2005

Why are salmon eggs red? An investigation of the benefits of red carotenoid-based pigmentation in the eggs and offspring of Chinook salmon (Oncorhynchus tshawytscha)

Selene Tracy Tyndale
University of Windsor

WHY ARE SALMON EGGS RED?

An investigation of the benefits of red carotenoid-based pigmentation in the eggs and offspring of Chinook salmon (*Oncorhynchus tshawytscha)*

by

Sélène Tracy Tyndale

A.Sc., Seattle Central Community College, 1995
B.Sc., University of Windsor, 2002

A Thesis
Submitted to the Faculty of Graduate Studies and Research
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

2005

Your file Votre référence
ISBN: 0-494-09845-7
Our file Notre référence
ISBN: 0-494-09845-7

Abstract

Red carotenoid-based pigmentation is characteristic of salmonid eggs. Though this incurs metabolic costs for maternal accumulation/allocation, and increases predation risk to the eggs, to date there has been no empirically supported adaptive explanation documenting benefits to offset the costs of red egg pigmentation in salmonids. This study investigates relationships between maternal egg carotenoid concentrations and measures of survival and immune function in larval and juvenile salmon. Chinook salmon (*Oncorhynchus tshawytscha*) possess a rare genetic polymorphism resulting in red- and white-fleshed phenotypes representing the most diverse range of naturally occurring flesh and egg carotenoid-based pigmentation known in any single salmonid species. In this study Chinook salmon eggs representing a wide range of carotenoid pigmentation were selected, fertilized and reared as maternal families following standard hatchery protocols with incubation survival measured. At the smolt stage, disease resistance was measured via interparitoneal injection with live *Listonella anguillarum*, and time to 10% mortality (LT10) in each family was used as a measure of resistance. Carotenoids and antibodies were extracted and concentrations (ug/g) measured by HPLC/UV-visible absorption and ELISA respectively in each family at different stages of embryonic development; unfertilized eggs (both), eyed-stage eggs (carotenoids only) and swim-up stage offspring (both). Astaxanthin was the primary carotenoid in all the eggs and the concentration declined in all families during development. Antibody levels varied widely among families at both stages measured, and in general were higher at the swim-up stage. A significant relationship was found between smolt stage disease resistance and mean egg carotenoid concentrations by family in a single regression, and this relationship was also significant in a multiple regression with independent covariates of egg carotenoid and

antibody levels, with egg carotenoids being the significant predictor. While no relationship was observed for incubation survival, a significant relationship was found between the decline in egg carotenoid and increase in antibody levels to the offspring swim-up stage. This study provides new evidence that carotenoid pigments and immunological function dynamics in salmon eggs and developing offspring are related in a complex fashion, reflecting a possible mechanism contributing to the published relationships between egg carotenoid and offspring fitness.

Dedication

This thesis is dedicated to:
- my son, Samuel Thomas Tyndale-Hersha, whose existence, hugs, kisses, smiles & curiosity has fueled my desire & dedication to pursue this degree to its completion

- Panda (1989 – 2004) my source of unconditional love, eternal confidence and constant companion for 14 ½ years – may you always rest in peace & grow the roses beautiful!

- my Babi, who inspired me to always believe in myself, follow my dreams, treat all life & our planet as precious & above all, to always be happy with who & what I am!

- my parents, whose assistance was immeasurable in understanding, emotional support, ensuring a functional computer and most importantly, for countless hours of child-care!

- most importantly, to my amazingly yummy & wonderful partner in life, love and explorations of science, nature and life's continual evolution - Brent W. Young

Acknowledgements

I gratefully acknowledge the following people and industry partners for their invaluable contributions to the research leading to this thesis:

Hoffman-LaRoche, Roche Vitamins, Canada for the generous donation of analytical chemically pure carotenoid standards of astaxanthin, zeaxanthin, lutein, canthaxanthin and β-cryptoxanthin.

Dr.'s John and Ann Heath and the staff and facilities of Yellow Island Aquaculture Ltd. for the use of experimental incubation stacks, freshwater hatchery rearing facilities and maintenance and tabulation of individual family progeny survival and providing facilities and advice on the mechanistics of the field experiment portions of this study.

Dr. Andrea Osborn, Pacific Biological Station, Nanaimo B.C. for providing a live culture of *Listonella anguillarum*, and consultation advice for the disease challenge experiment.

Dr. Hugh Fackrell, Professor, Biological Sciences, U. of Windsor, ON, for his invaluable and tireless consultation assistance in the development of protocols for (1) implementing a workable disease challenge with limited resources in the field location, and (2) a scientifically accurate ELISA analysis.

Ms. Anathea Albert, Technician for the Dixon Lab, U. of Waterloo, ON, for her practical demonstration and on-call assistance with conducting and analyzing ELISA results.

Dr. Eleanor Maticka-Tyndale, University Professor and Senior Canada Research Chair in Social Justice and Sexual Health Research, Dept. of Sociology and Anthropology &,

University of Windsor, Windsor, ON, for extensive assistance and advice on statistical analyses and software integration

The Social Justice and Sexual Health Research Lab and its coordinator Mr. Sandy Tyndale for the use of and help with computers, software programs and printers that made the writing and presentation of this thesis possible

Co-Advisors:
Dr. Daniel D. Heath, Professor and Junior Canada Research Chair in Conservation Genetics, Dept. of Biological Sciences, U. Windsor, ON, for providing office and lab space, consumable materials funding, the initial idea of this study, and some suggestions on analytical, writing and presentation formats.

Dr. Robert J. Letcher, Adjunct professor, Dept. of Chemistry, Carleton U., Great Lakes Institute for Environmental Research and Dept. of Chemistry & Biochemistry, U. of Windsor, Assoc. Faculty, Dept. of Environmental Biology, U. of Guelph, Research Scientist, Head of Environmental and Analytical Chemistry Research, National Wildlife Research Centre, Canadian Wildlife Service Environment Canada, Carleton University (Raven Road), Ottawa, ON, for providing office and lab space, equipment & materials funding, assistance with methodological development, applications & analyses, along with interactive advice on writing and presentation formats.

Committee members:
Dr. Dennis Higgs, Assistant Professor, Biological Sciences, U. Windsor, ON.

Dr. Otis Vacratsis, Assistant Professor, Chemistry and Biochemistry, U. Windsor, ON.

The following funding sources made this research possible:

Canada Research Chair funding,
National Sciences and Engineering Research Council (NSERC) operational finding &
Ontario Foundation for Innovation grants to my supervisors RJL & DDH &
President's Research Excellence Award (Prea) to DDH
Collectively covered lab equipment, supplies, partial costs of field work + 4 semesters of a partial monthly student stipend in 3 years of graduate enrollment!

Ontario Graduate Scholarship, Province of Ontario, Canada

Clemens-Rigler Travel Award to attend the Canadian Conference For Fisheries Research

Ian David Burks Memorial Scholarship for graduate students supporting dependents

Departments of Graduate Studies and Biological Sciences, University of Windsor, for 2 semesters each of graduate tuition wavers & teaching assistanceships + 1 semester of a general undergraduate level teaching assistanceship in 3 years of graduate enrollment!

Statement of Originality

I affirm that the research and writing embodied in this thesis is original and was conducted by myself (the author), with advice and input from my supervisors and others specifically acknowledged or cited.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations and Symbols

Ab = Antibody or immunoprotein

ATU = accumulated thermal units or degree days; the number of days times the average water temperature at which salmon eggs and offspring have been reared

DFO = Department of Fisheries and Oceans, British Columbia, Canada

Maternal phenotypes & origins of Chinook salmon progeny of this study
Also see Figure 1.2 map of sample origins:
WF = white-flesh, originating from the Chehalis River Enhancement Hatchery, tributary of the Harrison River in the Lower Fraser Watershed, S.W. mainland, British Columbia
RFw = red-flesh (wild), originating from the Quinsam River Enhancement Hatchery, Vancouver Island, British Columbia
RFd = red-flesh (domestic), from broodstock at Yellow Island Aquaculture Ltd., Quadra Island, British Columbia

Terms referring to Chinook salmon development stages of this study:
unfertilized egg = salmon eggs artificially spawned, not fertilized
eyed eggs = salmon eggs that have been fertilized and incubated until their eye spots are visible, ≈ 250-300 ATU
swim-up = salmon offspring that have hatched, completely absorbed their yolk-sac and are ready to begin exogenous feeding, in the wild this is when they would begin to "swim-up" out of the gravel, ≈1000 ATU
smolt = salmon offspring that are ready to enter the marine environment, ≈ 2000 ATU

Units of measure:
ug/g = microgram / gram, units of concentration or levels of compounds
% = percent, the amount remaining divided by the original amount then multiplied by 100
log = base 10 logarithm of the indicated unit of measure, applied when data under analysis displays non-homogeneous variances
log +1 = as above, with 1 added to the data prior to log transformation, applied when data to be transformed is less that 1
log +2 = as above, with 2 added to data prior to log transformation, applied when data in the set to be transformed is ≥ -1
wt = weight (g) or eyed egg wt = weight (g) at the eyed egg stage, used as a surrogate measure of maternal effects

CHAPTER #1

Why are salmon eggs red?

A General Introduction

1.1 Carotenoids and Retinoids

Carotenoids are a class of over 600 hydrophobic compounds characterized by a long (40) carbon chain with a conjugated π electron system as well as methyl-, and in some isoforms, oxygenated, side groups (Figure 1.1).

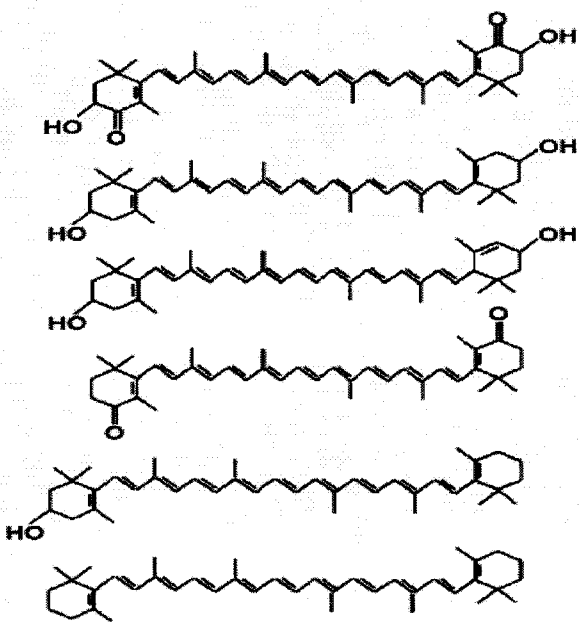


Figure 1.1: Examples of molecular carotenoid structures. top to bottom: astaxanthin, zeaxanthin, lutein, canthaxanthin, β-cryptoxanthin, and β-carotene. Hydrogen atoms have been omitted for clarity.

These molecular structural characteristics for carotenoids facilitate the absorption of radiation in the visible wavelength range of the electromagnetic spectrum (typically 440-490 nm), and the reflection of the characteristic red to yellow colour commonly associated with carotenoids. This is exemplified by the red to yellow colouration of salmonid and bird eggs and yolks (Craik 1985; Blount *et. al.* 2000).

Synthesized in plants and obtained by animals through their diet (Goodwin 1986; Stahl and Sies 2005), carotenoids serve a diverse range of critical biological functions. The most notable of these are carotenoid-based ornaments, often part of secondary sexual signals, are believed to convey health or fitness benefits to potential mates (Hamilton and Zuk 1982; Skarstein and Flostad 1996).

At the cellular level, carotenoids are metabolic precursors to vitamin A and other retinoids (Goodwin 1954; Tanumihardjo 2002; Mora *et. al.* 2004). Retinoids are well known for their roles in embryonic pattern formation (Kraft *et. al.* 1994) and disease resistance (Sommer 1989; Wolf 1996) through regulation of apoptosis (Sumantran *et. al.* 2000; Palozza *et. al.* 2004; Sacha *et. al.* 2005), chemotaxis (Demvinska-Kiec *et. al.* 2005) and intercellular communication (Stahl *et. al.* 2000).

Carotenoids are also powerful antioxidants (Mortensen *et. al.* 2001; though for conditions of carotenoids as pro-oxidant see Palozza 2005). In synergy with other dietary antioxidants (Böhm 1997; Stahl *et. al.* 1998) and their conjugated proteins (Bhosal and Bernstein 2005) carotenoids help maintain the functional stability/integrity of biological systems and organs (Kurashige et. al. 1990; Liebler *et. al.* 1997; Sujak *et. al.* 1999; Cantrell *et. al.* 2004). Xanthophyll carotenoids, with their polar end groups (Figure 1.1), span bi-lipid membranes, influencing membrane physical and structural integrity, similar to cholesterols (Wiesyaw *et. al.* 2005). Carotenoids and their retinoid metabolic derivatives can also act as transcription enhancers (Silveira and Moreno 1998) through interactions with promoter regions, or by stabilizing mRNA transcripts such as those of connexion 43, which contributes to gap junction intercellular communication, essential to normal development and cancer prevention (Zhang *et. al.* 1992; Hanusch *et. al.* 1995; Stahl *et. al.* 2000; Bertram and Vine 2005). Additionally, carotenoids and retinoids

stimulate oestrogen production (Ng *et. al.* 2000), which upregulates production of vitellogenin (vtg) and very low density lipoprotein (VLDL) (Speake *et. al.* 1998). These compounds (VLDL and vtg) then form complexes with maternal carotenoids and other yolk resources, assisting in their transport to developing oocytes (Ando *et. al.* 1986a, b & c), where vtg and VLDL then serve as precursors of protein and lipid resources (respectively) for developing embryos (Speake *et. al.* 1998; Hiramatsu *et. al.* 2002). The high metabolic rate associated with embryonic development generates elevated levels of oxidative by-products (Danapat *et. al.* 2003; Dreon *et. al.* 2004). Yolk carotenoids, in synergy with other antioxidants, mediate peroxidative damage to the lipid-rich embryonic tissues, membranes and organ systems (Speake *et. al.* 1998), as well as other maternal resources such as antibodies (Haq *et. al.* 1996), preserving their integrity for the developing embryo (Blount *et. al.* 2000). As most developmental resources are colourless, carotenoids offer a visual signal of the abundance and integrity of egg/yolk resources.

1.2 Carotenoids/Retinoids and Immune Function

Dietary carotenoids increase circulating carotenoid levels. Then, following vaccination, carotenoid supplemented individuals show increases in immune measures such as vaccine-specific antibody levels (Kiss *et. al.* 2003), and maintaining the integrity of red blood cells during free radical attack (Alonso-Alvarez *et. al.* 2004). Immune activation is rapidly mirrored by reductions in circulating carotenoids (Faivre *et. al.* 2003). Inverse correlations between circulating levels of carotenoid and antibody are frequently reported in adult birds (Saino *et. al.* 1999; Verhulst *et. al.* 1999; Ohlsson *et. al.* 2003; Kiss *et. al.* 2003; Peters *et. al.* 2004) and formed part of the immunocompetence

handicap hypothesis in studies of sexually mature threespine sticklebacks (Folstad and Karter 1992) and Arctic charr (Skarstein and Folstad 1996). The intensity of male secondary sexual carotenoid pigmentation used in female mate choice has been correlated with reduced parasite loads in guppies (Houde and Torio 1992) and the threespine stickleback (Milinski and Bakker 1990; Folstad et. al. 1994; Bakker et. al. 1999), and these correlated traits have been shown to be heritable in their offspring (Barber et. al. 2001) or confer other offspring fitness advantages (Reynolds and Gross 1992). In birds, maternal carotenoid supplements increase egg levels of maternally allocated carotenoids and vaccine-specific antibodies (Haq *et. al.* 1996a; Kiss *et. al.* 2003); as well as endogenous antibody production from *in vitro* mitogen challenge in the bursa of newly hatched chicks (Haq *et. al.* 1996b). Vitamin A and retinoids also boost the recovery potential from a number of diseases in humans (see reviews by Sommer 1989; Wolf 1996) and are well known for their interactive roles with immune cells and processes *in vitro* and other mammal species (Smith and Hayes 1987; Geissmann *et. al.* 2003; Langmann *et. al.* 2005). Carotenoids mediate oxidative DNA damage in leukocytes and cancer cells, decreasing *in vitro* proliferation (Dulinska et. al. 2005) and inducing *in vitro* apoptosis (Stacewicz-Sapuntzakis et. al. 2005) in cancer cells. Additionally, carotenoids and retinoids both act as cofactors inducing T-cell immune process cascades *in vivo* in humans (Alexander et. al. 1986; Jonasson et. al. 2003), mice (Jyonouchi et. al. 1994) and *in vitro* cell culture (Garbe et. al. 1992; Jyonouchi et. al. 1995; Stephensen *et. al.* 2002).

1.3 Evolutionary Processes: Carotenoids in Sexual vs. Natural Selection

Carotenoid-based pigments are often a vital part of secondary sexual signals in birds (Hamilton and Zuk 1982) and fish (Skarstein and Folstad 1996). Darwin first

proposed such ornamental colouration as being evolutionarily driven by sexual selection (Darwin 1871). Recently, several studies have documented correlations between the carotenoid-based pigment intensity of sexual ornaments and measures of fitness both within individuals (e.g. Alonso-Alvarez *et. al.* 2004) and from parent to offspring (e.g. Barber *et. al.* 2001). Salmonids develop nuptial ornaments at sexual maturity, some of which are carotenoid-based, such as the intense red skin colour of male sockeye salmon (*Oncorhynchus nerka*) or the red belly in both sexes of Arctic charr (*Salvelinus alpinus*). Mature Chinook salmon (*O. tshawytscha*) develop darker skin colour, though not predominantly red. Males grow a hump behind their head and a kype with sharp teeth used to fight for access to spawning females. Dominant males cue on the quiver and redd digging behaviours of gravid females, and approach to fertilize her eggs as they are laid. Neither male nor female see the eggs; hence egg pigmentation is not a sexually selected trait. Carotenoid pigments are highly visible, especially within cryptically coloured stream beds (pers. obs.). High visibility increases predation risk (Wieland and Koster 1996; Godin and McDonough 2003), and thus egg carotenoid pigmentation should be selected against. Additionally, maternal dietary intake, metabolism and allocation of carotenoids to maturing oocytes incur metabolic costs (Schiedt, et. al. 1985; Torrissen *et. al.* 1989; Metusalach, *et. al.* 1996). Evolutionarily speaking, salmon offspring should derive benefits of egg carotenoids, which offset the costs of maternal provisioning and egg predation risk; yet, to date, there has been no empirically supported explanation why salmonids allocate such high levels of carotenoids to their eggs.

1.4 Carotenoids in Salmonid Aquaculture: Early Survival and Disease Resistance

The characteristic red of salmon flesh and eggs drives consumer marketability and is due to the presence of dietary carotenoids (Storebakken *et. al.* 1987; March and MacMillian 1996). Traditionally, salmonid egg pigmentation has been interpreted as a sign of quality, thought to contribute to higher fertilization, hatching or offspring survival (Hubbs and Stavenhagen 1958; Yarzhombek 1964 and 1970). Relationships between egg yolk carotenoids and offspring survival are well established in birds (Blount *et. al.* 2000). Similar correlations have been documented in salmonid fish under conditions of oxidative stress syndromes such as M74 in the Baltic (Pettersson and Lignell 1999), Cyuga and early mortality syndrome (EMS) in the Finger Lakes of New York state, and in the North American Great Lakes (Palace *et. al.* 1998), all of which entail early yolk-sac fry mortality within affected maternal families. Investigations of healthy salmonid populations, however, have not empirically supported the egg carotenoid-offspring survival relationship (Torrissen 1984; Craik 1985; Tveranger 1986; Christainsen and Torrissen 1997). Craik (1985) proposed a hypothesis that a minimum egg carotenoid threshold level is necessary for optimal offspring survival, as opposed to the often tested linear relationship between these two variables, perhaps explaining some of the lack of empirical support for the egg carotenoid-offspring survival assertions. From a review of published data spanning decades, continents and numerous salmonid species, Craik (1985) proposed that in salmonids, a minimum carotenoid threshold range of 1-3 ug/g egg was required to ensure "acceptable" offspring survival rates, typically above 85%, while egg batches with carotenoid levels below this tend to experience rapid, exponential declines in offspring survival relative to their egg carotenoid concentrations. Similarly, studies of salmonid populations experiencing M74 and EMS proposed a minimum

carotenoid concentration range of 0.1-0.3 ug/g egg as being required for basic oxidative protection, as egg batches with carotenoid levels below this range typically experienced almost total offspring failure regardless of other resource levels (Lundström *et. al.* 1999).

1.5 Carotenoids: Genetic Differences in Absorption/Retention Capacity

Variations in the capacity for dietary carotenoid absorption, metabolism and deposition involve a heritable component in salmonids (see review within Blanc and Choubert 1993). While salmonid species worldwide are known for their red carotenoid-based flesh and egg pigmentation (Craik 1985; Ando *et. al.* 1989), Chinook salmon (*Oncorhynchus tshawytscha*) are an exception. Chinook are the only salmonid species to have two distinct carotenoid-based flesh colour phenotypes, termed red-fleshed and white-fleshed (Prince 1916; Milne 1964; Godfrey 1975), with egg pigmentation mirroring that of the maternal flesh colour type (Hard 1986; Withler 1986; Hard *et. al.* 1989).

In mammals "low responders" to dietary carotenoids have between 50% (Bowen *et. al.* 1993) to 99% (Stahl *et. al.* 1995) reductions in circulating carotenoid levels compared to conspecifics receiving identical diets (Chew *et. al.* 2000; Lin *et. al.* 2000; Hickenbottom *et, al,* 2002). Similarly, white-fleshed Chinook salmon can have flesh carotenoid levels as much as 95% below those of their red-fleshed counterparts when evaluated by visual comparison to carotenoid flesh-pigment colour charts (McCallum *et. al.* 1987) or by thin layer chromatography (Ando *et. al.* 1992). Flesh colour, when treated as a threshold trait, is highly heritable in Chinook salmon, with the mean heritability of dam and sire components estimated at 0.8 (Withler 1986). Controlled by a genetic polymorphism, Chinook flesh colour is believed to involve two loci, each requiring a dominant allele for expression of the red-flesh phenotype (Withler 1986). White-fleshed

individuals lack the specific molecules necessary for carotenoid transport and/or deposition/retention in the flesh (Ando *et. al.* 1994), and perhaps it is these molecular transport & deposition factors that are controlled by the two proposed loci, the lack of either leading to the white-fleshed phenotype. While the occurrence of white-fleshed Chinook is far less extensive than the more common red-fleshed variety (Hard *et. al.* 1989), white-fleshed Chinook have nonetheless long been recognized in native cultures and commercial fisheries (Prince 1916), and are thus presumed to represent a viable evolutionary strategy. Chinook salmon therefore provide an exceptional opportunity to test for correlations between carotenoids and measures of fitness in healthy fish, offering a wide range of egg pigmentation levels without the need for artificially manipulating the maternal diet.

1.6 White- and Red-Fleshed Chinook Salmon of this Study

For this study, eggs from wild populations of the two flesh-colour phenotypes were chosen, along with a domestic red-fleshed population. Since marketability is largely driven by flesh colour, domestic populations of white-fleshed Chinook salmon are currently unavailable. Eggs of wild stock were obtained from British Colombia DFO (Department of Fisheries and Oceans Canada) enhancement hatcheries; white-fleshed Chinook salmon from the Chehalis River (Fraser river tributary), and red-fleshed Chinook salmon from the Quinsam River (Vancouver Island). Domestic red-fleshed Chinook salmon were selected from broodstock at Yellow Island Aquaculture Ltd. (YIAL, Quadra Island, B.C.), where all rearing and field experiment took place (see figure 1.2).

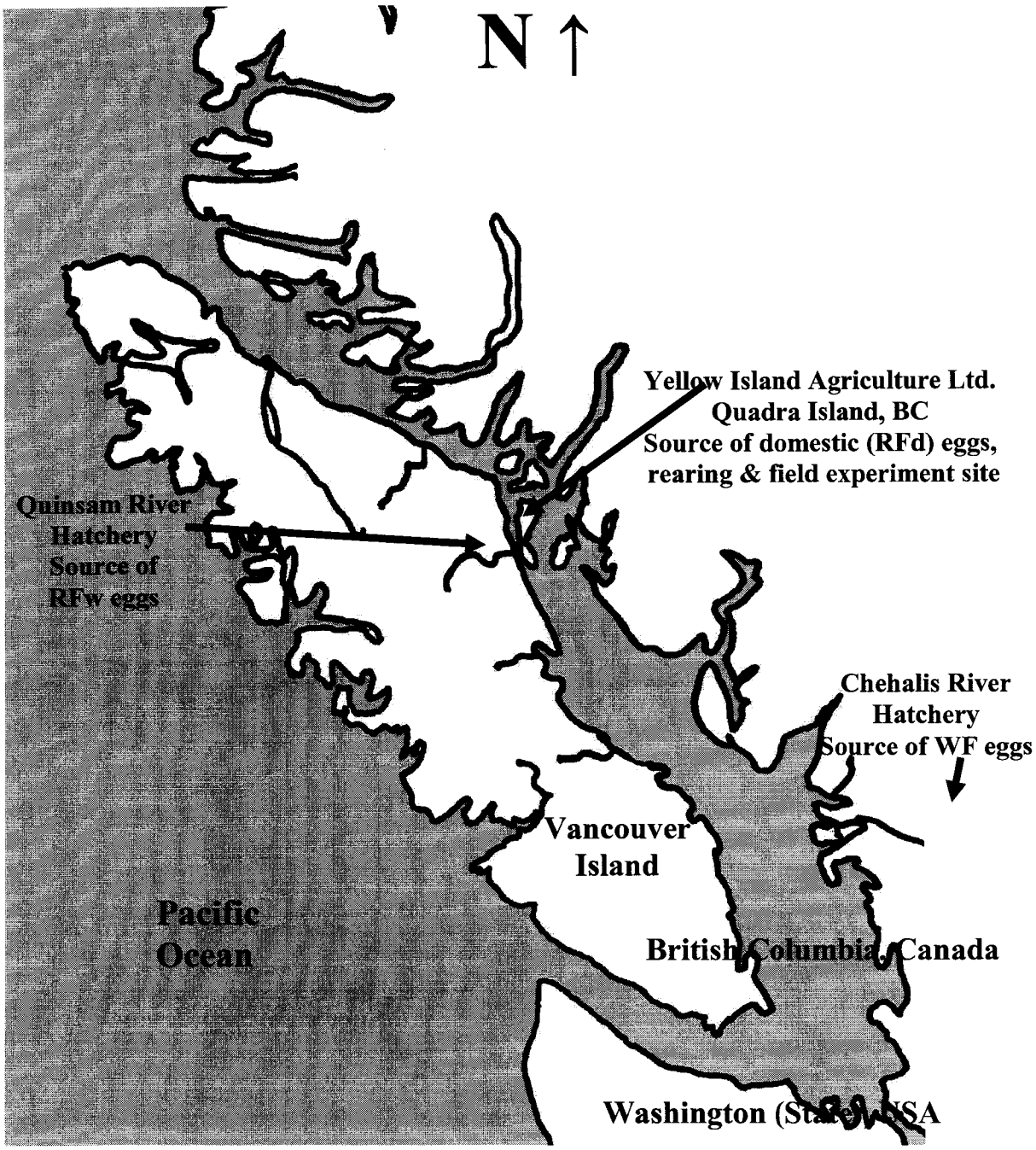


Figure 1.2: Sampling locations map, west coast of British Columbia, Canada.

WF = White-flesh, RFw = Red-flesh wild and RFd = domestic (red-flesh) Chinook salmon (*Oncorhynchus tshawytscha*)

1.7 Pilot Study: Novel Methodology

In a preliminary study, it was hypothesized that perhaps white-fleshed Chinook salmon may incorporate different carotenoid isoforms rather than simply reduced levels of astaxanthin, the carotenoid reported in a majority of salmonids (e.g. 16 species from 4 genera worldwide as examined in Ando *et. al.* 1989). However, a comprehensive review of the literature revealed that the most common method of carotenoid analysis for salmonid flesh and eggs involved either visually scoring with comparison to salmonid-flesh-colour scoring charts (Springate and Nickell 2000), by variations involving a crude acetone extraction and single wavelength spectral analysis compared to astaxanthin or β-carotene, or by limited simple separation using thin layer chromatography (Ando et. al. 1992). To date, no published methodologies were located which could adequately separate and quantify the closely related oxygenated carotenoid isoforms we hypothesized as potentially comprising the carotenoid profile of the white-fleshed and domestic Chinook salmon eggs. We therefore developed an analytical procedure to identify components of the carotenoid profile in Chinook salmon eggs, which was based on advanced separation by high performance liquid chromatography (HPLC) and coupled with either UV/visible absorption, mass spectrometric or electro-spray detection and quantification (Li *et al.* 2005). This study revealed that astaxanthin was the major carotenoid in the present Chinook salmon eggs, and thus the approach for carotenoid quantification in the present thesis was based on these findings. The portions of the analytical methodology utilized in this study are described in abbreviated form in the Methods and Materials, Carotenoid Determination section of Chapter 2. The abstract of this publication is included as appendix 2 at the end of this thesis.

