ENHANCEMENT OF THE NON-SPECIFIC IMMUNE RESPONSE, PIGMENTATION AND GROWTH OF FARMED CHINOOK SALMON (ONCORHYNCHUS TSHAWYTSCHA) FED A COMBINATION OF DIETARY FLAVONOIDS (GRAPE SEED EXTRACT, KPA®) AND ASTAXANTHIN

by

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Department of Animal Science

The University of British Columbia
Vancouver, Canada

Date Nov 27 / 2003
ABSTRACT

Salmon farming is a flourishing aquaculture industry throughout the world and relies on intensive husbandry practices and well balanced diets to succeed. As in agriculture, genetics, health, husbandry, and nutrition are the main pillars of a successful and sustainable industry. Astaxanthin, a xanthophyll pigment with antioxidant properties, is incorporated into farmed salmon diets to provide the desirable red colour in flesh. Dietary antioxidant supplements (i.e. vitamins, flavonoids) have been shown to fight free radicals normally formed during aerobic metabolism and generated in excess during some physiological and pathological processes (i.e. exercise, disease).

This research was conducted to investigate the role of dietary supplementation, with grape seed extract and astaxanthin, on pigmentation, growth and the immune response, of pre-smolt and post-smolt chinook salmon spp maintained in freshwater (FW) and saltwater (SW). Four experimental diets were formulated that contained uniform amounts of astaxanthin (60 ppm) and one of two concentrations (low and high) of grape seed extract (Kikkoman Proanthocyanidins, KPA®). The control diet (no astaxanthin, no KPA®), astaxanthin diet (astaxanthin, no KPA®), low KPA® diet (astaxanthin, 100 ppm KPA®), and high KPA® diet (astaxanthin, 1000 ppm KPA®) were fed for 32 days to pre-smolt chinook salmon in FW tanks, and 155 days to post-smolt chinook salmon in SW growout seacages.

Pre-smolts fed fortified diets with the antioxidants KPA® and astaxanthin, showed a significantly (p=0.043) larger weight gain than fish groups fed the control diet after 32 days. There were no significant weight differences among the groups ingesting the antioxidant-fortified diets. The specific growth rates (SGR) and feed conversion ratios (FCR) were not significantly different between groups fed the four experimental diets in FW. Conversely, muscle astaxanthin concentrations were significantly higher (p=0.019) in groups fed the astaxanthin-containing diets compared to baseline values and fish fed the control diet. There were no significant differences either in the concentration of astaxanthin in fish muscle or on the apparent astaxanthin retention coefficients (AARC) in salmon fed either the astaxanthin, low or high KPA® diets.

After a feeding period of 155 days in SW, post-smolts fed the high KPA® diet had a significantly higher deposition of astaxanthin in muscle (p=0.036), higher visual colour
score in fillets as measured by the Roche Salmofan® (p=0.029), and greater wet weight gain (p<0.001) than those fed the other three diets.

Following a disease challenge with *Vibrio anguillarum*, pre-smolts fed the high KPA® diet had a significantly lower (p=0.038) cumulative mortality among diet treatments, as well as a significantly greater (p=0.025) number of circulating leucocytes (p=0.025), neutrophils (p=0.018), and monocytes (p=0.034). Furthermore, the lysozyme activity both in plasma and head kidney, and the neutrophil respiratory burst activity were significantly greater in fish fed the high KPA® diet, (p=0.029, p=0.037, and p=0.027, respectively).

It is concluded that the addition of antioxidants (proanthocyanidins and astaxanthin) to a chinook salmon diet significantly enhanced the humoral and cellular non-specific immune response factors and was related to a lower cumulative mortality after the disease challenge. Furthermore, the deposition efficiency of the carotenoid astaxanthin, and growth-related variables in farmed chinook salmon were also significantly affected by the combination of dietary antioxidants.
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<table>
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<th>Description</th>
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<tr>
<td>AARC</td>
<td>apparent astaxanthin retention coefficient</td>
</tr>
<tr>
<td>$A^0KPA^0$</td>
<td>control diet, astaxanthin (0 ppm), KPA® (0 ppm)</td>
</tr>
<tr>
<td>$A^{60}KPA^{1000}$</td>
<td>high KPA diet, astaxanthin (60 ppm), KPA® (1000 ppm)</td>
</tr>
<tr>
<td>$A^{60}KPA^{100}$</td>
<td>low KPA diet, astaxanthin (60 ppm), KPA® (100 ppm)</td>
</tr>
<tr>
<td>$A^{60}KPA^0$</td>
<td>astaxanthin diet, astaxanthin (60 ppm), KPA® (0 ppm)</td>
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<tr>
<td>b.w.</td>
<td>body weight</td>
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<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CF</td>
<td>condition factor</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CL</td>
<td>chemoluminescence</td>
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<tr>
<td>DFO</td>
<td>Department of Fisheries and Oceans</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<tr>
<td>FCR</td>
<td>feed conversion ratio</td>
</tr>
<tr>
<td>FR</td>
<td>free radical</td>
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<td>FW</td>
<td>freshwater</td>
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<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
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<td>h</td>
<td>hour</td>
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<td>haemoglobin</td>
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<td>Hct</td>
<td>haematocrit</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>hydrogen peroxide</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>KPA®</td>
<td>Kikkoman proanthocyanidins (grape seed extract)</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MCH</td>
<td>mean corpuscular haemoglobin</td>
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<tr>
<td>MCHC</td>
<td>mean corpuscular haemoglobin content</td>
</tr>
<tr>
<td>MCV</td>
<td>mean corpuscular volume</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MS-222®</td>
<td>3-aminobenzoic acid ethyl ester methanesulfonate (tricaine)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium solution (0.2% in 0.85% saline)</td>
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NO  nitric oxide
*O²⁻  superoxide anion radical
¹O₂  singlet oxygen
*OH⁻  hydroxyl radical
OD  optical density
O. tshawytscha  *Oncorhynchus tshawytscha*
PA  proanthocyanidins
ppm  parts per million
PUFA  polyunsaturated fatty acids
RBC  red blood cells, erythrocytes
RCC  *Roche Colour Card*
ROS  reactive oxygen species
rpm  revolutions per minute
RSS  *Roche salmofan® score*
SD  standard deviation
SGR  specific growth rate
SOD  superoxide dismutase
SP  stress protein
SW  saltwater
TBARS  thiobarbituric acid reactive substances
TGC  thermal growth coefficient
TSA  *trypticase soy agar*
Vang  *Vibrio anguillarum*
VHDL  very high density lipoprotein
VLDL  very low density lipoprotein
vs.  versus
WBC  white blood cells
Wt  weight
Wtᵢ  initial weight
Wtf  final weight
WVL  *West Vancouver Laboratory*
YIAL  *Yellow Island Aquaculture Ltd*
®  registered
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DEDICATION

The completion of this thesis is dedicated to my families both South and North of the Equator line. I will always have one foot on each hemisphere. To Michelle and Oliver, your love and encouragement lightens my pace and comforts my spirit.

*Ad Astra Per Aspera. Je suis Prest.*
1. GENERAL INTRODUCTION

Aquaculture is the rearing of aquatic species under controlled or semi-controlled conditions. The salmon and trout farming have developed explosively in the last 20 years (FAO, 2000). In 1981, the industry produced 17,000 tonnes worldwide (3% of world’s total salmon production); in 2002 the industry supplied 1,445,000 tonnes (69% of world’s production) (Anonymous, 2003). Farmed salmon is the most important agricultural commodity in British Columbia (BC), generating revenues well over US$310 million in 2000 (Egan, 2001). Farmed salmon production in the province of BC is based on three species: Atlantic salmon (Salmo salar) 81%, chinook salmon (Oncorhynchus tshawytscha) 16% and coho salmon (Oncorhynchus kisutch) 3% (Egan, 2001).

As a highly intensive method of animal production, farmed salmon husbandry relies strongly on adequate feeding practices, both in feed quality and quantity; therefore, proper nutrition plays a critical role in maintaining normal growth and health of cultured fish. Infectious diseases are the major cause of economic loss in commercial aquaculture (Loyell 1996). Current problems with disease outbreaks in aquaculture include the limited number of government-approved antibiotics, improper administration, antibiotic resistance, and accumulation of drugs in the environment. Therefore the aquaculture industry needs to focus on prevention of diseases rather than on treatment.

The influence that dietary factors may have on a disease outbreak in cultured fish has been recognized for many years (Blazer, 1992). The effects of supplementing diets with vitamins (vitamin A, C, E), micronutrients (minerals), macronutrients (protein, carbohydrate and lipids), and immunostimulants (lipopolysaccharides, probiotics, β-glucans) have been the focus of investigation.

Enhancing the immune system through supplementation with dietary antioxidants is a common practice in human nutrition, and is gaining increased popularity in companion animal diets (Devlin et al. 2000). Such practice is almost non-existent in intensive animal farming. The effects of adding a potent natural antioxidant extracted from grape seeds (Kikkoman proanthocyanidins, KPA®) in the diet of farmed salmon could positively affect the immune system as well as their pigmentation and growth. Therefore, these studies were designed to address the potential beneficial effects of feeding farmed salmon diets fortified with one of two concentrations of KPA® and a uniform concentration of astaxanthin on deposition of the carotenoid astaxanthin in flesh, salmon growth and the fish immune response after a bacterial challenge.
The fish immune system:

The immune system works in a coordinated manner fighting foreign biotic and abiotic insults. The defense mechanisms can be differentiated into non-specific (innate) immunity and specific (acquired) immunity. Both use humoral (i.e. lysozyme, complement, C-reactive Protein (CRP), interferon, transferrin, lectin, lysines, agglutins, precipitins, and opsonins) and cellular (neutrophils, eosinophils, monocytes and macrophages) mechanisms to provide protection against foreign substances and pathogens.

The pathogenesis of disease include the generation of reactive oxygen species (ROS), during inflammation and the “oxygen-killing dependant mechanisms” (i.e. respiratory burst activity). A major innate defense mechanism consists of phagocytosis by monocytes, macrophages, neutrophils and eosinophils (Secombes, 1996). Once a pathogen is internalized in the body, phagocytic cells recognize and engulf antigenic particles, including bacteria, and damaged host cells in a three-step process involving attachment, phagocytosis, and digestion. This allows lysozomal enzymes to directly degrade the ingested particles. The respiratory burst activity produces toxic oxygen-, and nitrogen-free radicals, such as superoxide anion (\(\cdot O_2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)), reactive singlet oxygen (\(\cdot O_2\)), and nitric oxide (NO), respectively (reviewed by Secombes and Fletcher, 1992). These components are toxic to bacteria and protozoan parasites and occur within the cytoplasm of the phagocyte (phagosome). However, they may also be released extracellulary (Lamas and Ellis, 1994). The toxicity of these substances will not only affects microorganisms but will also affect the stability of lipid membranes of surrounding cells.

Farmed salmon are not only affected by the action of pathogens, but also by a number of chemical (i.e. pharmacological treatments) and environmental (i.e. pollutants, net antifouling, hyperoxic conditions) insults that expose fish to the actions of deleterious ROS. Free radicals are also produced under normal healthy conditions as part of the respiratory metabolism of aerobic organisms. These molecules are highly reactive and unstable, and when a biomolecule (lipid, nucleic acid, protein) reacts with a free radical, a new radical is created (chain reaction). Antioxidants work by suppressing ROS formation, inhibiting chain reactions, breaking chain propagation, and repairing and de novo synthesizing antioxidant enzymes.
The fish carotenoid deposition:

Carotenoids are a group of naturally occurring pigments that are responsible for the red, orange and yellow colour in the skin, flesh, shell and exoskeleton of aquatic animals. The most abundant natural pigments in the flesh of salmonids (Salmo spp., Oncorhynchus spp., and Salvelinus spp.) are the xanthophylls astaxanthin (3,3' – dyhydroxy-4, 4'- diketo-β-carotene) and canthaxanthin (4-4'-diketo-β-carotene).

Salmonids are incapable of synthesizing carotenoids de novo (Weedon, 1971; Simpson, 1982; Storebakken and No, 1992) or converting one carotenoid pigment into another (Torrissen et al. 1989). Thus, flesh pigmentation of farmed salmon can only be achieved by supplementing the feed of the fish with natural and/or synthetic astaxanthin or canthaxanthin.

Deposition of carotenoids in salmonid flesh occurs as a result of several processes: absorption of pigments in the digestive tract, transport of pigment in the blood, retention in the muscle and metabolism of carotenoids. These processes depend on several factors such as fish age, diet, genetic and husbandry conditions. Among dietary factors, lipid and antioxidant levels in feed are known to play important roles. Achieving a deep red flesh colour is critical for maximizing the price of salmon in the marketplace and is therefore a crucial aspect of salmon husbandry. Well-pigmented flesh is regarded second after freshness as the most important flesh quality criteria of farmed salmon (Torrissen, 1995; Schiedt, 1998).

In addition to their role as biological pigment, carotenoids possess strong antioxidant properties as shown by Burton (1989) and later confirmed in vivo in rainbow trout by Nakano et al. (1995).

The antioxidant network:

Antioxidants break the chain reaction caused by free radicals on biomolecules. Moreover, they scavenge and neutralize reactive oxygen metabolites before they can do significant damage to cells, tissues and organs. The antioxidant defense system includes enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), free radical scavengers (vitamin C, vitamin E, carotenoids, flavonoids), and metal chelators. These molecules work together in a highly organized and cooperative manner to regulate the equilibrium between the production of oxygen radicals and the counteracting defense mechanisms of antioxidant scavenging systems.
and repair enzymes. The components of the antioxidant system exert their actions either on the lipid (lipophilic) or the aqueous (hydrophilic) phase of the cell membranes and there have been recognized synergistic interactions and regenerations between antioxidants of both phases (i.e. Vitamin E-Vitamin C, carotenoids-Vitamin E, flavonoids-Vitamin E) (Aruoma, 1994; Halliwell, 1996) (Appendix 1 and 2-A).

Flavonoids are polyphenolic antioxidants naturally present in fruits, vegetables, nuts, seeds, flowers and barks. Proanthocyanidins (PA) are a group of flavonoids present in high concentration in the seeds of grapes. Proanthocyanidins have significantly more potent antioxidant activity than vitamin C, E or β-carotene (Buettner, 1993; Bagchi et al. 1997; Bagchi et al. 1998) singly, or in combination (Bagchi et al. 2000). In addition, PA exerts a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory actions (Buening et al. 1981; Afanas’ev et al. 1989; Kolodziej et al. 1995). Proanthocyanidin regulates the activity of enzymes participating in inflammation, including cyclooxygenase and lipoxygenase (Kolodziej et al. 1995), and have been shown to prevent lipid peroxidation and oxidation of low-density lipoproteins (LDL) (Frankel et al. 1993).

The Kikkoman Corporation (Noda city, Chiba, Japan) has extracted natural flavonoid PA from grape seeds (KPA®), which have been experimentally tested as a photo-oxidative protector in salmon fillets. The colour of salmon fillets can fade with time due to the action of light and oxygen. Soaking salmon fillets in a KPA®-saline solution has been shown to preserve the fillet colour compared to a saline control solution (Kikkoman Corporation, personal communication).

**Rationale:**

Farmed salmon are raised in confined high-density production systems and they are fed balanced diets that contain high levels of protein and lipid together with supplemental micronutrients and carotenoids. They are exposed to a variety of deleterious stimuli that promote the production of ROS. In this study it was postulated that supplementation of salmon diets with antioxidants (i.e. grape seed extract and astaxanthin) would either correct a deficiency of or restore the balance between pro-oxidants and antioxidants, and consequently enhance body functions, namely immune responses, growth and pigmentation.
The effects of supplementing fish feed with KPA® have not been described yet. The supplementation of KPA® in salmon feed might have positive impact on carotenoid pigment (astaxanthin) deposition efficiency in their flesh. In addition, the cell-mediated and humoral non-specific immune responses of salmon could be enhanced due to the fortification of their feed with a combination of both antioxidants. This is the first study that has used a combination of astaxanthin and a natural flavonoid supplement in salmon diet in an attempt to enhance the immune responses of the fish against a bacterial challenge, while concurrently improving their flesh pigmentation and growth under practical salmon farming conditions.
2. GENERAL HYPOTHESIS

H₀: Fortification of a salmon diet with a combination of grape seed extract (KPA®) and astaxanthin has no effect on the immune response, pigmentation and growth of farmed chinook salmon (O. tshawytscha).

H₁: Fortification of a salmon diet with a combination of grape seed extract (KPA®) and astaxanthin has an effect on the immune response, pigmentation and growth of farmed chinook salmon (O. tshawytscha).

2.1 Specific hypotheses

Experiments A and C: Assessment of flesh pigmentation and growth of pre-smolt and post-smolt farmed chinook salmon fed diets with one of two concentrations of a natural flavonoid antioxidant and a single astaxanthin level or no supplemental antioxidants.

1. H₀: KPA®-supplemented diet does not increase astaxanthin retention in muscle of pre-smolt and post-smolt chinook salmon (O. tshawytscha).

H₁: KPA®-supplemented diet does increase astaxanthin retention in muscle of pre-smolt and post-smolt chinook salmon (O. tshawytscha).


H₁: KPA®-supplemented diet does improve growth performance of pre-smolt and post-smolt chinook salmon (O. tshawytscha).

Experiment B: Non-specific immune response of pre-smolt chinook salmon fed diets with one of two concentrations of a natural flavonoid antioxidant and a single astaxanthin level or no supplemental antioxidants after a disease challenge.

1. H₀: Dietary KPA® does not enhance immune response in pre-smolt chinook salmon after disease challenge with Vibrio anguillarum by i.p injection.

H₁: Dietary KPA® does enhance immune response in pre-smolt chinook salmon after disease challenge with Vibrio anguillarum by i.p injection.

2. H₀: Dietary KPA® does not increase disease resistance in pre-smolt chinook salmon after disease challenge with an i.p injection of Vibrio anguillarum.

H₁: Dietary KPA® does increase disease resistance in pre-smolt chinook salmon after disease challenge with an i.p injection of Vibrio anguillarum.
3. GENERAL MATERIALS AND METHODS

3.1 FISH

Half-siblings of chinook salmon (O. tshawytscha) were hatchery reared in freshwater tanks at Yellow Island Aquaculture Ltd (YIAL, Quadra Island, BC, Canada), and then transferred to growout seacages at the same site.

3.2 EXPERIMENTAL DESIGN

3.2.1 Experiment A.

The experiment was carried out as a completely random design with four replicate groups of four diet treatments. In May 2000, 6400 chinook salmon alevins (mean weight 1.35 ± 0.3 g) were randomly distributed into 16 circular plastic tanks (200 L capacity each), each one containing 400 hundred fish (stocking density 1 kg/m³). Feeding trials started in June 2000 (mean fish weight 2.95 ± 0.2 g). The fish groups were hand-fed three times daily, at a feeding rate between 3.0% to 3.5% of body weight (b.w.) according to Lovell (1998b). Prior to the start of the experiment, fish were sampled for initial weight, length and astaxanthin concentration in muscle (10 fish/tank). During the experiment, non-lethal samples of fish were weighed and measured (fork length) on a weekly basis over 32 days. At the end of the feeding trial, muscle samples (n=12/diet group) were analyzed by HPLC to determine the amount of astaxanthin in tissue. Feed amounts were carefully weighed and recorded; uneaten feed was weighed and recorded daily. Collected data served to estimate weight gain, Fulton's condition factor (CF), feed conversion ratio (FCR), specific growth rate (SGR), and apparent astaxanthin retention coefficient (AARC) in FW hatchery. The feeders did not know the diet that they were feeding to each tank which reduced any hand-feeding bias to specific groups. All groups were fed 3 times a day with three feeding opportunities each time. This protocol was adopted so that all fish ingested their daily feed ration with a minimum of feed wastage. The amount of feed was initially estimated by the projected weight given by the thermal growth coefficient (TGC) (Iwama and Tautz, 1981) and was corrected according to the observed weight gain of fish after every weekly weight sampling. Fish weight projections were estimated using the UBC Aquaculture Production Analysis Computer Program, developed by Dr. G. Iwama and Dr. L. Fidler. The software was run using actual water temperature for the trial period and the recommended growth coefficient (Gc) for the month/temperature/species under
investigation (Iwama, 1996). Changes in feed size were made over a period of time, with small pellet sizes gradually replaced by larger ones according to the size increased of the fish.

