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Why are salmon red? Proximate and ultimate causes of flesh pigmentation in Chinook salmon

Sarah Jean Lehnert
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WHY ARE SALMON RED? PROXIMATE AND ULTIMATE CAUSES OF FLESH PIGMENTATION IN CHINOOK SALMON

By

Sarah Jean Lehnert

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Great Lakes Institute for Environmental Research
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the Degree of Doctor of Philosophy
at the University of Windsor

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2016

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Why are salmon red? Proximate and ultimate causes of flesh pigmentation in Chinook salmon

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19 December 2016
DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is the result of joint research, as follows:

I am the principle author on all chapters, and I primarily developed the major ideas, experimental design, executed the sampling and laboratory work and completed the statistical analyses, interpretation and writing. Drs. Daniel Heath, Trevor Pitcher and Robert Devlin are co-authors on all data chapters (Chapter 2-6) as they contributed to the key ideas, experimental design, interpretation, editing of the manuscript and provided funding and logistical support for the research described therein. For Chapter 2, parts of the experimental design and sampling were completed by co-authors Drs. Robert Devlin and Wendy Vandersteen, who also contributed to the interpretation and editing of the manuscript. Additionally, co-author Dr. John Heath provided facilities and logistical support and contributed to the editing of the manuscript. For Chapter 3, co-author Dr. Christina Semeniuk contributed to the experimental design, as well as interpretation and editing of the manuscript. For Chapter 4, co-authors Dr. Kyle Garver and Jon Richard contributed to the experimental design, provided expertise, facilities and logistical support and contributed to the interpretation and editing of the manuscript.

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ABSTRACT

Carotenoids are responsible for the characteristic red eggs, skin and flesh of salmonids. Although carotenoids are thought to provide salmon with many benefits, carotenoid pigmentation has not evolved in all, or even the majority of fishes, thus highlighting our lack of understanding of the evolutionary costs and benefits associated with the pigment. In nature, Chinook salmon (*Oncorhynchus tshawytscha*) exhibit extreme variation in carotenoid utilization due to genetic polymorphisms that affect carotenoid deposition into the flesh, skin and eggs, consequently resulting in two colour morphs (red and white). Chinook salmon are thus an ideal model species to study carotenoid pigmentation evolution. Using red and white Chinook salmon, I examined the proximate (genetic) and ultimate (fitness) mechanisms involved in carotenoid pigmentation. By examining these mechanisms, my thesis also determined evolutionary processes responsible for maintaining this unique colour polymorphism.

In my thesis, I first examined the proximate mechanisms responsible for carotenoid pigmentation using a genome-wide association study, where I identified 90 single nucleotide polymorphisms (SNPs) associated with carotenoid pigmentation. Several SNPs mapped to locations in the Atlantic salmon (*Salmo salar*) genome near candidate genes for pigmentation, including genes associated with carotenoid absorption, metabolism and binding. This work therefore showed that several genes throughout the genome are responsible for carotenoid colouration in Chinook salmon.

Second, I examined the effects of maternal carotenoids on early life fitness in Chinook salmon. I determined that increased carotenoids in salmon eggs can increase predation risk, where using choice trials, I show that rainbow trout (*O. mykiss*) predators showed a significant bias for red eggs over white eggs. Additionally, I showed that
increased carotenoids in salmon eggs does not lead to benefits on offspring performance, as I found no significant difference in offspring of red and white females (i.e., eggs) in survival, size and immune, stress and oxidative stress responses. My results indicate that high levels of carotenoids are not required in Chinook salmon eggs.

After determining that colour morphs were not reproductively isolated (i.e., no genetic divergence), I examined pre- and post-spawning sexual selection in red and white Chinook salmon. Under experimental breeding trials that quantified colour assortative mating, I found that red females mated assortatively with red males, whereas white females did not mate based on colour. Next, I examined post-spawning processes (sperm competition and cryptic female choice (CFC)), where, first, I found that red males had marginally faster sperm relative to white males, suggesting that carotenoid storage may affect sperm performance. However, although sperm velocity was important for predicting in vitro fertilization success under competitive fertilization trials, CFC was also a key mechanism that affected fertilization. Overall, colour assortative mating was important for red and white Chinook salmon, however red and white females employ different strategies (pre- versus post-spawning) to bias paternity in favour of males that are the same colour as themselves.

In conclusion, my thesis determined that salmon are red because of several genes that influence carotenoid pigmentation. However, salmon are not red because it increases offspring performance or reproductive fitness, in fact, I demonstrated that during early life carotenoids can pose a cost rather than a benefit. Therefore, my thesis demonstrates that not all salmon need to be red, and therefore this unique colour polymorphism can be stably maintained in nature. My thesis chapters show a pattern of interactions between natural and sexual selection that promote the maintenance of both phenotypes.
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CHAPTER 1: GENERAL INTRODUCTION

Genetic variation in nature

The remarkable diversity of organisms that exists today is the product of evolution, where the interaction of genotype and environment is responsible for much of this variation (Lynch and Walsh 1998). Not only is there variation among species, but variation also exists within species and within populations. This heritable variation is fundamental to the process of evolution, as it represents the adaptive potential of the species and the raw material for which selection can act upon. In nature, natural selection can act in a directional manner to reduce variation (Rieseberg et al. 2002; Albertson et al. 2003) or selection can act to maintain genetic variation, where processes such as balancing selection may favour the maintenance of different genotypes (Hedrick 2006; Gray and McKinnon 2007; Charlesworth 2006). In addition to natural selection processes, traits also evolve in the context of sexual selection, and similarly, sexual selection can act to maintain or reduce variation depending on the mating strategy adopted by the organism (Hoekstra et al. 2001; Roulin and Bize 2007; Maan and Seehausen 2011). Processes that maintain genetic variation within or among species or populations are important as they can provide insight into evolutionary mechanisms that promote local adaptation, population divergence and ultimately speciation (Coyne and Orr 2004).

Some of the most notable variation that exists in nature includes variation in colouration (Gray and Mckinnon 2007; Seehausen et al. 2008; Hubbard et al. 2010; Wellenreuther et al. 2014). Colour traits have been long been studied given that they represent clearly visible phenotypes that can be used as markers for evolution (Hoekstra 2006). Indeed, classic Mendelian genetics began with Mendel studying inheritance of colour traits (among others) in pea plants. Over the last few decades, one colour trait that
has garnered considerable interest in evolutionary biology is carotenoid pigmentation (Svensson and Wong 2011). Carotenoids represent one of the most abundant types of pigments used in animal signaling and carotenoids play an important role in several biological processes, thus carotenoid traits are often studied in the context of ecology and evolution as these traits are subjected to forces of both natural and sexual selection (Svensson and Wong 2011).

**Carotenoids**

Carotenoids are pigments responsible for many vibrant colours observed in the animal kingdom such as the pink feathers of a flamingo, the red spawning colour of a sockeye salmon and the orange beak of a zebra finch. Carotenoids are lipophilic hydrocarbons that are produced by photosynthetic organisms and microorganisms; therefore, animals cannot synthesize carotenoids *de novo* but must acquire the pigment through their diet (Goodwin 1984). Carotenoids are consumed, absorbed intestinally, and subsequently transported in the bloodstream, metabolized in the liver then circulated in the blood to various tissues (Parker 1996). Carotenoids can contribute to the overall fitness of an animal in diverse ways, as there may be both costs and benefits associated with carotenoids utilization (Olson and Owens 1998) (see Table 1.1 for examples of costs and benefits associated with carotenoids). Natural selection may favour carotenoid use as carotenoids can improve physiological functions, including antioxidant defense and immune response (described below) (Svensson and Wong 2011). Sexual selection may also favour the expression of carotenoid pigmentation during mating, as carotenoid colouration has been correlated to male mating success in several species (Hill 1991; Lozano 1994; Blount et al. 2003; Evans et al. 2004; Pike et al. 2007; Simons and Verhulst 2011; Yang et al. 2013). Conversely, the acquisition and metabolism of carotenoids can
be energetically costly and the expression of carotenoid signals may lead to increased predation risk (Olson and Owens 1998; Godin and McDonough 2003). Although the benefits of carotenoids have been widely demonstrated and carotenoid colouration has evolved in many species (Svensson and Wong 2011), variation still exists within and among species in the degree of carotenoid utilization (Withler 1986; Craig and Foote 2001; Svensson et al. 2009; Deeming and Pike 2013).

**Carotenoids and natural selection**

Carotenoids can act as antioxidants by quenching reactive oxygen species (ROS) (Krinsky 2001). The production of ROS in the body can occur through different means; however, ROS are primarily produced during normal cell metabolism via the formation of ATP, while ROS are also produced during the immune response (Chen et al. 2003; Costantini and Verhulst 2009; Nathan and Cunningham-Bussel 2013). When there is an abundance of ROS relative to antioxidants, ROS can cause damage to lipids, proteins and DNA and therefore lead to oxidative stress (Costantini and Verhulst 2009). Thus, the ability of individuals to utilize carotenoids may be indicative of their capacity to deal with oxidative stress. Additionally, by increasing the regulation of ROS produced in the body, carotenoids may also allow an individual to mount a greater immune response.