1.8 Thesis Objectives

This thesis examines measures of fitness in Chinook salmon offspring in relation to measures of maternally allocated egg/embryonic carotenoid levels. Measures of carotenoids include concentrations (ug/g) at three stages of embryonic development; (a) unfertilized eggs, (b) as the embryonic eye spots become visible in the egg (eyed stage eggs) and (c) at the end of yolk-sac absorption, just prior to the start of exogenous feeding (swim-up stage offspring). Measures of fitness are described below and in the thesis results chapters, each of which has been organized with the intent that the sections will stand alone as manuscripts for submission and publication in peer-reviewed scientific journals; any overlap in content or wording of these thesis chapters is solely for this purpose.

The objectives of this thesis are to examine possible offspring benefits of red carotenoid-based pigmentation in the eggs of Chinook salmon, manifest as:

1) Correlations between measures of egg carotenoids and offspring survival including, levels (ug/g) of egg carotenoids and embryonic survival (%) during the first 4 months post-hatch, and survival following a challenge with a common marine bacterial pathogen (*Listonella anguillarum*) reported as (LT10) resistance at the smoltification stage (8 months post-hatch, just prior to salt-water introduction).

and

2) Correlations between measures of egg/embryonic carotenoids and immunological function. Selected immune measures include levels of (1) maternally allocated antibody (ug/g) in the unfertilized eggs, (2) offspring antibody levels (ug/g) at swim-up, up to the

point where endogenous antibody expression begins, and (3) the relationship of the above variables to survival following a disease challenge at the smolt stage, 8 months post-hatch.

1.9 References

Alexander,M., H.Newmark, and R.G.Miller, 1986. Oral beta-carotene can increase the number of OKT4+ cells in human blood. Immunological letters 9: 221-224.

Alonso-Alvarez,C., S.Bertrand, G.Devevey, M.Gaillard, J.Prost, B.Faivre, and G.Sorci, 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. The American Naturalist 164: 651-659.

Ando,S., T.Takeyama, and M.Hatano, 1986a. Transport associated with serum vitellogenin of carotenoids in chum salmon (*Oncorhynchus keta*). Agricultural Biological Chemistry 50: 557-563.

Ando,S. and M.Hatano, 1986b. Carotenoids in an egg yolk protein of chum salmon *(Oncorhynchus keta).* Agric. biol. chem. 50: 1043-1044.

Ando,S., T.Takeyama, and M.Hatano, 1986c. Isolation and characterization of a carotenoid-carrying lipoprotein in the serum of chum salmon *(Oncorhynchus keta)* during spawning migration. Agricultural Biological Chemistry 50: 907-914.

Ando,S., K.Osada, M.Hatano, and M.Saneyoshi, 1989. Comparison of carotenoids in muscle and ovary from four genera of salmonid fishes. Comparative Biochemistry and Physiology B 93: 503-508.

Ando,S., H.Yamauchi, M.Hatano, and W.R.Heard, 1992. Comparison of muscle compositions between red- and white-fleshed chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 103: 359-365.

Ando,S., N.Fukuda, Y.Mori, A.Sugawara, and Q.R.Heard, 1994. Characteristics of carotenoid distribution in various tissues from red- and white-fleshed Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). Aquaculture and Fisheries Management 25: 112-120.

Bakker, T.C.M.; R. Künzler and D. Mazzi 1999. Condition-related mate choice in sticklebacks. Nature 401: 234.

Barber,I., S.A.Arnott, V.A.Braithwaite, J.Andrew, and F.A.Huntingford, 2001. Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasitic infections. Proceedings of the Royal Society of London B 268: 71-76.

Bertram,J.S. and A.L.Vine, 2005. Cancer prevention by retinoids and carotenoids: independent action on a common target. Biochimica et Biophysica Acta - molecular basis for disease 1740: 170-178.

Bhosale,P. and P.S.Bernstein, 2005. Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. Biochimica et Biophysica Acta - molecular basis for disease 1740: 116-121.

Blanc,J.-M. and G.Choubert, 1993. Genetic variation of flesh color in pan-size rainbow trout fed astaxanthin. Journal of Applied Aquaculture 2: 115-123.

Blount,J.D., D.C.Houston, and A.P.Møller, 2000. Why egg yolk is yellow. Trends in Ecology and Evolution 15: 47-49.

Böhm,F., R.Edge, E.J.Land, D.McGarvey, and T.G.Truscott, 1997. Carotenoids enhance vitamin E antioxidant efficiency. Journal of American Chemical Society 119: 621-622.

Bowen, P.E.; M. Garg; M. Stacewicz-Sapuntzakis; L. Yelton; R.S. Schreiner 1993. Variability of serum carotenoids in response to controlled diets containing six servings of fruits and vegetables per day. Annals New York Academy of Sciences 691: 241-243.

Cantrell, A., D.J.McGarvey, T.G.Truscott, F.Rancan, and F.Böhm, 2004. Singlet oxygen quenching by dietary carotenoids in a model membrane environment. Archives of Biochemistry and Biophysics 412: 47-54.

Chew, B.P., J.S.Park, T.S.Wong, H.W.Kim, B.B.C.Weng, K.M.Byrne, M.G.Hayek, and G.A.Reinhart, 2000. Dietary β-carotene stimulates cell-mediated and humoral immune response in dogs. Journal of Nutrition 130: 1910-1913.

Christiansen,R. and O.J.Torrissen, 1997. Effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (*Salmo salar* L.). Aquaculture 153: 51-62.

Craik,J.C.A., 1985. Egg quality and egg pigment content in salmonid fishes. Aquaculture 47: 61-88.

Danapat,J., G.B.N.Chainy, and K.J.Rao, 2003. Lipid peroxidation and antioxidant defense status during larval development and metamorphosis of giant prawn, *Macrobrachium rosenbergii*. Comparative Biochemistry and Physiology C 135: 221-233.

Darwin,C., 1871. The Descent of man and selection in relation to sex, J. Murray, London.

Dembinska-Kiec,A., 2005. Carotenoids: risk or benefit for health. Biochimica et Biophysica Acta - molecular basis for disease 1740: 93-94.

Dreon,M.S., G.Schinella, H.Heras, and R.J.Pollero, 2004. Antioxidant defense system in the apple snail eggs, the role of ovorubin. Archives of Biochemistry and Biophysics 422: 1-8.

Dulinska,J., D.Gil, J.Zagajewski, J.Hartwich, M.; Bodzioch, A.Cembinska-Kiec, T.Langmann, G.Schmitz, and P.Laidler, 2005. Different effect of beta-carotene on proliferation of prostate cancer cells. Biochimica et Biophysica Acta - molecular basis for disease 1740: 189-201.

Faivre,B., A.Grégoire, M.Préault, F.Cézilly, and G.Sorci, 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science 300: 103.

Folstad, I.; A.M. Hope; A. Karter and A. Akorping 1994. Sexually selected color in male sticklebacks - A signal of both parasite exposure and parasite resistance. IOKOS 69(3):511-515.

Folstad, I. and A.J. Karter 1992. Parasites, bright males and the immunocompetence handicap. The American Naturalist 139(4): 603-622.

Garbe,A., J.Buck, and U.Hämmerling, 1992. Retinoids are important cofactors in T-cell activation. Journal of Experimental Medicine 176: 109-117.

Geissmann,F., P.Revy, N.Brousse, Y.Lepelletier, C.Folli, A.Durandy, P.Chambon, and M.Dy, 2003. Retinoids regulate survival and antigen presentation by immature dendritic cells. Journal of Experimental Medicine 198: 623-634.

Godfrey, H. Review of the occurrence of red- and white-fleshed chinook salmon in British Columbia with particular reference to Fraser River fish present off the west coast of Vancouver Island. 1359, 1-49. 1975. Pacific Biological Station, Manama, BC, Fisheries Research Board of Canada. Manuscript Report Series.

Godin,J.-G.J. and E.McDonough, 2003. Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioral Ecology 14: 194-200.

Goodwin,T.W., 1954. conversion of carotenoids into vitamin A: Conclusions. In: Goodwin,T.W. (ed.), Carotenoids: their comparative biochemistry, Chemical Publishing Co., Inc., NY, pp. 269-289.

Goodwin,T.W., 1986. Metabolism, nutrition and function of carotenoids. Annual Review of Nutrition 6: 273-297.

Hamiltion,W.D. and M.Zuk, 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Hanusch,M., W.Stahl, W.A.Schulz, and H.Sies, 1995. Induction of gap junctional communication by 4-oxoretinoic acid generated from its precursor canthaxanthin. Archives of Biochemistry and Biophysics 317: 423-428.

Haq,A., A.Chinnah, and C.A.Bailey, 1996. Effect of ß-carotene, canthaxanthin, or lutein on lymphocyte proliferation (*in vitro*) of newly hatched chicks. Avain Diseases 40: 823-827.

Hard,J.J., 1986. Flesh color variation in Chinook salmon (*Oncorhynchus tshawytscha*) at Little Port Walter, Alaska. NOAA Technical Memorandum NMFS F/NWC 109: 1-31.

Hard,J.J., A.C.Wertheimer, and W.F.Johnson, 1989. Geographic variation in the occurrence of red- and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) in Western North America. Canadian Journal of Fisheries and Aquatic Sciences 46: 1107-1113.

Hickenbottom,S.J., J.R.Follett, Y.M.Lin, S.R.Dueker, B.J.Burri, T.R.Neidlinger, and A.J.Clifford, 2002. Variability in conversion of beta-carotene to vitamin A in men as measured by using a double-tracer study design. American Journal of Clinical Nutrition 75: 900-907.

Hiramatsu,N., K.Hiramatsu, K.Hirano, and A.Hara, 2002. Vitellogenin-derived yolk proteins in a hybrid sturgeon, bester (*Huso huso x Acipenser ruthenus*): Identification, characterization and course of proteolysis during embryogenesis. Comparative Biochemistry and Physiology A 131: 429-441.

Houde, A.E. and A.J. Torio 1992. Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology 3(4): 346-351.

Hubbs,C. and L.Stavenhagen, 1958. Effects of maternal carotenoid deficiency on the viability of darter (*Osteichthyes*) offspring. Physiological Zoology. 31: 280-283.

Jonasson,L., A.Wikby, and A.G.Olsson, 2003. Low serum β-carotene reflects immune activation in patients with coronary artery disease. Nutrition and Metabolism in Cardiovascular Disease 13: 120-125.

Jyonouchi,H., L.Zhang, M.Gross, and Y.Tomita, 1994. Immunomodulating actions of carotenoids: Enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutrition and Cancer 21: 47-58.

Kiss,Z., L.Bárdos, C.Szabó, L.Lengyel, and M.Szabó, 2003. Effect of β-carotene supplementation on plasma and yolk IgY levels induced by NDV vaccination in Japanese quail. International Journal of Vitamin Nutritional Research 73: 285-289.

Kraft,J.C., T.Schuh, M.Juchau, and D.Kimelman, 1994. The retinoid X receptor ligand, 9-cis-retinoic acid, is a potential regulator of early *Xenopus* development. Proceedings of the National Academy of Science, USA 91: 3067-3071.

Kurashige,M., E.Okimasu, M.Inoue, and K.Utsumi, 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiological Chemistry Phys. & Med. NMR 22: 27-38.

Langmann,T., G.Liebisch, C.Moehle, R.Schifferer, R.Dayoub, S.Heiduczek, M.Grandl, A.Dada, and G.Schmitz, 2005. Gene expression profiling identifies retinoids as potent

inducers of macrophage lipid efflux. Biochimica et Biophysica Acta - molecular basis for disease 1740: 155-161.

Li,H.A., S.T.Tyndale, D.D.Heath, and R.J.Letcher, 2005. Determination of carotenoids and all-trans-retinol in Chinook salmon (*Oncorhynchus tshawytscha*) eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. Journal of Chromatography B, 816: 49-56.

Liebler,D.C., S.P.Stratton, and K.L.Kaysen, 1997. Antioxidant actions of β-carotene in liposomal and microsomal membranes: Role of carotenoid-membrane incorporation and α-tocopherol . Archives of Biochemistry and Biophysics 338: 244-250.

Lin,Y.M., S.R.Dueker, B.J.Burri, T.R.Neidlinger, and A.J.Clifford, 2000. Variability of the conversion of beta-carotene to vitamin A in women measured by using a double-tracer study design. American Journal of Clinical Nutrition 71: 1545-1554.

Lundström,J., B.Carney, P.Amcoff, A.Pettersson, Borjeson.H, L.Forlin, and L.Norrgren, 1999. Antioxidative systems, detoxifying enzymes and thiamine levels in Baltic salmon (*Salmo salar*) that develop M74. Ambio 28: 24-29.

March,B.E. and C.MacMillan, 1996. Muscle pigmentation and plasma concentrations of astaxanthin in rainbow trout, chinook salmon, and Atlantic salmon in response to different dietary levels of astaxanthin. Progressive Fish Culturist 53: 178-186.

McCallum,I.M., K.Cheng, and B.E.March, 1987. Carotenoid pigmentation in two strains of chinook salmon (*Oncorhynchus tshawytscha*) and their crosses. Aquaculture 67: 291-300.

Metusalach, J.Synowiecki, J.Brown, and F.Shahidi, 1996. Deposition and metabolism of dietary canthaxanthin in different organs of Arctic charr (*Salvelinus alpinus* L.). Aquaculture 142: 99-106.

Milinski, M. and C.M. Bakker 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. Nature 344: 330-333.

Milne, D. J. The chinook and coho salmon fisheries of British Columbia. 142, 1-45. 1964. Ottawa, Ontario, Canada, Fisheries Research Board of Canada Report.

Mora, O., Kuri-Melo, L., González-Gallardo, A., Meléndez, E., Morales, A., Shimadal, A., and Varela-Echavarría, A. A Potential Role for beta-Carotene in Avian Embryonic Development. International Journal of Vitamin Nurtitional Research 74(2), 116-122. 2004.

Mortensen,A., L.H.Skibsted, and T.G.Truscott, 2001. The interaction of dietary carotenoids with radical species. Archives of Biochemistry and Biophysics 385: 12-19.

Ng, J-H, Nesaretnam, K., Reimann, K., and Lai, L. C. Effect of retinoic acid and palm oil carotenoids on oestrone sulphatase and oestradiol-17 hydroxysteroid dehydrogenase activities in MCF-7 and MDA-231 breast cancer cell lines. International Journal of Cancer 88, 135-138. 2000.

Ohlsson,T., H.G.Smith, L.Råberg, and D.Hasselquist, 2003. Effects of nutrition on sexual ornaments and humoral immune responsiveness in adult male pheasants. Ethology, Ecology & Evolution 15: 31-42.

Palace,V.P., S.B.Brown, C.L.Baron, J.Fitzsimons, B.Woodin, J.J.Stegeman, and J.F.Klaverkamp, 1998. An evaluation of the relationships among oxidative stress, antioxidant vitamins and early mortality syndrome (EMS) of lake trout (*Salvelinus namaycush*) from Lake Ontario. Aquatic Toxicology 43: 195-208.

Palozza,P., S.Serini, F.Di Nicuolo, and G.Calviello, 2004. Modulation of apoptotic signalling by carotenoids in cancer cells. Archives of Biochemistry and Biophysics 430: 104-109.

Palozza,P., 2005. Can β-carotene regulate cell growth by a redox mechanism? An answer from cultured cells. Biochimica et Biophysica Acta - molecular basis for disease 1740: 215-221.

Peters,A., A.G.Denk, K.Delhey, and B.Kempenaers, 2004. Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. Journal of Evolutionary Biology 17: 1111-1120.

Pettersson,A. and A.Lignell, 1999. Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. Ambio 28: 43-47.

Prince,E.E., 1916. On the red color of the flesh in the salmons and trouts. Transactions of the American Fisheries Society 46: 50-61.

Reynolds, J.D. and M.R. Gross 1992. Female mate preference enhances offspring growth and reproduction in a fish, *Poecilia reticulata.* Procceedings of the Royal Society of London B 250: 57-62.

Sacha,T., M.Zawada, J.Hartwich, Z.Lach, A.Polus, M.Szostek, E.Zdzilowska, M.Libura, M.Bodzioch, A.Dembinska-Kiex, A.B.Skotnicki, R.Góralczyk, K.Wertz, G.Riss, C.Moele, T.Langmann, and G.Schmitz, 2005. The effect of β-carotene and its derivatives on cytotoxicity, differentiation, proliferative potential and apoptosis on the three human acute leukemia cell lines: U-937, HL-60 and TF-1. Biochimica et Biophysica Acta - molecular basis for disease 1740: 206-214.

Saino,N., R.Stradi, P.Ninni, E.Pini, and A.P.Møller, 1999. Carotenoid plasma concentration, immune profile, and plumage ornamentation of male barn swallows (*Hirundo rusitca*). The American Naturalist 154: 441-448.

Schiedt,K., F.J.Leuenberger, M.Vecchi, and E.Glinz, 1985. Adsorption, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. Pure & Applied Chemistry 57: 685-692.

Silveira,E.R. and F.S.Moreno, 1998. Natural retinoids and β-carotene: From food to their actions on gene expression. Journal of Nurtitional Biochemistry 9: 446-456.

Skarstein,F. and I.Folstad, 1996. Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. OIKOS 76: 359-367.

Smith,S.M. and C.E.Hayes, 1987. Contrasting impairments in IgM and IgG responses of vitamin A-deficient mice. Proceedings of the National Academy of Science, USA 84: 5878-5882.

Sommer,A.1989. New Imperatives for an old vitamin (A). Journal of Nutrition 119:96-100.

Speake,B.K., A.M.B.Murray, and R.C.Noble, 1998. Transport and transformations of yolk lipids during development of the avian embryo. Progress in Lipid Research 37: 1-32.

Stacewicz-Sapuntzakis,M. and P.E.Bowen, 2005. Role of lycopene and tomato products in prostate health. Biochimica et Biophysica Acta - molecular basis for disease 1740: 202-205.

Stahl, W., W., Schwarz, J., von Laar, and H. Sies, 1995 All-trans β-carotene preferentially accumulates in human chylomicrons and very low density lipoproteins compared with the 9-cis geometrical isomer. Journal of Nutrition 125(8), 2128-2133. 1995.

Stahl,W., A.Junghans, B.de Boer, E.S.Driomina, K.Briviba, and H.Sies, 1998. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. FEBS -- Letters 427: 305-308.

Stahl,W., J.von Laar, H.-D.Martin, T.Emmerich, and H.Sies, 2000. Stimulation of gap junctional communication: comparison of acyclo-retinoic acid and lycopene. Archives of Biochemistry and Biophysics 373: 271-274.

Stahl,W. and H.Sies, 2005. Bioactivity and protective effects of natural carotenoids. Biochimica et Biophysica Acta - molecular basis for disease 1740: 101-107.

Stephensen,C.B., R.Rasooly, X.Jiang, M.A.Ceddia, C.T.Weaver, R.A.S.Chandraratna, and R.P.Bucy, 2002. Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. Jounral of Immunology 168: 4495-4503.

Storebakken,T., P.Foss, K.Schiedt, E.Austreng, S.Liaaen-Jensen, and U.P. Manz, 1987. Carotenoids in diets for salmonids IV. Pigmentation of Atlantic salmon with astaxanthin, astaxanthin dipalmitate and canthaxanthin. Aquaculture 65: 279-292.

Sujak,A., J.Gabrielska, W.Grudzinski, R.Borc, P.Mazurek, and W.I.Gruszecki, 1999. Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: The structural aspects. Archives of Biochemistry and Biophysics 371: 301-307.

Sumantran,V.N., R.Zhang, D.S.Lee, and M.S.Wicha, 2000. Differential regulation of apoptosis in normal versus transformed mammary epithelium by lutein and retinoic acid. Cancer Epidemiology, Biomarkers & Prevention 9: 257-263.

Torrissen,O.J., 1984. Pigmentation of salmonids -- effects of carotenoids in eggs and start-feeding diet on survival and growth rate. Aquaculture 43: 185-193.

Torrissen,O.J., R.W.Hardy, and K.D.Shearer, 1989. Pigmentation of salmonids - Carotenoid deposition and metabolism. Reviews in Aquatic Sciences 1: 209-225.

Tveranger,B., 1986. Effect of pigment content in broodstock diet on subsequent fertilization rate, survival and growth rate of rainbow trout (*Salmo gairdneri*) offspring. Aquaculture 53: 85-93.

Verhulst,S., S.J.Dieleman, and H.K.Parmentier, 1999. A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. Proceedings of the National Academy of Science 96: 4478-4481.

Wieland, K. and Koster, F. W. Size and visibility of Baltic cod eggs with reference to size-selective and stage-dependent predation mortality. Journal of Applied Ichthyology 12(2), 83-89. 1996.

Wiesyaw I.Gruszeckia,K.S., 2005. Carotenoids as modulators of lipid membrane physical properties. Biochimica et Biophysica Acta - molecular basis for disease 1740: 108-115.

Withler,R.E., 1986. Genetic variation in carotenoid pigment deposition in the red-fleshed and white-fleshed chinook salmon (*Oncorhynchus tshawytscha*) of Quesnel River, British Columbia. Canadian Journal of Genetic Cytology 28: 587-594.

Wolf, G. 1996. A history of vitamin A and retinoids. FASEB Journal 10: 1102-1107.

Yarzhombek,A.A., 1964. Carotenoids and trout farming. Sb. Tekhnicheskoy Informatsii VNIRO 6: 20-25.

Yarzhombek,A.A., 1970. Carotenoids in salmonidae and their relation to reproduction of these fishes (from "Problems of fish physiology"). Proceedings of the All-Union Research Institute of Maritime Fisheries and Oceanography (translated title, original in Russian) 69: 234-267.

Zhang,L.X., R.V.Coonery, and J.S.Bartram, 1992. Carotenoids up-regulate connexin 43 gene expression independent of their provitamin A or antioxidant properties. Cancer Research 52: 5707-5712.

CHAPTER #2

Why are salmon eggs red?

Egg carotenoids and offspring survival in Chinook salmon (*Oncorhynchus tshawytscha*)

2.1 Introduction

The eggs of salmonid fish are coloured red by carotenoids, as are the yellow to red egg yolks of many bird and reptile species (Blount 2000). Carotenoids (Figure 1.1) are antioxidant compounds synthesized in plants and obtained by animals through the diet (Goodwin 1986; Stahl and Sies 2005). Marketability in salmonids is largely driven by the characteristic colour of their flesh (Prince 1916) which is due primarily to the xanthophyll carotenoid astaxanthin (Skrede and Storebakken 1986; Nickell and Springate 2001). Traditional salmon hatchery practices were based on beliefs that the redness of eggs is indicative of egg quality, i.e. redder eggs confer increased fertilization, hatching success, and offspring survival (Hubbs and Stavenhagen 1958; Yarzhombek 1970; Czeczuga 1975). Indeed, studies of salmonid populations under oxidative stress (such as M74 in the Baltic, Cyuga Syndrome in the New York Finger Lakes or early mortality syndrome (EMS) in North America's Great Lakes) have found that maternal egg batches pale in colour were both low in carotenoids and incurred the highest rates of yolk-sac stage offspring mortality (Palace, et. al. 1998; Pettersson and Lignell 1999). Studies of healthy salmonids however, have found no similar correlations (Torrissen 1984; Craik 1985; Tveranger 1986; Christiansen and Torrissen 1997). Carotenoid pigmentation such as in salmonid eggs is highly visible to potential predators, especially against streambed

aggregate (pers. obs.); the colour and shape is renowned as fisherman's bait. High visibility invariably increases predation risk (Wieland and Koster 1996; Godin and McDonough 2003), hence egg carotenoid pigmentation should be selected against. Additionally, the degree to which animals absorb, metabolize, allocate and retain carotenoids from the diet is heritable (Blanc and Choubert 1993; Araneda 2005) and involves metabolic costs (Goodwin 1986; Olsen and Owens 1998). Therefore, salmonid offspring ought to derive sufficient benefit from carotenoid pigments to offset the costs of both maternal accumulation and allocation, as well as increased egg predation. However, to date, there has been no empirically supported adaptive explanation why salmonids allocate such high levels of carotenoids to their eggs, which begs the question, why are salmon eggs red?

Birds provide some of the most brilliant and best studied examples of visible carotenoid-based structures (Darwin 1871; Hamilton and Zuk 1982; Gray 1996; Møller *et. al.* 2000), and relationships between maternal/egg yolk carotenoid levels and reproductive fitness/offspring survival are well established (reviewed in Blount *et. al.* 2000). The nutritional properties and developmental processes of bird eggs and embryos have been the best studied of any animal, and have proved to be useful models of embryonic physiology and development among vertebrate taxa (Speake *et. al.* 1998). Recent studies have suggested several potentially overlapping mechanisms for carotenoid-fitness correlations, of which some may be involved in salmonid egg carotenoid allocation. Carotenoids and their metabolic retinoid derivatives have been shown to increase oestrogen production in cancer cells *in vitro* (Ng et. al. 2000). Anthropogenically sourced estrogen mimic compounds are known to stimulate vitellogenin (vtg) production in other species (Tyler et. al. 1996; Boon et. al. 2002), while

maternal estrogen levels are related to vitellogenin (vtg) production in rainbow trout (Van Bohem and Lambert 1981), and maternal oestrogen stimulates production of vtg and very low density lipoprotein (VLDL) in birds (Speake *et. al.* 1998). Maternal resources (including carotenoids) are bound by vtg for transport to developing oocytes in salmon (Ando et. al. 1986). Xanthophyll carotenoids such as astaxanthin easily exchange between lipoproteins such as vtg and particularly VLDL (Tyssandier et. al. 2002), which are essential precursors of egg yolk proteins and lipids (respectively) in developing embryos of birds (Speake *et. al.* 1998) and fish (Matsubara et. al. 1999; Reith et. al. 2001; Hiramatsu et. al. 2002). So effectively, maternal carotenoids levels may influence expression levels of vtg and VLDL, these compounds in turn influence the levels of carotenoids and other resources transported to developing oocytes, themselves becoming the most important resources for developing embryos and oxidatively protected by carotenoids and other antioxidants.

The high metabolic rate of embryonic development elevates the production of oxidative by-products (Blount et. al. 2000). Yolk carotenoids, in synergy with other antioxidants (Böhm et. al. 1997), mediate the subsequent peroxidative damage, preserving the integrity of the lipid-rich embryonic tissues and developing organs systems (Speake, et. al. 1998), membranes (Sujak et. al. 1999) and maternally allocated resources (Haq et. al. 1996) of the developing embryo. Cross-fostering experiments in birds indicate that availability of carotenoids in early development, both via endogenous egg levels and/or exogenous early post-hatch parental feeding, increases offspring capacity for carotenoid absorption and use into adult life (Fitze et. al. 2003a and b; Koutos et. *al.* 2003; Blount *et. al.* 2003). In effect, early high carotenoid availability appears to "prime" an individual's

metabolic capacity for more efficient carotenoid use throughout life than is possible with low early life carotenoid levels.

The majority of egg carotenoid-offspring survival studies in salmonids have experimentally manipulated maternal diets with various levels of carotenoids (Harris 1984; Tveranger 1986; Christiansen and Torrissen 1997), or have compared records of egg carotenoid pigmentation and offspring survival from different hatcheries, each with different rearing protocols, physical conditions, species of salmonid and in many cases at least somewhat subjective scoring of pigmentation (Craik 1985). In this study we eliminated potential bias due to differences in species, manipulations of maternal diet, physical rearing conditions and subjectivity by (1) taking advantage of the exceptional opportunity provided by the carotenoid-based flesh and egg colour phenotypes of Chinook salmon (Hard 1989), (2) rearing all experimental fish in a common hatchery, and (3) chemically extracting, analyzing and quantifying carotenoid content (Li et. al. 2005). Controlled by a genetic polymorphism resulting in reduced carotenoid accumulation capacity (Withler 1986), Chinook salmon flesh-colour phenotypes have the most diverse range of carotenoid levels in flesh and eggs of any salmonid species known (Craik 1985; Ando et. al. 1989; 1991; and 1994; Bjerkeng 2000).