**Experimental conditions:** artificial photoperiod regime during the experiment was set at 16L:8D. Well water was supplied to all tanks and flow and quality during the experiment were as follows: water flow 10 L/min; temperature 9.10 ± 0.25°C (Appendix 3); dissolved oxygen 8.0 ± 1.0 mg/L; oxygen saturation level 70 ± 15%. Temperature was recorded by an electronic probe every 4 h, and dissolved oxygen and oxygen saturation were recorded daily. Water flow was checked on a weekly basis.

### 3.2.2 Experiment B.

After 28 days of feeding, disease challenge and sham injection groups were established. The numbers of fish were reduced from 400 fish/tank to 2 sets of 40 fish/tank (tank replicates 1 and 2), and 2 sets of 80 fish/tank (tank replicates 3 and 4) and these were fed for another 4 days, completing the 32-day feeding period. Four additional tanks, each containing a pool of 200 fish from each diet treatment, were prepared as sham injection control groups (Table 3.2.1-A and 3.2.1-B).

Once fish were injected either with a bacterial suspension (disease challenge) or sterile peptone saline (sham injection), daily mortality was recorded and tissue samples were undertaken for a period of 10 days. Replicate tanks 1 and 2 served as mortality controls and replicate tanks 3 and 4 provided blood and tissue samples throughout the challenge experiment. Six fish (n=6/tank) from each diet treatment were sampled every day for haematology, immunocompetence and tissue analyses, as numbers permitted. Whole blood was analysed for haematocrit, haemoglobin, total erythrocyte and leucocyte counts, and differential leucocyte counts. Neutrophil respiratory burst activity was also estimated from fresh blood. Plasma was frozen on dry ice, and stored at -80°C for later analyses of lysozyme activity. Liver and head kidney were rapidly dissected, frozen on dry ice, and stored at -80°C for later analyses of stress protein 70 Kd (SP70) and lysozyme activity, respectively. Fish injected with peptone saline were sampled daily (n=3/diet group) for all the variables mentioned above.
3.2.3 Experiment C.

Chinook salmon smolts from experiment A were transferred from the hatchery of YIAL to on-site growout seacages in August 2000. Four thousand and four hundred smolts (mean weight 7.57 ± 0.3 g) were distributed into four 5 x 5 x 10 m adjacent seacages (1100 fish/pen) suspended in a floating wooden cage.

Fish groups were fed manually to apparent satiation every day (estimated between 2–2.5% b.w). As in freshwater, the feeding regime was blind-designed to reduce the hand-feeding bias to specific groups. The amount of feed each group received was estimated according to the biomass in each seacage and was increased weekly based on weight gain prediction. The feeding regime was adjusted monthly based upon the actual weight gain of each group after every weight sampling. The feeding period lasted for 155 days in saltwater.

Water temperature fluctuated from 11.6 ± 0.8°C in August to 8.6 ± 0.2°C in January (Appendix 4). Dissolved oxygen was 8.0 ± 1.0 mg/L, and oxygen saturation level was 70 ± 15%. Temperature was monitored by an electronic probe every 4 h, and dissolved oxygen and oxygen saturation were recorded daily. Mortalities were collected by a scuba diver every 7 to 14 days, counted and disposed of in the YIAL disposal pit.

Fish weights and fork lengths were recorded before transferring smolts to seacages, and every month thereafter, for 155 days (n=25 fish/diet group). Astaxanthin concentrations as determined by HPLC at the end of the freshwater period were considered as the baseline for muscle concentration of pigment for the saltwater phase. Flesh samples (n=14 fish/group) for astaxanthin analyses were also taken in the middle (October) and at the end (January) of the experiment. Data regarding fish weight and length, fed intake and muscle pigmentation were used to estimate CF, FCR, SGR, and AARC. Quantification of Roche Salmofan® scores (n=14 fish/group) was only possible in the final sampling time (January), when the sizes of fish fillets were large enough to avoid background interference.
Table 3.2.1 Experimental protocol layout for the disease challenge (3.2.1-A) and sham injection (3.2.1-B) groups

A. Bacterial suspension injection groups (*Vibrio anguillarum*, 0.1 mL, \(10^9\) cfu/mL).

<table>
<thead>
<tr>
<th>Groups</th>
<th>(A^0KPA^0)</th>
<th>(A^{60}KPA^{1000})</th>
<th>(A^{60}KPA^{100})</th>
<th>(A^{60}KPA^0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>40 fish</td>
<td>40 fish</td>
<td>40 fish</td>
<td>40 fish</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>40 fish</td>
<td>40 fish</td>
<td>40 fish</td>
<td>40 fish</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>80 fish</td>
<td>80 fish</td>
<td>80 fish</td>
<td>80 fish</td>
</tr>
<tr>
<td>Replicate 4</td>
<td>80 fish</td>
<td>80 fish</td>
<td>80 fish</td>
<td>80 fish</td>
</tr>
</tbody>
</table>

B. Sham injection groups (Sterile peptone saline, 0.1 mL)

<table>
<thead>
<tr>
<th>Groups</th>
<th>(A^0KPA^0)</th>
<th>(A^{60}KPA^{1000})</th>
<th>(A^{60}KPA^{100})</th>
<th>(A^{60}KPA^0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 fish</td>
<td>200 fish</td>
<td>200 fish</td>
<td>200 fish</td>
</tr>
</tbody>
</table>
3.3 DIET PREPARATION

Diet treatment groups were based on the supplemental concentrations of grape seed extract (KPA®) and astaxanthin (Carophyll Pink®) (Table 3.3.1). Carophyll Pink® and KPA® were weighed on an air-dry basis, and added to the fish feed as active ingredient. An experimental chinook salmon diet was formulated by Dr. Dave Higgs and manually prepared by A. Schlicht and M. Rowshandeli at the West Vancouver Laboratory; DFO (West Vancouver, BC, Canada). All diets were formulated to contain equivalent concentrations of protein, lipid and energy based upon ingredient proximate analyses.

The composition of the basal diet formulation, as well as the vitamin and mineral premix are described in Table 3.3.2. Briefly, Austral fishmeal was ground in a hammer mill (Fitzpatrick Co., Fitz-Mill®, JT model, IL, USA) to standardize particles size. Vitamins were individually weighed and thoroughly blended by hand in a mortar and then mixed altogether in raw starch in an electric mixer for 30 min. Minerals, astaxanthin and KPA® were also weighed individually and ground manually in a mortar and mixed in α-cellulose for 30 min in a mixer (Blakeslee, model B-12, Chicago, IL, USA). All ingredients of the basal formulation were weighed on an air-dry basis and thoroughly mixed (Hobart mixer, M 802, OH, USA) for 30 min and then in a commercial paddle blender (Marion mixer, Iowa, USA) for another 45-60 min. Diets were steam-pelleted (maximum temperature 80°C for less than 5 sec.) in order of increasing pigment and antioxidant concentration, i.e. control diet, astaxanthin diet, low KPA® diet, and high KPA® diet, respectively using a laboratory pellet mill (model CL-type 2, California Pellet Mill Co., San Francisco, CA, USA) and following pellet size recommendation given by Lovell (1998b).

Astaxanthin (Carophyll Pink® 2%, Hoffman-La Roche, Basel, Switzerland) was incorporated at the same proportion on an air-dry basis in all diets, except the control diet. The concentration of astaxanthin among the three diets containing astaxanthin was not significantly different (p=0.117) as measured by HPLC in duplicate samples (Table 3.3.1-B). Grape seed extract (KPA®) containing 42.7 mg/g (as active ingredient) of the polyphenolic antioxidant PA (oligomeric flavan-3-ol) was incorporated in a high (1000 ppm) and low (100 ppm) concentration in diets designated as high and low KPA®, respectively.
After pelleting, the diets were sprayed with a mix of fish and vegetable oil (anchovy oil: canola oil 5:2, 122 g/kg of pellets) and stored in dark bags at room temperature until use. Fish feed was prepared no longer than 8 weeks from one batch to another.
Table 3.3.1 Experimental diet groups supplemented with pigment (astaxanthin, 0 and 60 ppm) and grape seed extract (KPA®, 100 ppm or 1000 ppm), respectively (3.3.1-A); and final concentrations of astaxanthin in the test diets (3.3.1-B).

A. Experimental diet treatments

<table>
<thead>
<tr>
<th>Experimental Diet</th>
<th>Grape Seed Concentration (KPA®, ppm)</th>
<th>Astaxanthin Concentration (Carophyll Pink®, ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Diet (A°KPA°)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High KPA Diet (A°KPA°1000)</td>
<td>1000</td>
<td>57</td>
</tr>
<tr>
<td>Low KPA Diet (A°KPA°100)</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>Astaxanthin Diet (A°KPA°)</td>
<td>0</td>
<td>57</td>
</tr>
</tbody>
</table>

B. Mean (± SD) HPLC concentrations of astaxanthin in the test diet (µg/g) fed to chinook salmon (Oncorhynchus tshawytscha); pooled data for different batches.

<table>
<thead>
<tr>
<th>Diet</th>
<th>A°KPA°</th>
<th>A°KPA°1000</th>
<th>A°KPA°100</th>
<th>A°KPA°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin Concentration</td>
<td>0.21 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.49 ± 1.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.20 ± 2.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.57 ± 1.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
### Table 3.3.2 Formulation of the basal diet and vitamin and mineral premix composition.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/kg as fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austral fish meal</td>
<td>536.29</td>
</tr>
<tr>
<td>Blood flour; spray-dried</td>
<td>51.26</td>
</tr>
<tr>
<td>Squid meal</td>
<td>71.89</td>
</tr>
<tr>
<td>Wheat gluten meal</td>
<td>50.90</td>
</tr>
<tr>
<td>Pregelatinized wheat starch</td>
<td>52.38</td>
</tr>
<tr>
<td>Raw wheat starch</td>
<td>21.77</td>
</tr>
<tr>
<td>Vitamin premix(^1)</td>
<td>18.93</td>
</tr>
<tr>
<td>Mineral premix(^2)</td>
<td>18.93</td>
</tr>
<tr>
<td>Anchovy oil; stabilized(^3)</td>
<td>84.88</td>
</tr>
<tr>
<td>Canola oil</td>
<td>37.86</td>
</tr>
<tr>
<td>Soybean lecithin</td>
<td>9.47</td>
</tr>
<tr>
<td>Choline chloride (60%)</td>
<td>4.73</td>
</tr>
<tr>
<td>Vitamin C (monophosphate, 42%)</td>
<td>0.95</td>
</tr>
<tr>
<td>Permapell(^®)</td>
<td>9.47</td>
</tr>
<tr>
<td>Finstimm(^®)</td>
<td>9.47</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>1.89</td>
</tr>
<tr>
<td>Carophyll pink and/or α-cellulose</td>
<td>9.47</td>
</tr>
<tr>
<td>KPA(^®) and/or α-cellulose</td>
<td>9.47</td>
</tr>
</tbody>
</table>

\(^1\). **Vitamin premix composition (amount/kg dry diet):** D-calcium pantothenate, 168.40 mg; pyridoxine HCl, 49.30 mg; riboflavin, 60 mg; folic acid, 15 mg; thiamine mononitrate, 56 mg; biotin, 1.50 mg; Vitamin B\(_{12}\) (cyanocobalamin), 0.09 mg; menadione (as vitamin K), 18 mg; vitamin E (\(α\)-tocopherol), 50 IU; vitamin D\(_3\), 2400 IU; vitamin A, 5,000 IU; inositol, 400 mg; niacin, 300 mg; BHT, 22 mg; raw starch (carrier).

\(^2\). **Mineral premix composition (mg/g dry diet):** Mn (as MnSO\(_4\)\*H\(_2\)O), 75 mg; Zn (as ZnSO\(_4\)\*7 H\(_2\)O), 60 mg; Co (as CoCl\(_2\)*6 H\(_2\)O), 3 mg; Cu (as CuSO\(_4\)*5 H\(_2\)O), 7 mg; Fe (as FeSO\(_4\)*7 H\(_2\)O), 100 mg; I (as KIO\(_3\)), 5 mg; I (as KI), 5 mg; F (as NaF), 5 mg; Na (as NaCl), 1,500 mg; Se (as Na\(_2\)SeO\(_3\)), 0.20 mg; Mg (as MgSO\(_4\)*7 H\(_2\)O), 400 mg; K (as K\(_2\)SO\(_4\)), 850 mg; K (as K\(_2\)CO\(_3\)), 850 mg; α-cellulose, (carrier).

\(^3\). Stabilized with 500 ppm Santoquin
3.4 FEED ANALYSIS

3.4.1 Feed KPA® analysis

Random samples (250 g) of each diet were vacuum packed in oxygen-impermeable bags and frozen at -40°C until further analysis. Feed samples (20 g) were diluted in 400 mL of methanol. The mixture was extracted with methanol at 65°C for 2 h by gently stirring, and then filtered through cellulose powder. The filtered mixture was then evaporated and the residue was dissolved in 50 mL of methanol prior to measuring the content of PA in the extract by the Porter method. The Porter method measures the procyanidin oligomers/polymers as the absorbance of a 1% w/v solution of methanol soluble sample material at 550 nm.

Results are reported as Porter Value Units (PVU) rather than as percent, which correlates to the procyanidin content. Monomers are not included in the measurement and the degree of polymerization is not differentiated. Absorbance generally increases with average phenolic content. On a molar basis, hexamers will have higher PVUs than dimers. Therefore, if a grape seed extract is comprised of primarily larger polymers, it will have a higher PVU than an extract comprised of the same molar concentration of dimers. In our experience, grape seed extracts have a range from 25 to 325 PVU. The analysis of proanthocyanidins in feed were performed in the laboratory of Kikkoman Corporation in Japan (Noda City, Chiba, Japan), and unfortunately this laboratory failed to report the content of KPA® in the final diet.

3.4.2 Astaxanthin extraction from feed and muscle

Feed samples were pre-dissolved in warm water bath (<50°C) while muscle samples were extracted directly. Samples of 10 g of feed were ground in a coffee grinder (Braun, type 4014) and astaxanthin was extracted using the Bligh and Dyer (1959) method for lipid extraction. One-gram (± 0.005 g) samples of ground feed were suspended in 10 mL of deionised water in a thick-glass test tube. The suspension was warmed up in a water bath (Precision Scientific Co., Winchester, VA, USA) at 80°C for 30 min with occasional shaking, allowing the release of astaxanthin from its gelatin matrix and cornstarch coating. The feed suspension was then extracted three times in a separatory funnel using a 2:2:1.8, chloroform: methanol: deionised water mixture. The chloroform layer from each subsequent extraction was pooled in a round-bottom flask
for rotatory evaporation under vacuum on a water bath (50°C). Following evaporation, the oil-film coat containing carotenoids was dissolved in 2 mL of n-hexane (HPLC grade) and re-evaporated, and re-dissolved in one mL of n-hexane (HPLC grade). An aliquot of the solvent containing pigment was put in a 1.0 mL glass HPLC vial (Wheaton, USA) and immediately analyzed by HPLC. Duplicate samples of feed from each diet batch were analyzed for astaxanthin concentration.

Muscle samples were weighed (1.0 ± 0.005 g), and then each one was placed directly in a separatory funnel and astaxanthin was extracted following the same protocol as described for feed astaxanthin analysis.

### 3.4.3 HPLC analysis

Astaxanthin was quantitatively analyzed by HPLC according to Kiessling et al. (1995). Hexane solution (150 µL) (see 3.4.2.1) was injected into a Waters HPLC-module 1, equipped with a stainless steel silica column (μPorasil 125’, 10 µm, 3.9 mm X 30 cm). The system was equipped with a 600E Powerline™ Controller pump, a 715 Ultra WISP™ autoinjector, and a Waters 486 Tunable Asorbance Detector (Table 1.3). The mobile phase was hexane:acetone (83:17) with a flow rate of 1 mL/min. Authentic astaxanthin standards were prepared from crystalline all-E astaxanthin (Hoffman-La Roche, Basel, Switzerland). The absorbance was measured with spectrophotometer at 472 nm, and the concentration of astaxanthin was calculated according to the formula used by Schierle and Hardi (1992):

\[
\text{Astaxanthin (mg/L)} = \text{Absorption} \times \frac{10,000}{2100}
\]

A standard curve was generated, and HPLC peaks detected at 472 nm were integrated and quantified using Waters Millennium® software. The HPLC retention times for the pigment isomers (XZ, all-E, 9Z and 13Z) were estimated, and the amounts of the different isomers were calculated and corrected for their differences in extinction coefficients. In regard to the latter, the quantities of the three Z astaxanthin isomers were multiplied by 1.6, 1.2, and 1.1 (ratio of the isomer extinction coefficient for the 13Z, 9Z and XZ isomers relative to the all-E isomer coefficient, 2100) to account for their lower specific absorption. Total astaxanthin concentration in each sample was reported as the sum of all four isomers.

\[2100 = E(1%/1 \text{ cm}) = \text{standard absorption of a 1% astaxanthin solution (w/v) in a 1 cm cuvette at 470 nm in n-hexane} \]
3.5 PIGMENTATION ASSESSMENT

The deposition of astaxanthin in the muscle of chinook salmon was assessed for both the FW and SW phases of the study. Baseline values for astaxanthin concentration in muscle of salmon grown in freshwater were determined before the start of the feeding trial in June 2000. Astaxanthin deposition efficiency in pre-smolt chinook salmon was calculated after 32 days in freshwater. In this regard, a random sample of 40 alevins (10 fish/dietary treatment, mean weight 2.90 ± 0.21 g) were killed by a lethal dose of anaesthetic (MS-222®), skinned and filleted. Salmon fillets were pooled and ground altogether and ten samples of 1 g were analyzed by HPLC to establish the baseline concentration of astaxanthin in muscle. After 32 days of feeding, astaxanthin concentration in flesh was assessed on individual fillets for each dietary treatment group (mean weight 5.31 ± 0.36 g).

HPLC results reported at the end of experiment A were considered as the initial flesh concentration of astaxanthin in smolts that were transferred to SW (beginning of experiment B). A second muscle sample (n=7/diet group) was taken after 70 days, and the final sample (n=7/diet group) was taken at the end of the experiment, after 155 feeding days.

All samples for astaxanthin quantification were immediately vacuum-sealed in oxygen-impermeable bags after filleting, wrapped in tin foil, frozen on dry ice, and then stored at -80°C until HPLC analysis.

3.5.1 Muscle astaxanthin analysis

Chinook salmon were killed by a lethal dose of anaesthetic, skinned and filleted. Fillets were wrapped in tin foil, rapidly frozen on dry ice, vacuum-packed in oxygen impermeable bags and stored at -80°C until further analysis. Fillets were first thawed and then ground by hand in a mortar and pestle in order to achieve an even consistency and a homogeneous sample of flesh. A 1.0 g (± 0.005 g) sample of fillet homogenate was extracted directly following the procedures outlined in sections 3.4.2 and 3.4.3.