The benefits of carotenoids on oxidative status and immune function have been explored in the framework of both maternal effects on offspring fitness as well as effects of dietary supplementation on individual fitness. First, the maternal provisioning of carotenoids to eggs has evolved in many oviparous species (Craik 1985; Blount et al. 2000), possibly to protect cells of rapidly developing embryos from oxidative damage by reducing lipid peroxidation (Surai and Speake 1998; Blount et al. 2000; Blount et al. 2002; McGraw et al. 2005), as the rate of embryonic development has been correlated
with egg yolk carotenoid content across bird species (Deeming and Pike 2013). The increased protection from oxidative damage via carotenoids, may thus improve egg survival as demonstrated in fishes (Tyndale et al. 2008) and birds (McGraw et al. 2005), and perhaps lead to later benefits in offspring performance such as offspring growth (Bazyar Lakeh et al. 2010) and later offspring survival (McGraw et al. 2005).

Furthermore, carotenoid concentration of egg yolk has been correlated with enhanced offspring immune function in birds and fishes, where increased carotenoids can increase the T-cell mediated immune response in barn swallow (Hirundo rustica; Saino et al. 2003), increase the ability of hihi (Notiomystis cincta) nestlings to cope with parasitic infection (Ewans et al. 2009) and increase disease resistance in Chinook salmon fry (Oncorhynchus tshawytscha; Tyndale et al. 2008). Not only are maternally provisioned carotenoids important, but individuals also gain benefits from dietary carotenoids. Many studies have demonstrated these benefits using carotenoids supplementation experiments, where, for example, dietary carotenoids increased antioxidant status and/or immune function in both birds (McGraw and Ardia 2003; Butler and McGraw 2013; Alonso-Alvarez et al. 2004; Hörak et al. 2007; Lucas et al. 2014; Biard et al. 2009; McGraw et al. 2011) and fishes (Christiansen et al. 1995; Amar et al. 2004; Pike et al. 2007; Amar et al. 2012; Pham et al. 2014).

Although carotenoids can have benefits at the physiological level, it is possible that carotenoids may also incur costs to the individual. While the empirical evidence supporting the cost of carotenoids is more limited, some studies have demonstrated that carotenoids can increase predation risk. For example, in copepods (Eurytemora affinis), increased carotenoid pigmentation can improve antioxidant status, however it also leads to greater predation risk (Gorokhova et al. 2013). Similarly in fishes, carotenoids can
improve sexual attractiveness (as described below), however the use of carotenoids for secondary sexual traits can also increase predation risk as demonstrated in guppies (*Poecilia reticulata*; Godin and McDonough 2003) and sticklebacks (*Gasterosteus aculeatus*; Moodie 1972). Additional costs of carotenoids could come in the form of energetic costs associated with their acquisition and metabolism, which could potentially influence growth rates. However studies have shown that carotenoids improve growth rates rather than hinder them (Torrissen 1984; Bazyar Lakeh et al. 2010; Gorokhova et al. 2013).

**Carotenoids and sexual selection**

In addition to the role of carotenoids in natural selection processes, carotenoid pigments have been widely studied in the context of sexual selection (Hill 1991; Houde and Torio 1992; Lozano 1994; Blount et al. 2003; Evans et al. 2004; Yang et al. 2013), where carotenoids may be important during both pre- and post-copulatory sexual selection processes. This is because, given that carotenoids play a role in various biological functions, it has been hypothesized that carotenoid colouration should indicate male quality as only healthy individuals should be capable of allocating carotenoids to signal expression (Hamilton and Zuk 1982; McGraw and Hill 2000; Barber et al. 2001; Blount et al. 2003; Simons and Verhulst 2011). Several studies have found that male carotenoid colouration can signal aspects of male quality (i.e., disease/parasite resistance, parental care, fertility) and influence female choice (Hill 1991; Houde and Torio 1992; Blount et al. 2003; Smith et al. 2014). Indeed the pattern of female preference for brightly coloured male traits has been demonstrated in many species (Evans et al. 2004; Pike et al. 2007; Simons and Verhulst 2011; Yang et al. 2013).
In addition to pre-copulatory sexual selection, post-copulatory sexual selection may also operate based on carotenoid pigmentation (Evans et al. 2003; Pilastro et al. 2004). Post-copulatory sexual selection can occur through sperm competition (Parker et al. 1998) and cryptic female choice (CFC) (Eberhard 1996). Carotenoids may influence sperm competition because carotenoids may be important for ensuring sperm quality as the testes can be especially susceptible to oxidative damage due to the high production of free radicals (Blount et al. 2001; Helfenstein et al. 2010). Oxidative stress in the testes may affect sperm performance (including velocity or density), as well as the structural integrity of the DNA within the gametes (Blount et al. 2001). Indeed, male carotenoid signals have been correlated with sperm quality in some species including mallard (Anas platyrhynchos; Peters et al. 2004), European bitterling (Rhodeus amarus; Smith et al. 2014), guppy (Locatello et al. 2006), stickleback (Pike et al. 2010) and redside dace (Clinostomus elongates; Beausoleil et al. 2012). These studies represent indirect links between carotenoids and sperm quality; however, a direct relationship has been demonstrated in goldfish (Carassius auratus), where carotenoid supplementation increased the concentration of carotenoids in semen and subsequently led to higher sperm motility, sperm density and fertilization rate (Tizkar et al. 2015). Although sperm performance can be a good predictor of fertilization success, CFC can influence the outcome of sperm competition where females may bias fertilization in favour of a specific male that will confer the greatest fitness benefit for her offspring (Eberhard 1996; Birkhead 1998; Neff and Pitcher 2005). Few studies have examined CFC in relation to carotenoid pigmentation (Evans et al. 2003; Pilastro et al. 2004), however given that pre-copulatory female choice has evolved to select males based on carotenoid displays, it is plausible that post-copulatory processes should reinforce pre-copulatory decisions. For
example, under sperm competition, CFC in the guppy has been demonstrated to favour males with greater carotenoid colouration (Evans et al. 2003; Pilastro et al. 2004); however the direct mechanism for this selection is unknown. Nonetheless, mechanisms of both pre- and post-copulatory mate choice may favour the expression of carotenoid signals.

**Study system: Salmon**

**The role of carotenoids in salmon**

Carotenoids are responsible for the characteristic red flesh, eggs and skin of salmon. The main carotenoid found in salmon and other aquatic organisms is astaxanthin (Guerin et al. 2003; Rajasingh et al. 2007). Astaxanthin is a very powerful antioxidant and also has pro-vitamin A activity in fishes (Guerin et al. 2003). Although the deposition of these antioxidant pigments into the skin and eggs is not unique to salmonids, flesh pigmentation has evolved almost exclusively in salmonid species, where only four genera of Salmonidae (*Oncorhynchus, Salvelinus, Salmo* and *Parahucho*) exhibit flesh pigmentation (Rajasingh et al. 2007). The accumulation of carotenoids in the flesh may act for storage of these pigments during the marine phase in anadromous salmonids, where during the spawning phase these carotenoid stores can be utilized to enhance somatic maintenance during this stressful life stage when salmon travel upstream, expend energy and cease feeding (Rajasingh et al. 2007). Although the long migration associated with the anadromous life history is not unique to species exhibiting flesh pigmentation, extensive nest building (i.e., redd construction) is unique to the four genera of flesh-pigmented salmonids (Rajasingh et al. 2007). The coupling of these obligatory behaviours (migration and nest building) during reproduction may thus lead to oxidative damage, however the movement of carotenoids from degrading white muscle tissue into the
bloodstream to preserve vital functions may explain why evolution has favoured flesh pigmentation in salmonid species (Rajasingh et al. 2007).

During spawning, the occurrence of excess carotenoids mobilized into the bloodstream may have also formed the basis for, or enhanced, reproductive characteristics in salmon such as the deposition of carotenoids into skin and eggs (Rajasingh et al. 2007). Maternal allocation of carotenoids into the eggs has been correlated with offspring survival and immune function in salmon (Tyndale et al. 2008; but see Christiansen and Torrissen 1997). The accumulation of carotenoids in the skin resulting in red external colouration has often been considered important for mate choice in salmonids (Fleming and Gross 1994; Skarstein and Folstad 1996; Craig and Foote 2001). However the evolutionary mechanisms promoting mate choice for red colouration remains unknown, though red colouration may be favoured due to sensory bias (Craig and Foote 2001) or good genes. Although carotenoids may provide many important benefits, carotenoid pigmentation has not evolved in the majority of fishes, thus underscoring our lack of understanding of carotenoid evolution in fishes.

**Chinook salmon: a carotenoid anomaly**

In nature, some Chinook salmon populations may exhibit extreme variation in carotenoid pigmentation, which can result in the occurrence of two colour morphs (red and white) that differ in carotenoid utilization (see Figure 1.1). The difference between red and white Chinook does not reflect diet preference but results from genetic polymorphisms (Withler 1986). White Chinook salmon have little or no ability to deposit carotenoids into their flesh, eggs and skin, and thus white Chinook salmon have white (or pale) eggs and flesh, and appear uncharacteristically grey in colour during spawning (Figure 1.1). The extreme dichotomy in carotenoid utilization exhibited in Chinook
salmon make them an ideal model species for the study of carotenoid pigmentation evolution and ecology in salmonids.

Current knowledge on red and white Chinook salmon populations is limited. There is substantial geographic variation in the proportion of white Chinook salmon in western North America (Hard et al. 1989). For example, in British Columbia, the Harrison River is composed of almost 100% white Chinook (although red individuals have been appearing in recent years) and the Quesnel River contains approximately equal proportions of red and white morphs (Withler 1986). River systems on Vancouver Island maintain little or no white Chinook salmon, similar to river systems in Washington and Oregon (Hard et al. 1989). There is strong evidence that shows that the phenotype is highly heritable, however the number of loci and the specific genes involved in flesh pigmentation for Chinook salmon remains unknown (Withler 1986; Rajasingh et al. 2008).