In this study Chinook salmon maternal egg batches were selected to maximize the range of carotenoid levels naturally found in healthy fish. Carotenoids were measured through development and were regressed against measures of incubation survival and specific disease resistance in smolt stage offspring. This analysis provides a test of the early life survival benefits resulting from maternally-derived red egg carotenoid pigments

2.2 Methods and Materials

2.2.1 Reagents, Standards and Solvents

Chemically pure analytical standards of astaxanthin were obtained from Alexis Corp. (through Fisher Scientific), and β-carotene, lutein, zeaxanthin, canthaxanthin and β-cryptoxanthin were generously donated by Roche Vitamins Canada Inc. HPLC grade methanol, methyl-*tert*-butyl ether (MtBE) and acetone were obtained from Merck (Darmstadt, Germany). All other reagents and solvents were of high analytical grade supplied by VWR Scientific Products (Suwanee, GA, USA). Water was obtained from a Milli-Q (Millipore, San Jose, CA, USA) filtration system equipped with a 0.22 μm filter

2.2.2 Rearing and Sampling

In October and November 2002, eggs from 31 mature female Chinook salmon were selected from 3 spawning populations chosen for their diverse range of carotenoid accumulation capacity. Eggs of ocean-returning (wild) Chinook salmon were obtained from DFO enhancement hatcheries in British Columbia, Canada. Maternal egg batches representing the red-fleshed (wild) phenotype (RFw) were obtained from the Quinsam River (Vancouver Island) and the white-fleshed (wild) phenotype (WF) from the Chehalis River, tributary of the Harrison, in the lower Frasier River system, known to have among the highest proportions of white- to red-fleshed Chinook salmon spawners (Hard et. al. 1989). Eggs of red-fleshed domestic Chinook salmon (RFd) were also included, selected from broodstock at Yellow Island Aquaculture Ltd. (YIAL, Quadra Island, B.C.), where all rearing and field experiments took place (see Figure 1.2 for locations map).

Eggs were taken from 16 females on October 31st (10 RFw, 6 RFd), and from 15 females on November 13th (10 WF, 5 RFd), 2002. Subsets of 25 eggs per female were frozen (-20°C) for shipping, then stored at -80 °C until carotenoid extraction. Remaining eggs (N ± sd = 359 ± 25 per female) were fertilized with sperm from a common domestic male (1 per spawn date), creating 2 sets of half-sib families. Fertilized eggs from each family were held in separate compartments of a vertical incubation stack and reared under standard hatchery conditions in fresh water at approximately 8°C. As the eye spots of developing embryos became visible at the eyed egg stage (accumulated thermal units/degree days (ATU) ± sd = 341 ± 9.0), eggs were subjected to a mechanical shock, and all unfertilized/dead eggs were removed and counted. A subsample of 25 eyed eggs from each family were frozen for shipping and preserved at -80 °C until carotenoid extraction. The eggs of two RFd families were entirely non-viable, having overgrown with fungus and were removed from the experiment. The eggs of two WF families located in incubation compartments immediately adjacent to the non-viable, fungal infected RFd families also suffered heavy losses due to the fungus, with survival rates of 31% and 13% respectively at the eyed egg stage. Offspring survival in these families were found to be statistically low ($p \leq 0.05$) and extremely low ($p \leq 0.01$) outliers (Dixon 1950) compared to the remaining WF group (mean ± sd = 76.24 ± 9.83, n = 8), and the entire data set (WF, RFw and RFd family mean ± sd = 83.99 ± 10.77, n = 27), and hence were removed from this stage of analysis. Survival of these two families from the eyed egg to swim-up stage was within the normal range (89.39% and 93.94%) of both the WF group (family survival % mean ± sd = 88.62 ± 7.94, N=10) and the entire data set (WF, RFw and RFd family survival % mean ± sd = 91.29 ± 8.91%, n = 29). No other statistical survival outliers were identified and subsequent analyses from eyed egg stage onward

included n ± sd = 264 ± 20 eggs in each of 29 families. Eggs were checked 3-4 times each week, with mortalities removed and tabulated by family. Once offspring had completely absorbed their yolk-sacs just prior to the start of endogenous feeding (swim-up stage, ATU ± sd =1045 ± 9.0, family survival % mean ± sd = 91.29 ± 8.91) an additional 20 offspring per family were weighed and frozen (as above). Remaining offspring (N ± sd = 163.28 ± 18.24) from each of the 29 families were transferred to individual 200 L barrels for the remainder of freshwater rearing. Each barrel received approximately 250 mL of Organic Chinook Fry Grower (Taplow feeds, Victoria, BC) daily, was cleaned and mortalities removed 2-3 times per week.

2.2.3 Carotenoid Determination

Carotenoids were extracted and quantified in duplicate batches of approximately one gram (2-4) eggs per maternal family. Appropriate recovery tests and internal standards were applied, with extraction, resolution and measurement of carotenoids as described in Li et. al. (2005). Briefly, under dimmed lights the crude egg homogenate was extracted with acetone, followed by phase separation by acetone-water/methyl-*tert*-butyl-ether (MtBE). The majority of all carotenoids partition into the MtBE layer, which was then condensed, filtered, sealed under nitrogen and stored at -80 °C until analysis. Extracts were analyzed in triplicate at 3 injection volumes using a Waters 2695 HPLC. A gradient elution of methanol (MeOH) and MtBE over 25 minutes was used, and eluting carotenoids were detected and quantified via UV-visible absorption (λ maximum of 480 nm). Carotenoids were identified and quantified by comparison to the retention times and signal responses of the carotenoid standards. External standards included astaxanthin,

lutein, zeaxanthin, canthaxanthin and β-carotene. β-Cryptoxanthin was used as internal calibration standard at a concentration of 50 ppm with 100 uL added per gram of sample and a 90% mean recovery. Peak areas were averaged by injection volume, concentrations calculated (ug carotenoid/g original sample) and averaged by extract and family at each development stage. All quality assurance and control assessments for the generation of accurate and precise quantitative data are described in Li *et al.* (2005).

All possible laboratory precautions were taken to minimize potential carotenoid degradation in the samples and extracts to ensure as accurate a measure of sample carotenoid levels as possible. However, due to logistical issues the discrepancy between shipping and laboratory storage temperatures (-20°C and -80°C respectively) and the fact that the sample carousel of the HPLC injector did not have cooling capabilities with which to keep the extracts below room temperature during analyses raises the possibility that some thermal degradation could have occurred. The extent of possible sample carotenoid degradation is expected to be minimal, as storage of the working stocks of external and internal carotenoid standards in both pure and mixed isoforms solutions were stored at -20°C, and underwent the same treatment as the sample extracts. These working standard aliquots were periodically analysed for concentration accuracy, and were reliable to over 95% accuracy during typical one month periods. As subsamples initially were frozen to -20°C over a 24 hour period, shipped by next day courier packed in gel packs or dry ice and all were received frozen, to be immediately placed in the -80°C laboratory storage, any possible degradation would be expected to be minimal. As well, during analysis, the sample extracts were left in the injection carosel no longer than necessary, and for equal time as the standards. Any thermal or other degradation which may have

occurred would be expected to be relative among all families and populations of a sample period, and therefore our reported analyses are expected to be reliable. Future researchers should note however that there does exist the possibility that if different carotenoid isoforms were present among the eggs of the white-fleshed (WF) Chinook salmon phenotype (as proposed in a preliminary hypothesis) and if these potentially different isoforms were to undergo more rapid oxidation compared to the isoforms detected, that the carotenoid profile reported for these WF families may not be 100% accurate. Future researchers could ameliorate this possibility by collaborating with an analytical lab close to the field site, where a small subset of the white-fleshed eggs could be extracted and analysed immediately, or at least properly stored at the more appropriate -80°C temperature until such time as this is possible.

2.2.4 Disease Challenge

At smoltification (ATU ± sd = 2057 ± 11.9, weight ± sd = 5.45 ± 0.40 g), 714 fish from 23 families were included in an experimental challenge with the causative agent of vibriosis, a common Pacific marine pathogen. Briefly, a live vibrio culture was obtained (Dr. A. Osborn, Pacific Biological Station, Nanaimo B.C.), and verified (Bergy's Manuel of Determinative Bacteriology, 2001), then grown on marine agar at 25°C for approximately 30 hours, until a homogeneous lawn of bacteria covered the agar. A stock suspension was prepared in PBS (phosphate buffered saline, 0.85% saline, 0.1% peptone, pH 7.04) and diluted to an approximate bacterial concentration of 10^5 cfu/mL according to the McFarland field standards method (McFarland 1907). The stock was kept at 4°C to retard growth, while working aliquots were removed and kept on ice during experimental injections (10-12 mL working aliquot dosed 4-6 families). Fish (N = 31 ± 1/family) were

netted, anaesthetized by brief exposure to tricane methanosulfate (30-45 sec., 50 mg/L H_2O, 2:1 sodium bicarbonate buffer), weighed and injected with 0.1 mL of either the vibrio suspension (N = 21 ± 1) or a PBS blank (N = 10). Mortalities were removed every 2-6 hours, with mortality number and time since the start of the challenge calculated and recorded for each family. Dose concentration was verified by dilution series of each dosing aliquot and the stock at the beginning and end of each challenge day. Briefly, 20 uL/dilution was aliquoted, dropped and smeared on a marine agar plate with a modified glass rod in triplicate and incubated at 25 °C for 16 to 20 hours, at which time the number of colonies/plate (ie: the # of colonies per 20uL) were counted. Replicates were averaged and dilutions calculated to obtain a dose concentration in colony forming units (cfu) for each injection batch of families. Dose concentration averaged 3.12 ± 1.7 $x10^5$ cfu/mL (± 1 sd) throughout the duration of the challenge. A previous study involving a similar vibrio challenge at this facility resulted in extreme mortality with little variation in the LT_{50} resistance measured among families (Bryden *et. al.* 2004). In an effort to obtain a more realistic measure of bacterial resistance, the current study expanded the measured/reported range of disease resistance in two ways. The time to and total mortality number/family was decreased and variability among families increased by lowering the bacterial challenge dose from 10^6 cfu/mL (Bryden et. al. 2004) to 10^5 cfu/mL in the present study. This lower challenge dose greatly reduced the absolute mortality number/family such that, although some families still experienced virtually total mortality of all challenged individuals, other families incurred 3 or less mortalities over the entire 2 week course of the experiment. As such, the time to second death within families or LT_{10} was chosen as a robust measure of bacterial resistance. Differences in dose were statistically accounted for by calculating the mean LT_{10}/injection batch and

reporting family challenge survival as the ratio of family LT_{10}/mean LT_{10} of injection batch.

2.2.5 Statistical Analyses

Differences in spawning dates were corrected by calculating the % survival, weight and change in carotenoid concentration/ATU by family and converting these to common ATU equivalents for each development stage (288 and 992 for eyed egg and swim-up stages respectively). Dixon's (1950) outlier test was used to examine highest and lowest values of each data set for outlier status. Statistical outliers (at $p \leq 0.05$ for calculated critical value) were removed prior to further analyses (Dixon 1950). All statistical analyses were preformed using the statistical software SPSS 12.0 for Windows, including Levene's homogeneity of variances test was used (as it is a standard output of SPSS analyses used) to evaluate normality distribution within and between data sets. When data to be compared had significantly non-homogeneous variances (reported as (p) of Levene's critical value), data were transformed sequentially by arcsin ($\sqrt{}$) (for % values), then by log (or log + 1 for values less than 1) with Levene's test significance examined following each transformation, or non-parametrically rank ordered when assumptions of variance homogeneity could not be met (Zar 1999). Differences in family means (reported as mean and standard deviation) were examined between maternal origins (WF, RFw, RFd) for carotenoid levels (ug/g) at unfertilized, eyed egg and swim-up fry, and for % survival from unfertilized to eyed egg and from eyed egg to swim-up stages by either t-test (for WF-RFw only) or by ANOVA (for WF, RFw RFd comparisons), with post-hoc Tukey's Honestly Significant Differences (HSD) test (p values reported) for any F value indicating significant differences (Zar 1999). Then, due

to non-homogeneity of variances following all parametric data transformations and non-parametric tests for eyed egg to swim-up fry survival, this survival stage was examined by student's T-test (t and p values reported) between the (a) two flesh-colour phenotypes (ie: RF (w+d) vs WF), and (b) between wild (RFw + WF) and domestic (RFd) groups.

Empirical relationships of measures of survival on egg carotenoids were examined by single and multiple regression models. The dependent variables included measures of survival during embryonic incubation (ie: % survival from (1) unfertilized egg to eyed egg stages and (2) eyed egg to swim-up fry stages) as well as at smolt stage following (3) a live vibriosis disease challenge (LT_{10}). The independent variables included levels (ug/g) of maternally derived egg carotenoids at different stages of embryonic incubation (ie: (1) unfertilized eggs, (2) eyed eggs and (3) swim-up fry). Each set of dependent versus independent variables were examined in both a single as well as a multiple regression model including the co-variate of eyed egg weight (g) as a surrogate of maternal effects (Heath and Blouw 1998) to extract any possible confounding interactions this may have had on these relationships. Results of each analysis are reported as co-efficients of (1) signed Pearson's correlation (+/- PC) with corresponding 1-tailed directional ($p_{(1)}$) significance denoting the direction, relative correlational contribution and its significance in that direction for individual factors in the analysis, and (2) regression (R^2) with corresponding 2-tailed ($p_{(2)}$) significance denoting the relationship and corresponding model significance contributed by each additional co-variate added to the model (Zar 1999).

2.3 Results

2.3.1 Egg Carotenoid Level Differences through Embryonic Incubation.

The main carotenoid detected in all eggs was astaxanthin. Maternally derived egg carotenoids levels declined by the swim-up stage in all families (Figure 2.1).

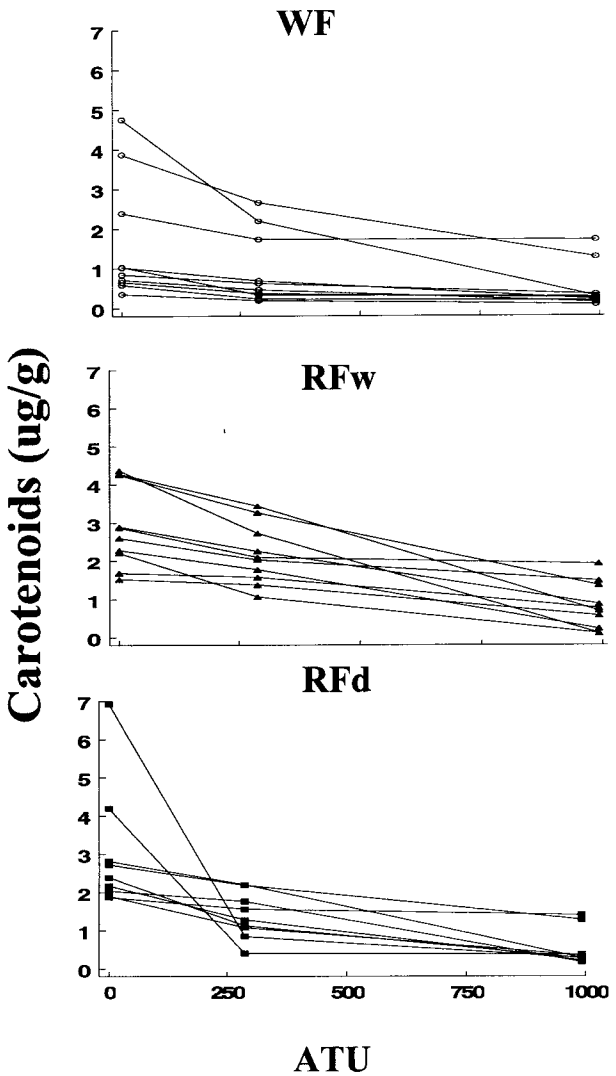


Figure 2.1: Family mean declines in maternally derived egg carotenoids (ug/g) through embryonic development as accumulated thermal units (ATU) in Chinook salmon offspring representing maternal flesh-colour phenotypes and a domestic populations. (WF) white-fleshed (wild), (RFw) red-fleshed wild, and (RFd) red-fleshed domestic.

2.3.2 Family Survival Differences through Embryonic Incubation:

Offspring survival was examined between the different maternal origins (Table 2.1). ANOVA results revealed RFw families had significantly higher incubation survival compared to their WF counterparts at all stages examined, and RFW had significantly higher survival compared to the RFd families both from the unfertilized to eyed egg stages and from the unfertilized egg through to swim-up (Table 2.1).

Table 2.1: Statistical comparisons of offspring incubation survival differences among Chinook salmon maternal flesh-colour phenotypes and a domestic population

(i) ANOVA comparing offspring survival from unfertilized to eyed egg stage

origin	n	mean	sd
WF [a]	8	76.241	9.828
RFw [b]	10	92.138	6.234
RFd [a]	9	81.899	10.021

$F = 7.787$, $p = 0.002$

Tukey's HSD post-hoc results: RFw significantly different from WF ($p = 0.002$) and from RFd ($p = 0.042$)

(ii) Offspring survival from eyed egg to swim-up stage

WF	10	88.615	7.94
RFw	10	96.172	1.83
RFd	9	88.828	12.55

variances significantly non-homogeneous following all data transformations (see appendix 2, Table 2.1aiii-v), therefore data was re-analyzed non-parametriucally below.

(iii) Non-parametric analyses of eyed egg to swim-up survival among maternal origins

WF[a]	10	9.10
RFw[b]	10	20.40
RFd[ab]	9	15.56

$X^2 = 8.862$, asymptotic $p = 0.012$

Mann-Whiney U test results: RFw significantly different from WF ($p = 0.001$)
RFd not significantly different from either WF ($p = 0.221$) or RFw (0.414)

(iv) ANOVA comparing offspring survival from unfertilized egg to swim-up

WF[a]	9	59.481	14.665
RFw[b]	10	88.621	6.414
RFd[a]	9	73.464	16.920

$F = 11.509$, $p = 0.000286$

Tukey's HSD post-hoc results: RFw significantly different from WF ($p = 0.00018$) and from RFd ($p = 0.050$)

Regression analyses of incubation survival versus carotenoid levels revealed no significant relationships at either the unfertilized to eyed egg nor the eyed egg to swim-up stages (Table 2.2). Carotenoids remaining at the eyed egg stage were significantly related in a 2-tailed manner to survival throughout the entire embryonic period from unfertilized egg to swim-up, and unfertilized egg carotenoid levels showed a 1-tailed directional significant correlation with survival during this period. This indicates that while a unidirectional relationship likely exists such as that lower levels of carotenoids correlate with lower survival, that higher egg carotenoids do not necessarily correlates with higher survival. In multiple regression analyses the maternal effect (eyed egg weight) covariate emerged consistently as a significant predictor of survival (Table 2.2). Relationships are well established in salmonids between survival and maternal effects (see Heath and Blouw 1998) and in particular between offspring survival and egg weight in juvenile Chinook salmon (Fowler 1972). As this was not the focus of these experiments, weight-survival correlations will not be discussed further (Table 2.2).

2.3.2 Disease Challenge Survival and Egg Carotenoids

Disease resistance (LT_{10}) following the live vibriosis challenge at the smolt stage was positively correlated with egg carotenoid concentrations (ug/g) at both the unfertilized egg ($R^2 = 0.183$, $p \leq 0.041$) and eyed egg ($R^2 = 0.197$, $p \leq 0.034$) stages among all Chinook salmon maternal origins (Figure 2.2, Table 2.2).

Table 2.2: Hierarchical OLS regression results* examining measures of Chinook salmon egg and offspring survival on measures of maternally derived egg carotenoids (ug/g) and maternal effects as eyed egg weight (g) at different developmental stages.

Survival measures	Hierarchical Models	Independent variable(s)			Model Summary		
			r	$p_{(1)}$	n	R^2	$p_{(2)}$
Survival % U-E	1	Carotenoid U	0.286	0.074	27	0.082	0.148
	2	Carotenoid U	0.286	0.074	25	0.195	0.092
		Eyed egg weight	0.376	0.032			
Survival % E-S	1	Carotenoid U	0.205	0.143	29	0.042	0.286
	2	Carotenoid U	0.205	0.143	27	0.106	0.26
		Eyed egg weight	0.28	0.079			
	1	Carotenoid E	0.112	0.281	29	0.013	0.562
	2	Carotenoid E	0.112	0.281	27	0.079	0.374
		Eyed egg weight	0.28	0.079			
	1	Carotenoid S	0.023	0.453	29	0.001	0.907
	2	Carotenoid S	0.023	0.453	27	0.081	0.361
		Eyed egg weight	0.28	0.079			
Survival % U-S	1	Carotenoid U	0.363	0.029	28	0.132	0.057
	2	Carotenoid U	0.363	0.029	26	0.219	0.059
		Eyed egg weight	0.342	0.042			
	1	Carotenoid E	0.390	0.020	28	0.152	0.040
	2	Carotenoid E	0.390	0.020	26	0.203	0.074
		Eyed egg weight	0.342	0.043			
	1	Carotenoid S	0.162	0.206	28	0.026	0.411
	2	Carotenoid S	0.162	0.206	26	0.122	0.223
		Eyed egg weight	0.342	0.043			
Disease Challenge	1	Carotenoid U	0.428	0.021	23	0.183	0.041
	2	Carotenoid U	0.428	0.021	21	0.184	0.16
		Eyed egg weight	0.032	0.446			
	1	Carotenoid E	0.444	0.017	23	0.197	0.034
	2	Carotenoid E	0.444	0.017	21	0.212	0.117
		Eyed egg weight	0.032	0.446			
	1	Carotenoid S	0.154	0.242	23	0.024	0.484
	2	Carotenoid S	0.154	0.242	21	0.024	0.806
		Eyed egg weight	0.032	0.446			

*Hierarchical ordinary least squares regression model results include: signed correlations (r) and 1-tailed significance ($p_{(1)}$) to denote directional contributions of each individual variable to the model; single followed by multiple regression (R^2) with corresponding 2-tailed significance ($p_{(2)}$) to denote the overall relationship between model 1 variable (single R^2) and model 1 + 2 variables (multiple R^2) to the model. Development stages denoted as: U = unfertilized eggs, E = eyed stage eggs, S = swim-up stage offspring, U-E = survival between unfertilized and eyed egg stages, E-S = survival between eyed egg and swim-up stages, U-S = survival between unfertilized egg to swim-up stages, disease challenge measured as $LT_{(10)}$; significant relationships at $p \leq 05$.

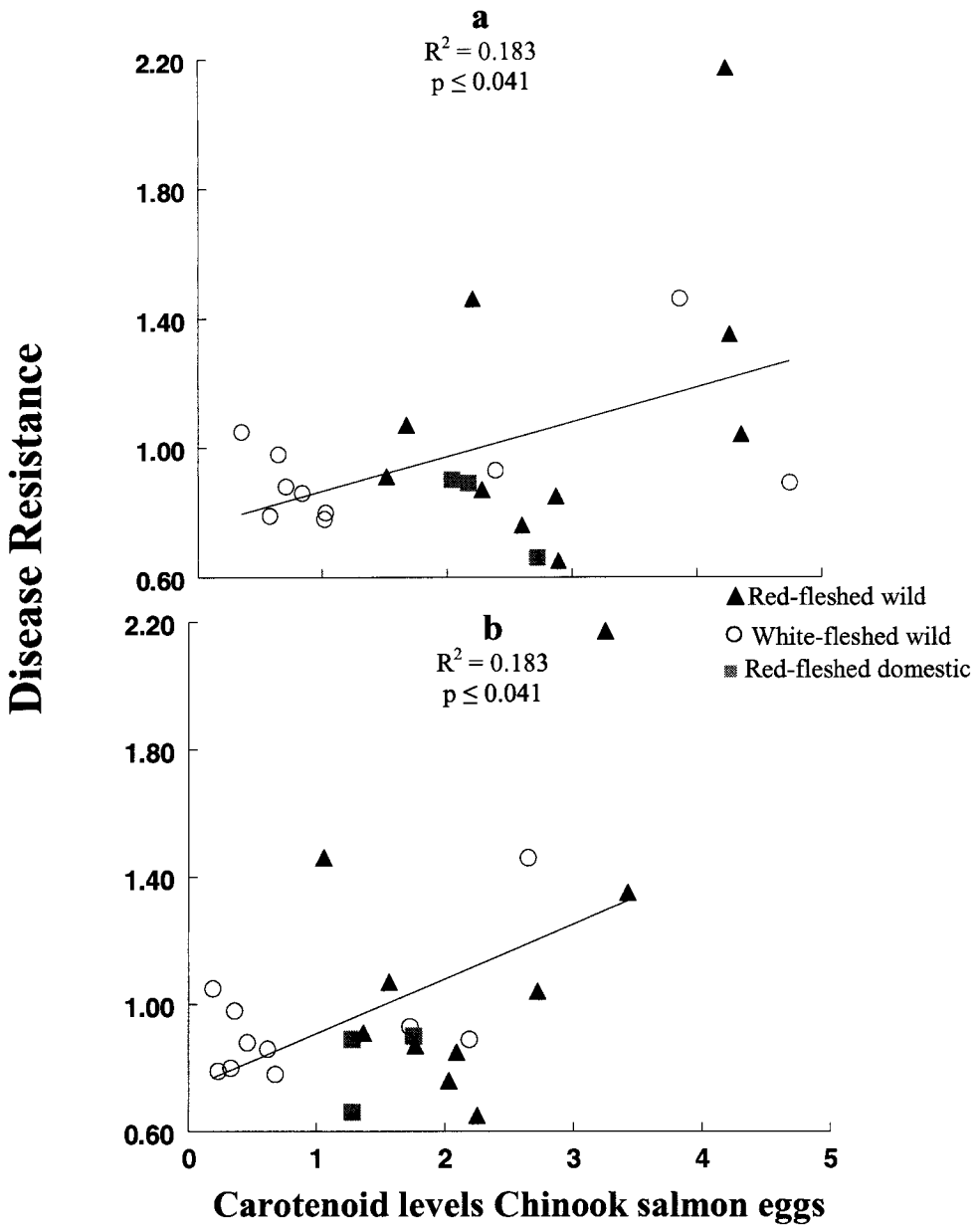


Figure 2.2: Relationship between disease resistance (LT_{10} following vibriosis challenge) vs carotenoid levels (ug/g) at two development stages of eggs in Chinook salmon maternal populations; **(a)** unfertilized eggs, and **(b)** eyed egg stage.

Maternal effects as eyed egg weight (g) was examined as a covariate with the carotenoid concentrations in multiple regression analyses, however, this was not a

significant predictor of smolt stage disease resistance, nor were the carotenoid concentrations at the swim-up stage (Table 2.2), or any other variable examined (appendix 2, Table 2.2).

2.4 Discussion

This study reports new evidence supporting traditional beliefs that in salmonids, carotenoid-based egg pigmentation is indicative of egg "quality" (Hubbs and Stavenhagen 1958; Yarzhombek 1970; Czeczuga 1975). While this study reports higher egg carotenoid and survival levels among the progeny of the RFw families, this is likely a population effect, as regression analyses showed no empirical 2-tailed significance between survival and egg carotenoid levels. These results support studies reporting no empirical relationship between egg carotenoids and early offspring survival among various salmonid species under hatchery conditions (Torrissen 1984; Craik 1985; Craik and Harvey 1986; Tveranger 1986; Christiansen and Torrissen 1997). Additionally, Hard et. al. (1989) reviewed of decades of DFO hatchery records for 30 Chinook salmon spawning populations from N. California through S.W. Alaska and the Asian Pacific Rim, as well as a worldwide literature review of other salmonid species, they speculated that egg carotenoid-offspring survival correlations may only become pronounced when incubation conditions become suboptimal, and hence may not be evident in optimal hatchery conditions. Similar to a study by Hard (1986), results of this study found that while survival may have been obscured by egg size no significant differences in offspring embryonic incubation survival could be directly attributed to maternal egg carotenoid levels at any development stage examined (Tables 2.1 and 2.2).