3.5.2 Apparent astaxanthin retention coefficient (AARC)

AARC in chinook salmon fillets was calculated according to the formula used by Nickell and Bromage (1998):

\[
AARC: \frac{((A_{xf} - A_{xi}) (W_{tf} - W_{ti}))}{DAX} \times 100
\]
Where \( A_{xf} \) is the final mean flesh astaxanthin concentration (\( \mu g/g \)), \( A_{x_i} \) is the initial flesh astaxanthin concentration (\( \mu g/g \)), \( W_{tf} \) is mean final fish weight, \( W_{ti} \) is initial mean fish weight, and \( DAX \) means the total amount of dietary astaxanthin over the experimental period (\( \mu g/g \)).

### 3.5.3 Roche Salmofan\textsuperscript{®} fillets scores (RSS)

Immediately after filleting, the opposite halves of the salmon fillets sampled for flesh astaxanthin quantification were each placed in a one-side-open white box illuminated with two fluorescent light tubes (Spectralite\textsuperscript{®} F40T12 fluorescent lamp, Philips Lighting, USA). Two judges scored independently the colour of fillets using the Roche Salmofan\textsuperscript{®} (scale 20-34, Appendix 5). Scores were assessed following the cut recommended by the Norwegian general standardizing body as stated by Sigurgisladottir et al. (1997). Briefly, each RSS reading was taken just under the dorsal fin at an equidistant point between the dorsal ridge and the pelvic fins. The colours of the individual fillets were compared with the Salmofan and a score was assigned according to the matching colour of the Salmofan tiles (20-34). Fillets of the control diet group, as well as some of the smaller fillets from fish fed astaxanthin-fortified diet groups showed flesh colour below the minimum score of 20 (ranging from the palest green to the palest peach), and they were assigned a score of 20.

### 3.6 GROWTH ASSESSMENT

#### 3.6.1 Wet weight gain

Random samples of fish were taken from all diet treatment groups. Fish were anaesthetized (MS-222\textsuperscript{®} and NaHCO\textsubscript{3}, 50 mg/L and 100 mg/L, respectively) and weights and lengths were recorded individually (n=20). This permitted us to calculate the mean weight increase per dietary group. The fork length of each fish was determined by measuring from the tip of the snout to the fork of the tail (fork length).

#### 3.6.2 Specific growth rate (SGR)

SGR (percent of increase in weight/day) values were calculated according to the formula:

\[
SGR = \left( \frac{\ln W_{tf} - \ln W_{ti}}{t} \right) * 100
\]
Where, $W_{tf}$ is mean final weight, $W_{ti}$ is initial mean weight, and $t$ is time (in days) between samplings.

### 3.6.3 Feed conversion rate (FCR)

FCR values were estimated according to the formula:

$$\text{FCR} = \frac{\text{amount of fed food (kg)}}{\text{fish biomass increase (kg)}}$$

Where, feed was the weight of feed fed to the fish groups population and biomass increase was the difference between the initial and final mean weights of the fish in each group.

### 3.6.4 Fulton’s condition factor (CF)

Condition factor was estimated according to the formula:

$$\text{CF} = \frac{W_t}{\text{Length}^3} \times 100$$

Where, $W_t$ is the mean weight of the fish population (g), and length is the mean fork length (cm) of the fish population.

### 3.7 DISEASE CHALLENGE IN FRESHWATER

A virulent strain of *Vibrio anguillarum* (*Vang*) that was originally isolated from an adult chum salmon (*Oncorhynchus keta*) which exhibited signs of vibriosis was used in this experiment (isolate 98055, Pacific Biological Station, DFO, Nanaimo, B.C). The isolates were grown on tripticase soy agar (TSA) plates supplemented with 1.5% NaCl. Bacteria in their log phase (24 h at 20°C) were transferred into sterile peptone-saline (PS, 0.2% peptone and 1.5% saline, pH 7.2) and vortexed to suspend the bacteria thoroughly in the solution. The concentration of *Vang* used to inoculate the fish was estimated from absorbance measurements made by spectrophotometer at 540 nm (one OD unit at 540 nm was estimated to contain $10^9$ cfu/mL). The suspension was diluted to reach a final concentration of $1 \times 10^9$ cfu/mL, as determined by drop-plate counts.

#### 3.7.1 Bacteria injection protocol

A volume of 0.1 mL of a bacterial suspension of *Vang* ($10^9$ cfu/mL) was injected into the peritoneal (i.p) cavity of fish. Sham fish groups were inoculated with 0.1 mL i.p of sterile peptone saline, following the same procedure as that described for the disease challenge groups. All injection procedures were carried out in fish anaesthetized in a
buffered solution of MS-222® and NaHCO₃ (50 mg/L and 100 mg/L, respectively) and the bacterial suspension and sham solutions were maintained on ice during the duration of the inoculation procedure.

3.7.2 Bacterial re-isolation and identification from moribund fish

*Vang* was re-isolated and identified from moribund fish from the challenge groups. Samples from liver and head kidney were grown on trypticase soy agar (TSA) (Difco Laboratories, Oakville, ON, Canada) and 0.85% NaCl for 20-24 h. Well isolated suspicious colonies (smooth, opaque, creamy colour, medium size) were picked, smeared onto a microscope slide and Gram stained. Small Gram-negative bacilli (often curved) were considered *Vibrio anguillarum* suspicious. Biochemical confirmation of the bacteria was performed using API® 20-E kit (BioMérieux, Missouri, MO, USA) (Appendix 6). A single well-isolated colony was removed from the agar plate and tested for an oxidase positive reaction. Another similar single colony was placed in 0.85% NaCl and emulsified to obtain a homogeneous suspension. Thereafter, this suspension was used to fill all 20 chambers of an API® 20-E strip. Colonies exhibiting oxidase (+), gelatinase (+), oxidation and fermentation of glucose (sorbitol, sucrose, arabinose) (+), and Voges Proskauer (+) were identified as *Vang* positives.
4. CHAPTER ONE

Assessment of flesh pigmentation and growth of pre-smolt farmed chinook salmon fed one of two concentrations of a natural flavonoid antioxidant and astaxanthin in the diet
4.1 INTRODUCTION

The effects of feeding juvenile chinook salmon diets containing a combination of natural dietary antioxidants, other than antioxidant vitamins (i.e. vitamin A, vitamin C, vitamin E), and astaxanthin on flesh pigmentation and growth variables have not been described previously. Kikkoman proanthocyanidins are natural flavonoid antioxidants that have been shown to exert beneficial effects both in vitro and in vivo. Amongst other attributes, PA have been shown to enhance the growth and viability of gastric mucosal cells in humans and macrophages in rats (Ye et al. 1999). KPA® has also been shown to provide photo-oxidative protection of flesh colour in salmon fillets soaked in a KPA®-saline solution (Kikkoman Corporation, personal communication).

Astaxanthin is a naturally occurring carotenoid pigment that provides salmonids with their characteristic red-pink colour to various tissues. Salmonids must obtain carotenoids from a dietary source. They are deposited into the muscle, skin, and gonads according to the physiological state of the fish (Torrissen, 1985). Carotenoids are an essential nutritional supplement in the diets of farmed salmonids during their early development (Christiansen et al. 1994). A minimum of 5 ppm of astaxanthin is required in the diet to ensure fish survival, optimal development and growth in Atlantic salmon swim-up fry (Salmo salar) during first-feeding.

There are conflicting results in the literature regarding the ability of small salmonids to deposit carotenoid pigment in their flesh. Some reports indicate either no presence of astaxanthin in muscle or very little deposition of pigment. For instance, Christiansen et al. (1995b) reported negligible deposition of astaxanthin in the muscle of Atlantic salmon fry (weight ~4 g). Alternatively, Thomas (1999) and Wieruszewski (2000) reported some deposition of astaxanthin in the muscle of small chinook and Atlantic salmon (<100 g), respectively. The deposition of pigment in the flesh of young salmonids is not completely understood. Absorption, metabolism, and deposition of carotenoids in salmon depend on several endogenous and exogenous factors. In addition to the role of astaxanthin in pigmentation, astaxanthin has been shown to have an in vivo antioxidant activity in rainbow trout (Nakano et al. 1995). It may be speculated that feeding a combination of KPA® and astaxanthin might increase the overall redox status in salmon and thereby exert a “sparing effect” on the availability of astaxanthin as a flesh pigmentation agent.
In this study, I compared the efficiency of astaxanthin deposition in the muscle of pre-smolt juvenile chinook salmon (initial mean weight 2.95 g) when the fish had been fed one of four experimental diets that contained either a uniform amount of astaxanthin and one of two levels of grape seed PA (KPA®) or no supplemented astaxanthin or KPA® for 32 days. In addition, the growth performance, as well as their condition factor and food conversions were assessed during the feeding trial.

4.2 MATERIAL AND METHODS
4.2.1 PIGMENTATION ASSESSMENT

Muscle astaxanthin concentration, AARC, RSS

The concentration of astaxanthin in flesh was measured before the start and after 32 days of feeding the experimental diets. Collected data regarding feed and muscle astaxanthin concentration, and total consumed feed by fish served to estimate AARC. Pigmentation analysis and formulas were outlined in section 3.4 of the General Materials and Methods. Pigmentation analysis using the Roche Salmofan® was not estimated at this time due to the small size of the fish.

4.2.2 GROWTH ASSESSMENT

Wet weight gain, length, CF, FCR, and SGR

Random samples of 20 fish were taken from all diet treatment groups. The salmon were anaesthetized (MS-222® and NaHCO₃, 50 mg/L and 100 mg/L, respectively), then individually weighed and measured for fork length. Collected data was used to calculate all growth variables (CF, FCR, SGR) according to the formulae described of section 3.6 in the General Materials and Methods section.

4.3 STATISTICAL ANALYSES

Each diet was fed to four replicate groups and the results for all the parameters have been expressed as the mean ± one standard deviation (SD). Quantitative data were analysed by ANOVA (p<0.05), and the significant differences among means were detected using Student-Newman-Keul’s or Dunn’s test where appropriate. All statistical analyses were performed by SigmaStat™ software (SSPS Inc., Chicago, IL, USA).
4.4 RESULTS

Mortality

Mortality reached 3.5% during the freshwater phase of the experiment. There were no statistically significant differences in fish mortality between the diet treatment groups (p=0.872).

Fish Growth and feed conversion

The initial mean weights of the groups were not significantly different, averaged 2.95 ± 0.21 g (Fig 4.1). On day 32, fish fed the diet astaxanthin alone (astaxanthin diet) or together with a high or low dose of KPA® had mean weights of 5.35 ± 1.34 g, 5.43 ± 1.18 g, and 5.66 ± 1.33 g, respectively. And these values were statistically equivalent and higher than fish fed the diet without any supplement KPA® and astaxanthin (mean weight 4.75 ± 0.86 g).

Specific growth rate values for the groups were uninfluenced by the diet treatment and varied between 2.16%/day for the control diet and 2.40%/day for chinook ingesting the diet with the high dose of KPA® together with astaxanthin (Fig. 4.2-B). Likewise, the feed to gain ratios were also not significantly influenced by diet treatment and ranged from 1.36 ± 0.28 (low KPA® diet) to 1.90 ± 0.54 (high KPA® diet).

Fulton’s condition factor was recorded weekly throughout the feeding trial for all groups, but values for the parameter were generally not affected by diet treatment, except for fish fed diet with high KPA which had a significantly higher CF (p=0.047) than noted for all the other fish groups on day 21 (Fig. 4.3).

Pigmentation assessment

Muscle astaxanthin

Pre-smolt chinook salmon fed the control diet exhibited a statistically significant (p=0.019) lower concentration of astaxanthin in muscle after 32-day feeding period, compared with treatment groups fed diets containing 60 ppm astaxanthin (Figure 4.4). Moreover, fish fed the control diet had similar flesh astaxanthin concentration compared with the fish on day 0 (0.0290 µg/g vs. 0.0285 µg/g, respectively). Chinook fed the astaxanthin, low KPA® and high KPA® diet had an overall three-fold increase in flesh
astaxanthin concentration after 32 days (mean values ranged from 0.098 µg/g to 0.104 µg/g).

Correlation between dietary astaxanthin (DAX), apparent astaxanthin retention coefficient (AARC) and HPLC astaxanthin concentration in muscle (Figure 4.5-A and 4.5-B)

A significant positive correlation was detected both between DAX (µg/g) and the concentration of astaxanthin in muscle (Figure 4.5-A, r²= 0.996, p=0.029, n=4); and DAX and AARC (Figure 4.5-B, r²= 0.994, p=0.004, n=4).

Apparent astaxanthin retention coefficient, AARC

Fish groups fed the high KPA® diet for 32 days showed a higher, but not significant (p=0.709) apparent astaxanthin retention coefficient (0.069%) than fish groups fed low KPA® (0.068%), and astaxanthin diet (0.060%), respectively (Table 4.1).
Figure 4.1 Wet weight increase in pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets containing various levels of antioxidant supplementation (A°KPA°, A°KPA°, A°KPA°, A°KPA°) after a 32-day feeding period. Letters indicate a statistical difference between dietary groups (n=20/diet treatment, four replicates/diet, p=0.043).
Figure 4.2 Mean (± SD) apparent values for FCR (4.2-A) and SGR (4.2-B) in pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets after a 32-day feeding period. Different letters indicate significant differences (n=20/diet treatment, four replicates/diet, p<0.05) between diet groups.
Figure 4.3 Mean (± SD) Fulton's condition factor for pre-smolt chinook salmon (O. tshawytscha) during a 32-day feeding period fed four experimental diets containing various levels of antioxidant supplementation (A°KPA°, A°60KPA°, A°60KPA1000°, A°60KPA100°). Solid bars represent dietary group mean, error bars indicate SD of diet group. Different letters indicate significant differences (n=20/diet treatment, four replicates/diet, p<0.05) between the diet groups.
Figure 4.4 Mean (± SD) astaxanthin concentration in flesh of pre-smolt chinook salmon (O. tshawytscha) after a 32-day feeding period with four experimental diets supplemented with astaxanthin and two levels of KPA®. Solid bars represent dietary group mean, error bars indicate ± 1 SD. Different letters indicate significant differences (n=20/diet treatment, four replicates/diet, p<0.05) between diet groups. Horizontal hatched bars (far right) represent the astaxanthin concentration in the fish flesh prior to the start of the experiment.
Figure 4.5 Correlation between total accumulated dietary astaxanthin (DAX) and flesh astaxanthin concentration in muscle (4.5-A), and apparent astaxanthin retention coefficients (4.5-B). Four experimental diets supplemented with astaxanthin and two levels of grape seed extract in chinook salmon in freshwater tanks for 32 days.
Table 4.1 Apparent Astaxanthin Retention Coefficient (AARC) in muscle of pre-smolt chinook salmon (O. tshawytscha) fed control diet (A°KPA°), astaxanthin diet (A60KPA°), High KPA° diet (A60KPA1000) and low KPA° diet (A60KPA100). Four replicate groups were fed each of the test diets for 32 days at 3.5% body weight (b.w.). Different letters indicate significant differences (n=6/diet treatment, 4 replicates/diet, p<0.05) between diet groups. (n.a. not analyzed)

Feeding Period: June 19, 2000 – July 20, 2000

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<th>Diet Group</th>
<th>Wi (g)</th>
<th>Wf (g)</th>
<th>Axd (μg/g)</th>
<th>Feed (kg)</th>
<th>DAx (mg)</th>
<th>Axi (μg/g)</th>
<th>Axf (μg/g)</th>
<th>AARC (%)</th>
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4.5 DISCUSSION

Farmed salmon are housed in enclosed growing facilities (tanks, floating seacages) and must be fed diets containing balanced amounts of macronutrients and micronutrients. Astaxanthin is an important micronutrient that is required for proper growth, development, and survival of early stages of fish life. Further, astaxanthin is an essential nutrient in salmon diets for its provitamin A (Christiansen et al. 1994), biological colorant (Torrissen, 1985) and in vivo antioxidant properties (Nakano et al. 1999). Kikkoman PA added singly in cell culture has been shown to promote the growth and viability of normal human gastric mucosa cells and macrophages (Ye et al. 1999). There is no information on the use of KPA® as an antioxidant supplement in diets for farmed animals.

Besides a well-formulated diet and water temperature, feeding level (amount of given food as percentage of fish b.w.) is a very important variable affecting fish growth and feed conversion efficiency. Pre-smolts fed diets containing astaxanthin alone or in combination with each of two levels of KPA® at a feeding rate of 3.5% b.w did not show significant differences on growth (weight gain and SGR), feed utilization or condition factor after a short feeding period. Chinook salmon fed the control diet (no KPA®, no astaxanthin) did show a significantly (p=0.019) depressed weight gain at the end of the 32-day feeding trial, however, values for SGR, FCR and CF were not significantly different from those observed for fish fed the other three diets.

In this study, feeding rate was set at 3.5% b.w. per day following recommendations by Lovell (1998b). This rate was chosen because smaller fish have faster growth rates than larger fish due to their relatively higher metabolic rate per unit of body weight, relatively larger stomach capacity, and faster gut evacuation (Pennell and McLean, 1996). Maintenance feeding level in salmonids has been established at 1% of fish body weight, and this is the ration where the nutrients and energy contained in feed meet the metabolic and activity needs of the fish (Lovell, 1998b). Beyond this point, there is a net gain in weight and length, and the growth curve is almost linear until the feeding level reaches the maximum levels of feed consumption. Once maximum feeding level has been reached, the fish cannot consume any more feed and there is wastage.

The significantly lower growth (weight gain) of salmon fed the diet without astaxanthin in this study agrees with the results obtained by Christiansen et al. (1995a)
for Atlantic salmon parr over a 10.5 month period. Contrary to the findings of Christiansen and co-workers (1995a), the fish sampled weekly for weight and length in my study did not show any signs of malnutrition or poor appetite. However, the cumulative mortality was slightly higher (cumulative mortality 3.5%, no statistical difference between treatment groups) than the mortality noted by Christiansen and co-workers in their study (1% mortality).

Thermal growth coefficient has proven to be a very useful growth predictor tool in aquaculture systems and it may be more accurate predictor than the SGR (Cho, 1992). The weights increase of chinook salmon in this study predicted by the thermal growth coefficient (TGC) was exceeded by the observed weights obtained by all diet treatment groups. The difference was more evident in fish fed the astaxanthin diet (+13%), followed by the high KPA® (+8%), low KPA® (+8%), and diet control diet (+5%), respectively. These differences might be due to the high feeding ratio given to the fish, which was close to maximum ration and hence resulted in improved growth.

Specific growth rate is most useful and accurate when used to compare growth of fish of the same specie, size, and reared simultaneously with the same growing conditions (i.e. water temperature, stocking density, feed formulation, etc). The supplementation of KPA® in salmon diet did not result in significant SGR or FCR differences when compared to diets containing the combination of antioxidants and no antioxidants.