The occurrence and persistence of white Chinook in certain populations is not understood. Although carotenoids in eggs, skin and flesh may provide fitness benefits during various life stages (Craig and Foote 2001; Rajasingh et al. 2007; Tyndale et al. 2008; Bazyar Lakeh et al. 2010), white Chinook salmon must obtain certain fitness benefits to persist in such high frequencies in some populations. In white Chinook salmon, limited carotenoids in eggs may protect inconspicuous eggs against predation risk and the absence of deposition of carotenoids into the flesh may incur lower energetic costs and promote greater growth resulting in larger body size (Godfrey 1968; Rajasingh et al. 2007). Additionally, although red colouration is expected to enhance mating success, the mating dynamics in genetic colour polymorphic species could favour the maintenance of genetic variation through assortative mating for colour (Roulin and Bize
2007), however mating dynamics has not been characterized in Chinook salmon populations where red and white individuals co-exist and the degree of genetic divergence between red and white individuals remains unknown.

My project primarily focuses on Chinook salmon from the Quesnel River, BC, because the proportions of red and white individuals are approximately equal (Withler 1986). In the Quesnel River, red and white morphs represent stable evolutionary stable strategies (ESS), as their frequencies have remained stable throughout the last few decades. The expected distribution of colouration in the Quesnel River is presented in Figure 1.2, which demonstrates the variation in colouration that is expected within morphs. Although the trait can be considered dichotomous in terms of pigmented (red) versus unpigmented (white), variation can still exist within the morphs. Red individuals are characterized by a higher degree of carotenoid pigmentation, however this pigmentation can undoubtedly be influenced by environmental factors (i.e., access to carotenoids). Conversely, less variation should exist within the white morph given that white individuals have limited ability to deposit carotenoids into their tissues. Intermediate colouration (pink) is not expected to be prevalent in the population, possibly due to selection against the intermediate phenotype (i.e., disruptive selection); however this is most likely due to non-additive genetic effects (i.e., dominance) influencing the colour phenotype (Withler 1986). Throughout my PhD, the external colour phenotype that salmon exhibit (red versus white) is expected to be directly correlated with carotenoid content, where red individuals are rich in carotenoids and white individuals are poor in carotenoids. This difference in carotenoid content is expected to be present in the skin, eggs and flesh of the salmon. By using red and white Chinook salmon from the same population, I can examine the role of natural and sexual selection processes in
shaping carotenoid colouration in salmon, while identifying the cost and benefits of the pigment. Therefore, by examining fitness differences between red and white Chinook salmon throughout their life, I can determine why salmon evolved to use carotenoids pigments in the way that they do.

**Thesis objectives**

As the title of my thesis indicates, the overarching objective of my PhD is to determine why salmon have evolved to use carotenoids by determining the proximate and ultimate causes of carotenoid pigmentation in Chinook salmon. The outcomes of my research will also help determine the evolutionary mechanisms that operate to maintain the unique colour polymorphism in Chinook salmon populations. First, for a trait to be shaped by evolutionary processes, it must be heritable, and thus knowledge of the inheritance and the genetic basis of the trait is important. My thesis therefore begins with a genetic study. In **Chapter 2**, I use a genome-wide association study to address the genetic mechanisms responsible for flesh pigmentation. The objective of this chapter is to identify loci in the genome of Chinook salmon that are significantly associated with flesh pigmentation and characterize the nature of the genetic processes that control red pigmentation in Chinook salmon. After confirming that pigmentation is indeed a phenotype that is controlled by genetics, I then examine differences in the fitness of red and white Chinook salmon throughout life to understand both the fitness cost and benefits of carotenoids. I begin with the egg stage, where in **Chapter 3**, I examine how the maternal provisioning of carotenoids can impact offspring fitness through predation. The eggs of red and white Chinook salmon are visibly different in colour (i.e., carotenoid content) (see Figure 1.1B), and therefore may experience differences in predation risk if red eggs are more conspicuous to predators. The objective of this chapter is to determine
the difference in predation on red and white eggs using rainbow trout (*O. mykiss*) as an egg predator. If indeed highly visible red eggs experience greater predation risk, this would provide a cost of carotenoids in salmon during early life. Next, the maternal provisioning of carotenoids may not only impact offspring fitness through predation but also in diverse ways through their role as antioxidants. In **Chapter 4**, I examine performance differences in the offspring of red and white females from the egg stage to a later juvenile stage. The objective of this chapter is to determine how maternal carotenoids can impact egg and offspring survival, offspring growth and offspring immune, stress and oxidative stress responses. By examining a wide range of fitness related measures in salmon eggs and fry derived from the eggs of red and white females, I can determine the relationship between egg carotenoid content and offspring performance in Chinook salmon. After examining early life stages, I then examine how carotenoids can impact reproductive fitness in adult Chinook salmon. In **Chapter 5**, I examine how mating operates in a population of red and white Chinook salmon by first determining if red and white Chinook salmon interbreed in the population using neutral genetic markers (microsatellites) and measuring genetic divergence between morphs. I then use experimental spawning channels to quantify assortative mating in red and white Chinook salmon. Furthermore, in this chapter, I also examine the role of natural selection in shaping the red and white phenotypes by quantifying selection at important immune genes (major histocompatibility complex, MHC). Given the role of carotenoids in immune function, it is plausible that white Chinook salmon have evolved mechanisms to compensate for their lack of carotenoids, potentially through differences at the MHC genes. Therefore, the objectives of this chapter are three-fold, and include 1) determine whether red and white Chinook salmon are genetically divergent at neutral markers, 2)
determine whether mating occurs based on colour (i.e., colour assortative mate choice) in the population, and 3) determine whether selection operates differentially on immune genes (MHC) based on colour. The outcomes of this chapter will be important for understanding pre-copulatory sexual selection in red and white Chinook salmon, as well as lend insight into some of the potential evolutionary mechanisms maintaining the polymorphisms. Next, although pre-spawning decisions can be important for reproductive success, post-spawning sexual selection can also operate in Chinook salmon to bias fertilization success. Therefore, in Chapter 6, I examine how post-spawning sexual selection, including sperm competition and cryptic female choice, operate in red and white Chinook salmon. The objective of this chapter is to determine whether carotenoid pigmentation corresponds to sperm performance in Chinook salmon males and whether post-spawning processes operate based on colour in the population.

**Significance**

Through my PhD research, I will aim to not only characterize the proximate mechanisms (genetics) involved in carotenoid pigmentation, but also the ultimate causes (sexual and natural selection), which in turn will allow me to answer the question: why are salmon red? My research will further our understanding of salmon evolution, as well as address the fundamental evolutionary question of how genetic variation is maintained in populations. The co-existence and persistence of red and white Chinook salmon in nature remains puzzling, however my thesis will aim to resolve the evolutionary mechanisms responsible for the persistence of the two morphs. My research not only has important implications for salmonid evolution but also has consequences for industry and conservation practices. Understanding the genetic mechanisms controlling flesh colour in salmon is a major goal of aquaculture breeding programs, as colour is an economically
valuable trait that consumers associate with quality and taste. By identifying loci associated with flesh pigmentation, marker-assisted selection could be employed to efficiently maximize flesh colour in farmed salmon. Furthermore, flesh colour is a trait that is often not considered in hatchery or management practices, and thus the synthesis of my research can aid in conservation efforts to increase offspring viability and maximize production and management efforts. In conclusion, the integrative nature of my thesis, from genes to behaviour to population genetics and physiology, is essential to addressing the overarching evolutionary questions of my project to further our knowledge of salmon ecology and evolution, as well as contribute to innovation and application in salmonid research.