Listonella anguillarum is a common bacterial pathogen in the Pacific marine environment, representing an environmentally encountered threat. As such, our results support a relationship between egg carotenoids and offspring survival in the face of environmental stress, such as those reported by Palace *et. al.* (1998) and by Pettersson and Lignell (1999). Our results most closely match those of Christiansen *et. al.* (1995) and a pilot study (Bryden and Tyndale unpublished data). Both these studies demonstrate significant relationships between salmonid early carotenoid availability (carotenoid levels in the early freshwater diet and eggs respectively) and offspring resistance to common marine bacterial pathogens (*Aeromonas salmonicida* and *Listonella anguillarum* respectively) at the smolt stage, when young salmonids first enter the marine environment and those in hatchery settings are typically vaccinated against the above pathogens to enhance survival (Erdal and Reitan 1992; Akhlaghi 1999 respectively).

Vibriosis is a ubiquitous bacterial disease threat to saltwater Pacific salmonids, causing financial losses in aquaculture (Egidius 1987) and thus, standard hatchery vaccination protocols are common prior to saltwater introduction of smolt stage offspring (Akhlaghi 1999). Carotenoids and their metabolic retinoid derivatives are well known to enhance resistance to, and recovery from, numerous diseases in humans (Sommer 1989; Wolf 1996), while interactions between carotenoids and immune function have been documented in birds (Saino et. al. 1999; Peters et. al. 2004) and mammals (Alexander et. al. 1986; Bendich and Shapiro 1986; Chew et. al. 1999, 2000; Kim et. al. 2000); Additionally, carotenoids and their metabolic retinoid derivates have been shown to protect valuable host resources via antioxidant mediation, such as; a) red blood cells in immune challenged Zebra finch (Alonso-Alvarez *et. al.* 2004), b) skeletal and cardiac muscle following prolonged exercise in dogs (Baskin et. al. 2000; Aoi et. al. 2003) and c)

liposomal membrane lipids in humans (Bhosal and Bernstein 2005). Our results support those of Craik (1985) and others where egg carotenoids are metabolised through embryonic development. Results clearly suggest that available carotenoid levels during these metabolically sensitive initial 4 months post-fertilization are correlated with disease resistance at smoltification (8 months post fertilization) as evidenced by the relationship between disease resistance versus egg carotenoid levels at both the unfertilized and eyed stages found in this and a pilot study (Bryden and Tyndale unpublished results). The enhanced resistance to vibriosis comes as offspring prepare to enter the marine environment, and hence may represent an important survival benefit to offspring with high maternal carotenoid allocation.

Following yolk-sac absorption, swim-up stage salmon offspring begin exogenous feeding, replacing depleted maternal-egg carotenoids (and other resources) from their freshwater diet. As the assimilation of dietary carotenoids begins around 150 g (Hard 1986), the ratio of feed supplement costs to marketable flesh-pigment returns results in most hatcheries not supplementing carotenoids until saltwater stages (Storebakken and Choubert 1991; Nickell and Bromage 1998). Underyearling Chinook salmon in Alaskan lakes have been observed to have red flesh, facial bones and fin rays at as little as 10 g in size (pers.obs. Hard 1986). These observations are supported by freshwater carotenoid supplementation studies in Coho and Atlantic salmon where flesh pigmentation was more dependent on consumption rate than fish size (Spinelli and Mahnken 1978; Christiansen et. al. 1995). Christiansen et. al.'s (1995) carotenoid supplemented offspring also showed significantly increased resistance to an Atlantic marine bacterial pathogen, *Aeromonas salmonicida*, causative agent of furnunculosis (Erdal and Reitan 1992), which is among the first study to investigate effects of freshwater carotenoid supplementation on juvenile

salmon bacterial disease resistance. Our results are also among the first documented relationships between egg carotenoid levels and bacterial disease resistance in young salmon. While freshwater offspring feed in this study was not supplemented with carotenoids, small amounts are known to be present (pers. Comm. B. Hicks, Taplow Feeds, Victoria, B.C.). If correlative relationships reported in birds of early carotenoid availability enhancing metabolic absorption and use capacity for carotenoids throughout life (Koutsos et. al.2003; Fitze et. al. 2003a and b; Blount et. al. 2003) apply in salmonids, then offspring from eggs with higher carotenoid levels may have been better able to absorb the limited amounts available from their freshwater diet, thus leading to increased carotenoid availability to affect disease resistance. This may in part account for the 8 month time differential between measured egg carotenoid levels and smolt stage disease resistance. This study underscores the importance of early life carotenoids in young salmonids for development of effective survival mechanisms when faced with immunological challenge such as bacterial pathogens.

We propose that maternal allocation of carotenoid-based pigmentation in salmonid eggs represents an adaptation for increased offspring survival, though not necessarily in the timeframe of embryonic incubation. Egg carotenoids may perform two non-exclusive functions in young salmon. First, salmonid egg carotenoid pigmentation may be indicative of the overall resource base of the egg, thus darker pigmentation indicates better provisioned eggs, leading to fitter offspring who are then better equipped to overcome environmental stress, including immunological challenges. Additionally, egg carotenoid levels in salmonids may influence the metabolic capacity of offspring (priming the organism's metabolism) to better absorb/incorporate carotenoids from the diet throughout ontogeny, thus leading to a superiorly provisioned antioxidant system for

the organism throughout life, as has been shown in birds (Koutos *et. al.* 2003; Fitze et. al. 2003a and b; Blount *et. al.* 2004), better enabling offspring to deal with environmental or immunological stress.

This study provides compelling evidence that early carotenoid availability in the red pigmentation of salmonid eggs results in higher offspring survival through increased disease resistance at the smolt stage. This is the first report of survival benefits offsetting the costs of maternal carotenoid allocation and the associated increased egg predation risk, costs inherent to the nearly universal red eggs of salmon. These results suggest that hatchery protocols could be modified to ameliorate losses to smolt stage bacterial outbreaks by selecting for redder pigmented eggs at spawning and by supplementing dietary carotenoids to salmon fry. Our results further suggest that carotenoids affect survival in Chinook, at least in part, through interactions with immune function. Further research should focus on the nature of the immunological implications of maternally allocated carotenoids in salmonids, not only for commercial and government aquaculture, but also to better understand the relationship between carotenoids and health across animal taxa.

2.6 References

Akhlaghi,M., 1999. Passive immunization of fish against vibriosis, comparison of intraparitoneal, oral and immersion routes. Aquaculture 180: 191-205.

Alexander, M.; H. Newmark; R.G. Miller 1986. Oral beta-carotene can increase the number of OKT4+ cells in human blood. Immunological letters 9: 221-224.

Alonso-Alvarez,C., S.Bertrand, G.Devevey, M.Gaillard, J.Prost, B.Faivre, and G.Sorci, 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. The American Naturalist 164: 651-659.

Ando,S., T.Takeyama, and M.Hatano, 1986. Isolation and characterization of a carotenoid-carrying lipoprotein in the serum of chum salmon (*Oncorhynchus keta*) during spawning migration. Agricultural Biological Chemistry 50: 907-914.

Ando,S., K.Osada, M.Hatano, and M.Saneyoshi, 1989. Comparison of carotenoids in muscle and ovary from four genera of salmonid fishes. Comparative Biochemistry and Physiology B 93: 503-508.

Ando,S., N.Fukuda, Y.Mori, A.Sugawara, and Q.R.Heard, 1994. Characteristics of carotenoid distribution in various tissues from red- and white-fleshed Chinook salmon, *Oncorhynchus tshawytscha* (W.). Aquaculture and Fisheries Management 25: 112-120.

Aoi,W., Y.Naito, K.Sakuma, M.Kuchide, H.Tokuda, T.Maoka, S.Toyoduni, S.Oka, M.Yasuhara, and T.Yoshikawa, 2003. Astaxanthin limits exercise-induced skeletal and cardiac muscle damage in mice. Antioxidants & Redox Signaling 5: 139-144.

Araneda,C., R.Neira, and P.Iturra, 2005. Identification of a dominant SCAR marker associated with colour traits in Coho salmon (*Oncorhynchus kisutch*). Aquaculture 247: 67-73.

Baskin,C.R., K.W.Hinchcliff, R.A.DiSilvestro, G.A.Reinhart, M.G.Hayek, B.P.Chew, J.R.Burr, and R.A.Swenson, 2000. Effects of dietary antioxidant supplementation on

oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs. American Journal of Veterinary Research 61: 886-891.

Bendich, A. and S.S. Shapiro 1986. Effects of ß-carotene and canthaxanthin on the immune responses of the rat. Journal of Nutrition 116: 2254-2262.

Bergey, J.G.Holt, N.R.Krieg, and P.H.A.Sneath, 1994. Bergey's Manual of Determinative Bacteriology.

Bhosale,P. and P.S.Bernstein, 2005. Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. Biochimica et Biophysica Acta - molecular basis for disease 1740: 116-121.

Bjerkeng, B.; B. Hatlen; M. Jobling 2000. Astaxanthin and its metabolites idoxanthin and crustaxanthin in flesh, skin, and gonads of sexually immature and maturing Arctic charr (*Salvelinus alpinus* (L.)). Comparitive Biochemistry and Physiology B 125: 395-404.

Blanc,J.-M. and G.Choubert, 1993. Genetic variation of flesh color in pan-size rainbow trout fed astaxanthin. Journal of Applied Aquaculture 2: 115-123.

Blount,J.D., D.C.Houston, and A.P.Møller, 2000. Why egg yolk is yellow. Trends in Ecology and Evolution 15: 47-49.

Blount,J.D., N.B.Metcalfe, K.E.Arnold, P.F.Surai, G.L.Devevey, and P.Monaghan, 2003. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. Procceedings of the Royal Society of London B 270: 1691-1696.

Böhm,F., R.Edge, E.J.Land, D.McGarvey, and T.G.Truscott, 1997. Carotenoids enhance vitamin E antioxidant efficiency. Journal of American Chemical Society 119: 621-622.

Boon, J.P.; J.J. Zanden; W.E. Lewis; B.N. Zegers and A.E. Arukwe 2002. The expression of CYP1A, vitellogenin and zona radiata proteins in Atlantic salmon (*Salmo salar*) after oral dosing with two commercial PBDE flame retardant mixtures: absence of short-term responses. Marine Environmental Research 54: 719-724.

Bryden,C.A., J.W.Heath, and D.D.Heath, 2004. Performance and heterosis in farmed and wild Chinook salmon (*Oncorhynchus tshawytscha*) hybrid and purebred crosses. Aquaculture 235: 249-261.

Chew, B.P.; M.W. Wong; J.S. Park; T.S. Wong 1999. Dietary β-carotene and astaxanthin but not canthaxanthin stimulate splenocyte function in mice. Anticancer Research 19: 5223-5228.

Chew, B.P.; J.S. Park; T.S. Wong; H.W. Kim; B.B.C. Weng; K.M. Byrne; M.G. Hayek; G.A. Reinhart 2000. Dietary β-carotene stimulates cell-mediated and humoral immune response in dogs. Journal of Nutrition 130: 1910-1913.

Christiansen,R., J.Glette, Ø.Lie, O.J.Torrissen, and R.Waagbø, 1995. Antioxidants and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. Journal of Fish Diseases 18: 317-328.

Christiansen,R. and O.J.Torrissen, 1997. Effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (*Salmo salar L.*). Aquaculture 153: 51-62.

Craik,J.C.A., 1985. Egg quality and egg pigment content in salmonid fishes. Aquaculture 47: 61-88.

Craik,J.C.A. and S.M.Harvey, 1986. The carotenoids of eggs of wild and farmed Atlantic salmon and their changes during development to the start of feeding. Journal of Fish Biology 29: 549-565.

Czeczuga,B., 1975. Carotenoids in fish. *V. Salmonidae* and *Thymallidae* from Polish waters. Hydrobiologia 46: 223-239.

Darwin,C., 1871. The Descent of man and selection in relation to sex, J. Murray, London.

Dixon WJ (1950) Analysis of extreme values, *Annals of Mathematical Statistics*, **21**, 488-506.

Egidius,E., 1987. Vibriosis: Pathogenicity and pathology. A review. Aquaculture 67: 15-68.

Erdal,J.I. and L.J.Reitan, 1992. Immune response and protective immunity after vaccination of Atlantic salmon (*Salmo salar* L.) against furnunculosis. Fish & Shellfish Immunology 2: 99-108.

Fitze,P.S., M.Kölliker, and H.Richner, 2003a. Effects of common origin and common environment on nestling plumage coloration in the great tit (*Parus major*). Evolution 57: 144-150.

Fitze,P.S., B.Tschirren, and H.Richner, 2003b. Carotenoid-based colour expression is determined early in nestling life. Oecologia 137: 148-152.

Fowler, L.G. 1972. Growth and mortality of fingerling Chinook salmon as affected by egg size. Progressive Fish Culturist. 34: 66-69.

Godin,J.-G.J. and E.McDonough, 2003. Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioual Ecology 14: 194-200.

Goodwin,T.W., 1986. Metabolism, nutrition and function of carotenoids. Annual Review of Nutrition 6: 273-297.

Gray,D.A., 1996. Carotenoids and sexual dichromatism in north american passerine birds. The American Naturalist 148: 453-480.

Hamiltion,W.D. and M.Zuk, 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Haq,A., C.A.Bailey, and A.Chinnah, 1996. Effect of β-carotene, canthaxanthin, lutein, and vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. Poultry Science 75: 1092-1097.

Hard,J.J., 1986. Flesh color variation in Chinook salmon (*Oncorhynchus tshawytscha*) at Little Port Walter, Alaska. NOAA Technical Memorandum NMFS F/NWC 109: 1-31.

Hard, J.J.; A.C. Wertheimer; W.F. Johnson 1989. Geographic variation in the occurrence of red- and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) in Western North America. Canadian Journal of Fisheries and Aquatic Sciences 46: 1107-1113.

Harris,L.E., 1984. Effects of a broodfish diet fortified with canthaxanthin on female fecundity and egg color. Aquaculture 43: 179-183.

Heath, D.D. and Blouw, D.M. 1998. Are maternal effects in fish adaptive or merely physiological side-effects? In: Mousseau, T.A. and Fox, C. (eds.) Maternal Effects as Adaptations. Pp. 178-201. Oxford University Press

Hiramatsu,N., K.Hiramatsu, K.Hirano, and A.Hara, 2002. Vitellogenin-derived yolk proteins in a hybrid sturgeon, bester (*Huso huso x Acipenser ruthenus*): Identification, characterization and course of proteolysis during embryogenesis. Comparative Biochemistry and Physiology A 131: 429-441.

Hubbs,C. and L.Stavenhagen, 1958. Effects of maternal carotenoid deficiency on the viability of darter (*Osteichthyes*) offspring. Physiological Zoology. 31: 280-283.

Kim, H.W.; B.P. Chew; T.S. Wong; JS. Pard; B.B.C. Weng; K.M. Byrne; M.G. Hayek; G.A. Reinhart 2000. Modulation of humoral and cell-mediated immune responses by dietary lutein in cats. Vetrinary Immunology and Immunopathology 73: 331-341.

Koutsos,E.A., A.J.Clifford, C.C.Calvert, and K.C.Klasing, 2003. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). Journal of Nutrition 133: 1132-1138.

Li,H.A., S.T.Tyndale, D.D.Heath, and R.J.Letcher, 2005. Determination of carotenoids and all-*trans*-retinol in Chinook salmon (*Oncorhynchus tshawytscha*) eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. Journal of Chromatography B.

Matsubara,T., N.Ohkubo, T.Andoh, C.V.Sullivan, and A.Hara, 1999. Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte

maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. Developmental Biology 213: 18-32.

McFarland,J., 1907. The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. Journal of the American Medical Association 49: 1176.

Møller,A.P., C.Ciard, J.D.Blount, D.C.Houston, P.Ninni, N.Saino, and P.F.Surai, 2000. Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? Avian and Poultry Biology Reviews 11: 137-159.

Ng, J-H, Nesaretnam, K., Reimann, K., and Lai, L. C. Effect of retinoic acid and palm oil carotenoids on oestrone sulphatase and oestradiol-17 hydroxysteroid dehydrogenase activities in MCF-7 and MDA-231 breast cancer cell lines. International Journal of Cancer 88, 135-138. 2000.

Nickell,D.C. and N.R.Bromage, 1998. The effect of dietary lipid level on variation of flesh pigmentation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 161: 237-251.

Nickell, D. C. and Springate, J. R. C. Pigmentation for farmed salmonids. Biological Sciences Database , 58-75. 2001.

Olson,V.A. and I.P.F.Owens, 1998. Costly sexual signals: are carotenoids rare, risky or required? Trends in Ecology and Evolution 13: 510-514.

Palace,V.P., S.B.Brown, C.L.Baron, J.Fitzsimons, B.Woodin, J.J.Stegeman, and J.F.Klaverkamp, 1998. An evaluation of the relationships among oxidative stress,

antioxidant vitamins and early mortality syndrome (EMS) of lake trout (*Salvelinus namaycush*) from Lake Ontario. Aquatic Toxicology 43: 195-208.

Peters,A., A.G.Denk, K.Delhey, and B.Kempenaers, 2004. Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. Journal of Evolutionary Biology 17: 1111-1120.

Pettersson,A. and A.Lignell, 1999. Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. Ambio 28: 43-47.

Prince,E.E., 1916. On the red color of the flesh in the salmons and trouts. Transactions of the American Fisheries Society 46: 50-61.

Reith,M., J.Munholland, J.Kelley, R.N.Finn, and H.J.Fyhn, 2001. Lipovitellins derived from two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). Journal of Experimental Zoology (Molecular Developmental Evolution) 291 : 58-67.

Saino,N., R.Stradi, P.Ninni, E.Pini, and A.P.Møller, 1999. Carotenoid plasma concentration, immune profile, and plumage ornamentation of male barn swallows (*Hirundo rusitca*). The American Naturalist 154: 441-448.

Skrede,G. and T.Storebakken, 1986. Instrumental colour analysis of farmed and wild Atlantic salmon when raw, baked and smoked. Aquaculture 58: 279-286.

Sommer,A 1989. New Imperaitves for an old vitamin A. Journal of Nutrition 119:96-100.

Speake,B.K., A.M.B.Murray, and R.C.Noble, 1998. Transport and transformations of yolk lipids during development of the avian embryo. Progress in Lipid Research 37: 1-32.

Spinelli,J. and C.Mahnken, 1978. Carotenoid deposition in pen-reared salmonids fed diets containing oil extracts of red crab (*Periscopes platies*). Aquaculture 13: 213-223.

Stahl,W. and H.Sies, 2005. Bioactivity and protective effects of natural carotenoids. Biochimica et Biophysica Acta - molecular basis for disease 1740: 101-107.

Storebakken,T. and G.Choubert, 1991. Flesh pigmentation of rainbow trout fed astaxanthin or canthaxanthin at different feeding rates in freshwater and saltwater. Aquaculture 95: 289-295.

Sujak,A., J.Gabrielska, W.Grudzinski, R.Borc, P.Mazurek, and W.I.Gruszecki, 1999. Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: The structural aspects. Archives of Biochemistry and Biophysics 371: 301-307.

Torrissen,O.J., 1984. Pigmentation of salmonids -- effects of carotenoids in eggs and start-feeding diet on survival and growth rate. Aquaculture 43: 185-193.

Tveranger,B., 1986. Effect of pigment content in broodstock diet on subsequent fertilization rate, survival and growth rate of rainbow trout (*Salmo gairdneri*) offspring. Aquaculture 53: 85-93.

Tyler, C.R.; B. Van der Eerden; S. Jobling; G. Panter and J.P. Sumpter 1996. Measurement of vitellogenin, a biomarker for exposure to osetrogenic chemicals, in a wide variety of cyprinid fish. Journal of Comparative Physiology B 166: 418-426.

Tyssandier, V., Choubert, G., Grolier, P., and Borel, P. Carotenoids, mostly the xanthophylls, exchange between plasma liproproteins. International Journal of Vitamin Nurtitional Research 72[5], 300-308. 2002.

van Bohem,C.G. and J.G.D.Lambert, 1981. Estrogen synthesis in relation to estrone, estradiol, and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, *Salmo gairdneri*. General and Comparative Endocrinology 45: 105-114.

Wieland, K. and Koster, F. W. Size and visibility of Baltic cod eggs with reference to size-selective and stage-dependent predation mortality. Journal of Applied Ichthyology 12[2], 83-89. 1996.

Withler,R.E., 1986. Genetic variation in carotenoid pigment deposition in the red-fleshed and white-fleshed chinook salmon (*Oncorhynchus tshawytscha*) of Quesnel River, British Columbia. Canadian Journal of Genetic Cytology 28: 587-594.

Wolf,G., 1996. A history of vitamin A and retinoids. FASEB Journal 10: 1102-1107.

Yarzhombek,A.A., 1964. Carotenoids and trout farming. Sb. Tekhnicheskoy Informatsii VNIRO 6: 20-25.

Zar, J.H.1999. Biostatistical analysis, 4th edition. Prentice Hall, N.J. p. 208-228, 324-443.

CHAPTER #3

Why are salmon eggs red?

Carotenoids and immune function in the eggs and offspring of Chinook salmon (*Oncorhynchus tshawytscha*)

3.1 Introduction

Carotenoids are antioxidant compounds that absorb radiation in the visible wavelength region of the electromagnetic spectrum and depending on their specific structures reflect in the yellow-red colour spectrum. Synthesized in plants and obtained by animals through the diet, carotenoids serve a diverse range of critical biological functions, including: (a) vitamin A pre-cursors (Goodwin 1954 and 1986), (b) sexually selected ornaments believed to signal superior health/fitness (Hamilton & Zuk 1982; Folstad and Karter 1992; Zuk *et. al.* 1995; Møller *et. al.* 2000), (c) transcription enhancers of metabolic enzymes (Sharoni *et. al.* 2004), developmental (Blount 2004) and immunological (Garbe et. al. 1992) resources, as well as (d) the mediation or enhancement of immune responses (Chew and Park 2004).

Domestic animal feed is typically supplemented with carotenoids to enhance either (a) pigment driven marketability, such as the red colour of salmon flesh and eggs (Craik 1985; March and MacMillan 1996) and pigmentation of poultry feathers, flesh and egg yolks (Schiedt et. al. 1985), or b) vitamin A status, general health and survival in mammals (Goodwin 1954 and 1986; Chew 1995). Birds provide the most brilliant of the

visible carotenoid-based ornamental displays, and studies in birds have led to many findings of cost-benefit tradeoffs in carotenoid physiology (e.g. Hamilton and Zuk 1982; Møller *et. al.* 2000). In fish, carotenoids have been primarily studied for their role in the pigmentation of flesh (Ando *et. al.* 1989) and eggs in relation to offspring survival and fitness (as in Chapter 2 and Craik 1985).

Published studies of carotenoid-immune function relationships in salmonids are relatively rare, with a few exceptions (Christainsen et. al. 1995; Thompson et. al. 1995; Skarstein and Folstad 1996; Amar et. al. 2001 and 2004), and to the knowledge of the author, no studies of carotenoid-immune function relationships in the eggs and offspring of healthy salmonids have yet been published. Comparisons in the present study on Chinook salmon are therefore largely limited to taxa other than salmonids, though whenever possible, examples from salmonids or at least other fish taxa have been incorporated. However, it is likely reasonable to assume that general mechanisms of carotenoid physiology can be applied to salmonid eggs and their offspring, given the conservation of cellular, molecular, physiological and transcriptional regulation mechanisms related to carotenoids and their retinoid derivatives in diverse vertebrate taxa.

Maternal dietary carotenoid intake correlates highly with yolk carotenoid levels in birds (Blount et. al. 2000) and fish (Harris 1984). It is well established in birds that maternal/yolk carotenoid levels enhance offspring survival (e.g. Blount et. al. 2000). Traditionally, fish hatchery practices have been based on beliefs that similar relationships between egg carotenoid pigmentation and measures of offspring performance exist in salmonids, such as increasing fertilization, hatching success or survival (Hubbs and Strawn 1957; Hubbs and Stavenhagen 1958; Yarzhombek 1964 & 1970). Indeed, in

salmonid populations experiencing oxidative stress positive correlations have been documented between carotenoid-based egg pigmentation and early offspring survival. Examples of this include: M74 syndrome in the Baltic (Pettersson and Lignell 1998 and 1999; Pickova *et. al.* 1998 and 1999), Cyuga Syndrome in the Finger Lakes region of New York state, and early mortality syndrome in the North American Great Lakes (Palace *et. al.* 1998; Lundström *et. al.* 1999).

Positive correlations between measures of carotenoids and immune function have been documented in guppies (Houde and Torio 1992), Arctic charr (Skarstein and Flostad 1996) and Atlantic salmon (Christainsen *et. al.* 1995), as well as in birds (Ruff *et. al.* 1974; Dufva and Allander 1995; Saino *et. al.* 1999), and mammals (Alexander *et. al.* 1986; Bendich and Shaprio 1986; Chew 1995; Chew *et. al.* 1999 and 2000). These diverse studies suggest that carotenoids have a conserved or basic role in immune function. The current paradigm is that carotenoids act in synergy with other antioxidants (Böhm *et. al.* 1997; Burke *et. al.* 2000), mediating host damage from toxic oxygen radical species (Burton 1989; Chew 1995; Krinsky and Yuem 2003; Chew and Park 2004). Such radicals may be produced during an initial innate immune response (Ellis 1988), through physiological changes such as aging (Gale *et. al.* 2001; Massimino *et. al.* 2003) or reproduction (Alonso-Alvarez *et. al.* 2004), exposure to environmental factors such as organohalogen organic contaminants (Asplund *et. al.* 1999) or ultraviolet sunlight radiation (Obermüller-Jevic *et. al.* 1999). In effect, carotenoids protect the integrity of the biological resources of an organism from oxidative degradation, providing health benefits. Another hypothesis is that carotenoids enhance the specific immune response by directly influencing the production of antibody, as suggested by increased antigen-

specific antibody titers with dietary carotenoid supplementation in mammals (Jyonouchi *et. al.* 1994; Chew *et. al.* 2000) and birds (Kiss *et. al.* 2003).

Antibodies are immune recognition proteins produced (in part) as a specific immunological response to foreign antigens (Bernstein *et. al.* 1998). Maternal antibodies are allocated to developing oocytes both for embryonic passive immune protection and as a metabolic resource (Takahashi and Kawahara 1987; Mor and Avtalion 1988 and 1990; Takemura and Takano 1997). Maternally allocated fish egg antibody is metabolised during embryonic development (Chantanachookhin, et. al. 1991), and endogenous offspring antibody expression begins at approximately four weeks post-hatch in rainbow trout (*Oncorhynchus mykiss*) (Tatner & Manning 1983; Razquin *et. al.* 1990), Atlantic salmon (*Salmo salar*) (Ellis 1977 and 1988) and Chum salmon (*Oncorhynchus keta)* (Nagae *et. al.* 1993). In birds, maternal carotenoid supplementation increases egg allocated levels of both carotenoids and vaccine-specific antibody titres (Kiss *et. al.* 2003), and enhances endogenous antibody production after *in vitro* mitogen challenge of the bursa from newly hatched chicks (Haq *et. al.* 1996). Additionally, early carotenoid availability, whether via endogenous egg and/or exogenous post-hatch parental feeding, has been shown to increase offspring capacity for carotenoid absorption and metabolism through ontogeny in chickens (Koutos *et. al.* 2003) and zebra finches (Blount *et. al.* 2003); in effect "priming" an organism's metabolic systems for more efficient absorption of available carotenoids throughout their lifetime.

Mammalian studies have identified "low responders" to dietary carotenoid supplementation (Bowen *et. al.* 1993; Stahl *et. al.* 1995; Chew *et. al.* 2000; Lin *et. al.* 2000; Hickenbottom *et. al.* 2002), who show reduced absorption and/or metabolic capacity for dietary carotenoids, ranging from 50% (Bowen et. al. 1993) to 99% (Chew

et. al. 2000; Lin 2000) reductions compared to con-specific responders receiving identical diets. Additionally, carotenoid "low responders" have also shown significantly reduced measures of immune function (Chew *et. al.* 1995). In salmonids, the degree to which dietary carotenoids are absorbed, metabolised and deposited in flesh is heritable (Blanc and Choubert 1993), though salmonids worldwide are characterized by red flesh and eggs (Prince 1916; Craik 1985; Ando *et. al.* 1989). Chinook salmon, however, are an anomaly, possessing a rare genetic polymorphism leading to two flesh-colour phenotypes, termed red-fleshed and white-fleshed (Prince 1916; Hard 1985; Withler 1986). Studies have shown that white-fleshed Chinook salmon flesh-carotenoid levels may be reduced by as much as 95% compared to their red-fleshed counterparts when measured by single wavelength spectral analysis of crude acetone extracts (Ando et. al. 1992), and that flesh-colour differences are mirrored in their eggs (March and MacMillian 1996). Results of this study were more conservative, finding a 45% reduction in family mean carotenoid levels (ug/g) in the eggs of the white-fleshed (mean ± sd = 1.62 ± 1.5, n = 10) compared to those of the red-fleshed (mean ± sd = 2.95 ± 1.3, n = 19) Chinook salmon populations (Chapter 2).