The increase of fish weight relative to the amount of food consumed is termed feed efficiency and feed conversion ratio is the reciprocal relationship. Achieving a low FCR is important to fish farmers because it helps to minimize production costs. In this study, the FCR in pre-smolt chinook salmon reared in freshwater tanks was quite elevated (ranged from 1.36 to 1.90). FCR for salmonids in FW have been reported to be roughly 1.0 or below. Feed conversion ratio has been reported to be more efficient in smaller, faster growing fish than in mature fish (Steffens, 1989), probably because more frequent feedings may result in a higher maximum daily ration and higher growth rate. In addition, small fish gain more protein and less fat than larger fish. According to Jobling (1994) deposition of 1 g of fat represents a weight increase of 1 g, whereas deposition of 1 g of protein leads to the gain of about 4 g of tissue. This is because muscle contains approximately 75% water while fat tissue contains no water (Lovell, 1998a).
High FCR index values indicate poor feed efficiency. High FCR values reported in this study likely result from feed wastage due to the high feeding rate that was used, which exceeded the maximum consumption ration of the fish. Feed conversion ratio relies on an accurate record of the amount of feed consumed. The feeding level chosen (3.5% b.w) was far above the 2.5% b.w feeding level recommended by Halver (1989), and by the Ewos feeding chart for chinook salmon parr in FW (1.6-2.0% b.w. at water temperatures between 8°C-10°C). The feeding design employed in this study failed to recognise the point of satiation in the fish, and consequently there was an increased quantity of uneaten food leading to an overestimation of the FCR values.

A practical salmon-farming tool used to assess the correct growth of fish is the Fulton’s condition factor. Condition factor provides an index of the weight to length relationship (Piper et al. 1982), and it is relatively constant for specific species. The greater the CF the greater the weight in relation to unit length. For rainbow trout (Oncorhynchus mykiss) normal CF is roughly 1.1 (1.0 – 1.2). For carps values for FCR vary between 2.0 and 2.5. In the present study, there was no significant difference in CF amongst diet treatment groups, although CF showed a marginal trend for increase at the end of the trial. This result is contrary to what is described in smolting salmon, and is difficult to explain. Chinook salmon are anadromous Pacific salmon with an early smoltification process, as early as 5 g in the “ocean-type” (Groot and Margolis, 1995; Clarke et al. 1996). Smoltification is the physiological process that some salmonids undergo to prepare them for life in the sea, and it is characterized by a boost of growth and an increase in the length-weight ratio. These changes result in a decrease in the CF of the fish (Hoar, 1988). Chinook salmon used in this experiment had clear external signs of smoltification, i.e. silvering and loss of parr marks (Hoar, 1988). It is possible that a decreasing CF trend would have been more evident at a latter stage of smoltification, i.e. at a larger fish size. Smolting chinook salmon were transferred to seacages when fish were over 7 g, two weeks after the last sampling in FW hatchery tanks. Salmonids that reach their smoltification state at a larger size show a clear decrease in CF, i.e. Atlantic salmon (~25 g to 40 g), coho salmon (~20 g to 35 g). Nordgarden et al. (2002) have described a decrease in CF in Atlantic salmon during smoltification (average weight ca. 40 g) from 1.25 to 1.10.

Despite the fact that the Fulton’s CF index was within the range described for the genus Oncorhynchus spp. in FW, there was a statistically significant reduction of CF
values on sampling day 7 and day 21 compared to baseline values for CF and values on sampling days 14, 28 and 32 (figure 4.3). These trends might have been due to the growth characteristic of juvenile salmonids. Experienced salmon farmers have noticed that salmon in FW exhibit a “differential” growth. Juvenile fish show a fast length increase, then a quick weight increase, and therefore CF fluctuates. It is likely that the statistically significant lower values for CF at the two preceding sampling times were not biologically significant, since the values fell within those reported for salmon. In addition, the reduced values were not associated with fish in an emaciated condition, nor was the situation prolonged.

In order to achieve a red-pink colour in flesh, salmonids have to ingest, transport and deposit biological pigments in their flesh (astaxanthin and/or canthaxanthin). In the wild, salmonids obtain most of their carotenoids by feeding on small crustaceans and/or invertebrates that contain them. In intensive farming systems, carotenoids are added to the feed mainly in the form of industrially synthesized compounds. The use of carotenoids in salmon farming has solely been of market and commercialization interest, until recent years. It is now appreciated that carotenoids are essential nutrients necessary for the growth, proper development and survival of salmon fry (Christiansen et al. 1994). Studies covering carotenoid deposition in the flesh of salmonids just after first-feeding are almost non-existent. This is mainly because of the very inefficient pigment deposition in salmonids smaller than 5 g (Christiansen et al. 1994).

The literature shows conflicting results regarding the ability of small salmonids, below a certain size, to deposit carotenoids efficiently in flesh. Fry and fingerlings have a limited capacity for carotenoid deposition in the flesh, while significant amounts are deposited in the skin (Torrissen et al. 1989; Storebakken and No, 1992). Christiansen et al. (1995b) did not find any traces of astaxanthin in the muscle of Atlantic salmon fry (~4 g) after 140 days of feeding. In the present study, chinook salmon slightly larger than the fish used by Christiansen and co-workers (average weight 4.79 g to 5.66 g) showed an average three-fold increase of astaxanthin in their flesh from the basal concentration obtained prior to feeding the different treatment diets (0.028 μg/g to 0.104 μg/g). Fish fed diets containing grape seed PA both in a high (1000 ppm) and low (100 ppm) concentration showed a marginally, but not significantly, larger concentration of astaxanthin in flesh and apparent increased pigment deposition efficiency (AARC) in flesh than fish fed the diet supplemented with only astaxanthin. Final astaxanthin
concentrations achieved in chinook salmon muscle after 32 days of feeding were in agreement with the results obtained by Bird and Savage (1989), who found levels of astaxanthin (mean weight 380 g) averaging 0.06 to 0.12 µg/g after 30 days of feeding.

The retention of carotenoid pigment in the muscle of salmonids depends on several interacting factors: 1) efficiency of absorption, 2) transport capacity, 3) efficiency of pigment deposition in various tissues, and 4) metabolism and excretion rate. These are also influenced by other exogenous factors, such as diet composition, feeding rate and duration, and type of carotenoid (reviewed by Torrissen et al. 1989; Storebakken and No, 1992).

Carotenoids are transported in the blood via lipoproteins, and this is dependent on the kind of ketocarotene and the physiological state of salmonids. The main carotenoid-carrying lipoprotein is HDL, 60.4% for canthaxanthin, 66.4% for astaxanthin; VLDL and LDL are minor carriers transporting less than 15% each (Choubert et al. 1992, 1994). Chapman et al. (1978), and Frémont and Marion (1982) showed that juvenile trout predominantly had circulating levels of VLDL and LDL, while in adults there was a shift to predominantly HDL, which accounted for more than the 60% of total circulating lipoproteins in plasma. These levels of circulating lipoprotein fractions might explain the inability of juvenile salmonids to efficiently deposit carotenoids even though their ability to absorb dietary carotenoids has been proven.

March et al. (1990) demonstrated that juvenile salmonids are able to absorb dietary carotenoids. These authors found that coho salmon weighing between 20-40 g were able to absorb astaxanthin from a single dose of pigment based on circulating levels of astaxanthin in blood. The astaxanthin levels found in the flesh of chinook salmon in my study support the findings described by March and co-workers (1990) and confirm that very small salmonids (well below 20 g) can absorb astaxanthin from dietary sources and deposit the carotenoid in muscle after a short feeding trial of 32 days.
5. CHAPTER TWO

Assessment of flesh pigmentation and growth of post-smolt farmed chinook salmon fed one of two concentrations of a natural flavonoid antioxidant and astaxanthin in the diet
## 5.1 INTRODUCTION

The effect of feeding a combination of a natural flavonoid and astaxanthin in a diet over a short period of time did not improve either the growth or pigment deposition in the flesh of pre-smolt chinook salmon. There was a trend for fish groups fed high KPA® diet (1000 ppm KPA®, 60 ppm astaxanthin) to have a higher growth rate (SGR), marginally higher concentration of astaxanthin in the muscle and improved astaxanthin deposition coefficient compared to fish fed the other diets.

Since we found that deposition of astaxanthin over the short feeding-period (32 days) was very limited in muscle (~ 0.1 µg/g) at a very small salmon weight (< 6 g), I hypothesized that bigger salmon might deposit astaxanthin in the actomyosin complex in muscle more efficiently than smaller ones. A longer feeding period would increase the amount of dietary astaxanthin consumed by the fish and potentially more astaxanthin could be deposited in flesh. March et al. (1990) and Chan et al. (2002) showed that carotenoid pigment deposition was positively correlated to the body weight of salmonids. Moreover, feeding experiments with juvenile coho salmon (O. kisutch) (Spinelli and Mahnken, 1978) and juvenile Atlantic salmon (Storebakken et al. 1987) have shown no pigment deposition in the flesh of fish weighing less than 80 g. The weight for commencement of pigment deposition in flesh has been reported to be 150 g for rainbow trout and chinook salmon, and greater (200-400 g) for Atlantic salmon. Wieruszewski (2000) reported pigmentation of less than 1.0 µg/g in Atlantic salmon weighing less than 200 g, meanwhile Thomas (1999) reported pigmentation ~1.3 µg/g in juvenile chinook salmon under 120 g.

In Experiment 2, astaxanthin deposition in muscle of post-smolt chinook salmon was assessed over a period of 155 days when fish were held in seacages. Fish were fed the four experimental diets that were described previously in the General Materials and Methods section. Additionally, growth performance variables (weight gain, FC, SGR, FCR) were recorded and assessed in relation to diet treatment.
5.2 MATERIAL AND METHODS

5.2.1 PIGMENTATION ASSESSMENT

Muscle astaxanthin concentration, AARC, RSS

Muscle astaxanthin concentrations reported in the dietary groups at the end of Experiment A were considered as the initial concentrations of astaxanthin in the smolts transferred to SW. Muscle samples were taken approximately halfway through the sea water phase of the experiment (day 70) and the last muscle samples were taken at the end of the experiment, after 155 days of feeding. The methodology that was used to extract the muscle astaxanthin concentration and for obtaining the values for AARC and RSS was outlined in sections 3.4.2, 3.4.3, and 3.5 in the General Materials and Methods section.

5.2.2 GROWTH ASSESSMENT

Weight gain, length, SGR, CF and FCR.

Random salmon samples were taken from each of the diet groups held in seacages (n=25) every month for 155 days. Anaesthetized fish were individually weighed and measured at each time. Collected data was used to calculate all growth and diet utilization variables (SGR, CF, FCR) according to the formulae described in the General Materials and Methods section.

5.3 STATISTICAL ANALYSIS

The experiment was performed using single treatment groups. It was not possible to have more seacages in the SW growout facility of YIAL. Due to the lack of replication of the diet treatments a statistical comparison of the results were incomplete. Results were analysed statistically by ANOVA to enable comparison between diet treatments. The data are presented as the mean of the sample ± one SD. The results presented in this chapter provide valuable biological information regarding the benefits of supplementing salmon diets with antioxidants (flavonoids and astaxanthin) under commercial conditions, and the findings should be confirmed in further research involving replicated groups per diet treatment.
5.4 RESULTS

Mortality

Fish mortality after 155-day feeding trial was not significantly different one diet to another, reaching a cumulated 3.1% in the control group, 2.0% in the low KPA® diet, 1.9% in the high KPA® diet and 1.7% in the astaxanthin diet (data not shown).

Growth assessment and feed utilization

a. Weight increase (Figure 5.1)

Fish fed diets containing the high concentration of KPA® and astaxanthin (high KPA® diet) had a significantly larger (p<0.001) mean weights after 155-day feeding period in seacages, compared to fish fed the diet supplemented with astaxanthin only (astaxanthin diet) or those fed the diet without astaxanthin and KPA® supplementation. Further, fish ingesting the low KPA® diet or astaxanthin diet also had higher mean weights than those fed the control diet (Fig. 5.1). The high KPA® diet group reached a final weight of 82.5 g, low KPA® diet group, 74.8 g; astaxanthin diet group, 69.4 g; and control diet group, 58.2 g. Diets containing supplemental KPA® and/or astaxanthin resulted in percent weight increases in chinook of a weight increase difference of 42%, 28% and 19%, respectively for the high KPA®, low KPA® and astaxanthin diets, relative to the fish fed the diet without KPA® and astaxanthin supplementation.

b. FCR (Figure 5.2-A), SGR (Figure 5.2-B), and CF (Figure 5.3)

Despite the preceding findings, the values of FCR and SGR were not significantly (p>0.05) affected by diet treatment during this part of the study (Fig. 5.2-A and 5.2-B). There were however, trends for improvements in fish growth rates and feed utilization when they were fed the high and low KPA® diet and the astaxanthin diet. The observed FCR after 155-day feeding period was 1.27 ± 0.58, 1.35 ± 0.47, 1.40 ± 0.52 and 1.54 ± 0.66, for diet high and low KPA®, astaxanthin only and control diet, respectively. The observed SGR was 1.58 ± 0.72, 1.44 ± 0.56, 1.43 ± 0.57 and 1.34 ± 0.65, for the same diet treatment.

Diet treatment had no significant effect on the Fulton’s condition factors of the fish during seawater phase of the study (Fig 5.3) regardless of the sampling time.
Pigmentation assessment

Muscle astaxanthin (Figure 5.4-A)

Post-smolt chinook salmon cultured in seacages for 155 days and fed diets either a combination of astaxanthin and KPA® or astaxanthin-alone had significantly higher deposition of astaxanthin in their muscle than those fed a control diet without supplemental astaxanthin (p<0.001). Fish fed the high KPA® diet had significantly more astaxanthin in their muscle (0.864 µg/g, p=0.036) than those fed the low KPA® (0.630 µg/g), although the difference was not significant when compared with those fed the astaxanthin diet (0.754 µg/g, p=0.543) (Figure 5.4-A).

Visual Roche Salmofan values (Figure 5.4 b)

Attempts to visually score the colour of the fillets using the Roche Salmofan® (Hoffman-La Roche, Basel, Switzerland) were unsuccessful at all sampling times in freshwater and saltwater except at the last weight sampling in January 2001, when all fish had reached the proper size to enable visual assessment of differences in the colour if the fillets. Post-smolts fed the high KPA® diet had a significantly higher fillet score (24.3 ± 1.8, p=0.029) than fish fed the diet with astaxanthin only (23.0 ± 0.8) or low KPA® (22.0 ± 1.7) or the control diet (20.0 ± 0.0). There were no statistical differences between the fillet scores of fish fed the low KPA® and astaxanthin diets (p=0.489). Fish fed the astaxanthin-containing diets had significantly higher scores than those fed the diet without astaxanthin (p<0.001).

Apparent astaxanthin retention coefficient, AARC (Table 5.1)

Fish fed the high KPA® diet for 155 days in SW showed a higher apparent astaxanthin retention coefficient (1.28%) than groups fed the astaxanthin-only diet (1.09%) and low KPA® diet (0.90%), respectively (Table 5.1).

Correlation between Roche Salmofan scores and astaxanthin concentrations in muscle (Figure 5.5)

A statistically significant positive correlation (r²=0.668, p=0.027) was detected between the visual salmofan scores in salmon fillets and the muscle concentrations of astaxanthin quantified by HPLC.
Figure 5.1 Weight gain in post-smolt chinook salmon (O. tshawytscha) fed four experimental diets containing various levels of antioxidant (astaxanthin and KPA®) supplementation (A°KPA°, A°50KPA°, A°60KPA1000, A°50KPA100) after a 155-day feeding period in seacages under commercial farming conditions. Different letters indicate significant differences (n=25/diet treatment, single diet groups, p<0.05) between diet groups.
Figure 5.2 Mean (± SD) values for apparent FCR (5.2-A) and SGR (5.2-B) of post-smolt chinook salmon (O. tshawytscha) fed diets supplemented with astaxanthin alone or together with one of two levels of grape seed extract, or no astaxanthin and grape seed extract (A<sup>60</sup>KPA<sup>0</sup>, A<sup>60</sup>KPA<sup>1000</sup>, A<sup>60</sup>KPA<sup>100</sup> and A<sup>0</sup>KPA<sup>0</sup>) after a 155-day feeding period in seacages under commercial farming conditions. Common letters indicate significant differences (n=25/diet treatment, single diet groups, p<0.05) between diet groups.
Figure 5.3 Temporal changes in mean (± SD) condition factors for post-smolt chinook salmon (O. tshawytscha) fed diets supplemented with astaxanthin alone or together with one of two levels of grape seed extract, or no astaxanthin and grape seed extract ($A^{60}$KPA$^0$, $A^{60}$KPA$^{1000}$, $A^{60}$KPA$^{100}$ and $A^{0}$KPA$^0$) after a 155-day feeding period in seacages under commercial farming conditions. Common letters indicate significant differences (n=25/diet treatment, single diet groups, p<0.05) between diet groups.
Figure 5.4 Muscle astaxanthin concentration of post-smolt chinook salmon (O. tshawytscha) assessed by HPLC (5.4-A) and visual estimation of Roche salmofan® scores for fillet (mean ± SD) (5.4-B) after a 155-day feeding period in seacages. Groups were fed diets supplemented with astaxanthin alone or together with one of two levels of grape seed extract, or no astaxanthin and grape seed extract (A60KPA°, A60KPA1000, A60KPA100 and A60KPA°) in seacages under commercial farming conditions. Different letters indicate significant differences (n=25/diet treatment, single diet groups, p<0.05) between diet groups.
Table 5.1 Apparent Astaxanthin Retention Coefficients (AARC) in muscle of post-smolt chinook salmon (O. tshawytscha) fed four experimental diets containing no antioxidant supplementation (control diet), astaxanthin (astaxanthin diet), astaxanthin and two concentrations of grape seed extract (high and low KPA\(^\circ\) diets, respectively). Single diet groups were raised in seacages for 155 days and fed between 2–2.5% b.w/day. Different letters indicate significant differences (n=25/diet treatment, single diet groups, p<0.05) between diet groups. (n.a. not analyzed)

**Feeding Period: September 2000 – January 2001**

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Wi (g)</th>
<th>Wf (g)</th>
<th>Axd ((\mu g/g))</th>
<th>Feed (kg)</th>
<th>DAx (mg)</th>
<th>Axi ((\mu g/g))</th>
<th>Axf ((\mu g/g))</th>
<th>AARC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(^0)KPA(^0)</td>
<td>7.3(^a) (1.7)</td>
<td>58.2(^c) (14.1)</td>
<td>0.21(^b) (0.01)</td>
<td>62.656</td>
<td>13.1</td>
<td>0.028(^b) (0.013)</td>
<td>0.047(^c) (0.018)</td>
<td>n.a</td>
</tr>
<tr>
<td>A(^6)KPA(^100)</td>
<td>7.4(^a) (1.6)</td>
<td>82.6(^a) (15.5)</td>
<td>60.4(^a) (1.51)</td>
<td>73.825</td>
<td>4466.4</td>
<td>0.104(^a) (0.033)</td>
<td>0.864(^a) (0.159)</td>
<td>1.28(^a)</td>
</tr>
<tr>
<td>A(^6)KPA(^100)</td>
<td>8.0(^a) (1.7)</td>
<td>75.8(^ab) (8.7)</td>
<td>56.20(^a) (2.43)</td>
<td>69.690</td>
<td>3916.6</td>
<td>0.101(^a) (0.027)</td>
<td>0.632(^b) (0.164)</td>
<td>0.90(^b)</td>
</tr>
<tr>
<td>A(^6)KPA(^0)</td>
<td>7.6(^a) (1.3)</td>
<td>69.4(^b) (10.9)</td>
<td>57.57(^a) (1.51)</td>
<td>64.635</td>
<td>3723.0</td>
<td>0.098(^a) (0.030)</td>
<td>0.754(^ab) (0.194)</td>
<td>1.09(^b)</td>
</tr>
</tbody>
</table>
Figure 5.5 Correlation between Roche Salmofan® scores (RSS) and concentrations of astaxanthin in chinook salmon muscle (HPLC) following a 155-day feeding period in seacages (n=28, \( r^2 = 0.668 \), p=0.027).
5.5 DISCUSSION

Growth, in simple terms, results from the elaboration of new tissue and is the principal aim of fish farming. Pre-smolt chinook salmon (<6 g) fed diets fortified with a combination of grape seed PA and astaxanthin for a short period of time in FW did not result in significant growth differences compared with fish fed diets containing astaxanthin alone, although a significant low weight gain was observed in fish fed diets containing no dietary astaxanthin and KPA®.