References


Table 1.1. Benefits and costs associated with either dietary consumption of carotenoids, maternal provisioning of carotenoids or carotenoid signals along with demonstrated examples of the effect. Note that this is not an exhaustive list of all potential costs and benefits or all studies that have demonstrated these effects.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Examples of species with demonstrated effect</th>
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<tbody>
<tr>
<td><strong>Benefits</strong></td>
<td></td>
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<tr>
<td>Survival</td>
<td>Chinook salmon (<em>Oncorhynchus tshawytscha</em>, Tyndale et al. 2008)</td>
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<tr>
<td>Immune function</td>
<td>Barn swallow (<em>Hirundo rustica</em>, Saino et al. 2003)</td>
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<td></td>
<td>Chinook salmon (Tyndale et al. 2008)</td>
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<td></td>
<td>Hihi (<em>Notiomystis cincta</em>, Ewans et al. 2009)</td>
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<td></td>
<td>Rainbow trout (<em>Oncorhynchus mykiss</em>, Amar et al. 2012)</td>
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<tr>
<td>Antioxidant protection</td>
<td>Atlantic salmon (<em>Salmo salar</em>, Christiansen et al. 1995)</td>
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<td></td>
<td>Three-spined stickleback (<em>Gasterosteus aculeatus</em>; Pike et al. 2007)</td>
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<tr>
<td></td>
<td>Zebra finch (McGraw et al. 2005)</td>
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<tr>
<td>Growth rate</td>
<td>Copepods (<em>Eurytemora affinis</em>, Gorokhova et al. 2013)</td>
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<td></td>
<td>Rainbow trout (Bazyar Lakeh et al. 2010)</td>
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<tr>
<td>Sexual attractiveness</td>
<td>Guppy (<em>Poecilia reticulata</em>, Evans et al. 2003; <em>P. parae</em>, Bourne et al. 2003)</td>
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<td></td>
<td>House finch (<em>Carpodacus mexicanus</em>, Hill 1991)</td>
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<td>Sockeye salmon (<em>O. nerka</em>, Craig and Foote 2001)</td>
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<td></td>
<td>Zebra finch (Blount et al. 2003)</td>
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<tr>
<td>Sperm performance/fertility</td>
<td>Goldfish (<em>Carassius auratus</em>; Tizkar et al. 2015)</td>
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<td>Guppy (Locatello et al. 2006)</td>
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<td>Three-spined stickleback (<em>Gasterosteus aculeatus</em>; Pike et al. 2009)</td>
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<td><strong>Costs</strong></td>
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<td>Predation</td>
<td>Copepod (<em>Eurytemora affinis</em>, Gorokhova et al. 2013)</td>
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<td>Guppy (Godin and McDonough 2003)</td>
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<td></td>
<td>Three-spined stickleback (Moodie 1972)</td>
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<tr>
<td>Pro-oxidant activity¹</td>
<td>American goldfinch (<em>Carduelis tristis</em>, Huggins et al. 2010)</td>
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<td></td>
<td>Eurasian kestrel (<em>Falco tinnunculus</em>, Costantini et al. 2007)</td>
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<tr>
<td>Foraging cost</td>
<td>To my knowledge, no studies demonstrate this cost</td>
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<tr>
<td>Energetic cost</td>
<td>House finch (Hill 2000)</td>
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<td>‘Social cost’ (i.e.,</td>
<td>Australian painted dragon lizards (<em>Ctenophorus pictus</em>, Healey and Olsson 2009)</td>
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<td>more aggressive encounters)</td>
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¹at high levels of carotenoids
Figure 1.1. Photographs showing red and white colour morphs of Chinook salmon (*Oncorhynchus tshawytscha*), where (A) shows external spawning colour of a white (top) and red (bottom) male, (B) shows eggs from red (top) and white (bottom) females and (C) shows differences in flesh pigmentation between red (top) and white (bottom) salmon during the ocean phase.
Figure 1.2. Expected frequency distribution of colouration (spawning or flesh colour) of Chinook salmon (*Oncorhynchus tshawytscha*) in the Quesnel River, British Columbia, where red and white morphs are expected to occur at the same frequency. The distribution above demonstrates that the variation in colouration within the red morph is expected to be greater than variation in colour in the white morph. Individuals exhibiting intermediate colouration (pink) are not expected to be present at high frequencies due to dominance effects influencing the colour phenotype.
CHAPTER 2: GENOME-WIDE ASSOCIATION STUDY (GWAS) FOR FLESH COLOURATION IN CHINOOK SALMON

Introduction

Salmonids are known for their characteristic bright red flesh, skin and egg colouration, which occurs as a result of the deposition of carotenoid pigments that salmon acquire through their diet (Rajasingh et al. 2007). Although carotenoid pigmentation in skin and eggs has evolved in other fishes, flesh pigmentation has evolved almost uniquely in salmonids, specifically in only four genera of Salmonidae including *Oncorhynchus, Salvelinus, Salmo* and *Parahucho* (Rajasingh et al. 2007). These members of Salmonidae exhibit the anadromous life history, where salmon are born and reside in freshwater streams for months to years then migrate to the ocean to grow for one or multiple years, and later return to freshwater streams to reproduce (Groot and Margolis 1991; Quinn 2005). Carotenoids can act as powerful antioxidants (Krinsky 2001); therefore, carotenoids in the flesh may act as an antioxidant “sink” during the marine phase and as a “source” during the spawning phase when carotenoids can be mobilized from the flesh to enhance protection of somatic tissues during this demanding life stage (Rajasingh et al. 2007). Upstream migration coupled with extensive nest construction exhibited by these four genera contribute to oxidative stress, thus the mobilization of carotenoids may provide a possible mechanism for positive selection acting to promote flesh pigmentation in salmon species (Rajasingh et al. 2007). Although carotenoids may provide important advantages to salmon (Christiansen et al. 1995; Rajasingh et al. 2007; Tyndale et al. 2008; Amar et al. 2012), the degree of carotenoid pigmentation can vary among and

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1 This manuscript is part of collaborative research project. Co-authors on the manuscript (in prep) include Lehnert SJ, Vandersteen WE, Heath DD, Pitcher TE, Heath JW, Devlin RH
within salmonid species; therefore, variation in carotenoid utilization may reflect evolutionary adaptations related to different environmental conditions and/or life history strategies (Rajasingh et al. 2007).

Not only is carotenoid pigmentation likely an evolutionary significant trait, carotenoid pigmentation is also important from an industry perspective. In both wild and aquaculture salmon, flesh colour is a commercially valuable trait as consumers often associate the degree of red colouration with quality of the salmon product (Alfnes et al. 2006). The primary carotenoid found in salmon flesh is astaxanthin (Christiansen et al. 1995; Garner et al. 2010), and in nature, salmon acquire this compound by feeding on prey that are rich in this pigment, such as shrimp, krill and squid. However, in the aquaculture environment, carotenoid ingredients are very expensive and astaxanthin and canthaxanthin (a related carotenoid) additives can account for up to 25% of feed costs (Torrissen 1995; Buttle et al. 2001; Alfnes et al. 2006). Aquaculture producers invest substantially in enhancing flesh colouration, yet individual variation still exists in the ability of a salmon to process and deposit carotenoids (Withler 1986; Torrissen and Naevdal 1988; Araneda et al. 2005), which causes variation in product appearance and hence value. Thus, improving the genetic capabilities of salmon strains to deposit pigments is an important objective for salmon producers. However, flesh colour is difficult to assess for selective breeding, as this trait must be assessed prior to sexual maturation and generally requires lethal sampling (Baranski et al. 2010). Therefore, understanding the underlying genomic factors affecting carotenoid deposition and flesh colouration in salmon is a major goal of the salmon aquaculture industry. The identification of loci responsible for carotenoid colouration would allow for marker
assisted selection (MAS) programs to improve pigmentation in salmon and enhance overall production efficiency.

Previous studies have found that flesh colour in salmonids is likely controlled by just a few major loci (Withler 1986; Araneda et al. 2005; Houston et al. 2009; Baranski et al. 2010; Tsai et al. 2015). In Atlantic salmon (Salmo salar), studies have identified a few loci as having genome- or chromosome-wide significance on flesh colour (Houston et al. 2009; Baranski et al. 2010; Tsai et al. 2015). In Coho salmon (Oncorhynchus kisutch), a single locus has been found to be associated with flesh colouration, where the marker amplified in 77% of the high-pigment fish and only 38% of the low pigment fish, thus suggesting that other loci were also involved in the trait (Araneda et al. 2005). Genes influencing multiple processes including carotenoid transport, metabolism and uptake could influence flesh colour variation; however, Rajasingh et al. (2006) proposed that genes affecting carotenoid uptake processes across the muscle membrane are the most likely source of variation. Nevertheless, several genes have been associated with carotenoid utilization processes in other species (e.g., Drosophilia melanogaster, Bombyx mori, Coturnix japonica, Mus musculus, Homo sapiens) including scavenger receptor class B (SCARB) genes (Kiefer et al. 2002; van Bennekum et al. 2005; Reboul et al. 2005; Sakudoh et al. 2010), steroidogenic acute regulatory (StAR) related lipid transfer proteins (Bhosale and Bernstein 2007; Li et al. 2011; Tsuchida and Sakudoh 2015) and beta-carotene oxygenase (bco) genes (Kiefer et al. 2001; Lobo et al. 2011). In salmon, no specific genes have been identified to contribute to carotenoid pigmentation; however, potential candidate genes may include SCARB1 and SCARB1-2 (novel paralog) genes, which are expressed in the mid-gut, muscle and liver of Atlantic salmon (Baranski et al. 2010; Sundvold et al. 2011). Additionally, SCARB1-2 has been mapped to a chromosome
that was found to contain a putative quantitative trait locus (QTL) for flesh colour in Atlantic salmon (Baranksi et al. 2010; Sundvold et al. 2011). Furthermore, a paralog of the bco2b gene showed high expression in the liver and intestine of Atlantic salmon, suggesting a potential role in astaxanthin metabolism (Helgeland et al. 2014).

To determine which loci are involved in a quantitative trait, offspring derived from the backcrossing of two inbred lines are often employed as a powerful approach for detecting associated loci (Wu et al. 2007). Chinook salmon (O. tshawytscha) is the ideal species for identifying loci associated with flesh colour because, unlike other salmonids, Chinook salmon exhibit extreme differences in flesh colouration (Withler 1986). Differences in flesh colour can range from white to bright red because of genetic polymorphisms affecting carotenoid deposition (Withler 1986). A previous study by Withler (1986) examined the genetic basis of flesh pigmentation in Chinook salmon from the Quesnel River where relatively equal proportions of red- and white-fleshed individuals exist. Withler (1986) determined that there were likely two major loci responsible for flesh colouration in Chinook salmon, where one red determining (dominant) allele must be present at both loci for a salmon to be pigmented. However, no studies have been undertaken to identify these loci in Chinook salmon.

In our study, F2 and F3 hybrid offspring derived from backcrossing two flesh colour populations (pure red-fleshed and pure white-fleshed), were used for a whole-genome association study. Individuals were assessed for flesh colour phenotype then genotyped by sequencing to obtain single nucleotide polymorphisms (SNPs) spanning the genome. In addition to backcrossed offspring, we also genotyped wild red- and white-fleshed Chinook salmon from the Quesnel River population to determine if our results could be applied to other systems. SNPs identified in our study were aligned to existing
NCBI sequences, as well as compared against the annotated Atlantic salmon genome, within which we could search for potential candidate genes located near our SNPs. The identification of SNPs and candidate genes associated with flesh pigmentation will not only be valuable to the aquaculture industry but also to our understanding of the underlying mechanisms responsible for flesh pigmentation in salmonids. By identifying the proximate mechanisms responsible for flesh pigmentation in salmon, we can provide an answer to the longstanding question: why are salmon red?

