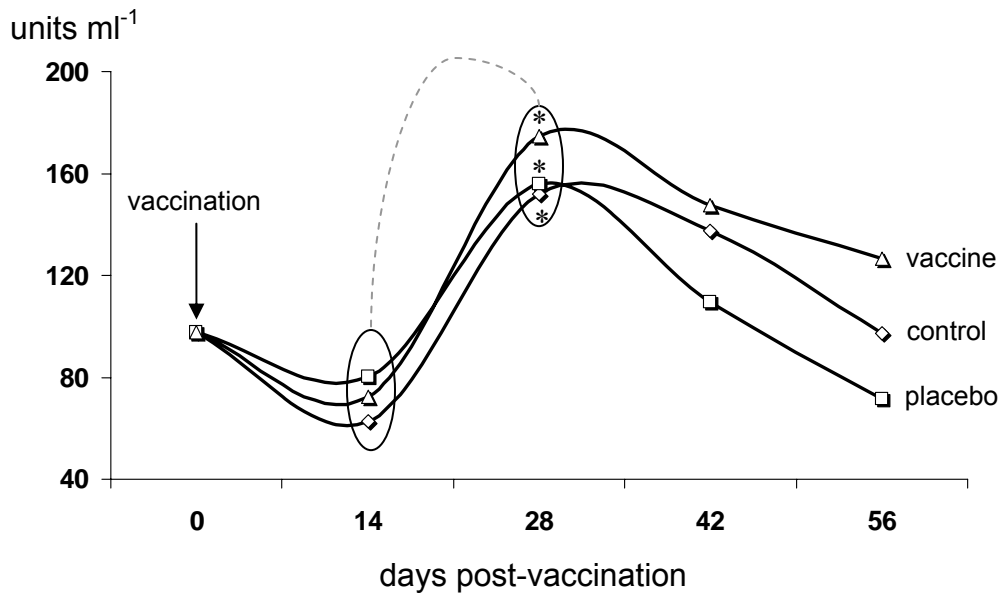
Chapter 5
Summary conclusions

Results of studies described in this thesis suggest that:

1) A fish farmer contemplating the use of Se enhancement as a means of producing a value-added functional food would benefit from using organic rather than the traditional inorganic sources because of differences in Se accumulation (i.e., favoring organic Se) in the fish fillet.

2) Additional studies are needed to determine the optimum finishing period and optimum dose of organic Se for the production of functional foods. A linear incorporation of Se was observed in both selenium studies up to the highest amount (3.2 mg/kg) and time (6 weeks) used, one of the highest amounts used in nontoxicological studies. Se would seem to be able to accumulate even further.

3) A period of only 6 weeks appears to be required for increasing muscle Se levels in HSB, indicating that the aquaculturist need consider Se dietary management only as a "finishing feed". Se enhancement offers a possible way for fish farmers to enter into the $10 B a year functional food market, due to the many possible health benefits that Se enhancement provides.

4) Muscle represents a more appropriate tissue to examine when evaluating the dynamics of Se absorption in fish than liver. In this study, there was a greater correlation observed with dietary selenium in muscle than liver. Moreover, the liver does not respresent and edible component of fish. Greatest Se accumulation occurred most rapidly in the liver after 0.1 mg/kg dietary Se and 0.3 mg/kg dietary Se. in fish fillets.

5) The experimental *Streptococcus iniae* vaccine used herein had no significant negative effects upon the production performance of cultured HSB, a finding that contrasts with much of the literature on salmonids. This observation indicated differences in the responsiveness of different fish species to vaccination. Previous oil-based vaccines have been associated with more severe side effects.

6) Preliminary studies using microarray techniques indicated that vaccination has significant and relatively long-lasting (6+ weeks) effects upon treated fish. Many genes were affected, but only four immune-related genes exhibited changes in mRNA expression. Genes, whose expression was affected, included those related to the production of lymphocytes, interferons and lysozyme.

7) Se supplementation may offer immune enhancement through enhanced circulating cerruloplasmin and glutathione peroxidase levels.

Biography

Paul Alfred Cotter was born on May 8, 1977 in Falls Church, VA., just outside Washington, D.C.; the youngest of seven siblings. His visits to Shenandoah National Park as a young boy stimulated his interest in the outdoors. He became interested in a career in fish and wildlife observing the forest rangers. He started out as a volunteer at the Patuxent National Wildlife Refuge, participating in amphibian surveys. He attended West Virginia University and earned a bachelor's degree in fisheries and wildlife in 2001. From there he went to the King & Queen state fish hatchery in Tappahannock, as a technician working the graveyard shift, involved in the Shad Restoration Project of the Chesapeake Bay. He then spent a year at the Fawn River State hatchery in Orland, Indiana, one of the largest producers of muskellunge and walleye for the state. While there he represented the Indiana Department of Natural Resources in the 2003 state fair in Indianapolis. He came to Virginia Tech in the winter of 2004 to conduct research for his masters degree.