The mean initial weights of post-smolts transferred to growout seacages were not significantly different among diet treatment groups (average 7.57 ± 2.26 g, p=0.389). The observed final mean weights of chinook salmon reared for 155 days in seacages, however, showed important diet differences. In this regard, the final mean weight in relation to diet treatment was as follows: high KPA® diet (82 g) > low KPA® diet (74 g) > astaxanthin diet (69 g) > control diet (58 g). Fish groups fed the high KPA®, low KPA® and astaxanthin only diet exhibited percent gain in weight of 42%, 30% and 18%, respectively, compared to that of fish fed the control diet.

As discussed in chapter 1, feeding level directly affects fish growth and feed conversion efficiency. The overestimated feeding ratio of 3.5% b.w a day in experiment A (fresh water trial) allowed pre-smolt chinook salmon to achieve maximum growth but adversely affected values for feed utilization (FCR). The daily feeding rate used for the post-smolt chinook salmon was established at 2.0-2.5% b.w a day. The Ewos (Surrey, BC, Canada) feeding chart recommendations for chinook salmon cultured in saltwater at water temperatures ranging from 8°C to 12°C varies between 1.46% to 1.85% b.w./day. Although this recommendation is based on higher energy diets than the employed in my research, hence the results support that the feeding rate chosen for the experiment in saltwater was more realistic for local salmon farming practice than that used in the experiment in freshwater.

The specific growth rates obtained for post-smolt chinook salmon in growout seacages ranged from 1.27% to 1.58%/day. Fish fed the high KPA® diet had the best growth rate (1.58%/day), followed by those fed the low KPA® and astaxanthin-only diet (both 1.40%/day). These results agree with those reported previously by Austreng et al. (1987) for both Atlantic salmon and rainbow trout (~1.60%) reared at comparable water temperatures (8°C to 12°C) and also under practical salmon farming conditions. My
results show better growth rates of chinook salmon than the observed by March and McMillan (1996), who reported a SGR value of 0.67%/day in chinook salmon fed for 25 weeks. Kreiberg (1991) found a growth rate of 0.70% per day for chinook salmon fed a commercial grower diet to satiation for 6 months during their first year in seawater (initial weight 70 g). The SGR values of this latter study agree with those previously observed by Kreiberg et al. (1989).

The predicted growth and estimated final weight in fish reared in seacages using the thermal growth coefficient (TGC) formula was achieved only by the fish group fed the high KPA® diet. The other diet groups showed less growth than the predicted weights and the percent differences were -11% in the low KPA®, -17% in the astaxanthin-only, and -30% in the control diet groups.

Best fed to gain ratios or utilization of feed for growth was attained by fish fed diets supplemented with the combination of astaxanthin and either high or low KPA®, 1.27 and 1.35, respectively. The fish group fed the astaxanthin-only diet showed a FCR value of 1.40, and those fed the control diet had a FCR of 1.54. Feed conversion ratios obtained in experiment C are within acceptable feed efficiency standards for cultured salmon in seawater at this life history stage. Thomas (1999) noted that the feed efficiency of chinook salmon ranging in weight from 40 g to 120 g was 1.1 after they were fed an experimental diet and held in SW.

The initial Fulton's condition factors in the present study varied from 1.10-1.15, and these values fall within the CF range reported for recently transferred post-smolt salmonids. This low weight to length ratio is a consequence of a transient reduction in appetite after transfer of smolts from FW to SW. (Usher et al. 1991; Jørgensen and Jobling, 1994). Afterwards, CF values showed an increasing trend towards the end of the feeding trial. The values ranged from 1.35-1.40. Such CF values have previously been reported for chinook salmon cultured in saltwater (Mazur, 1986). Furthermore, the observed pattern of increasing condition factors in the fish group fed the high KPA® diet suggests that well fed fish have higher condition factors than fish of the same length fed a less optimal diet (Piper et al. 1982).

Salmon diets contain large amounts of lipids and these are mainly of marine origin. Marine fish oils contain 20% to 25% polyunsaturated fatty acids (PUFA). PUFA of the n-3 family serve as structural building blocks in cell membranes, precursors of bioactive eicosanoids compounds, and as reservoirs of available energy.
Polyunsaturated fatty acids present in fish diets, biomembranes and plasma lipids are more susceptible to lipid peroxidation. The presence of dietary antioxidants has shown to reduce lipid peroxidation than less unsaturated fatty acids. Proanthocyanidins contained in red wine have also been shown to significantly reduce lipid peroxidation of LDL in humans (Frankel et al. 1993). Moreover, rainbow trout fed astaxanthin-containing diets had reduced lipid peroxidation in vivo (Nakano et al. 1999).

The salmon feed used in this experiment was stored at room temperature in a shed during late summer, fall and early winter. Heat and oxygen are environmental factors that are known to initiate, or to lead to intermediate metabolite participants in the oxidation of lipids and other oxidation-sensitive compounds. Autoxidation of unsaturated fatty acids produces a large number of free radicals and peroxide compounds, which are active pro-oxidants. These compounds may react with other diet ingredients (i.e. vitamins) and reduce their nutritional value, or after ingestion, react with oxidation-sensitive phospholipids in the cellular and subcellular membranes and cause oxidative damage. Roberts and Bullock (1989) have described that the ingestion of oxidised fish oils may reduce growth rate, provoke anaemia, nutritional muscular dystrophy, and ceroidosis in the liver of fish. The quality of the dietary lipid or extent of lipid peroxidation was not followed in this study but it is conceivable that a low level of lipid oxidation occurred during the experimental period especially in diets not supplemented with KPA®.

Grape seed PA have been shown to enhance the growth and viability of normal human gastric mucosal cells and murine macrophage J774A.1 cells (Ye et al. 1999). Cell health, communication, membrane fluidity, cell receptors, enzymes, and RNA and protein production might be protected by the actions of PA and their potential synergistic interaction with other antioxidants in the body. This overall cellular integrity might be reflected in greater ability for use of dietary protein, lipids and vitamins for new tissue growth. The dietary antioxidant combination effects were not evident after the short rearing time in freshwater tanks, but when they were fed for longer time in saltwater seacages, they were able to exhibit important differences in weight gain, growth rate. They appeared to be related to the dietary concentration of KPA® as described above.

The distinctive red-pink colour of salmon flesh, produced by the retention of astaxanthin in the muscle, provides an immediate indication of product quality and is, therefore, an essential aspect of fish farming, product marketing and commercial feed
production. As fish cannot synthesize these pigments, they must be provided in the diet. A deep and even red colour in fish muscle is more than a cosmetic effect and the consumer associates this characteristic with a high quality, healthy product (Schiedt, 1998).

Chinook salmon fry (< 7 g) housed in FW and fed astaxanthin-containing diets for 32 days had very limited capacity for carotenoid pigment deposition in their flesh. Diets fortified with astaxanthin (60 ppm), and astaxanthin with one of two levels of KPA® (100 and 1000 ppm) resulted in no significant deposition of astaxanthin in salmon muscle, although a higher level of astaxanthin was observed in both of the KPA®-astaxanthin fed groups (p=0.543).

Post-smolt chinook salmon were transferred to growout seacages and fed the same experimental diets for 155 days. At the conclusion of this feeding trial, astaxanthin concentrations in the muscle of chinook salmon fed the diets supplemented with astaxanthin with or without KPA® showed a six to eight-fold increase over initial values. Initial astaxanthin concentrations in the muscle of the fish consuming the astaxanthin-supplemented diets showed no significant differences, ranged from 0.098 to 0.104 µg/g, whereas the mean value of fish fed the astaxanthin free diet was 0.028 µg/g. Fish fed the high KPA® diet attained the highest deposition of astaxanthin in muscle (0.863 µg/g), followed by those fed the astaxanthin diet (0.751 µg/g), and the low KPA® diet (0.632 µg/g). Muscle astaxanthin deposition in fish fed the control diet remained negligible (0.047 µg/g).

As mentioned previously, salmon have to reach a certain size to deposit carotenoids efficiently in the actomyosin complex of their muscle. Torrissen (1985) and March et al. (1990) have positively correlated astaxanthin deposition in muscle of salmonids with an increase in body weight. Chinook salmon that were held in seacages and had an average final weight of less than 85 g showed little deposition of astaxanthin in their muscle (<1.0 µg/g) and estimated apparent astaxanthin retention coefficients were low (<1.3%). Several experiments have reported carotenoid concentrations in muscle of salmonids ranging between 1.0 µg/g and up to 3.0 µg/g in salmonids weighing less than 200 g (Christiansen and Wallace 1988; Bjerkeng et al. 1992; Smith et al. 1992; Hatlen et al. 1995; Thomas, 1999; Wieruszewski, 2000). Christiansen et al. (1995b) reported a two-fold increase of astaxanthin deposition from 1.2 µg/g to 2.7 µg/g
in Atlantic salmon parr muscle (initial to final weight, 16 g to 58 g, respectively) fed a diet fortified with 60 ppm astaxanthin for 10.5 months. However, these authors failed to mention the total dietary amount of astaxanthin ingested by the fish. In contrast, Spinelli and Mahnken, (1978) found no pigment deposition in coho salmon and Storebakken et al. (1987) also reported no pigment deposition in Atlantic salmon weighing less than 80 g and fed astaxanthin-containing diets (30, 50 and 90 ppm). These authors found that muscle pigment carotenoid deposition only began when fish reached a body weight over 100 g.

The reason for the meagre deposition of astaxanthin in the flesh of young salmonids is still unknown. The linear model hypothesized for carotenoid deposition by Torrissen et al. (1989) might explain the low final astaxanthin concentration in muscle of salmon that size. They propose that there is a linear increase in carotenoid concentration as the fish increases in weight, assuming there is also an increase in retention efficiency. This theory contemplates a 1% carotenoid retention in fish weighing 0.2 to 1 kg, reaching a maximum of 8.8% for fish weighing 4 to 5 kg (Torrissen et al. 1989). Also, juvenile salmonids show a preferential deposition of carotenoids in skin rather than in flesh (Torrisen et al. 1989). Finally, circulating levels of the main carotenoid-carrying lipoproteins (HDL and VDHL) are present in low quantities in juvenile salmonids (Choubert et al. 1992, 1994) compared to circulatory levels of these lipoproteins in adult salmonids.

The fortification of astaxanthin-containing diets with an antioxidant, such as KPA®, that has demonstrated strong FR scavenging properties, might have improved the deposition of astaxanthin in muscle by reducing the use of astaxanthin as an antioxidant ("sparing effect"), hence there may have been more astaxanthin available for deposition in the actomyosin complex. Nakano et al. (1999) showed the dual pigment-antioxidant properties of the carotenoid astaxanthin in rainbow trout. Other possibilities include an increased functionality of astaxanthin receptors in the muscle cell membrane, or a reduction in lipid peroxidation of astaxanthin-carrying lipoproteins due to the presence of KPA®.

The reduced efficiency of deposition of astaxanthin in salmon fed the experimental diets in this research might have been due to loss of astaxanthin during diet storage due to heat, oxygen decomposition and some lipid peroxidation in the food. Unfortunately astaxanthin concentrations in the feed at the end of the experiment were
not determined. Christiansen et al. (1995a) reported loss of astaxanthin from 59.6 ppm to 47.8 ppm (20%) in fish diets kept for 10.5 months at -20°C. The supply of freshly made fish feed in my research did not exceed more than 8 weeks between different feed batches. Thus the loss of astaxanthin during diet storage was probably minimal.

Regardless of the cause of the low astaxanthin deposition, the supplementation of high concentration of grape seed PA in the diet significantly increased both the efficiency of pigment deposition in chinook salmon muscle and the total final concentration level of astaxanthin in the flesh after 155-day feeding period.

Assessing flesh colour in salmonids at slaughter by using visual means is an industry standard. Roche Salmofan® scores observed for fillets from fish fed the high KPA® diet were significantly greater than those noted from the other 3 diets. Although there were no statistical difference between the low concentration of KPA® diet and astaxanthin-only diet, the Salmofan® score were significantly greater than those for fish fed the control diet. The visual assessment of the flesh colour obtained using the Roche Salmofan® agreed with the muscle astaxanthin concentration results obtained by chemical analysis (HPLC) of flesh. The use of RSS in chinook salmon (weights ranging between 50 to 80 g) fillets proved to be a helpful and practical tool to estimate and compare the colour of salmon flesh.

Visual pigmentation scores assessed by RCC have been reported to correlate well with HPLC concentrations of astaxanthin in the flesh of Atlantic salmon (Christiansen et al. 1995c), rainbow trout (Smith et al. 1992), and chinook salmon (Thomas, 1999). In the present study, a good correlation was found between Salmofan® and astaxanthin concentrations in muscle ($r^2=0.668$) as measured chemically by HPLC. This result supports the high coefficient regression found by Smith et al. 1992 ($r^2=0.997$), and Thomas, 1999 ($r^2=0.911$).
6. CHAPTER THREE

Non-specific immune responses of pre-smolt chinook salmon fed one of two concentrations of a natural flavonoid antioxidant and astaxanthin in the diet, to an intraperitoneal injection of *Vibrio anguillarum*
6.1 INTRODUCTION

The bacterial fish disease vibriosis is caused by *Vibrio anguillarum*. Vibriosis is one of the most economically important diseases of marine cultured and feral fish (Toranzo *et al.* 1997). *Vibrio anguillarum* is a ubiquitous, facultative pathogen of aquatic organisms living in marine and brackish environment worldwide (Groff and LaPatra, 2001).

The immune system in fish is comprised of precise defensive mechanisms to prevent and control disease caused pathogens entering, spreading and multiplying in the body. The immune system is composed of cells and molecules and when the body reacts to an infection, many powerful antimicrobial processes are brought into play (i.e. respiratory burst). These defense mechanisms result in the production of vast amounts of free radicals.

Dietary supplementation of antioxidants has been postulated to enhance the immune responses of animals by protecting cell membranes from the oxidative damage caused by free radicals, modulating the activity of molecules and enzymes that participate in the immune response (Halliwell *et al.* 1994), and working synergistically with one to another to exert a “sparing effect”. Fortification of diets with large amounts of antioxidant vitamins (i.e. vitamin E, C, β-carotene) has received considerable attention in fish (Sealey and Gatlin, 1999). Nakano *et al.* (1995, 1999) demonstrated the *in vivo* antioxidant activity of astaxanthin since lipid peroxidation was reduced in rainbow trout tissues when the diet was supplemented with this pigment source. Grape seed extract has been shown to exert great cellular protection by scavenging free radicals, reducing lipid peroxidation, and modulating the activity of inflammatory enzymes such as cyclooxygenase and lipooxygenase (Hollman and Katan, 1998). There has been no work documenting the use of antioxidants other than antioxidant vitamins, micronutrients and astaxanthin in fish diets within the current feeding practice of intensive salmon farming.

This study assessed the effects of feeding a combination of a natural flavonoid antioxidant (grape seed extract, KPA®) and astaxanthin in salmon feed on the non-specific immune responses of pre smolt chinook salmon after a disease challenge by an i.p injection with a bacterial suspension (*Vibrio anguillarum*).
6.2 MATERIALS AND METHODS

6.2.1 EXPERIMENTAL DESIGN, GENERAL HUSBANDRY AND DISEASE CHALLENGE PROTOCOL

Fish used in this experiment and challenge protocol were described in sub-section 3.1, 3.2.2, and 3.7 in the General Materials and Methods section. After bacterial suspension and sterile peptone saline (0.1 mL i.p.) were inoculated, samples (n=6 fish/tank) were obtained daily for blood and tissue analysis. The water temperature was 9.0 ± 0.5°C throughout the sampling period.

6.2.2 CLINICAL HAEMATOLOGY

Primary haematology indices (haemoglobin, haematocrit, and total red blood cell) and their derived indices (mean corpuscular volume, MCV; mean corpuscular haemoglobin, MCH; and mean corpuscular haemoglobin concentration, MCHC) were determined according to the protocols outlined by Houston (1990) and Klontz (1994).

Fish from each treatment group were rapidly sacrificed in a lethal buffered solution of anaesthetic (500 mg/L MS-222® + 500 mg/L NaHCO₃). Blood was collected in a microhaematocrit capillary tube after severing the caudal peduncle with a scalpel, a few millimetres posterior to the adipose fin.

Primary haematology indices

6.2.2.1 Haemoglobin (Hb)

Haemoglobin was analysed as cyanmethemoglobin according to the procedure described by Houston (1990) using Drabkin's reagent (Kit 525, Sigma, ON, Canada) and samples were read in a microplate spectrophotometer SpectraMAX 340pc (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The concentration of haemoglobin in the blood sample is directly proportional to the absorption read in the spectrophotometer.

6.2.2.2 Haematocrit (Hct)

Fish haematocrit was analysed according to Klontz (1994). Blood was directly sampled into a 75 mm heparinized microhaematocrit capillary tube (Fisherbrand; Fisher, Inc., Pittsburgh, PA, USA), sealed and centrifuged in a haematocrit centrifuge (IEC Micro-MB Microcentrifuge, Philadelphia, PA) at 11,500 rpm for 5 min.
6.2.2.3 Total red blood cell count (RBC)

The total numbers of circulating erythrocytes (RBC) were estimated using a haemocytometer according to the technique described by Houston (1990). The cell concentration for each sample was calculated according to Klontz (1994):

\[ \text{RBCs/mL} = \frac{\text{number of erythrocytes counted} \times 10}{\text{counts for depth of chamber, 0.1 mm} \times 5} \times 200 \times 200 \]

Secondary haematological indices

6.2.2.4 Mean Corpuscular Volume (MCV)

Expresses the mean volume of an individual erythrocyte.

\[ \text{MCV} = \frac{\text{Hct} \times 10}{\text{RBC}} \text{ (reported in nm}^3\text{, Houston 1990).} \]

6.2.2.5 Mean Corpuscular Haemoglobin (MCH)

Expresses the mean amount of haemoglobin contained within an erythrocyte.

\[ \text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC}} \text{ (reported in } \mu\text{g/cell, Houston 1990).} \]

6.2.2.6 Mean Corpuscular Haemoglobin Concentration (MCHC)

Expresses the relative amount of haemoglobin per unit volume of cell.

\[ \text{MCHC} = \frac{\text{Hb} \times 100}{\text{Hct}} \text{ (reported as } \text{g/100 mL, Houston 1990).} \]

6.2.2.7 Total leucocytes count (WBC)

Total circulating leucocytes were estimated according to Klontz (1994). The number of total leucocytes per mL was estimated as:

\[ \text{WBC/mL} = \frac{\text{number of total leucocytes counted} \times 10}{\text{counts for depth of chamber, 0.1 mm} \times 200} \frac{1}{4} \text{ (corrects for the chamber area of 4 mm}^2\text{).} \]

6.2.2.8 Differential blood cell count

Blood smears were stained in a modified Wright-Giemsa stain (Diff-Quick®, Dade diagnostics Inc, Aguada, PR, USA) according to the manufacturer's directions. Smears were air-dried before staining the slides using Diff-Quick, rinsed with deionized water, and air-dried before being examined under immersion oil microscopy. Cells that touched the outer boundaries of the microscope field were not included in the count. A thousand erythrocytes were counted per slide in ten randomly selected microscopic fields (min. 50
RBCs; max. 200 RBCs). In each field, the number of RBCs was tallied and leucocytes (lymphocytes, neutrophils, thrombocytes and monocytes) were differentiated and counted (Houston, 1990).