**Methods**

*Inbred strains for flesh colour*

Individuals from two populations of Chinook salmon that exhibit different flesh colours were used to create backcrosses in our study. Chinook salmon include individuals from a pure red-fleshed strain from Big Qualicum River, British Columbia, Canada and a pure white-fleshed strain from Harrison River, BC. In both populations, the other flesh colour phenotype is absent, thus individuals are expected to be homozygous at flesh colour loci.

*F2 backcrosses*

In the fall of 2008, two families of F2 backcrosses of Chinook salmon were created at DFO Centre for Aquaculture and Environmental Research facility (CAER) in West Vancouver, BC. Using four parents, two F2 backcross families were produced by backcrossing a F1 hybrid male (Big Qualicum red-fleshed x Harrison white-fleshed) to a Harrison white-fleshed female. Offspring were reared at CAER until smolting, and then between June 19 and July 20 2009, smolts were transferred from the CAER facility to a saltwater net pen at Yellow Island Aquaculture Ltd. (YIAL), Quadra Island, BC. All smolts were implanted with a visible implant elastomer tag unique to their family. Fish
were fed pellets that included offshore fish protein and naturally derived carotenoid pigments. In July 2010, when offspring reached approximately 200–400 grams, one F2 backcross family was sacrificed to assess flesh pigmentation. At this time, it is late enough that offspring will exhibit flesh pigmentation and early enough that any offspring that may precociously mature as “jacks” in the fall will still retain their pigmentation which can be lost closer to maturation (Withler 1986). A fillet was taken to quantify the level of pigmentation in the muscle of each fish through visual observation. The fish were given a score between 1 and 5 (± 0.5) as indicated by the Roche Colour Card for Salmonids. Liver samples were collected for genetic analysis. Individuals were also assessed for precocious maturation based on external colouration and gonad development, and all other individuals were sexed using PCR (Devlin et al. 2001).

**F3 backcrosses**

In November 2012 at YIAL, two sexually mature red F2 backcross females (from the other F2 family; see above) were mated with a Harrison white-fleshed male to create two F3 backcross paternal half-sib families. Fertilized eggs were moved to incubation trays, and after hatching, offspring were transferred to 200-L tanks. In June 2013, offspring from both F3 families (herein referred to as 18xHMB and 25xHMB) were weighed, measured, fin clipped for genetic analysis and PIT tagged for permanent individual identification (18xHMB n = 759; 25xHMB n = 748). The following month offspring were vaccinated for *Vibrio* and moved to a saltwater net pen at YIAL. In the net pen, offspring were fed the same diet as offspring from the F2 generation (see above). Offspring were monitored for survival and growth over time. In August 2014, fish were sacrificed to assess flesh colour. We used visual observation where the Roche Colour Card for Salmonids was used to score the fillet with a colour score between 0 and 5 (± 1).
Besides flesh pigmentation, individuals were also assessed for precocious maturation based on external colouration and gonad development.

**Wild red and white Chinook salmon: Quesnel River population**

A subsample of red and white adult Chinook salmon from the Quesnel River, BC was also included in our design. Chinook salmon captured by netting during the spawning season in 2013 and 2014 were characterized as red or white based on external spawning colouration (see Lehnert et al. 2016 for details), and colour scores were given as binary values where red was coded as 1 and white was coded as 0. Quesnel River individuals were included in the analysis to establish whether any significant loci detected in the backcrossed families would also be present in a different wild population that exhibit both red and white phenotypes.

**Genotype-by-sequencing**

DNA was extracted from fin or liver tissue using the standard phenol/chloroform extraction method. All genomic DNA samples were quantified and screened for quality by visual assessment on agarose gels and using Quant-iT PicoGreen dsDNA assay kit (ThermoFisher Scientific Inc.). All samples ranged between 50-100 ng/μL in concentration. Library preparation and genotype-by-sequencing (GBS) were carried out following the protocol described by Elshire et al. (2011). The restriction enzyme EcoT221 was used to digest genomic DNA during library preparation.

**SNP discovery**

SNP discovery was performed following the TASSEL-GBS pipeline described by Glaubitz et al. (2014) using TASSEL version 3 (Bradbury et al. 2007). Briefly, tags were identified as unique trimmed 64-bp sequences that were represented by one or multiple good barcoded reads (see Glaubitz et al. 2014). All tags were counted, and then merged
into a single file if represented by a minimum of 3 reads. Using BWA version 0.7.8-r455 (Li and Durbin 2010), merged tags were aligned to the reference genome for rainbow trout (*O. mykiss*) (Berthelot et al. 2014) obtained from [https://www.genoscope.cns.fr/trout/](https://www.genoscope.cns.fr/trout/). Alignment was performed with a total of 6,290,577 merged tags, where 39.9% aligned to unique positions, 10% aligned to multiple positions and 50.1% could not be aligned to the rainbow trout genome. Following alignment, a file with the physical position of tags was generated for downstream analyses. Next, the number of reads per tag per individual was tabulated to determine the distribution of tags across samples. With the counts generated and physical positions, tags were filtered to generate SNPs. To prepare SNP data for GWA analyses, a HapMap file of filtered SNPs was generated, where parameters included a minimum site coverage of 0.8, minimum individual coverage of 0.1 and minor allele frequency of 0.01, which produced a total of 27,881 SNPs.

**Statistical analyses**

**Genome-wide association (GWA) analyses**

TASSEL version 5.2.19 was used to conduct all GWA analyses (Bradbury et al. 2007). Prior to analyses, SNPs were further filtered for minor allele frequency (MAF) of 0.05 and minimum number of individuals genotyped was 80%. Using the F2 family, we identified loci showing a significant association with flesh colour variation using the mixed linear model (MLM) approach, where covariates (if significant contributor to variation) and kinship could be accounted for in the model. Genome-wide significance was determined by Bonferroni correction where the total number of SNPs included in the analysis was used to determine the alpha level ($p = 0.05$/total number of SNPs). Given that Bonferroni correction is highly conservative, we also considered loci to be
“suggestive” if the model for the marker had an R-squared greater than 0.05. Manhattan and Q-Q plots were created to visualize the results using qqman package (Turner 2014) in R software (R Core Team 2014).

Targeted SNP analyses

In the F3 generation, we used a targeted analysis where we tested only SNPs that were identified as significant or suggestive loci associated with colour in the F2 generation. SNPs in the F3 were analyzed using the MLM approach. We also used a targeted analysis for the wild population of sympatric red and white Chinook salmon from the Quesnel River, BC. The wild population served as a means to determine whether identified SNPs associated with colour could be applied to other populations. For the targeted analyses, we used an alpha level of 0.05 to determine significance.

BLAST of significant and suggestive loci

All significant and suggestive loci detected were compared against the NCBI sequence database using BLAST. Additionally, using the Atlantic salmon genome nucleotide BLAST, significant and suggestive SNPs were aligned to locations in the salmon genome. For all SNPs that aligned to the salmon genome, we scanned approximately 200 annotated features (genes) in both upstream and downstream directions of the alignment. Based on previous research on carotenoid pigmentation, we compiled a list of potential candidate genes located near SNP alignments.

Results

GWAS analyses for F2 backcross offspring family

Using ANOVA, we found no significant difference in colour between sexes (p = 0.32), furthermore there was no significant difference in the distribution (Kolmogorov Smirnov test) of colours between sexes (p = 0.73). We also found no significant
relationship between weight and colour ($p = 0.18$). Thus the MLM analyses accounted for kinship and no other factors or covariates were included in the model. A total of 183 offspring were genotyped in the F2 backcross family, and after filtering, our analyses included 13,031 SNPs. GWAS analyses revealed a total of 24 SNPs that were significant at the genome wide level (Bonferroni corrected $p < 3.84 \times 10^{-6}$; Figure 2.1 and Figure 2.2). Of the 24 SNPs that were significant at a genome wide level, half were mapped to chromosomes in the rainbow trout genome (at known or unknown positions), where nine of the SNPs mapped to Chromosome 10, two SNPs were mapped to Chromosome 26 and one SNP was mapped to Chromosome 5 (Figure 2.2A). The remaining 12 significant SNPs were not mapped to rainbow trout chromosomes (Figure 2.2B). Three of the 24 significant SNPs were located within the same tag sequence of another SNP (i.e., three separate tags each with two significant SNPs). The three SNPs located within the same tag as another SNP were mapped to chromosomes 10 and 26, as well as an unknown chromosome position. An additional 66 SNPs were considered suggestive loci based on their effect size ($R$-squared $> 0.05$).

**Targeted SNP analyses for F3 backcross offspring**

A total of 256 offspring were genotyped from the two F3 backcross families, which included 119 individuals from 25xHMB family and 137 individuals from 18xHMB family. Among the offspring, we found a significant correlation between weight and colour ($p < 0.001$), thus we included weight as a covariate in the MLM. All significant and suggestive F2 SNPs were filtered before MLM analyses, where 32 SNPs did not meet criteria (i.e., MAF $> 0.05$ and individuals genotyped $> 80\%$), which included 16 SNPs that were homozygous in both F3 families. A total of 58 SNPs were tested, and our analyses identified 3 SNPs that were significant (see Figure 2.3; $p < 0.05$). Two
significant SNPs mapped to chromosomes in the rainbow trout genome including Chromosomes 9 ($R^2 = 0.02; p = 0.045$; Figure 2.3A) and 10 ($R^2 = 0.02; p = 0.042$; Figure 2.3B) and the remaining SNP ($R^2 = 0.02; p = 0.02$; Figure 2.3C) was not mapped to the trout genome.