Chinook salmon flesh colour polymorphisms represent an exceptional opportunity to study relationships between egg carotenoids and measures of offspring immune function in healthy fish across a wide range of naturally occurring egg pigmentation levels without the need of maternal diet manipulation. In the current study, sources of Chinook salmon eggs were selected to maximize the range of egg carotenoid-pigment levels found in healthy fish. Carotenoids were measured at three stages of embryonic development; unfertilized eggs, eyed stage eggs and swim-up stage offspring and were regressed against three measures of immunological function: (1) levels of passively

acquired maternal antibody in the unfertilized eggs, (2) offspring antibody levels at the beginning at the swim-up stage, as endogenous antibody expression begins (just prior to the start of feeding) and (3) disease resistance at the smolt stage (just prior to saltwater introduction) in response to a live marine pathogen (*Listonella anguillarum*) challenge.

3.2 Methods and Materials

3.2.1 Chemical and Materials

As described in Chapter 2 Methods and Materials Chemicals and Materials.

3.2.2 Sample origins, rearing and collection protocols

In October and November 2002, eggs were obtained from 31 mature female Chinook salmon from 3 spawning populations chosen for their diverse range of carotenoid pigmentation (see General Introduction Chapter 1, section 1.6 for description). Briefly, eggs from wild populations of red-fleshed (RFw) and white-fleshed (WF) Chinook salmon were obtained from the Department of Fisheries and Oceans, Canada (DFO) enhancement hatcheries and domestic red-fleshed (RFd) Chinook salmon were selected from broodstock at Yellow Island Aquaculture Ltd. (YIAL, Quadra Island, B.C.) (see Figure 1.2 map), where all rearing and field experiments took place (see Chapter 2 for detailed description). Briefly, 31 sexually mature female Chinook salmon were artificially spawned, 16 on October 31st (10 RFw, 6 RFd), and 15 on November 13th (10 WF, 5 RFd), 2002. A subsample of 25 eggs per female were frozen (-20°C) and stored (-80 °C) until extraction of carotenoids and antibody. Remaining eggs were fertilized and each family was held in a separate compartment of a vertical incubation stack and reared under standard hatchery conditions. As the eye spots of developing embryos became

visible (eyed egg stage, ATU (accumulated thermal units or degree days) ± sd = 341 ± 9.0), eggs were mechanically shocked, and all unfertilized/dead eggs were removed and counted. Two RFd families with fungal infected, non-viable eggs were removed from analyzes. A second subsample (N = 25 eggs/family) were preserved as above, with n ± sd = 264 ± 20 eggs in each of 29 families remained. Eggs were checked 3-4 times each week and mortalities removed. Once offspring had completely absorbed their yolk-sacs just prior to the start of endogenous feeding (swim-up stage ATU ± sd = 1045 ± 9.0) an additional 20 offspring/family were preserved (as above). Remaining offspring were transferred to 200L tanks (1/family), each receiving *ca.* 250 mL of Organic Chinook Fry Grower (Taplow feeds, Victoria, BC) daily. Tanks were cleaned and mortalities removed 2-3 times per week until offspring reached smoltification stage and were ready to enter the marine environment.

3.2.3 Carotenoid Determination

Carotenoids were extracted in duplicate batches of approximately one gram of (2-4) eggs per maternal family, analyzed in triplicate by HPLC, identified and quantified by comparison to authentic and pure carotenoid standards, as described in Li *et. al.* (2005). See Chapter 2, Materials and Methods: Carotenoid Assays for a more detailed description.

3.2.4 Antibody Assays

Antibodies were extracted and quantified from approximately 1 to 3 grams of unfertilized egg, or 4 individual swim-up stage offspring per maternal family. To preserve the conformational integrity of the antibodies, all solutions were ice cold when used and

all incubation stages were conducted in a 4°C refrigerator. Eggs/offspring were weighed, counted and homogenized by first fine razor-blade chopping, then crushing by mortar and pestle. Homogenate was suspended in phosphate buffered saline (PBS, pH 7.04 with 0.02% sodium azide to suppress bacterial contamination) to a 10% concentration (e.g., 1 g egg in 10 mL PBS). The suspension was vortexed 1 minute and stored at 4°C overnight. The following day suspensions were vortexed another minute, centrifuged 10 minutes (2500 rpm, 4°C), and the supernatant was removed, sealed and stored at -80°C until analysis by enzyme linked immunosorbant assay (ELISA).

ELISAs for total antibodies were conducted on 96 well plates, though only 60 wells/plate were used, with the outer row on all sides filled with distilled water at each incubation stage to minimize evaporation effects on well concentrations. A dilution series of each extract was made in PBS (as above) and 100 uL of each dilution was added to individual wells in triplicate along with 100 uL of carbonate coating buffer (pH 9.6). Plates were incubated overnight and the following day the wells were washed 3 times with PBS. Bovine serum albumin (BSA) was used as a control to block the non-specific binding of the primary antibody to other proteins in the sample extracts, with 300 uL added to each well and incubated 2 to 3 hours. Wells were washed 3 times with PBS and 100uL of primary antibody (mouse anti-salmon Ig, 1 ug/mL, CLF003AP Cedarlane Labs Ltd., ON, Canada,) was added to each, and incubated 1 hour. Wells were washed 3 times again with PBS and 100 uL of HRPO-secondary antibody (horseradish peroxidase linked rabbit-anti-mouse-Ab, 5ug/mL PBS) was added to each well and incubated 1 hour. Wells were washed 3 times with PBS, 150uL of (ABTS) substrate specific to ARPO was added, incubated 1 hour in the dark and absorbance was read in a plate reader at 540 nm.

Absorbance readings were corrected by subtracting the mean of blank/control well readings, and triplicate extract dilutions were averaged.

Immunoprotein (antibody) concentrations were calculated for each sample as in appendix 3. Briefly, replicates of BSA (bovine serum albumin) were used as a blank and a row of primary antibody in dilution series was run simultaneously with replicate dilutions of each sample assayed. The antibody standard absorbance readings were averaged by dilution and the average absorbance of BSA blanks subtracted to generate an adjusted absorbance, which was plotted against the corresponding dilution concentrations to generate a scatterplot. A regression line of adjusted absorbance by dilution concentration of the antibody standard was calculated. The absorbance readings of the sample dilution replicates were averaged and the averaged BSA blank reading was subtracted, giving an adjusted absorbance for each dilution of the sample. The regression equation was then used to calculate the concentration of antibody in each dilution of sample, which was then multiplied by the dilution factor. The calculated values for each dilution factor which fell within the range of the calibration curve were averaged to obtain the concentration of antibody (ug/g) within each original sample.

3.2.5 Disease Challenge

At smolt (2057 ± 11.9 ATU, fish weighed approximately 5.45 ± 0.40 g), n = 714 fish from 23 families were included in an experimental challenge with the causative agent of vibriosis, a common Pacific marine pathogen (as described in Chapter 2 Material and Methods, Disease Challenge section). Briefly, a live vibrio culture was obtained (Dr. A. Osborn, Pacific Biological Station, Nanaimo B.C.), verified (Bergy's Manuel of Determinative Bacteriology, 2001) and grown to a homogeneous bacterial lawn, from

which a stock suspension was prepared in PBS and diluted to approximately 10^5 cfu/mL, (McFarland 1907). The stock was kept at 4°C and working aliquots on ice throughout the experiment. Fish (31 ± 1/family) were anaesthetized in tricane methanosulphate, weighed and injected with 0.1mL of either the vibrio suspension (N = 21 ± 1) or a PBS blank (N = 10). Mortalities were removed every 2-6 hours, with family and time since challenge recorded. Dose concentration was verified by growing a smear of 20 uL/series dilution from each dosing aliquot and the stock at the beginning and end of each challenge day. After 16-20 hours the colonies were counted and mean colony forming unit (cfu)/mL was calculated for each challenge day aliquot. Challenge dose concentrations averaged 3.12 (± 1.7) x10^5 cfu/mL throughout the duration of the challenge. Challenge response was quantified as time to second death (time to 10% mortality, or LT_{10}) within families. Differences in dose were statistically accounted for by reporting the ratio of family LT_{10}/mean LT_{10} of the appropriate injection batch.

3.2.6 Statistical Analyses

Data were tabulated, calculated and statistically evaluated as described in Chapter 2 Methods and Materials Statistical Analyses. Differences in family mean levels of carotenoid (reported as mean and standard deviation) were examined at the unfertilized egg stage between the two wild flesh-colour phenotypes (exhibiting normal distributions) by student's T-test (t and p values reported). Family mean carotenoid levels at the eyed egg stage and the change in carotenoid levels from unfertilized to eyed egg stage were examined among the three maternal population origins (exhibiting noremal distributions following log transformation) by ANOVA (F and p values reported), with any significant differences examined by post-hoc Tukey's Honestly Significant Differences (HSD) test (p

values reported) (Zar 1999). Family mean egg antibody (Ab) level differences between the wild origin flesh-colour phenotypes (distributions heterogeneous following all parametric transformations) by Mann-Whitney Test (meadians reported). Family mean Ab level differences at swim-up and the family mean changes to swim-up (normal distributions following log transformation) were examined by ANOVA (mean and sd reported) with Tukey's HSD post-hoc reported for differences between groups. Statistical analyses of mean family differences are tabulated as described above in table 3.1 for carotenoids and 3.2 for antibody. Ordinary least squares regression analyses (single and multiple) were preformed to examine the relative effects between variables ie: comparing measures of antibody versus carotenoids, or disease resistance versus carotenoids and antibody, with eyed egg weight included as a measure of maternal effects (Heath and Blouw 1998). Analyses were conducted and significant results reported in Table 3.3 (as in Zar 1999). All results (including non-significant relationships) tabulated in appendix 2 Table 3.3 at the end of the thesis.

3.3 Results

3.3.1 Carotenoids

The main carotenoid detected in all the eggs was astaxanthin, with small amounts of canthaxanthin in the eggs of the domestic families. Levels are reported as total carotenoids (astaxanthin + canthaxanthin) in ug/g. The eggs from the RFw group had the highest mean carotenoids levels, and compared to their WF counterparts, RFw eggs had significantly lower levels of carotenoids at both the unfertilized stage (RFw mean ± s.d.= 2.893 ± 1.063, N=10; WF mean ± s.d.= 1.619 ± 1.526, n = 10; $t = -2.157$, $p \leq 0.045$) and eyed stage (RFw mean ± s.d.= 2.155 ± 0.782; WF mean ± s.d.= 0.944 ± 0.899, n = 10; F

= 6.743, $p \leq 0.004$, Table 3.1 and appendix 2 Table 3.1ai). The mean egg carotenoid levels of the domestic families were a bit of an anomaly (Figure 3.1a). At the unfertilized egg stage both the family mean and variation (sd) of this group were highest of the study, with mean carotenoids being closer to those of the RFw families, though among family variation was closer to that found in the WF families, resulting in no significant differences in an ANOVA between the three groups (see appendix 1, Table 3.1aii).

Table 3.1: Significant differences in family mean egg carotenoid levels (ug/g) between Chinook salmon of different maternal flesh-colour phenotypes and a domestic population.

a) T-test comparing egg carotenoids (ug/g) between maternal Chinook salmon phenotypes

Development stage	Measure	Maternal Origin	n	Mean	sd
unfertilized eggs	carotenoids (ug/g)	WF [a]	10	1.619	1.536
		RFw[b]	10	2.893	1.063
				t = -2.157	p = 0.045

b) ANOVA comparing carotenoid measures between maternal origins

i) Eyed eggs	Carotenoids (ug/g)	WF[a]	10	0.944	0.899
		RFw[b]	10	2.155	0.782
		RFd[a]	9	1.286	0.522
				F = 6.743; p =.004	

RFw significantly different from WF (p = 0.004) and from RFd (p = 0.049) using Tukey's HSD test

ii) Unfertilized to eyed egg	Carotenoid % change (log +1)	WF [a]	10	1.619	0.141
		RFw[c]	10	1.347	0.243
		RFd[ac]	9	1.552	0.3
				F = 3.646; p =0.040	

RFw and WF significantly different (p =0.039) using Tukey's HSD test

Superscripted letters differentiate groups with significantly different means by Tukey's HSD post-hoc
WF = white-flesh, RFw = red-flesh wild and RFd = red-flesh domestic

Between the unfertilized and eyed egg stages the decline in family mean carotenoid levels was still highest and most variable among the domestic compared to the WF or RFw families (Figures 3.1a and 3.3a), resulting in significant non-homogeneity between the group variances, necessitating (log +1) data transformation, after which no

significant differences emerged (see appendix 1, Table 3.1bii). When the % carotenoid decline was examined, even though the mean carotenoid decline in the WF families was the lowest of the three groups, their low starting levels resulted in their having significantly higher % decline than the their RFw counterparts (WF % decline ± sd = 42.64 ± 14.36, n = 10; RFw % decline ± sd = 24.27 ± 12.973, n = 10, $t = 3.002$, $p \leq 0.008$; appendix 2, Table 3.1biii).

The similar % decline between the WF and RFd families, even given the much higher RFd variation among families (RFd % decline ± sd = 42.83 ± 29.44, n = 9) necessitated data transformation due to non-homogeneity of variance prior to ANOVA, revealing overall significant difference between the three WF, RFw and RFd groups ($F = 3.646$, $p \leq 0.04$) driven by the WF-RFw difference (Tukey's HSD $p \leq 0.039$) and no statistical differences between the RFd and either WF or RFw families (Table 3.1biv). These dynamics resulted in statistical differences between the three groups in family mean eyed egg carotenoid levels (mean ± sd for RFw = 2.155 ± 0.782, n = 10; for RFd = 1.286 ± 0.522, n = 9; and for WF = 0.944 ± 0.899, n = 10, $F = 6.743$, $p \leq 0.004$), with the RFw family means being significantly higher than any of the others (Tukey's HSD post-hoc comparisons of RFw-WF $p \leq 0.004$ and RFw-RFd $p \leq 0.049$), with no significant difference between the WF and RFd family carotenoid means at this stage (Table 3.1b).

By the swim-up stage maternal egg carotenoids had declined in all families (Figures 3.1a, 3.3a) and there were no statistical differences between the family groups in either means, amount or % decline (see appendix 1 and Table 3.1).

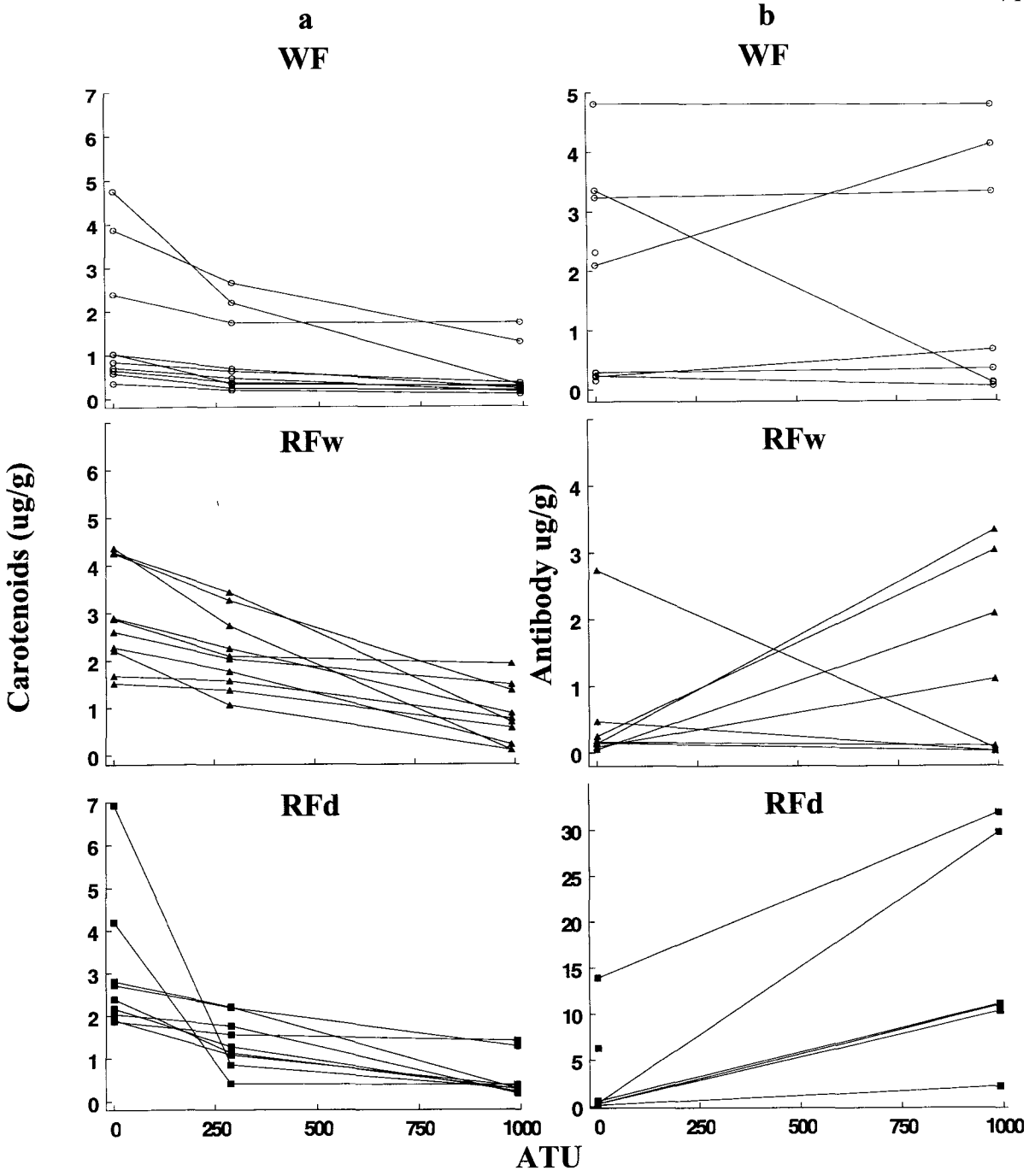


Figure 3.1: Changes in (a) maternally derived egg carotenoids and (b) antibody levels (ug/g) through embryonic incubation as accumulated thermal units/degree days (ATU) in Chinook salmon offspring representing maternal flesh-colour phenotypes and a domestic population. Lines represent individual maternal families and graphs maternal origins as (WF) white-fleshed (wild), (RFw) red-fleshed wild, and (RFd) red-fleshed domestic.

3.3.2 Antibody

Maternally allocated antibody (Ab) levels in the unfertilized eggs varied greatly among families both within and among different maternal origin groups (Figure 3.1b). Two domestic families had the highest mean egg Ab levels of the study (6.29 and 13.89 ug/g) which were revealed by Dixon's outlier test to be statistically high ($p \leq 0.05$) and extremely high ($p \leq 0.001$) outliers respectively (Dixon 1950). Even with their removal and parametric data transformation, variances in family egg Ab levels were still significantly non-homogeneous, necessitating non-parametric rank ordering prior to analyses. Aside from the two high outlier families, the majority of domestic family mean Ab concentrations were comparable to those of the RFw families (ie: < 0.6ug/g, see Figure 3.1b). In a non-parametric Kruskal-Wallis analysis no statistical differences were identified between the rank order of family median egg Ab levels among the three groups, wether or not the statistical high outliers were included (Appendix 2, Table 3.2a). Following this a Mann-Whitney test was used to examine mean family Ab differences between the wild origin maternal flesh-colour phenotypes. The rank order of egg Ab levels of the WF families were significantly higher than those of the RFw families (median of family Ab level rank of WF = 12.33, n = 9, of RFw = 6.67 n = 9, Z = -2.252, asymptotic (2-tailed) and exact (1-tailed) p = 0.024, Table 3.2a).

Antibody levels at swim-up had similarly non-homogeneous variances between the maternal origin groups. However, following log transformation the mean family Ab level variances among groups were then homogeneous and those of the domestic group were significantly highest and the most variable, with those of the two wild groups being not significantly different from eachother (see Table 3.2 bi).

Table 3.2: Significant differences in family mean antibody levels (ug/g) in eggs and offspring of Chinook salmon of different maternal flesh-colour phenotypes and a domestic population.

a) Mann-Whitney Test comparing egg antibody level rank orders between red-fleshed maternal Chinook salmon of wild and domestic origins

Development stage	Measure	Phenotype	n	Median
unfertilized egg	rank Ab	WF[a]	9	12.33
		RFw[b]	9	6.67
		Z = -2.252,asymptotic (2-tailed) & exact (1-tailed) p = 0.024		

b) ANOVA comparing measures of embryonic antibody between maternal Chinook salmon maternal origins

Development stage	Measure	Maternal Origin	n	Mean	sd
i) Swim-up offspring	Ab (log (ug/g) + 1)	WF [a]	9	0.774	0.234
		RFw[a]	9	0.722	0.350
		RFd[b]	7	1.663	0.562
				F = 14.08, p = 0.0001	
RFd significantly different from both WF (p = 0.013) and RFw (p =0.010) using Tukey's HSD test					
ii) egg-swim	Log (Ab change ug/g)	WF [a]	8	0.542	0.685
		RFw[a]	8	0.757	0.493
		RFd[b]	7	1.666	0.603
				F = 7.274; p = 0.004	
RFd significantly different from both WF (p = 0.005) and RFw (p =0.021) using Tukey's HSD test					

Maternal origins: WF = white-flesh, RFw = red-flesh wild and RFd = red-flesh domestic
Superscripted letters different for groups with significantly different means by Tukey's HSD post-hoc test

The mean change in family Ab levels from egg to swim-up among each maternal origin group increased (Table 3.2bii), though in a few individual families this decreased (Figure 3.1b). As many of the WF families initially had high egg Ab levels and increases were not as substantial as those of the other two groups, coupled with one WF family having a substantial decrease (Figrue 3.1b), resulted in the mean Ab level change of WF families being negative, and no significant differences were found between families of the WF and RFw phenotypes (see appendix 2, Table 3.2bi). The domestic families had a substantially higher mean Ab level increase between egg and swim-up than families of

the wild origin groups, with little variation in the increase among the domestic families (Figure 3.1b). This resulted in significant non-homogeneity of variances between the three groups (see appendix 2, Table 3.2bii). Following log transformation, the significant differences in the change of family mean antibody levels were driven by the significantly higher increases among the domestic families (Figure 3.1b), with no difference between the wild flesh-colour family phenotypes (Table 3.2bii). There were no significant differences in the % change in family mean antibody levels between any of the maternal origins (see appendix 2, Table 3.2).

3.3.3 Carotenoid-antibody correlations

As mentioned above, mean family levels of egg carotenoids declined (Figure 3.1a, Table 3.1bii) while those of antibody (Ab) rose (figure 3.1b, Table 3.2c) between the egg and swim-up stages in each maternal origin group of eggs and offspring, however there were no significant relationships between maternally derived egg levels of these compounds (see appendix 2, Table 3.3a). Instead, significant relationships emerged at the swim-up stage between measures of Ab and carotenoids in both levels (ug/g) at, change in levels (ug/g) to and percent (%) change in levels to swim-up stage (Figures 3.2, and Table 3.3).

Results of regression analyses were examined for dependent variables of swim-up stage measures of (log + 1 ug/g) Ab (Table 3.3a) and the change in Ab levels (log +2 transformed) to swim-up (Figure 3.2, Table 3.3b) versus the independent variables as measures of carotenoids as either levels (ug/g) remaining at or (%) decline to swim-up stage and the covariate for maternal effects of eyed egg weight. For antibody levels (log + 1 ug/g), egg weight (wt) was negatively correlated and the most significant predictor

(co-efficient -0.499, $p_{(1)} \leq 0.008$), with carotenoid (ug/g) levels and (%) decline also both being significant predictors, with levels being negatively correlated (co-efficient -0.398, $p_{(1)} \leq 0.024$, multiple R^2 with egg wt = 0.323, $p_{(2)} \leq 0.02$), and % decline being positively correlated (co-efficient = 0.454, $p_{(1)} \leq 0.011$, multiple R^2 with egg wt = 0.390, $p_{(2)} \leq 0.007$; Table 3.3a).

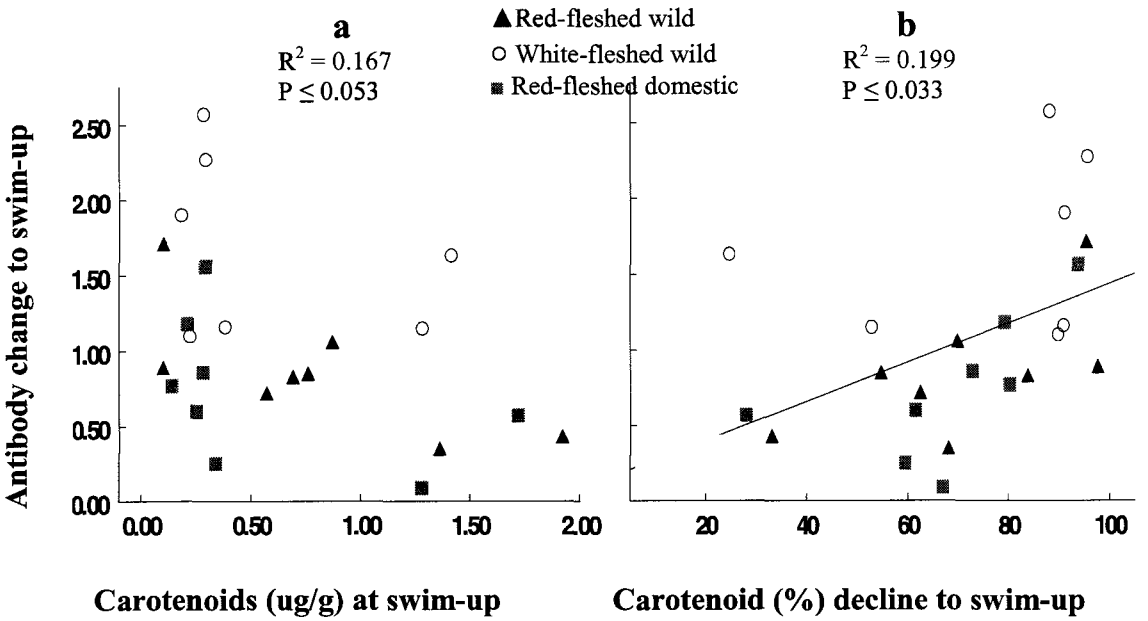


Figure 3.2: Relationships in swim-up stage offspring between the changes in mean family levels of antibody (log + 2 ug/g) vs carotenoids as **(a)** levels (ug/g) remaining in (no significant relationships) and **(b)** % decline to swim-up stage among offspring of different maternal Chinook salmon flesh-colour phenotypes and a domestic population.

For the change in antibody levels (log + 2 ug/g) from egg to swim-up, egg weight (wt) was no longer a significant predictor (co-efficient -0.293, $p_{(1)} \leq 0.099$, Table 3.3b). Higher carotenoid (ug/g) levels significantly predicted lower changes in antibody levels (co-efficient -0.409, $p_{(1)} \leq 0.026$), though the significance of the effect was unidirectional,

and not significant in a 2-tailed analysis ($R^2 = 0.167$, $p_{(2)} \leq 0.053$, Figure 3.2a). Greater (%) declines in egg carotenoids to swim-up significantly predicted a higher increase in antibody levels over the same period (co-efficient +0.446, $p_{(1)} \leq 0.016$). This effect was significant in both directions (single $R^2 = 0.199$, $p_{(2)} \leq 0.033$, Figure 3.2b), and this relationship was strong enough to remain significant in a multiple regression including the independently non-significant maternal effects predictor of eyed egg weight (g) (multiple $R^2 = 0.292$, $p_{(2)} \leq 0.045$; Table 3.3b).