6.2.3 NON-SPECIFIC IMMUNE RESPONSE ANALYSES

The non-specific immune responses were assessed by estimating the cellular and humoral immune responses in fish elicited by the bacterial pathogen Vang. Respiratory burst activity was measured in neutrophils, and lysozyme activity was assessed in plasma and head kidney tissue. In addition, the cellular stress response was estimated in hepatic tissue by measuring the production of stress proteins 70 kd (SP70) (Forsyth et al. 1997).

6.2.3.1 Neutrophil respiratory burst activity (NBT assay)

The detection of superoxide anion ($^\bulletO_2$) as a result of respiratory burst activity in neutrophils was estimated using the technique described by Anderson (1992a). Briefly, a drop of blood was placed directly onto a glass microslide with 4 reaction wells (Steinmetz, Surrey, BC, Canada). The slides were incubated for 30-60 min and the excess of cells was washed away with PBS (pH 7.4). A drop of nitro blue tetrazolium (NBT) solution (Sigma cat N° N-5514, ON, Canada) was placed onto the cells and covered with a glass coverslip. After incubating the slides for 30 min in a moist chamber, they were observed under a microscope (1000X). The total number of positive neutrophils (polymorphonuclear leucocytes with a purple-blue stain halo) were tallied and compared with negative neutrophils between the different diet treatment groups.

6.2.3.2 Lysozyme activity in plasma and head kidney

Plasma was removed from the 75 mm microhaematocrit tubes after reading haematocrit values of every blood sample. Subsequently the plasma was placed in a 0.5 mL microcentrifuge tube (cat N° 05-407-16, ON, Canada) and immediately frozen on dry ice and stored at -80°C until further analysis. The head kidney was dissected out and frozen on dry ice in a 1.5 mL microcentrifuge tubes, then stored at -80°C. Plasma and head kidney samples were thawed on ice. One mL of PBS was added into the tubes containing anterior kidney tissue and the suspension was sonicated in a Vibra-Cell™ (Sonics and Materials, Danbury, CT, USA) for 30 s. The lysates were centrifuged
at 13,000 rpm for 3 min at room temperature and the clear aliquot used for lysozyme assay.

Lysozyme activity in plasma and head kidney was measured by microplate assay adaptation of Litwack's method (1955) as applied by Muona and Soivio (1992) and Maule et al. (1996). The decrease in optical density (OD) readings at 450 nm over 20 min of incubation at 25°C was expressed as µg/mL equivalent of hen egg white lysozyme activity (HEWL, Sigma, cat N° L-6876, ON, Canada), which was used as the standard.

6.2.3.3 Liver SP70

Hepatic tissue was sonicated in lysis buffer kept on ice (contained 100 mM Tris-HCl, 0.1% sodium dodecyl sulphate (SDS), 1 mM ethylenediamine tetra acetic acid (EDTA), and protease inhibitors (1 µM Pepstatin, 1 µM α-Toluenesulfonyl fluoride (PMSF), 1 µM Leupeptin, and 0.01 µM Aprotinin). The homogenate was centrifuged in a microcentrifuge (MSE, Microcentaur, London, England) at 16,500 g for 2 min at room temperature. Protein concentration in the lysate was estimated in duplicate aliquots of homogenate using the Bicinchoninic acid assay (BCA, Sigma Diagnostics protein kit) and using bovine serum albumin (BSA) as a reference. Ten µL of supernatant was added to an equal volume of SDS-sample dilution buffer (Laemmli, 1970) and boiled for 3 min and then frozen at -80°C until further SP70 analysis.

Stress proteins 70 were separated by SDS-PAGE according to Laemmli (1970). Equal amounts of protein (10 µg) and control sample were loaded onto separate gel lanes (4% stacking and 12.5% resolving gel) on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). The molecular mass of each protein band in the electrophoretic gel was estimated using pre-stained molecular weight markers (Gibco-BRL, Burlington, ON, Canada). Protein samples from the control diet group were used to normalize the data.

After electrophoretic separation, strips of gel containing proteins in the 70-kda range were cut out and transferred onto nitrocellulose membranes (0.2 µm pore size, Bio-Rad, Hercules, CA, USA) for western immunoblotting according to Forsyth et al. (1997). Nitrocellulose membranes were incubated for 1 h in a polyclonal rabbit antibody raised against rainbow trout (RTG-2) SP70. Following the incubation in primary
antibody, membranes were incubated in an alkaline phosphatase conjugated goat anti-rabbit IgG (Gibco-BRL, Burlington, ON, Canada) secondary antibody for another 1 h. Both antibodies were diluted with 2% w/v skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5). The intensity of the SP70 bands was determined using a ScanJet II p (Hewlett Packard) and SigmaGel software (Jandel Scientific, CA, USA). Stress Protein 70 levels were expressed in relative units to a known positive control sample that was run simultaneously on every gel.

6.3 STATISTICAL ANALYSIS

Experiments were performed using replicates groups. Quantitative data were analysed by an analysis of variance (one-way ANOVA, α=0.05) using Student-Newman-Keuls’s and Dunn’s tests where appropriate. Data expressed as percentages (cumulative mortality and haematocrit) were arcsine square root transformed prior to performing ANOVA analysis. Correlation between circulatory neutrophils and lysozyme activity in plasma and head kidney were determined using the Pearson’s correlation test. Where significant differences were detected, all pair-wise multiple comparison tests were used to identify significantly different treatment means (p<0.05). All statistical analyses were performed using SigmaStat software (SSPS Inc., Chicago, IL, USA). Results were expressed as the mean ± one SD.

6.4 RESULTS

Mortality (Figure 6.1)

Fish fed the high KPA® diet had significantly lower (p=0.038) cumulative mortality (88.7%) compared with those ingesting the other 3 treatment diets (astaxanthin diet, 97.5%; control diet, 98.7%; low KPA® diet, 100%) following a disease challenge with Vang injection (Figure 6.1). There were no immediate mortalities at the end of the bacteria injection procedure in any group. Mortality started within 24 h and continued up to day 10 in all diet groups.

Mortality in the sham-injected groups ranged from 0.5-1.5% (up to 3 fish/tank) and was not significantly affected by diet treatment (p=0.677). There were no mortalities after 36 h following the injection with sterile peptone saline and no mortalities were attributed to Vibrio. There was a significantly lower mortality (p<0.001) in sham-injected groups compared to the bacterial injected groups.
Figure 6.1 Cumulative percent mortality in pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets containing various levels of antioxidant supplementation ($A^{0}{KPA}^{0}$, $A^{60}{KPA}^{0}$, $A^{60}{KPA}^{1000}$, $A^{60}{KPA}^{100}$) following an intraperitoneal injection with Vibrio anguillarum (0.1 mL, $10^9$ viable cells/mL) and sham injection (0.1 mL, sterile peptone saline). Different letters indicate a significant difference (n=6/diet treatment, two replicates tanks/diet treatment, p=0.038) between dietary groups and symbol ($\phi$) represents a significant disease difference (p<0.001) between bacteria injected groups and sham injected groups.
Clinical haematology
Primary indices
Hct, Hb, RBC (Table 6.1)

There were no significant diet differences in circulating red blood cells (RBC) at any sample time throughout the disease challenge (p=0.392 to p=0.926). A significant erythrocytopenia (p<0.001 to p=0.045) was observed from day 3 throughout day 6 between bacteria-injected and sham injected groups.

Haematocrit values began fluctuating at day 2 after Vang injection and no consistent significant differences were observed between fish groups in relation to dietary treatment. There was a trend towards a lower Hct in fish fed the astaxanthin diet during the disease challenge (p=0.076 to p=0.095).

There were no significant diet differences in Hb values between groups (p=0.221 to p=0.249). However, on day 3 of the challenge, those fish fed the control and low KPA® diet had significantly higher Hb values (p=0.025) compared to those fed the high KPA® and astaxanthin diets.

Secondary indices
MCV, MCH, MCHC (Figure 6.2)

Erythrocyte size (MCV) was uninfluenced by diet treatment (p=0.589 to p=0.975) and disease treatment (p=0.094 to p=0.533) following the bacteria and sham injections. There were significant differences in MCH after bacterial inoculation. The amount of Hb carried in erythrocytes was significantly (p=0.025) lower in fish fed the high KPA® diet from day 1 to day 3, and in fish fed the astaxanthin diet on day 3 (p=0.033) relative to the other groups after injection with Vang. The amount of haemoglobin per 100 mL (MCHC) was not significantly different between sham and bacteria inoculation groups (p=0.190). There was a significant decrease in MCHC in fish fed the high KPA® diet during the first 3 days of the challenge period (p=0.014 to p=0.028) compared to those fed the low and astaxanthin-only diet.
Table 6.1 Primary haematology indices in pre-smolt chinook salmon (*O. tshawytscha*) fed diets containing no supplemental astaxanthin and KPA® (control diet), astaxanthin (astaxanthin diet), or astaxanthin and one of two concentrations of grape seed extract (high KPA® and low KPA® diet, respectively) following a challenge with *Vibrio anguillarum* and sterile peptone saline (sham) injection. Letters indicate significant statistical differences (n=6/diet treatment, two replicates tanks/diet treatment p<0.05) between the means for diet treatment groups.

<table>
<thead>
<tr>
<th>Haematology Index</th>
<th>Dietary Group</th>
<th>Prior to challenge</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
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<tr>
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<tr>
<td><strong>Red Blood Cell</strong></td>
<td><strong>Count</strong></td>
<td><strong>(x 10⁶ cell/mL)</strong></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control Diet</td>
<td>n.a</td>
<td>1.25 ± 0.14</td>
<td>1.20 ± 0.29</td>
<td>1.07 ± 0.31</td>
<td>0.89 ± 0.27</td>
<td>0.77 ± 0.12</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>High KPA Diet</td>
<td>n.a</td>
<td>1.30 ± 0.12</td>
<td>1.24 ± 0.17</td>
<td>0.88 ± 0.39</td>
<td>0.98 ± 0.28</td>
<td>0.96 ± 0.31</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>Low KPA Diet</td>
<td>n.a</td>
<td>1.31 ± 0.26</td>
<td>1.26 ± 0.25</td>
<td>1.05 ± 0.29</td>
<td>0.98 ± 0.24</td>
<td>1.00 ± 0.32</td>
<td>0.78 ± 0.20</td>
<td>0.85 ± 0.34</td>
</tr>
<tr>
<td>Astaxant. Diet</td>
<td>n.a</td>
<td>1.18 ± 0.56</td>
<td>1.00 ± 0.24</td>
<td>0.97 ± 0.25</td>
<td>0.97 ± 0.26</td>
<td>0.96 ± 0.31</td>
<td>0.85 ± 0.34</td>
<td>0.85 ± 0.34</td>
</tr>
<tr>
<td>Sham Injection</td>
<td>n.a</td>
<td>1.46 ± 0.16</td>
<td>1.39 ± 0.17</td>
<td>1.37 ± 0.19</td>
<td>1.40 ± 0.23</td>
<td>1.35 ± 0.20</td>
<td>1.36 ± 0.40</td>
<td>1.36 ± 0.40</td>
</tr>
<tr>
<td><strong>Haematocrit (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Diet</td>
<td>48.9 ± 3.3</td>
<td>46.5 ± 7.64</td>
<td>42.8 ± 5.88</td>
<td>36.6 ± 5.38</td>
<td>32.6 ± 7.52</td>
<td>30.6 ± 1.28</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>High KPA Diet</td>
<td>50.4 ± 3.1</td>
<td>45.2 ± 3.95</td>
<td>44.2 ± 4.01</td>
<td>30.3 ± 8.11</td>
<td>36.5 ± 4.02</td>
<td>37.8 ± 3.22</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>Low KPA Diet</td>
<td>50.8 ± 2.6</td>
<td>46.7 ± 7.86</td>
<td>43.6 ± 6.35</td>
<td>38.7 ± 5.41</td>
<td>38.9 ± 4.32</td>
<td>39.1 ± 5.83</td>
<td>28.5 ± 4.00</td>
<td>31.2 ± 9.00</td>
</tr>
<tr>
<td>Astaxant. Diet</td>
<td>48.7 ± 1.7</td>
<td>36.8 ± 8.34</td>
<td>34.2 ± 5.54</td>
<td>36.5 ± 7.52</td>
<td>38.7 ± 3.43</td>
<td>34.7 ± 5.12</td>
<td>34.6 ± 2.30</td>
<td>45.8 ± 3.20</td>
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<tr>
<td>Sham Injection</td>
<td>n.a</td>
<td>45.4 ± 4.05</td>
<td>45.7 ± 3.89</td>
<td>46.4 ± 3.64</td>
<td>46.1 ± 2.70</td>
<td>46.3 ± 2.37</td>
<td>45.8 ± 3.23</td>
<td>45.8 ± 3.20</td>
</tr>
<tr>
<td><strong>Haemoglobin (g/dL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Diet</td>
<td>6.8 ± 1.8</td>
<td>7.5 ± 2.55</td>
<td>7.6 ± 2.23</td>
<td>9.0 ± 2.04</td>
<td>5.9 ± 2.11</td>
<td>5.5 ± 1.01</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>High KPA Diet</td>
<td>5.7 ± 0.7</td>
<td>6.7 ± 1.64</td>
<td>6.2 ± 0.65</td>
<td>5.1 ± 2.07</td>
<td>6.2 ± 2.33</td>
<td>6.0 ± 1.80</td>
<td>n.a</td>
<td>n.a</td>
</tr>
<tr>
<td>Low KPA Diet</td>
<td>6.6 ± 1.4</td>
<td>9.4 ± 2.00</td>
<td>8.9 ± 3.14</td>
<td>8.4 ± 1.00</td>
<td>5.4 ± 1.74</td>
<td>6.5 ± 2.98</td>
<td>5.6 ± 1.24</td>
<td>4.1 ± 2.10</td>
</tr>
<tr>
<td>Astaxant. Diet</td>
<td>6.2 ± 1.2</td>
<td>8.3 ± 2.55</td>
<td>6.5 ± 1.40</td>
<td>6.5 ± 2.40</td>
<td>6.3 ± 1.76</td>
<td>4.8 ± 2.10</td>
<td>4.1 ± 2.10</td>
<td>4.1 ± 2.10</td>
</tr>
<tr>
<td>Sham Injection</td>
<td>n.a</td>
<td>8.4 ± 2.11</td>
<td>6.9 ± 0.90</td>
<td>8.6 ± 2.40</td>
<td>7.2 ± 1.00</td>
<td>8.3 ± 1.50</td>
<td>7.8 ± 1.61</td>
<td>7.8 ± 1.61</td>
</tr>
</tbody>
</table>
Figure 6.2 Mean (± SD) secondary haematology indices in pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets containing different supplemental levels of KPA® and astaxanthin (A°KPA°, A°KPA°, A°KPA°, A°KPA°) following a disease challenge with Vibrio anguillarum (0.1 mL, 10⁹ cfu/mL) and sterile peptone saline injection (0.1 mL). Different letters indicate significant dietary difference (n=6/diet treatment, two replicates tanks/diet treatment p<0.05) between diet groups. Symbol (φ) indicates a statistical difference (p<0.05) among sham injection and challenge injection groups.
Total White Cell Count (WBC) (Figure 6.3)

There were a statistically significant (p<0.025) diet and disease treatment effect on WBC throughout the 4 day period after Vang and sham injection. Fish fed the high KPA® diet had significantly higher (p=0.003 to p=0.025) circulating numbers of WBC during day 3 to 5 of the disease challenge compared with ingesting the other the other 3 diets. There were no differences between fish receiving the control diet, low KPA® diet and astaxanthin diet between days 1 to 4 (p=0.104 to p=0.416), but on day 5, fish fed the control diet had a significantly lower WBC number (p=0.021) than those fed the other 3 diets.

Differential Leucocyte counts (Table 6.2)

Neutrophil cell counts

Fish fed the high KPA® diet had significantly higher numbers of circulating neutrophils in blood (p=0.018) on days 2, 3 and 4 after injection with Vang than noted for the other groups (Table 6.2). There were no significant differences among the fish groups fed the other 3 diets at any of the sampling times (p=0.155). Significantly higher numbers of circulating neutrophils (p=0.003) were observed in bacteria challenged groups compared to fish injected groups during the sampling period.

Lymphocyte cell counts

Fish fed the high KPA® diet on days 2, 3, 4 and 5 of the disease challenge period exhibited significantly greater numbers of circulating lymphocytes than the other groups. Circulating lymphocyte numbers in all infected fish were significantly higher (p=0.002) than found for sham-injected fish (Table 6.2).

Thrombocyte cell counts

A marked thrombocytopenia was evident when comparing bacteria-injected groups to sham-injected groups; the reduction of thrombocytes in blood was statistically significant (p=0.040) for the former groups. No significant diet differences were observed in numbers of circulating thrombocytes during the challenge sampling (p=0.104), but on day 1, fish fed the high KPA® diet, and on day 2 of fish fed the astaxanthin diet had significantly greater numbers of circulating cells (p=0.036 and p=0.046, respectively) than observed in other groups (Table 6.2).
Monocyte cell counts

Numbers of circulating monocytes were significantly affected by both the diet and disease treatments. Fish fed the high KPA® diet had significantly (p=0.021) greater numbers of monocytes in blood compared to those fed with the other 3 diets. Moreover, fish injected with sterile peptone saline had significantly fewer (p=0.044) circulating monocytes than fish injected with Vang (Table 6.2).
Figure 6.3 Total white blood cell counts (WBC) in pre-smolt chinook salmon (O. tshawytscha) fed four diets containing different concentrations of astaxanthin and KPA® for 6 days following an experimental challenge with Vibrio anguillarum (0.1 ml, 10⁹ cfu/mL) and pooled mean of sham injection groups (sterile peptone saline, 0.1 mL). Different letters indicate significant differences (n=6/diet treatment, two replicates tanks/diet treatment, p<0.05) between diet groups, and symbol (ϕ) means a statistical difference (p<0.05) between bacterial injected and sham-injected groups.
Table 6.2 Leucocyte differential counts in pre-smolt chinook (*O. tshawytscha*) salmon fed diets containing no supplemental astaxanthin and KPA® (control diet), astaxanthin (astaxanthin diet), or astaxanthin and one of two concentrations of grape seed extract (high KPA® and low KPA® diet, respectively) for six days after a challenge with *Vibrio anguillarum* and. Values for Sham-injected controls are also shown. Different superscripts indicate significant differences (n=6/diet treatment, two replicates tanks/diet treatment, p<0.05) between the means for the diet treatments. (n.a. not analyzed)