**Targeted SNP analyses in wild Quesnel River population (adult spawners)**

Given that a subsample of wild individuals was used in the analyses and sex ratio was not equal within the colours, we included sex as a factor in the MLM analyses to avoid potential associations due to sex. After filtering, 53 SNPs met our criteria, and MLM analyses revealed only 1 significant SNP (Figure 2.4; $R^2 = 0.16; p = 0.04$), which was not mapped to the rainbow trout genome.

**BLAST of significant and suggestive loci against nucleotide database**

Significant and suggestive loci were blasted against the NCBI nucleotide database, and a total of 14 of those 90 loci had alignment hits with a minimum alignment score of 80 (Appendix 1), where some SNPs aligned to the same NCBI sequences. Provided the short read length ($\leq 64$ bp) of our tag sequences, hits with higher eValues may be unreliable, thus results should be interpreted with caution. However, in our study, mean eValue for top hits was $1.57 \times 10^{-13}$ and mean query coverage was 95%.

**BLAST of significant and suggestive loci against Atlantic salmon genome**

A total of 31 out of 90 SNPs aligned to locations within the Atlantic salmon genome, which spanned a total of 13 different chromosomes (see Table 2.1 for results). Potential candidate genes related to carotenoid pigmentation were identified on all 13 chromosomes (Table 2.1).

**Discussion**
In our study, we aimed to identify loci underlying the evolutionarily and economically significant trait of flesh colour in salmon. Previous studies have identified flesh colour QTLs in Atlantic salmon, where one study found loci of genome wide significance (Baranski et al. 2010), and two other studies reported loci of chromosome wide significance (Houston et al. 2009; Tsai et al. 2015). In our study, we identified 24 SNPs of genome wide significance and an additional 66 suggestive SNPs that were associated with flesh pigmentation in a F2 backcross generation of Chinook salmon. Many SNPs of genome wide significance (n = 9) were mapped to Chromosome (Chr) 10 in rainbow trout. In salmonids, chromosome arms are relatively conserved across species, where Chr 10 arms (10p and 10q) in rainbow trout are homologous to one arm of Chr 4 (4q) and one arm of Chr 8 (8p) in Atlantic salmon (Phillips et al. 2009). Our alignment of SNPs to the Atlantic salmon genome also showed that many significant SNPs aligned to locations on Chr 4 in Atlantic salmon (see Table 2.1). Interestingly, Baranski et al. (2010) also found a flesh colour QTL of genome wide significance located on Chr 4 in Atlantic salmon. The comparable results between our study and Baranski et al. (2010) are promising and may indicate that flesh colour loci are conserved across salmonids.

In addition to our F2 backcross generation, we incorporated a F3 backcross generation for targeted SNP analyses. We expected that given an additional generation of recombination, we could increase our ability to detect effects at minor loci. In our analyses, we found that three significant and suggestive SNPs from the F2 were also significant in the F3 generation. The limited number of significant loci in the F3 may have resulted from several factors. In the F3 generation, flesh pigmentation was significantly correlated with weight, similar to a previous study (Baranski et al. 2010), which can reduce the power to detect significant flesh colour associations, yet even with
weight as a covariate, we still identified significant loci. Additionally, red alleles at major and minor colour loci in the F2 may be lost in the F3 depending on the segregation of pigmentation alleles from F1 to F2 and from F2 to F3. The loss of red alleles at potential colour loci is highlighted by the fact that many significant or suggestive loci from the F2 were homozygous in the F3 families (n = 16 SNPs). When we chose the parents for the F3 backcrosses, we did not know the genotype of the individual, and colour could not be assessed at sexual maturation (lethal sampling was performed at this stage), thus it was possible that the selected parents may have been homozygous for some of the loci associated with flesh colour. Based on the distribution of the flesh colour phenotypes in the F3 offspring (normal distribution), we expected major colour loci to be heterozygous in both F2 females chosen as parents (male parent was Harrison white) given that we saw a range of pigmentation scores from 0 (no pigment) to 5 (fully pigmented) in the offspring. Nonetheless, the few significant loci found in the F3 may suggest that alleles at major loci have been lost in this generation. Alternatively, our results may indicate that the colour trait is more polygenic than previously thought.

GWAS and QTL studies can be particularly valuable if loci detected are relevant to other populations of salmon. Therefore in our study, we also genotyped a wild population of mixed red and white Chinook salmon, where we found only one significant SNP out of the 90 identified SNPs from the F2. In the wild population, we assessed colour based on external spawning colouration, which is expected to reflect flesh colour (Withler 1986). The low number of significant SNPs may have resulted from our inability to assess colour quantitatively, but rather only red versus white based on skin colour. Furthermore, the results may reflect the complexity of the underlying genetics of the colour phenotype. Previously, Withler (1986) proposed a two-locus, two-allele model for
Chinook salmon colour genes in the Quesnel River population, where one red determining allele (dominant) is required at both of the colour loci for a salmon to be pigmented. However, that proposed model could not fully explain all the results of the breeding design (Withler 1986). Additionally, it is plausible that the white flesh phenotype arose independently in the Quesnel and Harrison populations and thus may be a result of polymorphisms at different loci, however this scenario seems unlikely.

Another important evolutionary question regarding flesh colour in Chinook salmon relates to how the polymorphism for pigmentation is maintained in nature. Given the importance of carotenoids to salmonids, the white-fleshed phenotype is expected to be at a disadvantage; however, one possibility is that white-fleshed Chinook salmon persist through compensating genetic mechanisms (Lehnert et al. 2016). Using BLAST against the nucleotide database, we found that many of our SNP sequences (6 out of 14 SNPs with alignment hits) aligned to immune related regions in salmon (see Appendix 1). For example, hits included the major histocompatibility genes (MHC class I and II), immunoglobulin heavy chain (IgH A) gene and interferon alpha 1-like gene in salmonids. Hence our results suggest the possibility of co-evolution of carotenoid deposition genes with immune genes, especially given the low recombination rates in salmon (Allendorf and Thorgaard 1984). The physical linkage is interesting given that carotenoids are often phenotypically correlated to immune function in salmon (Amar et al. 2004; Tyndale et al. 2008) and other species (Blount et al. 2003; Faivre et al. 2003; Clotfelter et al. 2007). Additionally, in Atlantic salmon, a QTL for flesh colour was found near a QTL for resistance to infectious pancreatic necrosis (Baranski et al. 2010; Houston et al. 2008). A recent study found significant differences between red- and white-fleshed Chinook salmon at two MHC genes (Lehnert et al. 2016), which further supports the functional
linkage between these categories of genes. The co-evolution of carotenoid and immune genes in salmon may provide an explanation for the persistence of white-fleshed Chinook salmon, if the lack of carotenoids could be counteracted by an enhanced immune response through the linkage of colour and immune genes.

When we aligned our candidate SNPs to the Atlantic salmon genome to assess nearby genes for their potential role in carotenoid pigmentation, candidate genes were identified (see Table 2.1) that may play a role in the absorption, transport, metabolism and binding of carotenoids. First, during digestion, dietary astaxanthin is incorporated into micelles then absorbed into the intestine (described in Rajasingh et al. 2006), and genes involved in the intestinal absorption of carotenoids may include scavenger receptor (SCAR) protein genes, including SCARB1 and SCARB3 (Kiefer et al. 2002; Reboul et al. 2005; van Bennekum et al. 2005; Yonekura and Nagao 2007; Sakudoh et al. 2010; Sundvold et al. 2011; Walsh et al. 2012). Although we did not find SNPs located near SCARB1, we did find a SNP located near platelet glycoprotein 4-like which is another name for SCARB3 (and also known as CD36) (see Silverstein and Febbraio 2009). Additionally, we found SNPs located near other SCAR genes of different classes, including class F (member 1-like and 2-like) and class A (member 3-like and 5-like) and scavenger receptor cysteine-rich domain-containing group B protein-like. The most significant SNP ($R^2 = 0.55$) that aligned to the genome from our study was located near SCARF member 2-like, which could be a potential protein related to astaxanthin specific absorption, as different lipid transporters can be associated with different carotenoids (Reboul 2013).

After astaxanthin is absorbed in the intestine, astaxanthin is incorporated into lipoproteins (chylomicrons), then moved into the bloodstream and transported to the liver
for metabolism (described in Rajasingh et al. 2006). Potentially strong candidate genes for astaxanthin metabolism in salmon that were located near SNPs in our study include beta-carotene oxygenase 2a (bco2a), 2b (bco2b) and 2b-like (bco2l) (see Helgeland et al. 2014). Interestingly, bco2b and bco2l were located on Chr 4 in Atlantic salmon, which has previously been identified as harboring QTLs of genome wide significance for flesh colour (Baranski et al. 2010), and in our study, 11 SNPs aligned to a 21Mbp region of Chr 4 within which bco2b and bco2l genes were located (including six significant SNPs). In Atlantic salmon, Helgeland et al. (2014) found that of the three bco2 genes, the bco2l gene had significantly higher expression in tissues (mainly liver and intestine), suggesting that bco2l may be the most important bco2 gene in salmon and likely evolved in teleosts for metabolizing a broader range of carotenoids present in the aquatic environment.