Table 3.3 Hierarchical OLS regression models* of significant relationships in Chinook salmon offspring immune measures vs measures of egg carotenoids at different development stages and maternal effects as eyed egg weight (g).

a) Offspring antibody (log + 1 ug/g) on measures of remaining egg carotenoids (ug/g or % ug/g) at swim-up and maternal effects as eyed egg weight (g)

Hierarchical Models	Independent variables	r	$p_{(1)}$	Model Summary n	R^2	$p_{(2)}$
1	*Carotenoid (ug/g) at swim-up*	*-0.398*	*0.024*	*25*	*0.158*	*0.049*
2	*Carotenoid (ug/g) at swim-up*	*-0.398*	*0.024*	*23*	*0.323*	*0.020*
	Eyed egg weight	*-0.499*	*0.008*			
1	*Carotenoid % decline egg to swim-up*	*0.454*	*0.011*	*25*	*0.207*	*0.022*
2	*Carotenoid % decline egg to swim-up*	*0.454*	*0.011*	*23*	*0.39*	*0.007*
	Eyed egg weight	*-0.499*	*0.008*			

b) changes in antibody egg to swim-up (log + 2 transformed ug/g) on measures of remaining egg carotenoids (ug/g or % ug/g) in swim-up offspring and maternal effects as eyed egg weight

Hierarchical Models*	Independent variables	r	$p_{(1)}$	Model Summary n	R^2	$p_{(2)}$
1	*Egg carotenoids remaining at swim-up*	*-0.409*	*0.026*	23	0.167	0.053
2	Egg carotenoids remaining at swim-up	*-0.409*	*0.026*	21	0.232	0.093
	Eyed egg weight (g)	-0.293	0.099			
1	*Egg carotenoid % change at swim-up*	*0.446*	*0.016*	*23*	*0.199*	*0.033*
2	*Egg carotenoid % change at swim-up*	*0.446*	*0.016*	*21*	*0.292*	*0.045*
	Eyed egg weight	-0.293	0.099			

c) Smolt stage offspring disease resistance LT_{10} to vibriosis on measures of egg carotenoids (ug/g) in unfertilized, eyed stage eggs or change in egg levels to swim-up with co-variants of antibody levels (ug/g) in unfertilized eggs and maternal effects as eyed egg weight (g)

Hierarchical Models*	Independent variables	r	$p_{(1)}$	n	Model Summary R^2	Model Summary $p_{(2)}$
1	*Carotenoid (ug/g) – unfertilized egg*	*0.428*	*0.021*	*20*	*0.183*	*0.041*
2	*Carotenoid (ug/g) – unfertilized egg*	*0.428*	*0.021*	*20*	*0.337*	*0.031*
	Antibody (ug/g) – unfertilized egg	0.299	0.101			
3	Carotenoid (ug/g) – unfertilized egg	*0.428*	*0.021*	19	0.346	0.087
	Antibody (ug/g) – unfertilized egg	0.299	0.101			
	Weight (g) – eyed egg	0.102	0.339			
1	*Carotenoid (ug/g) – eyed egg*	*0.444*	*0.017*	*20*	*0.197*	*0.034*
2	*Carotenoid (ug/g) – eyed egg*	*0.444*	*0.017*	*20*	*0.322*	*0.037*
	Antibody (ug/g) –unfertilized egg	0.299	0.101			
3	Carotenoid (ug/g) –eyed egg	*0.444*	*0.017*	19	0.324	0.109
	Antibody (ug/g) – unfertilized egg	0.299	0.101			
	Weight (g) – eyed egg	0.102	0.399			

*Hierarchical ordinary least squares regression model results include: signed correlations (r) and 1-tailed significance ($p_{(1)}$) to denote directional contributions of each individual variable to the model; single followed by multiple regression (R^2) with corresponding 2-tailed significance ($p_{(2)}$) to denote the overall relationship between model 1 variable (single R^2) and model 1 + 2 variables (multiple R^2) to the model; *significant relationships at $p \leq$ 05 in italics.*

3.3.2 Disease Challenge

Increased resistance time (LT_{10}) following a live ip vibriosis challenge at the smolt stage was positively correlated with higher egg carotenoid concentrations (ug/g) at both the unfertilized and eyed egg stages (Chapter 2, Figure 2.3). In a multiple regression analysis the co-variate of unfertilized egg Ab levels (ug/g) increased the significance of this relationship (from single $R^2 = 0.183$, $p_{(2)} \leq 0.041$ to multiple $R^2 = 0.337$, $p_{(2)} \leq 0.031$), even though egg Ab levels were not a significant predictor (co-efficient 0.299, $p_{(1)} \leq 101$, Table 3.3c). Similarly, the multiple regression of vibrio resistance versus co-variants of eyed egg carotenoid and unfertilized egg antibody levels was also significant, though the addition of unfertilized egg antibody levels reduced the significance of this relationship (from single $R^2 = 0.197$, $p_{(2)} \leq 0.034$ to multiple $R^2 = 0.322$, $p_{(2)} \leq 0.037$), with higher eyed egg carotenoid levels significantly predicting longer disease resistance times (co-efficient

$= 0.444, p_{(1)} \leq 0.021$). The maternal effect eyed egg weight (g) co-variate decreased the power of both multiple regressions to non-significance (Table 3.3c). Other variables and relationships examined with respect to vibrio resistance in this study did not improve or indeed show any other significant relationships (see appendix 2, Table 3.3 c).

3.4 Discussion

This study provides new insight into potential mechanism(s) of carotenoid-immune function relationships in juvenile salmon, in that the % decline in family egg carotenoid levels to the swim-up stage was significantly related to the increase in family mean Ab level during the same stage. Carotenoids have been proposed to be signals of superior immune function in birds (Hamilton and Zuk 1982; Blount 2004), mature Arctic charr (Skarstein and Folstad 1996), juvenile Atlantic salmon (Christiansen et. al. 1995) and mammals (Chew and Park 2004). One theory is that the antioxidant action of carotenoids (in synergy with other antioxidants) preserves the integrity of host resources (such as lipids, proteins, membranes, tissues and organ functions) against damage from oxygen radicals (Kurashige *et. al.* 1990; Krinsky and Yuem 2003), such as those generated during an innate immune response (Bernstein et. al. 1998). Thus carotenoids signal superior immunity through overall higher quality resources and better functioning systems leading to superior health. Other theories involve more direct effects of carotenoids on immunological measures. Studies have shown carotenoids and their metabolic retinoid derivatives to be involved in the mediation of T-cell immune processes, such as by (1) stimulating receptor pathways (Stephensen et. al. 2002), (2) triggering $G_{0\text{-}1}$ cell cycle activation, (3) as co-factors in transcription regulation and (4) in intercellular communication mechanisms imperative to adequate mediation of immune

responses, the maintenance of healthy or purging of foreign, damaged or diseased cells (Garbe et. al. 1992). Our results support other findings that carotenoids themselves influence antibody production (Alexander et. al. 1986; Bendich and Shapiro 1986; Jyonouchi et. al. 1994; Chew *et. al.* 2000; Kiss *et. al.* 2003; Peters et. al. 2004) and that measures of egg carotenoid levels influence disease resistance at smoltification, possibly with interactions of antibody, though the exact mechanisms remain to be investigated.

In the current study, the primary carotenoid in all eggs was astaxanthin, with small amounts of canthaxanthin present in the domestic family eggs. Egg carotenoid levels decreased with each subsequent developmental stage to swim-up in all families, while antibody levels rose to the swim-up stage in the majority of families compared to mean unfertilized egg levels of these compounds. Our results suggest that the relative decline in egg carotenoids through embryonic development in some way influences levels of endogenous antibody expression at the swim-up stage. Circulating measures of carotenoids and antibody have shown to be inversely correlated following immune activation in birds (Saino *et. al.* 1999; Verhulst *et. al.* 1999; Ohlsson *et. al.* 2003; Kiss *et. al.* 2003; Peters *et. al.* 2004) and mammals (Jyonouchi *et. al.* 1994; Chew *et. al.* 2000; Jonasson *et. al.* 2003) as well as at sexual maturity in fish (Skarstein and Folstad 1996), and perhaps a form of this effect was involved in observed correlations.

An interesting finding of this study is that maternal-egg allocation patterns were very different between the two Chinook phenotypes and population origins, not only for carotenoid levels (as was expected and discussed in Chapter 2) but also for levels of antibody (Figures 3.1 & 3.2). While no empirical linear correlations were found between the levels of these two groups of compounds in the eggs, the RFw families had significantly higher mean egg carotenoid levels than the WF families at the unfertilized

and eyed egg stages. Egg antibody levels showed significant non-homogeneity of variances between the groups following all parametric data transformations, necessitating a non-parametric rank ordering of their levels prior to statistical comparison. Following this, the RFw families had significantly lower level ranks than those of their WF family counterparts (Table 3.2a). Conversely, mammalian low responders to carotenoids show significantly reduced levels of circulating carotenoids, similar to the WF families of this study (Bowen et. al. 1993; Lin 2000), while mammalian low carotenoid responders also showed reduced levels of circulating antibody (Chew *et. al.* 2000); though the WF eggs of this study were both higher and substantially more variable in antibody levels than the RFw eggs (Figures 3.1 and 3.2, Table 3.2ai). While egg antibody and carotenoid levels together in a multiple regression correlated positively with disease resistance at the smolt stage (Table 3.3ci), the eggs of the white-fleshed group had both lower carotenoid and higher, more variable antibody levels both in the eggs and swim-up fry (Tables 3.1a and 3.2ai) than their RFw counterparts.

Skarstein and Folstad (1996) coined the "immunocompetence handicap hypothesis" in their studies of Arctic charr where they proposed that sex hormones mediate inverse correlations between circulating carotenoid and antibody levels during sexual maturation and spawning. They further speculated that higher carotenoid levels translated to better functioning of the innate immune system, and hence to lower levels of specific antibody required for immune defence. Here, it could be similarly interpreted for the present study that, as the antioxidant potential of carotenoids in Chinook salmon eggs were depleted, the requirement for antibody increased, and hence expressed levels of antibody increased. A possible mechanism for this proposed interaction could involve carotenoids and/or their derivatives as transcription enhancers. Retinoids (e.g. vitamin A)

are metabolic carotenoid derivatives known to boost recovery from a number of diseases in humans (see reviews by Sommer 1989; Wolf 1996), and are important regulators of the immune response (Garbe et. al. 1992; Stephensen et. al. 2002; Geissmann et. al. 2003). Dietary carotenoid supplementation boosts maternal production and egg allocation of vaccination induced antibody levels (Kiss *et. al.* 2003), while maternal carotenoids boost *in vitro* mitogen induced antibody production in the bursa of newly hatched chicks (Haq et. al. 1996). If carotenoid antioxidants mediate host damage from toxic oxygen by-products, they do so at the expense of their own oxidation. Since increases in circulating antibody correspond to decreases in circulating carotenoids (Faivre *et. al.* 2003; Jonasson et. al. 2003; Alonso-Alverez et. al. 2004) then perhaps as carotenoid antioxidant mediators are depleted, the subsequent rise in oxidative by-products may have triggered the observed increased production of antibody. Conversely, consider the relationship between retinoids, inflammatory cytokines (IC) and dendritic cells (DC), where in the presence of ICs, retinoids increase DNA binding, maturation and differentiation of DCs, expression of MHCII and co-stimulatory molecules and enhancement of antigen-specific T-cell responses, leading to increased antibody expression. In the absence of ICs, retinoids induce DC apoptosis, protecting the host from unwanted DC action (Geissmann *et. al.* 2003). A majority of carotenoid studies reporting immune activation relationships have found these to be mediated by T-cell mechanisms (Alexander *et. al.* 1986; Bendich and Shapiro 1986; Jyonouchi *et. al.* 1994; Jonasson *et. al.* 2003; Massimino *et. al.* 2003). Perhaps then, carotenoid-immune function relationships are mediated via carotenoid conversion to vitamin A or other retinoids, which then act as the direct immune activation factors. The results of this study lead to the proposition of two not necessarily exclusive possible hypotheses with respect to Chinook salmon:

1) Carotenoids act primarily as antioxidants and as this capacity is depleted, oxidative derivatives then stimulate the production of antibody.

2) Carotenoids act as antioxidants and are metabolised to retinoids, which then act as cofactors of T-cell activation, increasing circulating antibody levels.

Either of these possibilities could explain the observed relationship between carotenoid decline and increased antibody levels. Either of these could also explain the inverse relationships reported in other studies between carotenoid-pigmentation and infection, immune activation and/or antibody levels (Jyonouchi *et. al.* 1994; Skarstein and Folstad 1996; Saino *et. al.* 1999; Verhulst *et. al.* 1999; Chew *et. al.* 2000; Jonasson *et. al.* 2003; Ohlsson *et. al.* 2003; Amar *et. al.* 2004; Peters *et. al.* 2004). As well, since both hypotheses incorporate carotenoids as antioxidants this could in part account for some of the conflicting carotenoid-immune activation results reported by others. Some examples of conflicting relationships between carotenoid levels and measures of immune function include: (1) cell mediated but not humoral immune measures being correlated with carotenoids, (2) specific but not innate immune measures being correlated with carotenoids, or (3) certain types of immunoglobulin (e.g. IgG) but not others (e.g. IgM) being correlated with increased measures of carotenoids (e.g. Christiansen *et. al.* 1995; Thompson *et. al.* 1995; Hill 1999; Hartley and Kennedy 2004). Alternatively, the correlation between remaining egg carotenoids and expressed antibody at swim-up could be entirely a maternal effect, as suggested from the analysis of swim-up stage antibody when multiply regressed against the co-variants of eyed egg weight and remaining egg carotenoids or carotenoid % remaining. However, analyses examining the change in antibody from egg to swim-up when multiply regressed against the same carotenoid and egg weight co-variants revealed only the carotenoid measures to be significant predictors

with the maternal effects egg weight co-variate dropping entirely out of significance. If the correlations between different measures of egg carotenoid and antibody at swim-up were actually spurious, they would not be expected to show such similar results unless they were autocorrelated, which could also be the case here, although, if the two were autocorrelated it could be expected that this would represent a maternal effect. If these variables were really a maternal effect and not in factcorrelated (or autocorrelated with maternal effects) of their own accord, then the maternal effect co-variate should be the most significant predictor regardless of the analytical measure. The fact that these different measures of these two compounds are significantly related in all of the different analyses examined and all are more a maternal effect, except for the relative change of the two, which are significantly related to eachother though not related to maternal effects shows there is some connecting factor between carotenoids and antibodies in embryonic development, even though the mechanism of their interaction remains unclear and undefined.

Results of this study also show that levels of egg carotenoids and antibody together in a multiple regression significantly predict resistance to an intraperitoneal bacterial challenge with a marine pathogen (*Listonella anguillarum*) at the smolt stage. The inclusion of the egg antibody co-variate in the analysis of the relationship between disease resistance and egg carotenoids increases the R^2 and decreases the p value of the multiple versus single regression results (single regression of disease resistance versus egg carotenoids $R^2 = 0.183$, $p_{(2)} \leq 0.041$; multiple regression of disease resistance versus co-variants of egg carotenoid and antibody levels $R^2 = 0.337$, $p_{(2)} \leq 0.031$), though carotenoids were the only significant predictor. Similarly, Christiansen *et. al.'s* (1995) significant relationship between offspring freshwater dietary carotenoid supplementation

and a co-habitant disease resistance challenge found no difference between the dietary groups in vaccination induced antibody levels (measured by ELISA at 4 months post-vaccination), though did find that the supplemented offspring had red blood cells significantly more resistance to oxidative attack than the unsupplemented offspring. While the Tyndale and Bryden (unpublished) pilot study did not examine any specific immune measures such as antibody levels, no significant relationships were found between non-specific immune measures such as lysozyme or stress response and egg carotenoid levels; nor was the significant relationship between disease resistance and egg carotenoid levels enhanced by multiple regression analyses including these other non-specific immune measures as co-variants. The current study did not examine post-challenge antibody levels, and egg antibody levels only contributed as covariates with the significant egg carotenoid level predictor to an overall multiple regression of smolt stage disease resistance, and were not independent predictors of this relationship. Our results suggest that the % decline of egg carotenoids through embryonic incubation is related to the relative increase in antibody levels during the same time period, and that egg carotenoid levels affect offspring fitness in Chinook salmon via disease resistance at the smolt stage, possibly in part through interactions with egg antibody levels, though these were not independently correlated. This study is thus one of only a handful that addresses possible mechanisms behind the long asserted egg carotenoid – offspring survival relationships in salmonid fish.

3.5 References

Alexander,M., H.Newmark, and R.G.Miller, 1986. Oral beta-carotene can increase the number of OKT4+ cells in human blood. Immunological letters 9: 221-224.

Alonso-Alvarez,C., S.Bertrand, G.Devevey, M.Gaillard, J.Prost, B.Faivre, and G.Sorci, 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. The American Naturalist 164: 651-659

Amar,E.C., V.Kiron, S.Satoh, and T.Watanabe, 2001. Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow-trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Research 32: 162-173.

Amar,E.C., V.Kiron, S.Satoh, and T.Watanabe, 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish and Shellfish Immunology 16: 527-537.

Ando,S., K.Osada, M.Hatano, and M.Saneyoshi, 1989. Comparison of carotenoids in muscle and ovary from four genera of salmonid fishes. Comparative Biochemistry and Physiology B 93: 503-508.

Ando,S., H.Yamauchi, M.Hatano, and W.R.Heard, 1992. Comparison of muscle compositions between red- and white-fleshed chinook salmon *(Oncorhynchus tshawytscha*). Aquaculture 103: 359-365.

Asplund,L., M.Athanasisadou, A.Sjödin, and B.H.Bergman, 1999. Organohalogen substances in muscle, egg and blood from healthy Baltic salmon (*Salmo salar*) and Baltic salmon that produced offspring with M74 syndrome. Ambio 28: 67-76.

Bendich, A.; S.S. Shapiro 1986. Effects of ß-carotene and canthaxanthin on the immune responses of the rat. Journal of Nutrition 116: 2254-2262.

Bergey, J.G.Holt, N.R.Krieg, and P.H.A.Sneath, 1994. Bergey's Manual of Determinative Bacteriology.

Bernstein,R.M., S.F.Schluter, and J.J.Marchalonis, 1998. Immunity. The Physiology of Fishes, CRC Press LLC, pp. 215-242.

Bertram, J.S. and A.L. Vine 2005. Cancer prevention by retinoids and carotenoids: independent action on a common target. Biochimica et Biophysica Acta - molecular basis for disease 1740(2): 170-178.

Blanc,J.-M. and G.Choubert, 1993. Genetic variation of flesh color in pan-size rainbow trout fed astaxanthin. Journal of Applied Aquaculture 2: 115-123.

Blount,J.D., D.C.Houston, and A.P.Møller, 2000. Why egg yolk is yellow. Trends in Ecology and Evolution 15: 47-49.

Blount,J.D., N.B.Metcalfe, K.E.Arnold, P.F.Surai, G.L.Devevey, and P.Monaghan, 2003. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. Procceedings of the Royal Society of London B 270: 1691-1696.

Blount,J.D., 2004. Carotenoids and life-history evolution in animals. Archives of Biochemistry and Biophysics 430: 10-15.

Bowen,P.E., M.Garg, M.Stacewicz-Sapuntzakis, L.Yelton, and R.S.Schreiner, 1993. Variability of serum carotenoids in response to controlled diets containing six servings of fruits and vegetables per day. Annals New York Academy of Sciences 691: 241-243.

Böhm,F., R.Edge, E.J.Land, D.McGarvey, and T.G.Truscott, 1997. Carotenoids enhance vitamin E antioxidant efficiency. Journal of American Chemical Society 119: 621-622.

Bryden,C.A., J.W.Heath, and D.D.Heath, 2004. Performance and heterosis in farmed and wild Chinook salmon (*Oncorhynchus tshawytscha*) hybrid and purebred crosses. Aquaculture 235: 249-261.

Burke,M., R.Edge, E.J.Land, and T.G.Truscott, 2000. Characterization of carotenoid radical cations in liposomal environments: interaction with vitamin C. Journal of Photochemistry and Photobiology B 60: 1-6.

Burton,G.W., 1989. Antioxidant action of carotenoid. Journal of Nutrition 119: 109-111.

Chantanachookhin,C., K.Takano, T.Seikai, and M.Tanaka, 1991. Comparative study of the ontogeny of the lymphoid organs in three species of marine fish. Aquaculture 99: 143-155.

Chew,B.P., 1995. Antioxidant vitamins affect food animal immunity and health. Journal of Nutrition 125: 1804s-1808s.

Chew,B.P., M.W.Wong, J.S.Park, and T.S.Wong, 1999. Dietary β -carotene and astaxanthin but not canthaxanthin stimulate splenocyte function in mice. Anticancer Research 19: 5223-5228.

Chew,B.P., J.S.Park, T.S.Wong, H.W.Kim, B.B.C.Weng, K.M.Byrne, M.G.Hayek, and G.A.Reinhart, 2000. Dietary β-carotene stimulates cell-mediated and humoral immune response in dogs. Journal of Nutrition 130: 1910-1913.

Chew, B. P. and Park, J. S. 2004. Carotenoid action on the immune response. Krinsky, N. I. 257s-261s. Iowa State U., American Society for Nutritional Sciences. Functions and Actions of Retinoids and Carotenoids: Building on the vision of James Allen Olson. 21-6-2001.

Christiansen,R., J.Glette, Ø.Lie, O.J.Torrissen, and R.Waagbø, 1995. Antioxidants and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. Journal of Fish Diseases 18: 317-328.

Christiansen,R. and O.J.Torrissen, 1997. Effects of dietary astaxanthin supplementation on fertilization and egg survival in Atlantic salmon (*Salmo salar L.*). Aquaculture 153: 51-62.

Craik 1985. Egg quality and pigment content in salmonid fishes. Aquaculture 47: 61-88.

Craik,J.C.A. and S.M.Harvey, 1986. The carotenoids of eggs of wild and farmed Atlantic salmon and their changes during development to the start of feeding. Journal of Fish Biology 29: 549-565.

Dixon 1950. Analysis of extreme values. Annals of Mathematical Statistics 21:488-506.

Dufva,R. and K.Allander, 1995. Intraspecific variation in plumage coloration reflects immune response in Great Tit (*Parus major*) males. Functional Ecology □: 785-789.

Ellis,A.E., 1977. Ontogeny of the immune response in *Salmo salar*. Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leucocyte reactivity. In: Soloman,J.B. and J.D.Horton (eds), Developmental Immunobiology, Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 225-231.

Ellis,A.E., 1988. Ontogeny of the immune system in teleost fish. In: Ellis,A.E. (ed.), Fish Vaccination, Academic Press, London, pp. 20-31.

Faivre,B., A.Grégoire, M.Préault, F.Cézilly, and G.Sorci, 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science 300: 103.

Folstad,I. and A.J.Karter, 1992. Parasites, bright males and the immunocompetence handicap. The American Naturalist 139: 603-622.

Gale,C.R., N.F.Hall, D.I.W.Phillips, and C.N.Martyn, 2001. Plasma antioxidant vitamins and carotenoids and age-related cataract. Ophthalmology 108: 1992-1998.

Garbe,A., J.Buck, and U.Hämmerling, 1992. Retinoids are important cofactors in T-cell activation. Journal of Experimental Medicine 176: 109-117.

Geissmann,F., P.Revy, N.Brousse, Y.Lepelletier, C.Folli, A.Durandy, P.Chambon, and M.Dy, 2003. Retinoids regulate survival and antigen presentation by immature dendritic cells. Journal of Experimental Medicine 198: 623-634.

Goodwin,T.W., 1954. conversion of carotenoids into vitamin A: conclusions. In: Goodwin,T.W. (ed.), Carotenoids: their comparative biochemistry, Chemical Publishing Co., Inc., NY, pp. 269-289.

Goodwin,T.W., 1986. Metabolism, nutrition and function of carotenoids. Annual Review of Nutrition 6: 273-297.

Hamiltion,W.D. and M.Zuk, 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Haq,A., A.Chinnah, and C.A.Bailey, 1996. Effect of ß-carotene, canthaxanthin, or lutein of lymphocyte proliferation (in vitro) of newly hatched chicks. Avain Diseases 40: 823-827.

Hard,J.J., 1986. Flesh color variation in Chinook salmon (*Oncorhynchus tshawytscha*) at Little Port Walter, Alaska. NOAA Technical Memorandum NMFS F/NWC 109: 1-31.

Harris,L.E., 1984. Effects of a broodfish diet fortified with canthaxanthin on female fecundity and egg color. Aquaculture 43: 179-183.

Hartley,R.C. and M.W.Kennedy, 2004. Are carotenoids a red herring in sexual display? Trends in Ecology and Evolution 19: 353-354.

Heath and Blouw 1998. Are maternal effects in fish adaptive or merely physiological side effects? In: Mousseau T.A. and Fox, C.W. (ed.s) Maternal effects as adaptations, Oxford University Press, N.Y.p.178-201.

Hickenbottom,S.J., J.R.Follett, Y.M.Lin, S.R.Dueker, B.J.Burri, T.R.Neidlinger, and A.J.Clifford, 2002. Variability in conversion of beta-carotene to vitamin A in men as measured by using a double-tracer study design. American Journal of Clinical Nutrition 75: 900-907.

Hill,G.E., 1999. Is there an immunological cost to carotenoid-based ornamental coloration? The American Naturalist 154: 589-595.

Houde,A.E. and A.J.Torio, 1992. Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology 3: 346-351.

Hubbs,C. and K.Strawn, 1957. Survival of F_1 hybrids between fishes of the subfamily *Etheostominae*. Journal of Experimental Zoology 134: 33-62.

Hubbs,C. and L.Stavenhagen, 1958. Effects of maternal carotenoid deficiency on the viability of darter (*Osteichthyes*) offspring. Physiol. Zool. 31: 280-283.

Jonasson,L., A.Wikby, and A.G.Olsson, 2003. Low serum β-carotene reflects immune activation in patients with coronary artery disease. Nutrion and Metabolism in Cardiovascular Disease 13: 120-125.

Jyonouchi,H., L.Zhang, M.Gross, and Y.Tomita, 1994. Immunomodulating actions of carotenoids: Enhancement of *in vivo* and *in vitro* antibody production to T-dependent antigens. Nutrition and Cancer 21: 47-58.

Kiss,Z., L.Bárdos, C.Szabó, L.Lengyel, and M.Szabó, 2003. Effect of β-carotene supplementation on plasma and yolk IgY levels induced by NDV vaccination in Japanese quail. International Journal of Vitamin Nurtitional Research 73: 285-289.

Koutsos,E.A., A.J.Clifford, C.C.Calvert, and K.C.Klasing, 2003. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). Journal of Nutrition 133: 1132-1138.

Krinsky,N.I. and K.J.Yeum, 2003. Carotenoid-radical interactions. Biochemical and Biophysical Research Communications 305: 754-760.

Kurashige,M., E.Okimasu, M.Inoue, and K.Utsumi, 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiological Chemistry Phys. & Med. NMR 22: 27-38.

Li,H.A., S.T.Tyndale, D.D.Heath, and R.J.Letcher, 2005. Determination of carotenoids and all-*trans*-retinol in Chinook salmon (*Oncorhynchus tshawytscha*) eggs by liquid chromatography-electrospray ionization-tandem mass spectrometry. Journal of Chromatography B 816: 49-56.

Lin,S., L.Quaroni, W.White, T.Cotton, and G.Chumanov, 2000a. Localization of carotenoids in plasma low-density lipoproteins studied by surface-enhanced resonance Raman spectroscopy. Biopolymers (Biospectroscopy) 57: 249-256.

Lin,Y.M., S.R.Dueker, B.J.Burri, T.R.Neidlinger, and A.J.Clifford, 2000b. Variability of the conversion of beta-carotene to vitamin A in women measured by using a double-tracer study design. American Journal of Clinical Nutrition 71: 1545-1554.

Lundström,J., B.Carney, P.Amcoff, A.Pettersson, Borjeson.H, L.Forlin, and L.Norrgren, 1999. Antioxidative systems, detoxifying enzymes and thiamine levels in Baltic salmon (*Salmo salar*) that develop M74. Ambio 28: 24-29.

March,B.E. and C.MacMillan, 1996. Muscle pigmentation and plasma concentrations of astaxanthin in rainbow trout, Chinook salmon, and Atlantic salmon in response to different dietary levels of astaxanthin. Progressive Fish Culturist 53: 178-186.

Massimino,S., R.J.Kearns, K.M.Loos, J.Burr, J.S.Park, B.Chew, S.Adams, and G.Hayek, 2003. Effects of age and dietary β-carotene on immunological variables in dogs. Journal of Veterinary Internal Medicine 17: 835-842.

McFarland,J., 1907. The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. Journal of the American Medical Association 49: 1176.