<table>
<thead>
<tr>
<th>Days post <em>Vibrio anguillarum</em> injection</th>
<th>Dietary Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (x 10^3 cell/mL)</td>
<td>Control Diet</td>
<td>0.94 ± 0.10^b</td>
<td>1.35 ± 0.33^c</td>
<td>1.52 ± 0.19^c</td>
<td>1.60 ± 0.27^c</td>
<td>1.52 ± 0.21^b</td>
<td>n.a</td>
</tr>
<tr>
<td></td>
<td>High KPA Diet</td>
<td>1.12 ± 0.14^b</td>
<td>1.92 ± 0.34^b</td>
<td>2.65 ± 0.52^b</td>
<td>2.52 ± 0.49^b</td>
<td>2.33 ± 0.25^b</td>
<td>n.a</td>
</tr>
<tr>
<td></td>
<td>Low KPA Diet</td>
<td>1.03 ± 0.16^a</td>
<td>1.41 ± 0.30^c</td>
<td>1.83 ± 0.41^c</td>
<td>2.00 ± 0.31^c</td>
<td>1.83 ± 0.26^b</td>
<td>1.20 ± 0.20^c</td>
</tr>
<tr>
<td></td>
<td>Astaxant. Diet</td>
<td>0.98 ± 0.19^b</td>
<td>1.48 ± 0.35^c</td>
<td>1.78 ± 0.39^c</td>
<td>1.79 ± 0.24^c</td>
<td>1.85 ± 0.67^b</td>
<td>1.53 ± 0.37^c</td>
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<tr>
<td></td>
<td>Sham Injection</td>
<td>0.78 ± 0.14^a</td>
<td>0.93 ± 0.17^c</td>
<td>1.03 ± 0.36^c</td>
<td>1.09 ± 0.22^c</td>
<td>1.11 ± 0.27^a</td>
<td>1.18 ± 0.21^c</td>
</tr>
<tr>
<td>Lymphocytes (x 10^3 cell/mL)</td>
<td>Control Diet</td>
<td>12.03 ± 1.34^b</td>
<td>15.53 ± 3.34^c</td>
<td>15.03 ± 3.24^c</td>
<td>15.13 ± 1.34^b</td>
<td>10.03 ± 2.73^a</td>
<td>n.a</td>
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<tr>
<td></td>
<td>High KPA Diet</td>
<td>14.00 ± 3.04^b</td>
<td>19.87 ± 2.50^b</td>
<td>20.81 ± 2.77^b</td>
<td>18.73 ± 3.14^b</td>
<td>16.93 ± 3.23^b</td>
<td>n.a</td>
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<tr>
<td></td>
<td>Low KPA Diet</td>
<td>12.83 ± 1.51^a</td>
<td>15.79 ± 3.01^c</td>
<td>17.09 ± 2.85^c</td>
<td>13.04 ± 1.77^c</td>
<td>12.11 ± 1.49^c</td>
<td>12.56 ± 1.57^a</td>
</tr>
<tr>
<td></td>
<td>Astaxant. Diet</td>
<td>12.33 ± 2.12^b</td>
<td>13.16 ± 2.73^c</td>
<td>15.13 ± 2.34^c</td>
<td>15.23 ± 1.99^c</td>
<td>13.89 ± 2.06^c</td>
<td>13.23 ± 1.13^a</td>
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<tr>
<td></td>
<td>Sham Injection</td>
<td>10.00 ± 1.32^a</td>
<td>11.75 ± 1.24^a</td>
<td>12.95 ± 1.82^a</td>
<td>12.96 ± 1.98^c</td>
<td>11.58 ± 1.68^a</td>
<td>11.03 ± 1.81^a</td>
</tr>
<tr>
<td>Thrombocytes (x 10^3 cell/mL)</td>
<td>Control Diet</td>
<td>2.05 ± 0.32^b</td>
<td>1.78 ± 0.17^c</td>
<td>1.55 ± 0.30^b</td>
<td>1.50 ± 0.28^b</td>
<td>1.32 ± 0.32^b</td>
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<td>High KPA Diet</td>
<td>2.78 ± 0.35^a</td>
<td>1.26 ± 0.45^c</td>
<td>1.38 ± 0.30^b</td>
<td>1.34 ± 0.20^b</td>
<td>1.45 ± 0.31^b</td>
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<tr>
<td></td>
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<td>2.25 ± 0.28^b</td>
<td>1.50 ± 0.54^c</td>
<td>1.33 ± 0.41^b</td>
<td>1.30 ± 0.27^b</td>
<td>1.31 ± 0.28^b</td>
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</tr>
<tr>
<td></td>
<td>Astaxant. Diet</td>
<td>2.23 ± 0.25^b</td>
<td>1.97 ± 0.53^c</td>
<td>1.57 ± 0.57^b</td>
<td>1.18 ± 0.40^b</td>
<td>1.40 ± 0.46^b</td>
<td>1.45 ± 0.26^b</td>
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<tr>
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<td>Sham Injection</td>
<td>2.92 ± 0.46^a</td>
<td>2.58 ± 0.44^a</td>
<td>2.66 ± 0.56^a</td>
<td>2.59 ± 0.33^a</td>
<td>2.54 ± 0.44^a</td>
<td>2.59 ± 0.38^a</td>
</tr>
<tr>
<td>Monocytes (x 10^3 cell/mL)</td>
<td>Control Diet</td>
<td>0.116 ± 0.029^a</td>
<td>0.152 ± 0.089^a</td>
<td>0.253 ± 0.069^c</td>
<td>0.313 ± 0.120^c</td>
<td>0.216 ± 0.072^c</td>
<td>n.a</td>
</tr>
<tr>
<td></td>
<td>High KPA Diet</td>
<td>0.162 ± 0.061^a</td>
<td>0.288 ± 0.043^b</td>
<td>0.424 ± 0.054^c</td>
<td>0.440 ± 0.045^b</td>
<td>0.413 ± 0.079^b</td>
<td>n.a</td>
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<tr>
<td></td>
<td>Low KPA Diet</td>
<td>0.145 ± 0.081^a</td>
<td>0.247 ± 0.088^c</td>
<td>0.330 ± 0.093^c</td>
<td>0.385 ± 0.059^b</td>
<td>0.293 ± 0.085^c</td>
<td>0.243 ± 0.048^b</td>
</tr>
<tr>
<td></td>
<td>Astaxant. Diet</td>
<td>0.154 ± 0.059^a</td>
<td>0.123 ± 0.057^a</td>
<td>0.245 ± 0.133^c</td>
<td>0.207 ± 0.100^c</td>
<td>0.230 ± 0.113^c</td>
<td>0.271 ± 0.060^b</td>
</tr>
<tr>
<td></td>
<td>Sham Injection</td>
<td>0.132 ± 0.010^b</td>
<td>0.161 ± 0.030^a</td>
<td>0.167 ± 0.025^a</td>
<td>0.152 ± 0.021^a</td>
<td>0.134 ± 0.026^a</td>
<td>0.123 ± 0.010^a</td>
</tr>
</tbody>
</table>
NON-SPECIFIC IMMUNE RESPONSE

Neutrophil Respiratory Burst Activity

Glass-adherent NBT positive cells (Figure 6.4)

There was a significant difference (p<0.001) in the percentage of neutrophil-NBT positive cells after inoculation of peptone saline and bacteria suspension (Fig. 6.4). Neutrophils from fish fed the high KPA® diet had significantly more (p=0.032) glass adherent cells and they had a greater positive reaction to nitro blue tetrazolium in neutrophils than fish fed the other diets. In addition, the NBT-positive neutrophils sampled from the high KPA® fed groups showed statistically significant (p<0.001) increases overtime, which reached a peak on day 3 and then decreased on days 4 and 5 post challenge. In contrast, fish fed the astaxanthin diet showed the lowest neutrophil-NBT positive reaction on days 1 and 2 after bacterial injection (p<0.001). Control fish neutrophils had the lowest NBT positive reaction on days 4 and 5 (p<0.001).

Plasma Lysozyme activity (Figure 6.5-A)

Disease challenged fish showed a significantly higher plasma lysozyme activity (p<0.001) than sham injected fish at all sampling periods. Fish fed the diet supplemented with the high dose of KPA® had significantly higher plasma lysozyme activity than fish fed the other three diets on all samples days (Fig 6.5-A).

Head Kidney Lysozyme activity (Figure 6.5-B)

Disease challenged fish fed the experimental diets had statistically greater (p<0.05) activity of head kidney lysozyme at all samplings relative to the sham-injected controls. Further, fish fed the high KPA® diet had increased (p=0.037) head kidney lysozyme activity during the first 4 days following the bacterial challenge injection when compared to those fed the other diets. In addition, fish ingesting the control diet and astaxanthin diet had significantly lower head kidney lysozyme activity on day 2 after Vang injection than observed in the other groups, and this was also true for the control diet fed fish on day 3 (Fig. 6.5-B).

Correlation between lysozyme activity and circulating number of neutrophils (Figure 6.6)

The activity of lysozyme in the plasma and head kidney were found to be positively correlated with the numbers of circulating neutrophils in plasma (r²=0.941, p<0.001; and r²=0.909, p<0.001, respectively) (Fig. 6.6)
Cellular response to the disease challenge.

Hepatic SP70 (Figure 6.7)

Disease-challenged fish had significantly greater hepatic synthesis of SP70 on liver in days 2, 3, and 4 relative to the sham-injected fish ($p<0.001$). There were no significant effects of diet ($p=0.496$) on the production of hepatic stress protein after injection with Vang.
Figure 6.4 Number of glass-adherent, NBT-positive neutrophils from pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets that contained various levels of antioxidant supplementation ($A^0KPA^0$, $A^{60}KPA^0$, $A^{60}KPA^{1000}$, $A^{60}KPA^{100}$) after intraperitoneal injection of Vibrio anguillarum (0.1 mL, $10^9$ cfu/mL). Different superscripts indicate significant differences (n=6/diet treatment, two replicates tanks/diet treatment, p<0.05) between challenged groups. Symbol ($\phi$) denotes a statistical difference (p<0.05) sham vs. challenge-injected groups.
Figure 6.5 Mean (± SD) lysozyme activity in plasma (6.5-A) and head kidney (6.5-B) of pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets that contained various levels of antioxidant supplementation (A°KPA°, A°KPA°, A°KPA°, A°KPA°) for 6 days after disease challenge with Vibrio anguillarum (0.1 mL, 10⁵ cfu/mL). The responses of sham-injected control (sterile peptone saline, 0.1 mL) are also shown. Different letters indicate significant differences (n=6/diet treatment, two replicates tanks/diet treatment, p<0.05) between diet groups. Symbol (φ) indicates a statistical difference (p<0.05) between sham-injected and bacteria-injected fish.
Figure 6.6 Correlation between lysozyme activity (HEWL equivalent) in plasma (6.6-A) and anterior kidney (6.6-B), and the number of circulating neutrophils in blood from pre-smolt chinook salmon (*O. tshawytscha*) fed each of four experimental diets that varied in astaxanthin and KPA® supplementation levels following an experimental challenge injection of *Vibrio anguillarum*. 
Figure 6.7 Hepatic SP70 response in pre-smolt chinook salmon (O. tshawytscha) fed four experimental diets that contained various levels of antioxidant supplementation (A°KPA°, A°60KPA°, A°60KPA1000, A°60KPA100) following a bacterial challenge (Vibrio anguillarum). The responses of sham-injected control (sterile peptone saline, 0.1 mL) are also shown. Different letters indicate significant differences (n=6/diet treatment, two replicates tanks/diet treatment, p<0.05) between diet groups. Symbol (φ) indicates a statistical difference (p<0.05) between sham-injected and bacteria-injected fish.
6.5 DISCUSSION

The nutritional status of an organism may affect its immunocompetence. Furthermore, it is accepted that both nutritional deficiencies and excesses compromise the immune responses and disease resistance of animals (Chandra, 1993). Good nutritional status prior to an outbreak of infectious disease will increase disease resistance and reduce mortality during a period when feed intake is limited (Waagbø, 1994).

The use of immunostimulants can enhance protection against disease, by enhancing non-specific defense mechanisms (Anderson, 1992b). Natural antioxidants, including carotenoids and vitamins A, C and E, are among the most important nutrients that influence the immune system (Chew, 1996). Antioxidants are known to have synergistic interactions in fish (Hilton, 1982). Grape seed PA have been shown to have potent antioxidant capabilities singly and in combination with vitamins, and might interact synergistically with carotenoids and other antioxidants in fish (Bagchi et al. 2002). The effects of feeding farmed salmon with diets containing supplemental astaxanthin and concurrently fortified with proanthocyanidins flavonoids on their immunocompetence has not been tested previously.

Vibriosis is a serious bacterial disease that is responsible for severe losses in many aquatic species (Santos et al. 1996). Vibriosis is characterized by a haemorrhagic septicaemia. The early external clinical signs of this disease in salmonids include anorexia, discoloration of the skin, and inactivity. Internally, there are haemorrhages in the liver and kidney, and the gut becomes distended and filled with a clear-yellow fluid. As the disease progresses, the surface haemorrhages may become ulcerative, and the vent may be red and swollen.

The high concentration of bacterial suspension injected into the fish in this study was highly virulent and resulted in severe mortality. Fish mortalities started within 24 h after the injection challenge. *Vibrio anguillarum* was re-isolated from liver and head kidney of moribund fish, and identified based on Gram-stained smears, colony morphology on TSA + 1.5% NaCl plates, and biochemical reactions using an API-20 E® kit (BioMérieux, Missouri, MO, USA).

The severity of a disease outbreak in fish is modulated by a triage of pathogen-host-environment factors. It is known that the course of an infectious disease challenge is more severe when is caused by inoculation of fish with the pathogen rather than by
immersion of the fish in a bacterial suspension or cohabitation of uninfected fish with an infected fish (Balfry, 1997). The pathogenesis of vibriosis following intraperitoneal injection of bacteria was particularly severe in this study due to: 1) an immunologically naïve host (chinook salmon in FW) to the marine pathogen, 2) inoculation of a large concentration of bacteria, and 3) bypassing the primary immune barriers such as physical barriers (scales, integument, epithelial cilia) and humoral non-specific agents present in mucus (i.e. lysozyme, C-reactive protein, complement, etc).

Early fish mortalities did not exhibited any external or internal signs of infection. This likely was due to acute toxaemia because the overwhelming amounts of endotoxins and exotoxins present in *Vang* as a result of the very large number of bacteria injected to the fish. Peracute cases of vibriosis without evident pathological signs of disease have been reported in young fish by Anderson and Conroy (1970), and Horne et al. (1977). Furthermore, the fish used in this experiment were in the process of smoltification as noted by the loss of parr marks and silvering of the body. Smoltification is a physiological stage of anadromous salmonids that is characterized by physical, behavioural, and physiological changes that include increasing levels of circulating cortisol in the blood (Barton et al. 1985; Maule et al. 1987) and depression of immunocompetence variables (Maule et al. 1989). Hence, this might contributed to the observed findings.

In the latter course of infection, internal examination of fish from all diet groups showed clinical signs of *Vibrio* infection such as severe haemorrhaging and inflammation in the liver, spleen and kidney. These findings are consistent with the lesions that have been observed in chum, chinook and coho salmon infected with *Vang* as reported by Ransom et al. (1984), who found extensive vascular and haemorrhagic damage to different tissues during a vibriosis outbreak.

The primary haematology index parameters haematocrit (Hct), haemoglobin (Hb), and red blood cells number (RBC) test the oxygen-carrying capability of erythrocytes in the body. The values of RBC, Hct and Hb in this experiment fluctuated in relation to the diet treatment. Therefore no consistent diet differences were observed for these haematology variables. Ackerman and Iwama (2001) also reported fluctuating and insignificant haematology results in juvenile rainbow trout subjected to the same bacterial strain used in my disease challenge. Despite the lack of diet effect of treatment on the haematological variables, there was a significant effect of Vibriosis on
haematology and anaemia was observed in the fish 3 days post bacterial injection in all dietary groups compared with the haematology variables observed in the sham-injected groups. In agreement with these results, Cardwell and Smith (1971) reported significantly decreased values for all primary and secondary haematology variables during a natural outbreak of vibriosis in chinook salmon.

The progressive deterioration in RBC, Hct, and Hb values suggests either damage to the erythropoietic system or accelerated destruction of red blood cells due to the action of haemolysins in Vang as described by Lamas et al. (1994). Decreased values during the course of the infection could have also been due to to renal dysfunction, caused by destruction of renal excretory tissue of the posterior kidney due to the effect of Vang. Moreover, kidney damage will affect the osmoregulatory processes resulting in haemodilution in salmon housed in freshwater. Finally, decreased haematological values are inherently related to a decrease in plasma protein values that affect the osmotic contribution of plasma proteins to the fluid balance and homeostasis.

Secondary haematological indices are calculated from RBC, Hct and Hb values. They provide clinical insights regarding blood characteristics, and they identify pathological effects of a variety of conditions on the number and size of circulating erythrocytes, and the amount of haemoglobin contained within them. The observed normocytic (same erythrocyte size) and normochromic (normal amount of haemoglobin) anaemia is clinically related to haemorrhage and haemolysis of erythrocytes, total loss of blood from the circulatory system and haemodilution. Vibrio anguillarum possesses potent extracellular products such as haemolysins and proteases (Lamas et al. 1994), which may be responsible for such haemorrhagic anaemia.

There were significant differences in the differential leucocyte and monocyte counts throughout the infection period due to treatment. In this regard, the numbers of circulating neutrophils and monocytes in blood from fish fed the high KPA® diet were significantly greater than in the other 3 treatment groups. The increase in the number of circulating neutrophils and monocytes was associated with greater plasma lysozyme activity since the neutrophils synthesize and secrete the enzyme. The larger number of circulating neutrophils were also coincident with the significantly higher number of neutrophil-NBT positive cells measured by the nitro blue tetrazolium assay in the fish fed the high KPA® diet. The neutrophil cell counts in the blood were reduced in chinook salmon fed the control diet, which was not supplemented with astaxanthin and KPA®. In
addition, lysozyme activity in the plasma was also reduced in the fish fed the control diet.

KPA®-mediated antioxidant effects may have enhanced both the chemotactic and chemokinetic properties (increased production of cytokines) of neutrophils. Besides providing protection to the phagocytic membranes of neutrophils, KPA® may have increased the persistency and responsiveness of the reacting immune cells in the oxidative environment (oxygen-dependent killing) during the respiratory burst. Finally, grape seed antioxidant properties may have exerted important scavenging effects on the ROS produced by phagocytes to kill invading microbes, thus preventing damage the body's own cells and tissues and thereby reducing some immunopathological effects associated with infection and inflammation. It should be mentioned that astaxanthin-fed fish had a significantly lower neutrophil-NBT positive reaction on days 1 and 2 after bacterial injection, which coincided with the highest cumulative mortality during that period.

There was also a marked thrombocytopenia observed during the second day post challenge, although there was no statistical difference between treatment groups. Haemorrhagic anaemia is characterized by a decrease in the number of circulating thrombocytes, due to their active role in clotting processes.

The inflammatory response elicits a strong stress protein induction (reviewed by Jacquier-Sarlin et al. 1994). Increases in SP70 levels have been reported to occur in head kidney and liver during bacterial infections with *Renibacterium salmoninarum* (Forsyth et al. 1997) and *Vibrio anguillarum* (Ackerman and Iwama, 2001). In this experiment, there was an increase of hepatic SP70 throughout the course of the infection challenge, and values reached their highest levels on day 3 and day 4 after bacterial injection, although there was not a significant effect of diet treatment during the challenge period.
7. GENERAL DISCUSSION

The current depletion of wild fish stocks throughout the world in recent years has occurred concomitantly with an explosive increase in demand for fish by health conscious consumers and a rise in world population. This has created a need that can no longer be met by the existing wild fisheries and therefore commercial aquaculture production has increased to meet this growing demand for aquatic protein and lipid. Historically, the wild salmon catch has supplied the world market demand for salmon almost completely; however, in the last 2 decades salmonid farming (salmon and trout) has grown to such an extent that it has surpassed the gross tonnage supplied by salmon fisheries to the international market (FAO, 2000).

Proper nutrition plays a critical role in maintaining normal growth and health of cultured fish. Intensive fish farming demands not only precisely formulated and cost-efficient diets, but also diets that can mitigate the deleterious effects of diseases and improve pigmentation deposition efficiency. Dietary antioxidants can impart health benefits. The fortification of commercial diets with antioxidants other than vitamins is a novel nutritional practice in animals, despite their long history of health benefits in human nutrition.