In the liver, astaxanthin that is not metabolized is then packaged into very low-density lipoproteins (VLDL) and transported in the blood to the muscle (Rajasingh et al. 2006). Steroidogenic acute regulatory (StAR) related lipid transfer proteins (including StAR1, StAR3, StAR4, StAR5) have all been implicated in carotenoid binding and deposition in invertebrates and vertebrates (Bhosale and Bernstein 2007; Li et al. 2011; Walsh et al. 2012; Tsuchida and Sakudoh 2015). StAR proteins have been linked to carotenoid binding in the retina of humans (Li et al. 2011), in the midgut and silk gland of silkworms (Bombyx mori; Tsuchida and Sakudoh 2015) and StAR proteins were found to be expressed in the feather and bill of the red-billed quela (Quelea quelea; Walsh et al. 2012). In our study, SNPs aligned to locations near StAR3-like, StAR5, StAR13-like in the Atlantic salmon genome. The SNP that aligned near StAR3-like was also significant in the F3 generation.
In addition to the genes discussed above, genes encoding proteins involved in lipoprotein transport and recognition may also be important for carotenoid pigmentation. During transport to different tissues in salmon, astaxanthin is associated with different types of lipoproteins, including VLDL, low-density lipoproteins (LDL) and high-density lipoproteins (HDL), as well as serum albumin (see Rajasingh et al. 2006). Potential candidate genes related to lipoprotein transport and recognition located near SNPs in our study includes apolipoproteins (APO), APO binding protein, lipoprotein receptors, lipoprotein lipase, ATP-binding cassette (ABC) transporters and perilipin genes. APO genes may be important as APO are bound to lipoproteins and have been associated with carotenoid transport and may be important for receptor specific binding (Ando and Hatano 1988; Rajasingh et al. 2006; Wade et al. 2009). In chum salmon (O. keta), APOs have been isolated from carotenoid carrying lipoproteins (Ando and Hatano 1988). In our study, SNPs aligned to locations near three APO genes, as well as a gene for APO-A1 binding protein. Additionally, several SNPs were located near VLDL and LDL receptor proteins and one lipoprotein lipase-like protein. We also found three perilipin genes near SNPs in our study, and perilipins are associated with lipid storage droplets and may have a function in carotenoid storage (Londo et al. 1996; Walsh et al. 2012; Crawford 2013). Finally, several SNPs were located near ABC genes, some of which include classes A and G which may be involved in transport of astaxanthin (Herron et al. 2006; Iizuka et al 2012).

Here, we characterized significant and suggestive SNP loci, and subsequently identified candidate genes that may be informative for salmon aquaculture and evolution. Although we found fewer significant loci in the F3 families and wild Quesnel River population, our study contributes to the growing knowledge of the genetics of flesh.
pigmentation in salmon, providing support for the trait being controlled by a few major genes. Our results demonstrate that the genes for flesh colour may be conserved across salmonid species; therefore our SNPs may be useful for marker assisted selection (MAS) programs in other species, such as Atlantic salmon, which represent the largest sector (approximately 70%) of the salmon farming industry (2,326,288 tonnes produced in 2014; FAO 2014). In conclusion, our research adds to the growing understanding of the genetic mechanisms influencing flesh pigmentation in salmon, which can contribute to a reduction in cost for the aquaculture industry and further advance our comprehension of salmonid evolution.

References


novel insights into evolution after whole-genome duplication in vertebrates. Nat Commun. 5.


FAO Fisheries and Aquaculture Department. 2014. Species Fact Sheet: Atlantic salmon.


like (also known as CD36, -)

- ATP-binding cassette sub-family D (also known as CDF3, -)

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**Gene Descriptions:**
- S100_448927941: perilipin 2; ATP-binding cassette subfamily A member 1-like
- S100_18914485: ATP-binding cassette subfamily A member 1-like; ATP-binding cassette, subfamily E (OABP), member 1-like
- S100_325279022: low-density lipoprotein receptor class A member 3-like; scavenger receptor class A member 5-like; low-density lipoprotein receptor-related protein 11
- S100_15403293: scavenger receptor cysteine-rich domain-containing group B protein-like
- S100_277727465: ATP-binding cassette subfamily A member 1-like
- S100_96688987: very low-density lipoprotein receptor class A member 3-like
- S100_680289848: low-density lipoprotein receptor class A member 3-like
- S100_277727465: scavenger receptor cysteine-rich domain-containing group B protein-like
- S100_680289848: low-density lipoprotein receptor class A domain-containing protein 3-like
- S100_277727465: beta-carotene oxidase 2a; scavenger receptor class A member 1-like
- S100_680289848: beta-carotene oxidase 2a; scavenger receptor class A member 1-like
- S100_277727465: scavenger receptor class F member 2-like
- S100_680289848: low-density lipoprotein receptor class A domain-containing protein 3-like
- S100_277727465: scavenger receptor class F member 1-like; stAR-related lipid transfer protein 13-like
- S100_680289848: low-density lipoprotein receptor class A domain-containing protein 3-like
- S100_277727465: stAR-related lipid transfer protein 5
- S100_680289848: low-density lipoprotein receptor class A domain-containing protein 3-like
Figure 2.1. Quantile-quantile plot obtained from genome-wide association study (GWAS) analysis for flesh pigmentation in Chinook salmon (*Oncorhynchus tshawytscha*). Results are based on single nucleotide polymorphisms (SNPs) analyzed for a F2 family derived from the backcrossing of two flesh colour populations. SNPs above the dotted line represent loci with genome wide significance based on Bonferroni correction ($p < 3.84 \times 10^{-6}$).
Figure 2. Manhattan plot of all loci (single nucleotide polymorphisms, SNPs) analyzed for association with flesh pigmentation in a F2 family of Chinook salmon (Oncorhynchus tshawytscha) derived from the backcrossing of two flesh color populations. SNPs located above the red line indicate genome-wide significance based on Bonferroni correction (p < 3.84 x 10^-6). Chromosomes are represented by different colors, where (A) represents SNPs mapped to rainbow trout (O. mykiss) genome and (B) represents SNPs that were not mapped. Twelve significant SNPs are located on rainbow trout chromosome 5, 10 and 26 (A), whereas the twelve remaining SNPs could not be mapped to rainbow trout chromosome 5, 10 and 26 (B). No SNPs were mapped to Chr 25 (absent in panel A).
Figure 2. Mean (and standard error) colour scores for genotypes at three single nucleotide polymorphisms (SNPs) that were significantly associated with flesh colour in the F3 backcross generation of Chinook salmon (Oncorhynchus tshawytscha) derived from crossing two flesh colour populations. Significant SNPs include two that were mapped to Chromosome 9 (A) and Chromosome 10 (B) in rainbow trout (O. mykiss) and one SNP that was not mapped to the rainbow trout genome (C). Sample sizes represented by each genotype are presented in each panel.
Figure 2.4. Percentage of red and white Chinook salmon (*Oncorhynchus tshawytscha*) from the Quesnel River, British Columbia that were homozygous (AA) and heterozygous (AT) at a single nucleotide polymorphism (SNP; marker name: S100_567632636) that was significantly (*) associated with colour in the population (*p < 0.05*; see Methods for details).
CHAPTER 3: REDDER ISN’T ALWAYS BETTER: COST OF CAROTENOIDS IN CHINOOK SALMON EGGS

Introduction

Variation in carotenoid pigmentation has been widely studied in the context of both natural and sexual selection (reviewed in Svensson and Wong 2011). Higher carotenoid content has been correlated with enhanced immune function (McGraw and Ardia 2003; Amar et al. 2004), increased mating success (Blount et al. 2001; Craig and Foote 2001; Yang et al. 2013), improved antioxidant status (Pike et al. 2007; Hôrak et al. 2007) and higher offspring quality (Tyndale et al. 2008; Bazyar Lakeh et al. 2010). Indeed, appropriate levels of carotenoids appear to be important for fitness since high levels of carotenoids can also be detrimental in some cases (see Kolluru et al. 2006; Brown et al. 2016). Initial research on carotenoid signals was focused on the benefits for mate attraction and the potential cost of predation risk (Endler 1980). However, the understanding that carotenoids contributed to many physiological functions within the organism (Lozano 1994) resulted in a shift in research focus from predation to the potential trade-offs in carotenoid utilization within the animal (i.e., immune function versus secondary sexual traits) (Blount et al. 2003; Pike et al. 2007; Baeta et al. 2008).

While it is widely speculated that carotenoid pigmentation increases predation risk, aside from studies on a few species, there have been few direct tests of this hypothesis (Kotiaho 2001; Svensson and Wong 2011). For example, in guppies (Poecilia reticulata) and sticklebacks (Gasterosteus aculeatus), brightly coloured males are favoured by females, but experience greater predation risk relative to conspecific drab males (Moodie 1972; 1 Lehnert SJ, Devlin RH, Pitcher TE, Semeniuk CAD, Heath DD. Redder isn’t always better: cost of carotenoids in Chinook salmon eggs. Accepted in: Behav Ecol.
Godin and McDonough 2003). Similarly, in the copepod *Eurytemora affinis*, increased carotenoid pigmentation results in higher growth rates at the cost of increased predation risk (Gorokhova et al. 2013). Higher predation risk on carotenoid pigmentation found in these species may be driven by an innate preference for red or orange colouration (i.e., sensory bias) in the predator, which has been demonstrated across taxa (Rodd et al. 2002; Smith et al. 2004; Grether et al. 2005; Spence and Smith 2008). Although some species can respond to predation cues by reducing carotenoid colouration (van Der Veen 2005; Anderson et al. 2015), this is not an option for all species as carotenoid signals are not always phenotypically plastic traits.