Mor,A. and R.R.Avtalion, 1988. Evidence of transfer of immunity from mother to eggs in tilapias. The Israeli Journal of Aquaculture 40: 22-28.

Møller,A.P., C.Ciard, J.D.Blount, D.C.Houston, P.Ninni, N.Saino, and P.F.Surai, 2000. Carotenoid-dependent signals: Indicators of foraging efficiency, immunocompetence or detoxification ability? Avian and Poultry Biology Reviews 11: 137-159.

Nagae,M., H.Fuda, A.Hara, H.Kawamura, and K.Yamauchi, 1993. Changes in serum immunoglobulin M (Ig M) concentrations during early development of chum salmon (*Oncorhynchus keta*) as determined by sensitive ELISA technique. Comparative Biochemistry and Physiology A 106: 69-74.

Obermüller-Jevic,U.C., P.I.Francz, J.Frank, A.Flaccus, and H.K.Biesalski, 1999. Enhancement of UVA induction of haem oxygenase-1 expression by ß-carotene in human skin fibroblasts. Federation of European Biochemical Studies -- Letters 460: 212-216.

Ohlsson,T., H.G.Smith, L.Råberg, and D.Hasselquist, 2003. Effects of nutrition on sexual ornaments and humoral immune responsiveness in adult male pheasants. Ethology, Ecology & Evolution 15: 31-42.

Palace,V.P., S.B.Brown, C.L.Baron, J.Fitzsimons, B.Woodin, J.J.Stegeman, and J.F.Klaverkamp, 1998. An evaluation of the relationships among oxidative stress,

antioxidant vitamins and early mortality syndrome (EMS) of lake trout (*Salvelinus namaycush*) from Lake Ontario. Aquatic Toxicology 43: 195-208.

Palozza, P.; S. Serini; F. Di Nicuolo; G. Calviello 2004. Modulation of apoptotic signalling by carotenoids in cancer cells. Archives of Biochemistry and Biophysics 430: 104-109.

Palozza, P. 2005. Can □-carotene regulate cell growth by a redox mechanism? An answer from cultured cells. Biochimica et Biophysica Acta - molecular basis for disease 1740(2): 215-221.

Peters,A., A.G.Denk, K.Delhey, and B.Kempenaers, 2004. Carotenoid-based bill colour as an indicator of immunocompetence and sperm performance in male mallards. Journal of Evolutionary Biology 17: 1111-1120.

Pettersson,A. and Å.Lignell, 1998. Low astaxanthin levels in Baltic salmon exhibiting the M74 syndrome. In: McDonald,G., J.D.Fitzsimons, and D.C.Honeyfield (eds), Early Life Stage Mortality Syndrome in Fishes of the Great Lakes and Baltic Sea, American Fisheries Society, Bethesda, Maryland, pp. 26-30.

Pettersson,A. and A.Lignell, 1999. Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. Ambio 28: 43-47.

Pickova,J., A.Kiessling, A.Pettersson, and P.C.Dutta, 1998. Comparison of fatty acid composition and astaxanthin content in healthy and by M74 affected salmon eggs from three Swedish river stocks. Comparative Biochemistry and Physiology B 120: 265-271.

Pickova,J., A.Kiessling, A.Pettersson, and P.C.Dutta, 1999. Fatty acid and carotenoid composition of eggs from two nonanadromous Atlantic salmon stocks of cultured and wild origin. Fish Physiology and Biochemistry 21: 147-156.

Prince,E.E., 1916. On the red color of the flesh in the salmons and trouts. Transactions of the American Fisheries Society 46: 50-61.

Razquin,B.E., A.Castillo, P.López-Fierro, F.Alvarez, A.Zapata, and A.J.Villena, 1990. Ontogeny of IgM-producing cells in the lymphoid organs of rainbow trout, *Salmo gairdneri* Richardson: an immuno- and enzyme-histochemical study. Journal of Fish Biology 36: 159-173.

Ruff,M.D., W.M.Reid, and J.K.Johnson, 1974. Lowered blood carotenoid levels in chickens infected with coccidia. Poultry Science 53: 1801-1809.

Saino,N., R.Stradi, P.Ninni, E.Pini, and A.P.Møller, 1999. Carotenoid plasma concentration, immune profile, and plumage ornamentation of male barn swallows (*Hirundo rusitca*). The American Naturalist 154: 441-448.

Schiedt,K., F.J.Leuenberger, M.Vecchi, and E.Glinz, 1985. Adsorption, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. Pure & Applied Chemistry 57: 685-692.

Sharoni,Y., M.Danilenko, N.Dubi, A.Ben-Dor, and J.Levy, 2004. Carotenoids and transcription. Archives of Biochemistry and Biophysics 430: 89-96.

Skarstein,F. and I.Folstad, 1996. Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. OIKOS 76: 359-367.

Sommer, A. 1989. New Imperaitves for an old vitamin (A). Journal of Nutrition 119: 96-100.

Stahl, W., Schwarz, W., von Laar, J., and Sies, H. 1995. *All-trans* □-carotene preferentially accumulates in human chylomicrons and very low density lipoproteins compared with the 9-*cis* geometrical isomer. Journal of Nutrition 125[8], 2128-2133.

Stahl, W.; J. von Laar; H.-D. Martin; T. Emmerich; H. Sies 2000. Stimulation of gap junctional communication: comparison of *acyclo*-retinoic acid and lycopene. Archives of Biochemistry and Biophysics 373(1): 271-274.

Stephensen,C.B., R.Rasooly, X.Jiang, M.A.Ceddia, C.T.Weaver, R.A.S.Chandraratna, and R.P.Bucy, 2002. Vitamin A enhances *in vitro* Th2 development via retinoid X receptor pathway. Jounral of Immunology 168: 4495-4503.

Sumantran, V.N.; R. Zhang; D.S. Lee; M.S. Wicha 2000. Differential regulation of apoptosis in normal versus transformed mammary epithelium by lutein and retinoic acid. Cancer Epidemiology, Biomarkers & Prevention 9: 257-263.

Takahashi,Y. and E.Kawahara, 1987. Maternal immunity in newborn fry of the ovoviparous guppy. Nippon Suisan Gakkaishi 53: 721-725.

Takemura,A. and K.Takano, 1997. Transfer of maternally-derived immunoglobulin (IgM) to larvae in tilapia, *Oreochromis mossambicus*. Fish and Shellfish Immunology 7: 355-363.

Tatner,M.F. and M.J.Manning, 1983. The ontogeny of cellular immunity in the rainbow trout, *Salmo gairdneri* Richardson, in relation to the stage of development of the lymphoid organs. Developmental and Comparative Immunology 7: 69-75.

Thompson,I., G.Choubert, D.F.Houlihan, and C.J.Secombes, 1995. The effect of dietary vitamin A and astaxanthin on the immunocompetence of rainbow trout. Aquaculture 133: 91-102.

Torrissen,O.J., 1984. Pigmentation of salmonids -- effects of carotenoids in eggs and start-feeding diet on survival and growth rate. Aquaculture 43: 185-193.

Tveranger,B., 1986. Effect of pigment content in broodstock diet on subsequent fertilization rate, survival and growth rate of rainbow trout (*Salmo gairdneri*) offspring. Aquaculture 53: 85-93.

Verhulst,S., S.J.Dieleman, and H.K.Parmentier, 1999. A tradeoff between immunocompetence and sexual ornamentation in domestic fowl. Proceedings of the National Academy of Science 96: 4478-4481.

Withler,R.E., 1986. Genetic variation in carotenoid pigment deposition in the red-fleshed and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) of Quesnel River, British Columbia. Canadian Journal of Genetic Cytology 28: 587-594.

Wolf,G., 1996. A history of vitamin A and retinoids. FASEB Journal 10: 1102-1107.

Yarzhombek,A.A., 1964. Carotenoids and trout farming. Sb. Tekhnicheskoy Informatsii VNIRO 6: 20-25.

Yarzhombek,A.A., 1970. Carotenoids in salmonidae and their relation to reproduction of these fishes (from "Problems of fish physiology"). Proceedings of the All-Union Research Institute of Maritime Fisheries and Oceanography (translated title, original in Russian) 69: 234-267.

Zar, J.H. 1999. Biostatistical analysis 4th edition, Prentice Hall, N.J., p. 208-443.

Zuk, M.; S.L. Popma; T.S. Johnsen 1995. Male courtship displays, ornaments and female mate choice in captive red jungle fowl. Behaviours 132: 11-12.

CHAPTER 4:

Why are salmon eggs red?

Conclusions, Discussion and Future Directions

4.1. Conclusions and Disscussion

This thesis addresses the question "Why are salmon eggs red?"; specifically, what benefits do offspring of darker pigmented eggs gain to offset the costs of both maternal accumulation/metabolism/retention (Goodwin 1986; Olsen and Owens 1998) and egg predation risk (Wieland and Koster 1996; Godin and McDonough 2003; Hasson 2004; Van der Veen 2005) inherent to red eggs? While animals obtain carotenoids from plant synthesized dietary sources, many published correlative and mechanistic relationships are well accepted concerning health, survival and/or reproductive fitness benefits of carotenoids in life forms as diverse as plants, algae, copepods, shellfish, fish, birds and mammals. Carotenoid absorption and retention efficiency is hereditary in salmonids (see review by Blanc and Choubert 1993), especially so in the anomalous Chinook salmon with their genetic polymorphism for distinct red- and white-flesh colour phenotypes (Withler 1986). In contrast the sexual selection driven carotenoid- red nuptial skin colour in Sockeye salmon (*Oncorhynchus nerka*) has led freshwater populations (differentiated as the kokanee salmon morph, *O. nerka*) with limited dietary carotenoids to genetically diverge to maintain this sexually selected trait (Craig and Foote 2001; Foote et. al. 2004; Craig et. al. 2005). Non-anandromous Kokanee salmon possess a heritable genetic polymorphism leading to dietary carotenoid absorption/retention that is more than three

times as efficient as their anandromous Sockeye salmon counterparts who feed in the carotenoid-rich Pacific Ocean (Wood and Foote 1996; Craig and Foote 2001; Foote et. al. 2004; Craig et. al. 2005). Flesh-carotenoid colouration in Chinook salmon is not likely to be under sexual selection, and as salmon egg carotenoids in general are maternally derived and not seen by either sex during reproduction, egg carotenoids are also not likely to be under sexual selection. The red carotenoid pigmentation of Chinook salmon flesh and salmon eggs in general would be expected to be maintained by natural selection for benefits of the carotenoids themselves; benefits which should offset the costs of carotenoid accumulation/retention and egg predation risk. Egg carotenoids are metabolized through embryonic development, so it would seem that if naturally selected benefits for egg carotenoids exist, this would be their realm of influence. Empirical studies however, have found no correlations of red eggs or the carotenoid levels they represent increasing embryonic survival in the hatchery (see review by Craik 1985 and citations of previous chapters). In organohalogen-stressed environments, salmon egg carotenoid-embryonic survival correlations have been documented (Palace et. al. 1998; Pettersson and Lignell 1999), though offspring survival has been ameliorated by egg thiamine bathing (Amcoff et. al. 1998), irregardless of carotenoid levels (Hornung et. al. 1998). This leads us back the fundamental question addressed by this thesis; why are salmon eggs red?

Results of this thesis provide new insight into the benefits offspring acquire from darker pigmented eggs. Concurring with others (cited above & in previous chapters), this study found no definitive empirical relationship between levels of egg carotenoids and embryonic hatchery survival. Offspring benefits manifest at the smolt stage (just prior to saltwater transfer) following challenge with the live, marine bacterial pathogen of

vibriosis (see Chapter 2, Table 2.2). Similarly, Christiansen et. al. (1995) found that by carotenoid supplementation of freshwater diets, Atlantic salmon offspring were more resistant to the bacterial pathogen of furunculosis. Vibriosis represents a ubiquitous environmental threat to Pacific saltwater salmonids, and standard hatchery protocols include vaccination prior to saltwater introduction. Wild spawned salmon have no such advantage, and egg carotenoids were significant positive predictors of survival following vibrio encounter. Comparisons of carotenoid and antibody levels in eggs and progeny of the different maternal origins suggested these compounds may be related in an inverse, albeit complex fashion; though a regression analysis of these variables across all sample origins was non-significant. Smolt stage vibriosis resistance was significantly predicted by egg carotenoid levels. When disease resistance was multiply regressed on co-variate measures of egg carotenoid and antibody (Ab) levels, the relationship was still significant, with carotenoids as the significant positive predictor and Ab levels not showing any independent significant correlation with the observed disease resistance. This suggests that, if an interaction between levels of these compounds exists, the interaction is a complex one, with further research needed to elucidate an exact mechanism and its relation to disease resistance. Regression analyses of swim-up stage Ab levels on egg carotenoid measures of either (a) percent decline to and (b) levels remaining at the offspring swim-up stage showed significant relationships; though multiple regression including maternal effects co-variate of eyed egg weight revealed this to be obscured by egg weight or other maternal effects this represents. However, when the change in Ab levels between egg and swim-up stages was examined either as the change in absolute levels (ug/g) or as percent (%) antibody levels increased, the only significant predictor were the egg carotenoid measures, either as (a) % egg carotenoids declined to or (b)

carotenoid levels (ug/g) remaining at swim-up. Maternal effects as egg weight (g) were not significantly related to either the change in Ab level (ug/g) or % increase from egg to swim-up, nor to vibriosis resistance with egg carotenoids or egg carotenoids and Ab co-variants. The inclusion of the maternal effects co-variate in any of these multiple regression models reduced to the models to non-significance. This suggests that while the levels of antibody in swim-up stage offspring may be largely a maternal effect as indicated by egg weight being the most significant predictor, the relative amount (ug/g or %) of antibody gained (expressed) compared to the levels present in the eggs is in some way related to interactions with the relative decline in egg carotenoid levels simultaneously occurring, regardless of weight or other maternal effects this represents.

The significant findings of this thesis are that maternally derived egg carotenoids are statistically predictive of disease resistance in smolt stage Chinook salmon. Also, that the relative changes in levels of antibody and egg carotenoid during embryonic development are likely inter-related, regardless of maternal effects driving the absolute Ab levels at swim-up. This thesis provides new insight into benefits gained by salmon offspring offsetting the costs of red eggs, and together with a synthesis of the published literature, suggests some possible mechanisms of action.

Following breeding studies of the Chinook salmon phenotypes, consultations with fisheries personnel and their written records since their inception spanning the Northwest Pacific (northern California to northwest Alaska and Asia), and an extensive review of the worldwide salmonid literature, Hard et. al. (1989) proposed that egg carotenoids most likely increase the range of environments in which salmonid offspring are able to flourish. Chinook salmon, being the largest of salmonid species in adulthood, are ecologically specialized in their freshwater spawning habitats to larger freshwater drainages (Ricker

1972). White-fleshed Chinook salmon, which marine catch records indicate are consistently larger than their red-fleshed counterparts (Milne 1964; Godfrey 1975; Fraser et. al. 1982), are even further specialized in their freshwater spawning habitats. Of the 30 Chinook salmon populations examined by Hard et. al. (1989) spanning northern Oregon to northwest Alaska, those with the highest proportions of white- to red-fleshed Chinook salmon (reported as % white-flesh in brackets) spawn exclusively in either the short coastal rivers of southeast Alaska (2.6 - 37.1%), the larger coastal rivers of northern B.C. (primarily the Skeena 41.2%) or the lower tributaries of the Frasier river watershed (53.8%). Where populations predominated by red-fleshed Chinook salmon spawn in the same rivers as populations predominated by the white-flesh Chinook salmon, darker pigmented Chinook salmon eggs (indicative of red-fleshed dams) tend to be spawned either earlier in the season and/or in the upper reaches of these same rivers. Red-flesh Chinook salmon spawn throughout the rest of the Pacific Rim, whereas white-flesh Chinook salmon spawners are virtually unknown other than in those locations described. Coastal watersheds tend to maintain lower temperatures and receive higher precipitation than more inland streams, leading to conditions favouring higher water oxygenation; perhaps a requirement of white-fleshed Chinook offspring due to their relative lack of carotenoids. This theory could account for the lack of egg carotenoid-offspring survival results in hatchery experiments, as water quality (such as temperature and velocity, promoting optimal oxygenation) is typically maintained at optimal levels.

Egg carotenoid-offspring survival relationships are well established in birds (Blount et. al. 2000). With regards to the above proposed hypothesis for salmonids, bird embryos are relatively enclosed from the environment, with more limited oxygen transfer compared to salmonid eggs; and this could account for the more prevalently documented

egg carotenoid-offspring survival relationships. An integrated theory proposed by Blount et. al. (2000 and 2004) and discussed in Chapter 2 is that carotenoids are indicative of a higher overall resource base. Through integrated processes, carotenoids assist in upregulating the maternal expression of the critical egg resources vitellogenin (vtg) and very low density lipoprotein (VLDL), primary precursors of embryonic proteins and lipids (respectively). These compounds bind and transport other maternal resources, including carotenoids, to developing oocytes, dissociating as they are needed during embryonic development. The close proximity of carotenoids and other antioxidants protect precursor and formed proteins, lipids, membranes and developing systems from oxidative damage associated with the toxic by-products generated during the high metabolic rate of embryonic development (as proposed and referenced in Blount et. al. 2000 and 2004). Studies indicate that similar processes likely operate in salmonid fish (Ando et. al. 1986a, b and c). Studies have also shown that higher available levels of carotenoids during egg development and early post-hatch parental feeding in birds leads to life-long enhancement of dietary carotenoid absorption and metabolic efficiency (Fitze et. al. 2003a and b; Blount *et. al.* 2003; Koutos et. *al.* 2003). If similar mechanisms operate in salmonid fish, this could explain the 8 month time-lapse in the relationship between egg carotenoids and smolt stage vibriosis resistance; especially given that egg carotenoids were metabolised to low levels after only 4 months at swim-up. Though the freshwater feed of this study was not supplemented with carotenoids, small amounts are known to be present (pers. comm. B. Hicks, Research Co-ordinator, Taplow Feeds, Victoria, B.C.). If the carotenoid absorption capacity was more efficient in offspring of darker pigmented eggs, these offspring would have absorbed more of the limited

carotenoids from their freshwater diet than offspring of lighter pigmented eggs, resulting in greater carotenoid availability at smoltification to affect disease resistance.

Hamilton and Zuk (1982) first documented significant relationships between carotenoid-based pigmentation and lower body burdens of parasites in birds, suggesting carotenoids signal superior immune function or resistance mechanisms. A proposed mechanism involves inverse correlations between carotenoid-antioxidants and antibody, where antioxidants contribute to innate immune mechanisms, and if sufficient to ward off infection, spare the induction of costly specific immune cells and their antibody products as first proposed in the immunocompetence handicap hypothesis (Folstad and Karter 1992; Skarstein and Folstad 1996). Dietary carotenoids increase circulating carotenoid levels and boost vaccination induced antibody production (Kiss et. al. 2003). Immune activation is rapidly mirrored by reductions in circulating carotenoid levels (Faivre et. al. 2003). If carotenoid antioxidants do mediate non-specific immune responses (Amar et. al. 2004), they do so at the expense of their own oxidation. In this system, a possible trigger to up-regulate the production of antibody could be an oxidative derivative signalling the decline of carotenoid levels, and hence the need to upregulate specific antibody production. This theory could account for the observed relationship between greater percent decline (or lower remaining levels) of egg carotenoids correlating with higher offspring antibody levels.

One final line of reasoning involves sexual versus natural selection mechanisms operating on carotenoid pigmentation. Contrast the genetic polymorphisms related to carotenoid accumulation and retention in Chinook salmon with those of Sockeye/kokanee salmon. Under natural selection, Chinook salmon polymorphisms resulted in relaxed carotenoid absorption and retention mechanisms in certain populations, resulting in

distinct red- and white-flesh/egg phenotypes, even when both morphotypes receive carotenoid supplemented diets. Under sexual selection, Sockeye/kokanee salmon polymorphisms have increased carotenoid absorption and retention efficiency in the landlocked freshwater kokanee, resulting in visually indistinguishable bright red spawning colouration of both Sockeye and kokanee morphotypes; even though the diet of anandromous Sockeye are considered carotenoid rich, compared to the carotenoid poor freshwater diet of non-anandromous kokanee salmon. If the benefit of salmon egg-carotenoid pigmentation is largely to preserve offspring survival in oxidatively sub-optimal environments as proposed by Hard et. al. (1989), and red eggs incur predation costs (Wieland and Koster 1996; Godin and McDonough 2003; Hasson 2004; Van der Veen 2005), it would seem logical that populations adapted to spawn in optimal freshwater environments, such as where white-fleshed Chinook salmon are most prevalent, would most readily undergo selection for reduced egg carotenoid pigmentation, especially if carotenoid pigments are not being maintained by sexual selection pressures.

4.2 Future Research

One line of future research would be to investigate cost-benefit relationships of egg carotenoid pigmentation, as well as the theory that salmon egg carotenoids maintain offspring survival in sub-optimal conditions. Potential costs could be examined by a survey of the spawning streams of predominantely white- and red-fleshed Chinook salmon for the relative prevalence of common egg predators. If spawning streams where the white-flesh phenotypes prevail are found to have higher incidences of predators this could help drive natural selection toward reduced egg pigmentation. Potential benefits could be tested by obtaining Chinook salmon eggs from a wide range of pigmentation

levels. Subsets of each maternal egg batch could be analyzed for carotenoid levels and the rest fertilized and incubated under either control conditions or exposed to environmental stress, such as: different water quality parameters (e.g. temperature, flow rate, oxygenation level), or exposed to common pathogens of Chinook salmon eggs (e.g. fungus) or offspring (e.g. bacterial, Sauter et. al. 1987) and survival rates measured. Results would provide information either supporting or disproving Hard et. al.'s (1989) hypothesis, and provide insight into the cost-benefit relationships involved in red egg pigmentation during embryonic development in salmon.

Another line of research could investigate the plasticity of carotenoid pigment expression under sexual selection. If commercial hatcheries for Sockeye and/or Kokanee salmon exist, these would represent populations were sexual selection for carotenoid skin pigments is reduced or non-existent due to artificial spawning techniques commonly employed in hatcheries. Investigations could then examine responses of each morphotype to high and low dietary carotenoid levels by examining pigments in the flesh, skin and eggs for evidence of relaxed selection on carotenoid absorption and retention capacities. This would provide evidence of the effects of evolutionary selection mechanisms operating on carotenoid physiology in salmonids.

In conclusion the results of this thesis indicate that higher egg carotenoid levels provide offspring with the benefits of increased resistance to bacterial pathogen challenge, in particular vibriosis. Additionally, results in embryonic salmon support previous work with adult salmonids, birds and mammals that levels of antibody and carotenoids tend to be related in a complex, inverse fashion. Taken collectively these results suggest that egg carotenoids enhance survival in stress related conditions, such as with pathogen encounter, at least in part through interaction with immunological

mechanisms. This is perhaps one of the benefits maintaining red carotenoid-based pigmentation in the majority of salmonid species, phenotypes and populations worldwide.

4.3 References

Amar,E.C.; V. Kiron; S. Satoh and T. Watanabe. 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish and Shellfish Immunology 16: 527-537.

Amcoff, P.; H. Börjeson; R. Eriksson and L. Norrgren 1998. Effects of thiamine treatments on survival of M74-affected feral Baltic salmon, Chapter 5. *In* McDonald, G.; J.D. Fitzsimons and D.C. Honeyfield (ed.s). Early Life Stage Mortality Syndrome in Fishes of the Great Lakes and Baltic Sea. American Fisheries Society, symposium 21, Bethesda, Maryland, p. 31-40.

Ando,S. and M.Hatano, 1986a. Carotenoids in an egg yolk protein of chum salmon (*Oncorhynchus keta*). Agric. biol. chem. 50: 1043-1044.

Ando,S., T.Takeyama, and M.Hatano, 1986b. Transport associated with serum vitellogenin of carotenoids in chum salmon (*Oncorhynchus keta*). Agricultural Biological Chemistry 50: 557-563.

Ando,S., T.Takeyama, and M.Hatano, 1986c. Isolation and characterization of a carotenoid-carrying lipoprotein in the serum of chum salmon (*Oncorhynchus keta*) during spawning migration. Agricultural Biological Chemistry 50: 907-914.

Blanc J.-M. and G. Choubert 1993. Genetic variation of flesh color in pan-size rainbow trout fed astaxanthin. Journal of Applied Aquaculture 2(1): 115-123.

Blount,J.D., D.C.Houston, and A.P.Møller, 2000. Why egg yolk is yellow. Trends in Ecology and Evolution 15: 47-49.

Blount,J.D.; N.B. Metcalfe; K.E. Arnold; P.F. Surai; G.L. Devevey and P. Monaghan 2003. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. Procceedings of the Royal Society of London B 270: 1691-1696.

Blount, J.D. 2004. Carotenoids and life-history evolution in animals. Archives of Biochemistry and Biophysics 430: 10-15.

Christiansen,R., J.Glette, Ø.Lie, O.J.Torrissen, and R.Waagbø, 1995. Antioxidants and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. Journal of Fish Diseases 18: 317-328.

Craig, J.K. and C.J. Foote 2001. Countergradient variation and secondary sexual color: Phenotypic convergence promotes genetic divergence in carotenoid use between sympatric anadromous and nonanadromous morphs of sockeye salmon (*Oncorhynchus nerka*). Evolution 55(2): 380-391.

Craig, J.K.; C.J. Foote and C.C. Wood 2005. Countergradient variation in carotenoid use between sympatric morphs of sockeye salmon (Oncorhynchus nerka) exposes nonanadromous hybrids in the wild by their mismatched spawning colour. Biological Journal of the Linnean Society 84(2): 287-305.

Craik,J.C.A. 1985. Egg quality and egg pigment content in salmonid fishes. Aquaculture 47: 61-88.

Faivre,B., A.Grégoire, M.Préault, F.Cézilly, and G.Sorci, 2003. Immune activation rapidly mirrored in a secondary sexual trait. Science 300: 103.

Fitze,P.S.; M. Kölliker and H. Richner 2003a. Effects of common origin and common environment on nestling plumage coloration in the great tit (*Parus major*). Evolution 57(1): 144-150.

Fitze,P.S.; B. Tschirren and H. Richner 2003b. Carotenoid-based colour expression is determined early in nestling life. Oecologia 137: 148-152.

Folstad, I. and A.J. Karter 1992. Parasites, bright males and the immunocompetence handicap. The American Naturalist 139(4): 603-622.

Foote, C.J.; G.S. Brown and C.W. Hawryshyn 2004. Female colour and male choice in sockeye salmon: implications for the phenotypic convergence of anadromous and nonanadromous morphs. Animal Behaviours 76: 69-83.

Fraser, F.J.; P.J. Starr; and A.Y. Rederenko 1982. Review of the Chinook and coho salmon of the Fraser River. Canadian Technical Report, Fisheries and Aquatic Sciences 1126. 130 p.

Godfrey, H. 1975. Review of the occurrence of red- and white-fleshed Chinook salmon in British Columbia with particular reference to Fraser River fish present off the west coast of Vancouver Island. Fisheries Research Board of Canada Manuscript Report Series 1359: 49 p.

Godin,J.-G.J. and E.McDonough, 2003. Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioual Ecology 14: 194-200.

Goodwin,T.W., 1986. Metabolism, nutrition and function of carotenoids. Annual Review of Nutrition 6: 273-297.

Hamilton, W.D. and M. Zuk 1982. Heritable true fitness and bright birds: a role for parasites? Science 218: 382-387.

Hard 1986. Flesh color variation in Chinook salmon (*Oncorhynchus tshawytscha*) at Little Port Walter, Alaska. National Oceanic and Atmospheric Administration, Technical Memorandum, National Marine Fisheries Service, Northwest Fisheries Commission 109: 1-31.

Hard, J.J.; A.C. Wertheimer and W.F. Johnson 1989. Geographic variation in the occurrence of red- and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) in Western North America. Canadian Journal of Fisheries and Aquatic Sciences 46: 1107-1113.

Hasson, L.A. 2004. Plasticity in pigmentation induced by conflicting threats from predation and UV radiation. Ecology 85(4):1005-1016.

Hornung, M.W.; L. Miller; R.E. Peterson; S. Marcquenski and S.B. Brown 1998. Efficacy of thiamine, astaxanthin, β-carotene, and thyroxine treatments in reducing early mortality syndrome in Lake Michigan salmonid embryos, Chapter 13 in McDonald, G.; Fitzsimons, J.D. and Honeyfield, D.C. (ed.s) Early Life Stage Mortality Syndrome in

Fishes of the Great Lakes and Baltic Sea. American Fisheries Society, symposium 21, Bethesda, Maryland, p. 124-134.