There have been several studies on the effects of supplementing fish diets with very high doses of the antioxidant vitamins C and E, singly and in combination, on their immune system, and growth (Hamre et al. 1997; Mulero et al. 1998; Montero et al. 1999; Ortuño et al. 1999; Ortuño et al. 2000; Cuesta et al. 2001; Sealey and Gatlin. 2002). However, there are no reports on the benefits of the supplementing fish diets with antioxidants other than vitamins and carotenoids. Therefore the potential benefits of this nutritional strategy remain unknown. Proanthocyanidins are naturally occurring flavonoids in grape seeds, and PA extracts are readily available in the market (i.e. KPA®). Grape seed PA exhibit a broad spectrum of pharmacological, therapeutic and chemoprotective properties, while concurrently enhancing the growth and viability of normal cells (Reviewed by Bagchi et al. 2002; Joshi et al. 2000). Also, they are significantly more potent antioxidants than vitamin E, C and β-carotene (Buettner, 1993, Bagchi et al. 1998). Proanthocyanidins have highly protective effects against lipoperoxidative damage, which depend on the hydrogen-donating capacity of a hydroxyl group in their structure (Saija et al. 1995), as well as their incorporation rate
into cells, orientation in biomembranes (Thomas et al. 1992), and strong affinity in chelating iron (Afanas’ev et al. 1989).

It was not the intent of this research to unravel the mechanism of action of orally ingested proanthocyanidins and astaxanthin in chinook salmon, but rather to describe for the first time the possible beneficial effects of the preceding on chinook salmon immunocompetence, pigmentation and growth under practical farming conditions in British Columbia, Canada.

Diets fortified with flavonoid PA and astaxanthin improved growth and diet utilization variables, as well as astaxanthin deposition and retention coefficient in salmon muscle after 155-day feeding period in SW, but not after a short feeding period of 32 days in FW. In addition, the immune system and some of the non-specific immune factors were significantly enhanced by diets containing a combination of KPA® and astaxanthin in FW. As a result, the total cumulative mortality observed in pre-smolt chinook salmon fed the high KPA® diet after a disease challenge was significantly lower than observed for the other 3 dietary groups.

Observed pre-smolt chinook salmon growth in FW surpassed projections by TGC for the 32-day trial. Likely was due to the very high feeding rate (3.5% b.w./day) that was selected for the trial. The observed weight gain in FW exceeded the projected estimation by TGC by +5% (control diet group) and +13% (astaxanthin diet group). On one hand, this high feeding rate allowed fish to reach their maximum growth rate, but it also negatively depressed values noted for feed utilization. In contrast to the situation observed in FW, the final weight gain observed for chinook salmon in SW did not reach the projected weight estimated by TGC. Despite the fact that the growth and diet utilization variables (SGR and FCR) that were observed among groups were within farmed salmon standards, the projected weight gain estimated by TGC was not reached by fish fed the low KPA®, astaxanthin, or control diets. Fish fed the control, astaxanthin-only, and low KPA® diets grew less well, i.e. -41%, -21%, and -13%, respectively compared to their respective projected weights by TGC. Only the fish group fed the high KPA® diet attained the projected weight (no difference between projected and actual final wet weight). Indeed, the fish fed the diet with the high concentration of KPA® together with astaxanthin had significantly greater weight gain, but not values for SGR and FCR, related to those fed the other diets after 155 days in SW. In addition, this
group had significantly higher astaxanthin deposition in their muscle compared to the fish ingesting the other diets.

The relatively low carotenoid pigment deposition and weight gain in fish groups fed diets other than high KPA® diet after 155 days in SW may have been due to some oxidation of the PUFA-rich marine oil contained in the salmon diets during storage. Environmental conditions during storage (oxygen, heat) may have affected the nutritional composition of lipids, vitamins and the levels of astaxanthin in the diets. Carotenoid pigment breakdown in diets has been reported even under temperature-controlled storage (-20°C) after 10.5 months (Christiansen, 1995a). Thus, one may expect some degree of nutrient deterioration under room temperature storage. This was probably not extensive due to the frequent preparation of the diets.

The fortification of diets with grape seed PA may have protected the diets against oxidative deterioration. Proanthocyanidins have been shown to possess strong antioxidant properties (Ariga et al. 1988; Ricardo da Silva et al. 1991) and prevent lipid peroxidation. Also KPA® has exhibited protective photo-oxidative properties in reducing astaxanthin breakdown in salmon fillets exposed to refrigerated conditions for 5 days (Kikkoman personal communication). Therefore, it is conceivable that an increased level of grape seed PA with astaxanthin may have protected the highly susceptible PUFA-rich oils and astaxanthin in the feed.

Moreover, the increased astaxanthin concentration in muscle as well as the apparent pigment retention coefficient achieved by feeding the high KPA® diet over 155 days may be related to the antioxidant protection of PA. Pigmentation involves not only the occurrence of synthetic carotenoids in the feed, but also other factors like absorption, transport in blood and deposition. The impact that KPA® might have exerted on the deposition of astaxanthin in salmon muscle could have been due to several factors such as: 1) the reduction of astaxanthin oxidation in feed, 2) the reduction of PUFA-rich marine oil oxidation contained in salmon diets, thus reducing the breakdown of vitamins, triglycerides, phospholipids, etc, 3) the synergistic interaction with the antioxidant's network (vitamin A, C, E, glutathione, etc), thereby increasing astaxanthin's bioavailability, 4) the reduction of astaxanthin's use as a free radical scavenger in the gut of salmon, thus increasing availability and efficiency of absorption of astaxanthin from the digestive tract, and 5) reduced oxidation of transporting lipoprotein fractions (HDL and VHDL), thus increasing the quantity of xanthophyll carried...
in the blood. Finally, KPA® might have enhanced the non-specific carotenoid receptors in the actomyosin complex in salmon muscle due to a general improvement of the phospholipid fraction contained in the muscle cell membrane. Movileane et al. (2000) demonstrated a positive interaction between flavonoids affecting biomembranes and planar lipid bilayers.

The effect of grape seed PA on the immune system of pre-smolts was investigated by conducting a disease challenge test. Pre-smolts fed the high KPA® diet for 32 days had significantly enhanced non-specific immune response during the disease challenge. The total number of circulating white blood cells, circulating neutrophils and monocytes, number of NBT-positive neutrophils, and plasma and head kidney lysozyme activities were significantly higher in fish fed the high KPA® diet, when compared to the responses noted for the other diet groups.

Pathological processes such as infection, inflammation and tissue injury generate a pro-oxidant environment with the release of oxidative cellular components. The immune system is particularly sensitive to oxidative stress, primarily because the coordination of an effective immune response relies heavily on cell-to-cell communication. One of the events during the inflammatory response is the movement of phagocytic cells from the blood or surrounding tissue to the site of microbial invasion attracted by chemotactic factors (Secombes and Fletcher, 1992). Phagocytic cells described in fish are mononuclear cells (tissue macrophages and circulating monocytes) and polymorphonuclear granulocytes (particularly neutrophils)(Secombes, 1996).

Phagocytes attack the pathogen either through digestive enzymes (oxygen independent killing mechanism) or via the oxygen dependent mechanism. During oxygen dependent killing, oxygen is converted into a number of microbicidal ROS in a process termed the respiratory burst. The stimulation of the cell membrane activates the enzyme complex NADPH oxidase that is capable of the univalent reduction of molecular oxygen into $\text{O}_2^\cdot$ (Kang et al. 1994) and it has been demonstrated in fish by Secombes (1996). The nitro blue tetrazolium assay (NBT) reacts by reducing NBT by $\text{O}_2^\cdot$, producing a blue precipitate called formazan. The neutrophils that had a blue halo were counted as NBT positive cells, and these indicate the cells that were participating actively in the oxygen-dependent killing mechanism.

The NADPH is generated from the pentose phosphate shunt (Kang et al. 1994). Part of the $\text{O}_2^\cdot$ produced is dismutated to $\text{H}_2\text{O}_2$ either spontaneously or catalyzed by
SOD. Also \(^1\)O\(_2\) is produced during spontaneous dismutation and this is detectable by chemiluminescence (CL) measurement following the respiratory burst. Excess H\(_2\)O\(_2\) production can be regulated by catalase. The extremely harmful OH\(^-\) is produced when H\(_2\)O\(_2\) reacts with O\(_2\)^{*}. Further, there has been evidence for the existence of the myeloperoxidase (MPO) H\(_2\)O\(_2\)-halide system in fish granulocytes (not present in macrophages). MPO catalyzes the oxidation of halide ions by H\(_2\)O\(_2\) to form hypohalites and chloramines with enhanced microbicidal actions (Secombes, 1996). Finally, since the production of ROS increases during certain immune responses and during inflammation, and since oxidant enzymes are involved in the regulation of the respiratory burst, cellular antioxidants can be regarded as part of the non-specific immune system (Kang et al. 1994) illustrating the closer inter-relationship between the two protective systems.

Secombes (1996) pointed out the important participation of eicosanoids such as prostaglandins, leucotrienes and thromboxanes produced by leucocytes in the enhancement and development of the inflammatory response in fish. Eicosanoids are lipid mediators derived from arachidonic acid and have been shown to possess a number of non-specific and specific immune functions such as augmenting phagocytosis and increasing chemotaxis for neutrophils. KPA\(^\circ\) might have helped to control the excessive production of free radicals during the respiratory burst activity of neutrophils and therefore prevented the peroxidation of such eicosanoids. In addition, lipid peroxidation promotes the formation of peroxides that compromise the cellular membrane structure and function, thereby disrupting signal transduction through which cells communicate. As a result, a number of changes may indicate the possible loss in cell function, such as reduction in receptor binding (Nunez and Glass, 1982; Riley and Carlson, 1988), loss in membrane transport (Yuli et al. 1981) and alterations in enzyme activity (Kim and Yeoun, 1983; Riley and Carlson, 1987).

In addition to the reported individual effects of PA (reviewed by Bagchi et al. 2002), dietary antioxidants and antioxidant vitamins have been shown to act synergistically exhibiting a “sparing effect” against the damage caused by oxidative stress. Vitamin E, in particular \(\alpha\)-tocopherol, is regarded as the primary lipid-soluble antioxidant that operates synergistically with Vitamin C (ascorbic acid, which is a water-soluble antioxidant) to protect lipids against peroxidative damage (Burton et al. 1985; Sato et al. 1990; Buettner, 1993; Kamal-Eldin and Appelqvist, 1996). This interaction
has been demonstrated in phospholipid model membranes, microsomes, human platelets, and LDL against lipid peroxidation in vitro (Niki, 1987; Wefers and Sies, 1988; Chan et al. 1991; and Kagan et al. 1992). This synergistic interaction has also been demonstrated in juvenile Atlantic salmon by Hamre et al. (1997) by measuring vitamin concentrations in the liver. Shiau and Hsu (2002) measured and quantified plasma and liver vitamin concentrations, as well as hepatic TBARS in juvenile tilapia.

A combination of vitamins C and E has been shown to positively affect growth but not the immune system in juvenile hybrid striped bass (Sealey and Gatlin, 2002). In contrast, Hamre et al. (1997) showed that Atlantic salmon fed a combination of vitamins C and E had an improved immune system but growth was not affected.

Additionally, several recent studies have suggested that carotenoids, including β-carotene, astaxanthin and canthaxanthin possess potent antioxidant properties in cell membranes and operate synergistically with Vitamin E (Palozza and Krinsky, 1992a and 1992b; Nishigaki et al. 1994). The vitamin E and flavonoid regeneration in membranes has been discussed by Terao and Piskula (1998). Synergistic α-tocopherol and quercetin (also a natural flavonoid) regeneration has been shown in micellar solutions by Mukai et al. (1996); and the synergistic and regenerative activity between vitamin E and flavonoids have been shown by Pedrielli and Skibsted (2002).

In spite of the efforts made by the chemical analysis laboratory of Kikkoman Corporation in Japan in retrieving and quantifying the amount of PA in fish feed, the procedure was unsuccessful. This situation has not been unique, Yamakoshi et al. (1999) also failed to recover and quantify the amount of PA from blood in rabbits fed PA-containing diet, although they were able to recover and quantify PA when the grape seed extract was singly force-fed to rats. Biological properties of PA depend on their bioavailability. Proanthocyanidins from red wine are highly available due to their high rate and extent of intestinal absorption (Tapiero et al. 2002). It has also been shown by Jimenez-Ramsey et al. (1994) that PA solubles in water and ethanol, such as KPA®, are highly absorbed from the intestinal tract and extensively distributed in all tissues and plasma. Furthermore, Bravo (1998) demonstrated that plasma PA can be maintained following a regular intake of sufficient quantities of fresh fruits or supplementation with bioavailable PA’s. Based on the evidence from the studies mentioned above, one might expect the bioavailability of PA in chinook salmon fed diets fortified with KPA® to be high.
Finally, and supported by the literature cited above, it is conceivable that the combination of KPA$^\text{®}$ and other antioxidant vitamins and minerals in sufficient quantities in a balanced diet may have acted synergistically and exerted positive and beneficial effects on the immunological, pigmentation and growth variables in chinook salmon under the conditions of these studies.
8. CONCLUSION

Free radicals are normally produced under physiological conditions as part of the oxygen metabolism in aerobic organisms. Their levels increase significantly in specific situations (i.e. inflammation, as part of its regular defense functions). Farmed salmon are susceptible to environmental, chemical and pathological insults that will increase the production of these compounds. They possess different mechanisms to cope with the increasing production of free radicals (i.e. antioxidant enzymes, FR scavengers, metal chelators). The balance between oxidants and antioxidants is crucial for the integrity and functionality of cell membrane, signalling transduction and gene expression.

In addition to the endogenous enzymatic antioxidant defense, consumption of dietary antioxidants appears to be of great importance because of their protective antioxidant effects. Feeding dietary antioxidants other than antioxidant vitamins is a well-established practice in the human diet. By contrast, this is a novel practice in small animal diets (i.e. dogs and cats), and it is non-existent in livestock and aquaculture (i.e. salmon farming).

This is the first study that has assessed the effects of feeding farmed salmon diets supplemented with grape seed flavonoids (proanthocyanidins) in combination with astaxanthin with respect to their growth, flesh pigmentation and immune response. The effects of feeding a combination of the foregoing dietary antioxidants resulted in significant positive effects on immunocompetence after a short feeding period in FW (32 days). The circulating number of leucocytes (neutrophils and monocytes), oxidative burst activity, and the activity of lysozyme both in plasma and anterior kidney were significantly higher in fish fed the high KPA® diet after a disease challenge with *Vibrio anguillarum*. The increase of these non-specific immune factors may have contributed to the significantly lower cumulative mortality seen in this diet group. Muscle pigmentation and growth of pre-smolts (less than 7 g) were not significantly affected by feeding the antioxidant-fortified diets over the short trial in FW. However, when the feeding period was extended to 155 days in smolts transferred to saltwater (mean initial weight 7 g to final weight 80 g), significant increases were observed for growth (weight gain) and pigmentation (concentration of astaxanthin in muscle, AARC, RSS) variables in fish fed the high KPA® diet.

As farmed salmon production has increased every year in the world, the supply of salmon has at times exceeded demand and the market prices have declined. The
reduction in salmon prices has forced fish researchers to investigate new methods for improving general efficiency while reducing costs of production. The muscle retention of carotenoids represents only 1-5% of the ingested pigments (Choubert and Luquet, 1983; Torrissen et al. 1990). The price of extruded salmon feed supplemented with 40 to 80 ppm of astaxanthin comprises as much as 15-20% of the total feed costs, or about 6-8% of the total production cost (Torrissen, 1995). Therefore, reducing fish mortalities, and effective feed and pigment management are vitally important to the economics of intensive salmon aquaculture.

The results of this research indicate that the supplementation of 1000 ppm of KPA® in combination with 60 ppm of astaxanthin in a high quality salmon diet resulted in significant differences in valuable farmed salmon production traits, such as weight gain, flesh pigmentation and fish mortality. In order to realize the magnitude of the results obtained in this research we may extrapolate them into a full-scale farming situation. Seacages in SW are 15x15x15 m (a volume of 3,375 m³) and transferred chinook salmon smolts are held at stocking densities of 10-12 kg/m³ (total biomass between 33,750 to 40,500 kg).

Vibriosis caused by an immersion challenge in a Vang suspension has resulted in mortalities that have varied between 30 and 60% in coho salmon (Balfry, 1997). If one projects the results observed in fish fed the high KPA® diet in the disease challenge to a commercial situation (40,500 kg biomass/seacage), the use of the high KPA® diet would prevent the loss of 1,200 to 2,400 kg of biomass/seacage considering outbreak mortalities ranging from 30-60%. If the results observed in the SW experiment are considered, the attainment of an increase in SGR from 1.43%/day (astaxanthin diet) to 1.58%/day (high KPA® and astaxanthin diet) would increase the biomass between 50 to 60 kg/day/seacage, and after 155 growing days it will be between 7,850 to 9,420 kg/seacage. In addition, increasing the fish biomass by 1,000 kg would require 1,400 kg of food at a FCR of 1.40 (as seen in fish fed the astaxanthin diet) or 1,270 kg with a FCR of 1.27 (high KPA® diet), which represents a 10% saving in food. Also, feeding salmon with a diet fortified with a high concentration of KPA® increased significantly the retention coefficient of astaxanthin from 0.90% to 1.28%. Finally, the use of salmon diets fortified with a high concentration of KPA® would enable farmed salmon to reach a
market size faster than those fed diets without KPA\textsuperscript{©} supplementation and with higher concentrations of astaxanthin in their flesh.

While the findings in this study are very promising, it is important to conduct additional research to confirm the present findings at other stages of the life history and with extensive replication of each dietary treatment. Additionally, the relative efficiency of KPA as an antioxidant versus vitamins C and E should be considered in the future studies.
9. REFERENCES


Interactions between antioxidant systems:

Lipophilic and hydrophilic antioxidants do not stand-alone but interact with each other in multiple ways. Vitamin E scavenges radicals in the lipid phase (vitamin E cycle) and can be regenerated by vitamin C, which in turn can be regenerated by different substrates (vitamin E cycle). One of the main substrates is GSH, which can regenerate vitamin C enzymatically. Other Thiols fulfil similar functions. Thiols finally can be regenerated using metabolic energy (Thiol cycle). Thus antioxidants form a highly organized network.
Appendix 2

A. Role of dietary antioxidants in the defense against oxidative damage in biomembranes.

<table>
<thead>
<tr>
<th>Vitamin C: scavenging of water-soluble radicals</th>
<th>Regeneration of vitamin E</th>
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<tbody>
<tr>
<td>Flavonoids: scavenging of water-soluble radicals</td>
<td>Chelation of metal ions</td>
</tr>
<tr>
<td>Vitamin E: scavenging of chain-propagating radicals</td>
<td></td>
</tr>
<tr>
<td>Carotenoids: quenching of singlet oxygen</td>
<td></td>
</tr>
</tbody>
</table>

B. The overall mechanism of lipid oxidation consists of three phases (Newer, 1996)

1. Initiation: \[ RH + O_2 \rightarrow R^* + ^\cdot OH \]

2. Propagation: \[ R^* + O_2 \rightarrow ^\cdot + ROO^* \]
   \[ ROO^* + RH \rightarrow R^* + ROOH \]
   \[ ROOH \rightarrow RO^* + HO^* \]

3. Termination: \[ R^* + R^* \rightarrow RR \]
   \[ R^* + ROO^* \rightarrow ROOR \]
   \[ ROO^* + ROO^* \rightarrow ROOR + O_2 \]
Appendix 3

Freshwater temperature record (experiments one and three)

Record Date
(Week 1-4 = June 2000; Week 5-8 = July 2000)
Appendix 4
Saltwater temperature record (experiment C)