Kiss,Z., L.Bárdos, C.Szabó, L.Lengyel, and M.Szabó, 2003. Effect of β-carotene supplementation on plasma and yolk IgY levels induced by NDV vaccination in Japanese quail. International Journal of Vitamin Nurtitional Research 73: 285-289.

Koutsos,E.A.; A.J. Clifford; C.C. Calvert and K.C. Klasing 2003. Maternal carotenoid status modifies the incorporation of dietary carotenoids into immune tissues of growing chickens (*Gallus gallus domesticus*). Journal of Nutrition 133(4): 1132-1138.

Milne, D.J. 1964. The Chinook and coho salmon fisheries of British Columbia. Bulletin of the Fisheries Research Board of Canada, 142: 46p.

Olson, V.A. and Owens, I.P.F. 1989. Costly sexual signals: are carotenoids rare, risky or required? Trends in Evolution and Ecology 13(12): 510-514.

Palace, V.P.; Brown, S.B.; Baron, C.L.; Fitzsimons, J.; Woodin, B.; Stegeman, J.J. and Klaverakmp, J.F. 1998. An evaluation of the relationships among oxidative stress, antioxidant vitamins and early mortality syndrome (EMS) of lake trout (*Salvelinus namaycush*) from Lake Ontario. Aquatic Toxicology 43: 195-208.

Pettersson, A. and Lignell, A. 1999. Astaxanthin deficiency in eggs and fry of Baltic salmon (*Salmo salar*) with the M74 syndrome. Ambio 28(1): 43-47.

Ricker, W.D. 1972. Hereditary and environmental factors affecting certain salmonid populations, p. 19-60. In R.C. Simon and P.A. Larkin (ed.) The stock concept in Pacific salmon. H.R. MacMillan Lectures in Fisheries, University of British Columbia, B.C.

Sauter, R.W.; C. Williams; E.A. Meyer; B. Celnik; J.L. Banks and D.A. Leith 1987. A study of bacteria present within unfertilized salmon eggs at the time of spawning and their possible relation to early lifestage disease. Journal of Fish Diseases 10: 193-203.

Skarstein,F. and I.Folstad, 1996. Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. OIKOS 76: 359-367.

Van der Veen, I.T. 2005. Costly carotenoids: a trade-off between predation and infection risk? Journal of Evolutionary Biology 18: 992-999.

Wood, C.C. and C.J. Foote 1996. Evidence for sympatric genetic divergence of anadromous and nonanadromous morphs of Sockeye salmon (Oncorhynchus nerka). Evolution 50(3): 1265-1279.

Wieland, K. and Koster, F.W. 1996. Size and visibility of Baltic cod eggs with reference to size-selective and stage-dependent predation mortality. Journal of Applied Ichthyology 12(2): 83-89.

Withler, R.E. 1986. Genetic variation in carotenoid pigment deposition in the red-fleshed and white-fleshed Chinook salmon (*Oncorhynchus tshawytscha*) of Quesnel River, British Columbia. Canadian Journal of Genetic Cytology 28: 87-594.

APPENDIX 1:

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 816 (2005) 49–56
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of carotenoids and all-*trans*-retinol in fish eggs by liquid chromatography–electrospray ionization–tandem mass spectrometry

Hongxia Li, Sélène T. Tyndale, Daniel D. Heath, Robert J. Letcher*

Great Lakes Institute for Environmental Research (GLIER), University of Windsor, 401 Sunset Avenue, Windsor, Ont., Canada N9B 3P4

Received 24 June 2004; accepted 3 November 2004
Available online 28 November 2004

Abstract

A novel method was developed for the combined determination of carotenoids and retinoids in fish eggs, which incorporates prior analyte isolation using liquid-liquid partitioning to minimize analyte degradation, and fraction analysis using high-performance liquid chromatography–electrospray (positive)–quadrupole mass spectrometry (LC–ESI(+)–MS; SIM or MRM modes). Eggs from Chinook salmon (*Oncorhynchus tshawytscha*) were used as the model fish egg matrix. The methodology was assessed and validated for β-carotene, lutein, zeaxanthin, and β-cryptoxanthin (molecular ion radicals $[M]^{+\bullet}$), canthaxanthin and astaxanthin ($[M+Na]^+$ adducts) and all-*trans*-retinol ($[(M+H)-H_2O]^+$). Using replicate egg samples ($n=5$) spiked with β-cryptoxanthin and β-carotene before and after extraction, matrix-sourced ESI(+) enhancement was observed as evidenced by comparable %matrix effect and %process efficiency values for β-cryptoxanthin and β-carotene of 114–119%. In aquaculture-raised eggs from adult Chinook salmon astaxanthin, all-*trans*-retinol, lutein and canthaxanthin were identified and determined at concentrations of 4.12, 1.06, 0.12 and 0.45 μg/g (egg wet weight), respectively. To our knowledge, this is the first report on a method for LC–MS determination of carotenoids and retinoids in a fish egg matrix, and the first carotenoid-specific determination in any fish egg sample.

Keywords: LC–electrospray–tandem (quadrupole) MS; Carotenoids; Retinoids; Astaxanthin; Fish eggs; Chinook salmon

APPENDIX 2: Detailed Results of all Statistical Analyses

Table 2.1: Comparison of offspring survival between families of, WF = white-flesh, RFw = red-flesh wild and RFd = red-flesh domestic maternal Chinook salmon origins

a) ANOVA results comparing offspring survival of different maternal origins

incubation survival stage	measure	origin	N	mean	sd
(i) unfertilized to eyed egg	%	WF - a	8	76.241	9.828
		RFw - b	10	92.138	6.234
		RFd - a	9	81.899	10.021
				F = 7.787, p = 0.002	
RFw significantly different from WF (p =0.004) and from RFd (p =0.049) using Tukey's HSD test					
(ii) eyed egg to swim-up	%	WF	10	88.615	7.94
		RFw	10	96.172	1.83
		RFd	9	88.828	12.55
		variances non-homogeneous, data transformations required			
	arcsin	WF	10	1.108	0.15
		RFw	10	1.301	0.068
		RFd	9	1.154	0.26
		variances non-homogeneous, data transformations required			
	log	WF	10	1.946	0.043
		RFw	10	1.983	0.008
		RFd	9	1.944	0.067
		variances non-homogeneous, data transformations required			
	rank	WF	10	9.1	4.7
		RFw	10	20.4	5.7
		RFd	9	15.6	10.6
		variances non-homogeneous, data transformations required			

(b) t-test results comparing Chinook salmon offspring survival from eyed egg to swim-up of different maternal origin groups

groups compared	measure	groups compared	N	mean	sd
(i) red- vs white-fleshed	%	WF	10	88.615	7.94
		RF(w+d)	19	92.698	9.27
				t = -1.818, p = 0.248	
(ii) wild vs domestic	%	wild (WF+RFw)	20	92.394	6.82
		domestic	9	88.838	12.55
		variances non-homogeneous, data transformations required			
	arcsin	wild (WF+RFw)	20	1.204	0.15
		domestic	9	1.154	0.26
		variances non-homogeneous, data transformations required			
	log	wild (WF+RFw)	20	1.964	0.035
		domestic	9	1.944	0.067
		variances non-homogeneous, data transformations required			
	rank	wild (WF+RFw)	20	14.8	7.7
		domestic	9	15.6	10.6
				t = -0.232, p = 0.819	

Table 3.1a: Statistical comparisons of carotenoid levels (ug/g) in the eggs and offspring of Chinook salmon between maternal origins

i) T-test results comparing carotenoid concentrations (ug/g) in Chinook salmon eggs and offspring comparisons between maternal white- and red-fleshed phenotypes

development stage	phenotype	N	mean	sd
unfertilized eggs	WF	10	1.619	1.536
	RFw	10	2.893	1.063
				t = -2.157; p =0.045
eyed eggs	WF	10	0.944	0.899
	RFw	10	2.155	0.782
				t =-3.213; p =0.005
swim-up	WF	10	0.478	0.553
	RFw	10	0.807	0.616
			t =1.578; p =0.225	

ii) ANOVA results comparing carotenoid concentrations (ug/g) in Chinook salmon eggs and offspring between maternal origins of wild white- and red-fleshed phenotypes and a domestic red-fleshed population.

development stage	phenotype	N	mean	sd
unfertilized eggs	WF	10	1.619	1.536
	RFw	10	2.893	1.063
	RFd	9	3.002	1.637
				F = 2.846; p =0.076
eyed eggs	WF - a	10	0.944	0.899
	RFw - b	10	2.155	0.782
	RFd - a	9	1.286	0.522
			F = 6.743; p =0.004	
swim-up	WF	10	0.478	0.553
	RFw	10	0.807	0.616
	RFd	9	0.621	0.699
			F = 0.702; p =0.505	

RFw significantly different from WF (p =0.004) and from RFd (p =0.049) using Tukey's HSD test.

Table 3.1b: Statistical comparison of the decline in carotenoids through embryonic incubation in Chinook salmon eggs and offspring between maternal origins.

i) T-test results of absolute change in carotenoid levels (ug/g) between wild white- and red-fleshed phenotypes

development stage	phenotype	N	mean	sd
Δ caro unfert-eyed egg	WF	10	0.675	0.736
	RFw	10	0.738	0.458
			t = -0.230; p = 0.821	
Δ caro unfert-swim up	WF	10	1.141	1.342
	RFw	10	2.086	1.178
			t = -1.674; p = 0.111	

ii) ANOVA results comparing absolute change in carotenoid levels (ug/g) between wild white- and red-fleshed phentypes and a domestic population

development stage	maternal origin	N	mean	sd
Δ caro unfert-eyed egg	WF	10	0.675	0.736
	RFw	10	0.738	0.458
	RFd	9	1.612	1.988
			variances non-homogeneous, transformation required	
log (Δu-e + 1)	WF	10	0.196	0.151
	RFw	10	0.226	0.116
	RFd	9	0.337	0.258
			F = 1.54; 0 =0.233	
Δ caro unfert-swim up	WF	10	1.14	1.34
	RFw	10	2.08	1.17
	RFd	9	2.48	1.8
			F = 2.17; p =0.133	

iii) T-test results comparing % decline in carotenoid concentration (ug/g) of Chinook salmon eggs and offspring between wild white- and red-fleshed maternal phenotypes

development stage	phenotype	N	mean	sd
unfert-eyed egg	WF	10	42.640	14.362
	RFw	10	24.265	12.973
			t = 3.002; p = 0.008	
unfert-swim up	WF	10	68.568	17.398
	RFw	10	69.871	22.113
			t = -0.147; p = 0.885	

Table 3.1b (continued): Statistical comparison of the decline in carotenoids through embryonic incubation in Chinook salmon eggs and offspring between maternal origins.

iv) ANOVA results comparing % decline in carotenoid concentration (ug/g) of Chinook salmon eggs and offspring between wild white-, wild red- and domestic red-fleshed maternal origins

development stage	maternal origin	N	mean	sd
% Δ unfert-eyed egg	WF - a	10	42.640	14.362
	RFw - ab	10	24.265	12.973
	RFd - ac	9	42.827	29.438
		variances non-homogeneous, transformation required		
$\sin^{-1}$ (% Δ unfert-eyed egg)	WF - a	10	0.447	0.164
	RFw - ab	10	0.247	0.137
	RFd - ac	9	0.481	0.378
		variances non-homogeneous, transformation required		
log (% Δ unfert-eyed egg)	WF - ab	10	1.619	0.141
	RFw - c	10	1.347	0.243
	RFd - bc	9	1.552	0.3
			$F = 3.646; p = 0.040$	
		WF and RFw significantly different ($p = 0.039$) using Tukey's HSD test		
development stage	**phenotype**	**N**	**mean**	**sd**
% Δ unfert-swim up	WF	10	68.568	17.398
	RFw	10	69.871	22.113
	RFd	9	78.319	23.803
			$F = 0.582; p = 0.566$	

Table 3.2a: Statistical comparisons of antibody levels in the eggs and offspring of Chinook salmon.

i) T-test results comparing measures of antibody in Chinook salmon eggs and offspring between maternal wild white- and red-fleshed phenotypes

development stage	measure	phenotype	N	mean	sd
unfertilized eggs	Ab (ug/g)	WF	9	1.859	1.726
		RFw	9	0.47	0.859
		variances non-homogeneous, transformation required			
	log (Ab (ug/g) +1)	WF	9	0.377	0.286
		RFw	9	0.128	0.172
		variances non-homogeneous, transformation required			
	rank Ab	WF	9	16	7.18
		RFw	9	8.78	6.7
				$t = 2.206; p = 0.042$	
swim-up	Ab (ug/g)	WF	8	1.683	2.02
		RFw	9	1.088	1.38
				$t = 0.717; p = 0.484$	

ii) ANOVA results comparing measures of antibody in Chinook salmon eggs and offspring from wild white-fleshed, wild red-fleshed and domestic red-fleshed maternal origins

development stage	measure	phenotype	N	mean	sd
unfertilized eggs	Ab (ug/g)	WF	9	1.859	1.726
		RFw	9	0.4698	0.859
		RFd (-outliers)	6	0.3089	0.152
		variances non-homogeneous, transformation required			
	log (Ab (ug/g) +1)	WF	9	0.377	0.286
		RFw	9	0.128	0.172
		RFd (-outliers)	6	0.1145	0.235
		variances non-homogeneous, transformation required			

a) Kruskal-Wallis comparison of egg antibody level rank orders between maternal Chinook salmon maternal origins, with outliers

development stage	measure	Maternal origin	N	median
unfertilized egg	rank Ab	WF	9	16.33
		RFw	9	8.56
		RFd	8	15.88
			$X^2 = 5.768$, asymptotic p = 0.056	

ai) Kruskal-Wallis comparison of egg antibody level rank orders between maternal Chinook salmon maternal origins, with outliers

unfertilized egg	rank Ab	WF	9	16.33
		RFw	9	8.56
		RFd	6	12.67
			$X^2 = 5.449$, asymptotic p = 0.066	

swim-up	Ab (ug/g)	WF	8	1.683	2.01
		RFw	9	1.089	1.38
		RFd	6	15.975	11.96
		variances non-homogeneous, transformation required			
	log (Ab (ug/g) +1)	WF	9	0.745	1.387
		RFw	9	0.239	0.274
		RFd	6	1.112	0.373

Table 3.2b: Statistical comparison of the change in antibody through embryonic incubation in Chinook salmon eggs and offspring between maternal origins.

i) T-test results examining the change in antibody in Chinook salmon from eggs to swim-up between white- and red-fleshed maternal phenotypes

development stage	measure	phenotype	N	mean	sd
egg-swim	Δ Ab conc	WF	7	-0.129	1.569
		RFw	8	0.715	1.942
				t = -0.916; p = 0.376	
	Δ Ab %	WF	7	70.48	70.998
		RFw	8	1157.839	1515.18
		variances non-homogeneous, transformation required			
	arcsin (%Δ Ab)	WF	7	doesn't work for % increase	
		RFw	8		
	log (Δ % Ab +1)	WF	7	1.471	0.79
		RFw	8	2.581	0.778
				t = -2.739; p = 0.017	

ii) ANOVA results examining the change in antibody levels of Chinook salmon from eggs to swim-up between wild white- and red-fleshed phenotypes and a domestic population.

development stage	measure	phenotype	N	mean	sd
egg-swim	Δ Ab conc	WF	7	-0.129	1.569
		RFw	8	0.715	1.942
		RFd	6	13.39	0.311
		variances non-homogeneous, transformation required			
	log |(Δ Ab conc)+1|	WF - a	7	0.135	0.209
		RFw - a	8	0.233	0.323
		RFd - b	6	1.067	0.337
				F = 19.295; p = 0.000	
	RFd significantly different from WF (p=0.000) and RFd (p=0.000) using Tukey's HSD test				
	Δ Ab %	WF	7	70.48	70.998
		RFw	8	1157.839	1515.18
		RFd	5	3233.741	29.33.719
		variances non-homogeneous, transformation required			
	log (% Δ Ab +1)	WF - a	7	1.471	0.79
		RFw - b	8	2.581	0.778
		RFd - b	6	3.273	0.624
				F=9.872; p=0.001	
	WF significantly different from RFw (p=0.025) and RFd (p=0.001) using Tukey's HSD test				

Table 3.3: Hierarchical OLS regression of immunological parameters on maternally derived egg carotenoids in Chinook salmon eggs and offspring.

3.3 a) Regression analyses examining relationships between Chinook salmon egg/offspring antibody levels (ug/g) and measures of carotenoids and eyed egg wt. to account for maternal effects

				Model Summary				
Development stage	**Dependent variables**	**Hierarchical Model**	**independent variables**	**r**	**$p_{(1)}$**	**N**	**R^2**	**$p_{(2)}$**
i) unfertilized eggs	Ab (ug/g)	1	Car (ug/g) unfer	-0.112	0.201	24	0.013	0.602
		2	Car (ug/g) unfer			23	0.101	0.346
			wt (g) eye	-0.317	0.07			
ii) swim-up offspring	Ab (log+1)	1	Car unfertilized egg (ug/g)	0.229	0.136	25	0.052	0.271
		2	Car unfertilized egg (ug/g)			23	0.341	0.016
			wt (g) eye	-0.499	0.008			
		1	Car eye (ug/g)	-0.233	0.142	25	0.05	0.284
		2	Car eye (ug/g)			23	0.252	0.055
			wt (g) eye	-0.499	0.008			
		1	Car swim-up (ug/g)	-0.398	0.024	25	0.158	0.049
		2	Car swim-up (ug/g)			23	0.323	0.02
			wt (g) eye	-0.499	0.008			
		1	Δ car u-e (log + 1)	0.378	0.031	25	0.378	0.062
		2	Δ car u-e (log + 1)			23	0.384	0.008
			wt (g) eye	-0.499	0.008			
		1	Δ caro u-s	0.37	0.034	25	0.137	0.068
		2	Δ caro u-s			23	0.404	0.006
			wt (g) eye	-0.499	0.008			
		1	% Δ car u-e (log+1)	0.261	0.104	25	0.068	0.208
		2	% Δ car u-e (log+1)			23	0.288	0.034
			wt (g) eye	-0.499	0.008			
		1	% Δ car u-s	0.454	0.011	25	0.207	0.022
		2	% Δ car u-s			23	0.39	0.007
			wt (g) eye	-0.499	0.008			

Table 3.3 b) Regression analyses examining relationships between Chinook salmon egg to offspring change in antibody levels and measures of carotenoids and eyed egg wt. to account for maternal effects

			Model Summary				
Dependent variables	**Hierarchical Model**	**independent variables**	**r**	**$p_{(1)}$**	**N**	**R^2**	**$p_{(2)}$**
Δ Ab (log+1)	1	Car unfert egg (ug/g)	0.202	0.178	23	0.041	0.356
	2	Car unfert egg (ug/g)			21	0.109	0.353
		wt (g) eye	-0.23	0.158			
	1	Car eye (ug/g)	-0.244	0.131	23	0.059	0.262
	2	Car eye (ug/g)			21	0.085	0.451
		wt (g) eye	-0.23	0.158			
	1	Car swim-up (ug/g)	-0.427	0.021	23	0.183	0.042
	2	Car swim-up (ug/g)			21	0.196	0.14
		wt (g) eye	-0.23	0.158			
	1	car Δ u-e (log+1)	0.356	0.048	23	0.127	0.096
	2	car Δ u-e (log+1)			21	0.176	0.175
		wt (g) eye	-0.23	0.158			
	1	caro Δ u-s	0.347	0.052	23	0.121	0.105
	2	caro Δ u-s			21	0.181	0.165
		wt (g) eye	-0.23	0.158			
	1	% car Δ u-e (log+1)	0.262	0.114	23	0.069	0.227
	2	% car Δ u-e (log+1)			21	0.108	0.359
		wt (g) eye	-0.23	0.158			
	1	% car Δ u-s	0.434	0.019	23	0.188	0.039
	2	% car Δ u-s			21	0.213	0.115
		wt (g) eye	-0.23	0.158			
ii) % Δ Ab (log+1)	1						
		Car unfert egg (ug/g)	0.087	0.347	23	0.008	0.693
	2	Car unfert egg (ug/g)			21	0.008	0.928
		wt (g) eye	0.039	0.434			
	1	Car eye (ug/g)	-0.098	0.329	23	0.01	0.659
	2	Car eye (ug/g)			21	0.015	0.871
		wt (g) eye	0.039	0.434			
	1	Car swim-up (ug/g)	-0.365	0.044	23	0.133	0.087
	2	Car swim-up (ug/g)			21	0.146	0.207
		wt (g) eye	0.039	0.434			
	1	log (Δu-e + 1)	0.203	0.177	23	0.041	0.354
	2	log (Δu-e + 1)			21	0.044	0.635
		wt (g) eye	0.039	0.434			
	1	Δ caro u-s	0.213	0.165	23	0.045	0.33
	2	Δ caro u-s			21	0.046	0.654
		wt (g) eye	0.039	0.434			
	1	log (% car Δ u-e)	0.289	0.091	23	0.083	0.181
	2	log (% car Δ u-e)			21	0.089	0.432
		wt (g) eye	0.039	0.434			
	1	% car Δ u-s	0.366	0.043	23	0.134	0.086
	2	% car Δ u-s			21	0.145	0.245
		wt (g) eye	0.039	0.434			

Independent co-variate	Hierarchical Model	independent variables	r	$p_{(1)}$	Model Summary N	R^2	$p_{(2)}$
i) levels of carotenoids and Antibody	1	Car (ug/g) unfer	0.428	0.021	20	0.183	0.041
	2	Car (ug/g) unfer			20	0.337	0.031
		Ab (ug/g) unfer	0.299	0.101			
	3	Car (ug/g) unfer			19	0.346	0.087
		Ab (ug/g) unfer					
		wt (g) eye	0.102	0.339			
	1	Car (ug/g) unfer	0.428	0.021	23	0.183	0.041
	2	Car (ug/g) unfer			21	0.233	0.092
		Ab rank unfert	0.205	0.187			
	3	Car (ug/g) unfer			21	0.236	0.194
		Ab rank unfert					
		wt (g) eye	0.032	0.446			
	1	Car eye (ug/g)	0.444	0.017	20	0.197	0.034
	2	Car eye (ug/g)			20	0.322	0.037
		Ab (ug/g) unfer	0.299	0.101			
	3	Car eye (ug/g)			19	0.324	0.109
		Ab (ug/g) unfer					
		wt (g) eye	0.102	0.339			
	1	Car swim-up (ug/g)	0.154	0.242	23	0.024	0.484
	2	Car swim-up (ug/g)			21	0.03	0.761
		Ab swim-up (log+1)	-0.134	0.281			
	3	Car swim-up (ug/g)			21	0.032	0.902
		Ab swim-up (log+1)					
		wt (g) eye	0.032	0.446			
ii) change in carotenoid (log (ug/g)+1) from unfertilized to eyed egg, rank order of unfertilized egg antibody and eyed egg weight	1	Carotenoid	0.305	0.079	23	0.093	0.158
	2	Δ car u-e (log + 1)			21	0.165	0.197
		Ab unfer rank	-0.134	0.281			
	3	Δ car u-e (log + 1)			21	0.18	0.323
		Ab unfer rank					
		wt (g) eye	0.032	0.446			
	1	Δ caro u-s	0.395	0.031	23	0.156	0.062
	2	Δ caro u-s			19	0.389	0.019
		Δ Ab conc |(log+1)|	-0.316	0.094			
	3	Δ caro u-s			21	0.403	0.046
		Δ Ab conc |(log+1)|					
		wt (g) eye	0.032	0.446			
iii) log (%) change in (a) carotenoid levels from unfertilized to eyed egg stages, (b) antibody to swim-up and	1	Carotenoid	-0.058	0.397	23	0.003	0.794
	2	Carotenoid			19	0.104	0.417
		Antibody to swim-up	0.316	0.094			
	3	Carotenoid to eyed egg			21	0.105	0.633
		Antibody to swim-up					
		Eyed egg weight	0.032	0.446			

(c) eyed egg weight							
iv) log (%) change in carotenoid and antibody levels from unfertilized egg to swim-up stage and eyed egg weight	1	Carotenoid to swim-up	0.16	0.233	23	0.026	0.465
	2	Carotenoid to swim-up			19	0.225	0.13
		Antibody to swim-up	-0.357	0.067			
	3	Carotenoid to swim-up			21	0.237	0.242
		Antibody to swim-up					
		Eyed egg weight	0.032	0.446			

Table 3.3 c) OLS regression analyses examining relationships between offspring disease resistance and measures of carotenoids, antibody and maternal effects as eyed egg weight (g) in Chinook salmon egg and offspring

Appendix 3: Sample calculations for conversion of raw ELISA data into sample antibody concentrations (ug/g)

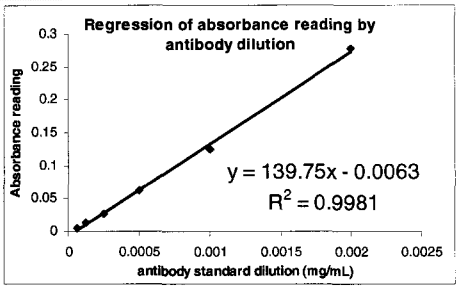
ELISA raw absorption data

	blank	std Ab dilution	Sample Q3-4
replicate 1	0.106	0.108[b]	0.104[c]
	0.11	0.119	0.112
	0.109	0.136	0.127
	0.112	0.175	0.146
	0.11	0.229	0.176
	0.105	0.383	0.272
replicate 2	blank	Ab	Q3-4
	0.094	0.106[b]	0.1[c]
	0.103	0.111	0.11
	0.102	0.126	0.116
	0.112	0.166	0.136
	0.105	0.214	0.163
	0.106	0.388	0.269
replicate 3	blank	Ab	Q3-4
	0.104	0.12[b]	0.112[c]
	0.105	0.129	0.12
	0.098	0.137	0.141
	0.103	0.164	0.152
	0.108	0.25	0.193
	0.109	0.378	0.259

Blank ave 0.105611[a]

abs vs dilution regression data

std dilution x (Ab mg/mL)	absorbance y (adj abs)
0.000062	0.0057[(b-a)]
0.000125	0.0141
0.00025	0.0274
0.0005	0.0627
0.001	0.1463
0.002	0.2774
range	**0.0057 - 0.2774**



Ab conc'n = (adj abs +0.0062)/139.75

calculation of antibody (ug/g) in sample from data

sample dilution	adj abs	Ab conc'n (diluted)	Multiplied by dilution factor
0.03125	-2.77778E[(c-a)]	too low	
0.0625	0.008388	1.05108E	6.56927E
0.125	0.022388	2.05287E	2.56609E
0.25	0.039055	3.24548E	8.11370E
0.5	0.071722	5.58299E	2.79149E
1	0.161055	0.001197	0.001197
	average of dilutions smpl (mg/mL)		3.18010E
conversion: mg/mL sol'n to ug/g sample			3.180103

antibody level of sample Q3-4 = 3.18 ug Ab / g sample

Vita Auctoris

Sélène Tracy Tyndale was born & raised in Montréal, Québéc, Canada until moving to Calgary, Alberta, where she graduated from Sir Winston Churchill High School in 1988 with an Advanced-Science High School Diploma. She spent the next several years travelling throughout North America playing music and operating partnership & personal small businesses in the food, craft & horticulture industries. Then, while working as a Recreation Therapy Assistant in Rehabilitative Nursing Facilities, she began her post-secondary academic career as a mature, part-time student and obtained a University-Transfer Associate of Science Degree with Phi Theta Kappa Honour's in 1995 from Seattle Central Community College (associated with the University of Washington), Seattle, Washington, USA. Following the birth of her son in 1998 she moved to Windsor, Ontario, Canada where, with the help of family childcare assistance, returned to academia. She completed an Honour's Batchelor of Science Degree with Research and Thesis, with a major in Biological Sciences and a minor in Biochemistry with Distinction from the University of Windsor in the Fall of 2002, while simultaneously beginning graduate work at the Great Lakes Institute for Environmental Research, University of Windsor. Having passed her defense with all revisions approved, she will graduate with a Master's of Science Degree from the Department of Biological Sciences, University of Windsor in the Fall of 2005. Her immediate plans include working in wildlife or nature conservation and research while paying off her student loans, following which she and her partner plan to start an international eco-tourism business that will encompass both recreation and education concerning sensitive ecosystem issues and localities worldwide.