Chinook salmon (*Oncorhynchus tshawytscha*) represent an ideal system to study fitness consequences of carotenoids because a genetic colour polymorphism results in red and white morphs that differ in their ability to deposit carotenoids (primarily astaxanthin; see Tyndale et al. 2008; Garner et al. 2010) into their eggs (Figure 3.1A,B), flesh and skin (see Lehnert et al. 2016), therefore resulting in colouration differences between morphs during multiple life stages. Although individuals can be categorized discretely as red (pigmented) and white (unpigmented), variation in the degree of pigmentation may exist across red individuals. Chinook salmon are the only salmonid species that exhibit this colour polymorphism, and it is suggested that the white colour phenotype may have first appeared during the last glaciation (Hard et al. 1989). In natural populations, morphs vary in frequency, where white individuals can represent 0-100% of the population. However, the evolutionary mechanisms responsible for the maintenance of the polymorphism remains unknown. Yet the persistence of the polymorphism underlies the mystery of why some genera within the family Salmonidae alone among teleosts have evolved such dramatic red colouration. One plausible explanation is that natural selection operates
differentially on red and white Chinook salmon across life stages resulting in potential fitness trade-offs between colour morphs that balance lifetime fitness in the two morphs. In this case, white Chinook salmon may experience different fitness benefits than red Chinook salmon if the lack of carotenoids can provide advantages at certain life stages or if white Chinook salmon have evolved compensatory mechanisms to counteract the expected handicap (see Lehnert et al. 2016). For example, in sockeye salmon (*O. nerka*), incipient speciation has led to a non-anadromous morph (known as kokanee) that have limited access to environmental carotenoids (Craig and Foote 2001). Kokanee have evolved mechanisms to counteract a low carotenoid diet by increasing the efficiency of carotenoid sequestration thus allowing both morphs to display similar red spawning colouration despite strong differences in carotenoid availability (Craig and Foote 2001). Similarly, white Chinook salmon may have evolved other means to counteract the absence of carotenoids, such as through functional genetic mechanisms where selection may operate differentially on immune genes between morphs (see Lehnert et al. 2016) or white Chinook salmon may replace carotenoids with other antioxidants as suggested in Gobidae species with striking differences in egg carotenoid content (Svensson et al. 2009).

The cost of carotenoids has been overlooked throughout all life stages in salmon, and in particular, the egg stage represents an important developmental stage where strong selection pressures can operate (Heath et al. 2003). For example, maternal allocation of carotenoids can increase salmon egg survival, as Tyndale et al. (2008) demonstrated that eggs from red Chinook salmon females experienced greater incubation survival relative to eggs from white females. However carotenoid pigments (primarily astaxanthin as it represents >95% of the total carotenoids found in the eggs (see Tyndale et al. 2008)), are
clearly visible in salmon eggs thus making them highly conspicuous to predators. In nature, many fish prey on salmon eggs such as sculpins (Foote and Brown 1998) and other salmonids including rainbow trout (O. mykiss), coho salmon (O. kisutch), and Dolly Varden (Salvelinus malma) (Kline et al. 1990; Willson and Halupka 1995). These predators can consume salmon eggs either during spawning, or when eggs are mobilized from redds (nests) by digging females or hydraulic actions of rivers. If predators have a bias for red Chinook salmon eggs, this could provide one mechanism by which white Chinook salmon may gain a relative fitness advantage from the absence of carotenoids (and may help explain why not all fish store carotenoids as salmonids do). To test this hypothesis, we used rainbow trout, an ecologically relevant predator (Kline et al. 1990) capable of colour discrimination (Ginetz and Larkin 1973), in choice experiments with red and white Chinook salmon eggs.

Methods

Predator: Rainbow trout

In August 2015, rainbow trout derived from hatchery stock populations of two wild strains were transported from the Fraser Valley Trout Hatchery in Abbotsford, British Columbia, Canada to the Center for Aquaculture and Environmental Research (CAER) in West Vancouver, BC. All rainbow trout used in our study were one generation from the wild and had no prior experience with salmon eggs (i.e., naïve to salmon eggs as a food source). The wild sources of the rainbow trout were Pennask Lake and Blackwater River both of which are riverine spawners. Pennask Lake (GPS coordinates 49°59’24”N and 120°05’57”W) is part of the Thompson River drainage and is located southeast of Merritt, BC, whereas Blackwater River (GPS coordinates 53°18’38”N and 122°52’32”W) is part of the Fraser River drainage and is located northwest of the mouth.
of the Quesnel River, BC. The two trout populations differ in their overlap with spawning Chinook salmon populations, as Blackwater River trout will have contact with spawning Chinook salmon as the river is located near the Quesnel River where both red and white morphs occur at equal frequency, whereas Pennask Lake does not support a spawning Chinook salmon population. Trout were held in 200 L tanks supplied with aerated fresh water at CAER and were fed a commercial salmon pellet diet (Skretting Canada Ltd.) until the experiment was initiated (approximately two months).

**Eggs: Chinook salmon gamete collection**

Eggs used in the study were collected from adults captured from a wild population of red and white Chinook salmon in the Quesnel River, Likely, BC (GPS coordinates 52°36’23’’N and 121°32’57’’W; see Lehnert et al. 2016). Eggs from red and white Chinook salmon females were fertilized on September 21, 2015 at the Quesnel River Research Center, Likely, BC. Eggs of three red and four white females were fertilized with mixed milt from two males to ensure high fertilization success. The colour of the male is not expected to influence egg colour, however all eggs were fertilized with sperm from paired red and white males, with the exception of the eggs of one red female which were fertilized by two white males. Egg weight of red females ranged from 0.247-0.296 grams (mean = 0.273 grams) and egg weight of white females ranged from 0.209-0.296 grams (mean = 0.268 grams). Egg weight did not differ significantly between red and white females in our study (t = 0.25, df = 4.88, p = 0.81). Additionally, to assess egg size differences between the morphs in the population overall, we compared egg weight for red and white females (n = 19 red; 18 white) collected from the Quesnel River population over three spawning seasons (2013 to 2015). We found no significant difference in egg weight between red and white females (t = 0.15, df = 34.56, p-value = 0.88), where mean
egg weight was 0.281 and 0.279 grams for red and white females, respectively. Following fertilization, eggs were treated for 10 minutes with 100 ppm free iodine disinfectant solution (Ovadine; DynamicAqua Supply, Canada). This treatment does not affect egg colouration, however disinfection is necessary when eggs will be transported to other locations (i.e., to reduce spread of disease). Eggs were then incubated in vertical stack trays with eggs separated by colour. At 15 days post-fertilization (approximately 150 accumulated thermal units), eggs were transported to the CAER and placed in vertical stack incubation trays until the experiment. Any dead eggs were removed and not used in the experiment.

**Egg predation experiment**

On October 14, 2015, a total of 16 rainbow trout from each population were divided into four 200 L tanks (8 trout/tank/population) that were light blue in colour. The groups of eight fish represent the predator populations. Artificial light (gold fluorescent light) was used during the experiment, where the spectral distribution ranged between wavelengths of approximately 500 to 750 nm, with peak intensity occurring at 575 nm. The light conditions used here would allow discrimination between red and white eggs, as red carotenoids reflect light of longer wavelengths greater than 600 nm. Additionally, the eyes of rainbow trout have visual pigments that are sensitive to this range of wavelengths (Hawryshyn and Hárosi 1994; Sabbah et al. 2013). Fish were given approximately 22 hours to acclimate to the new tank environment, and thus the experiment began on the morning of October 15. Prior to each experimental period, GoPro™ cameras were set up at the top of each tank to record trout behaviour during the experiment. After 10 minutes of recording, a red and white Chinook salmon egg were released in air (using transfer pipettes) simultaneously less than 20 cm apart from the top of the tank near the water
surface. When eggs were released and entered the water, they moved through the water column to the bottom of the tank, which contained no substrate. This design was chosen to simulate a spawning event, as eggs would be released from the female and sink towards to the gravel where during this time predators would have the opportunity to consume eggs. However, this design can also reflect the alternative situation when spawning females or river turbulence dislodge eggs from the river bottom. In this case, trout will need to move in quickly to consume eggs when the eggs are still in the water column. Eggs were simultaneously released every two minutes for a total of five trials during a 10-minute time span. During each trial, red and white eggs were released from alternating sides (left/right) of the tank, and we note that side of tank had no effect on the time required to consume an egg (p = 0.31; see below), as well as no effect on which egg was consumed first during the trials (n = 126 left side and 124 right side; \( \chi^2 = 0.016, p = 0.90 \); see below). The trials were repeated three times each day, with trials occurring during morning (start time between 8:30 and 8:45), at noon (start time between 12:04 and 12:19) and during the afternoon (start time between 15:12 to 15:30). On October 19, a total of 14 sets of trials were recorded (no trials October 19 in afternoon), after which rainbow trout from all four tanks were sampled for weight. Mean weight (± standard error) of Pennask and Blackwater rainbow trout were 50.4 (± 4.04) and 33.5 (± 2.54) grams, respectively.

**Statistical analyses**

From video recordings during each trial, the amount of time required for a trout to approach and either consume or attempt to consume each egg was recorded. We note that attempts to consume eggs may not result in egg consumption; however both consumption and attempts could still lead to egg mortality, as attempts to bite an egg could result in
egg damage, move an unfertilized egg away from fertilization opportunities or alert other predators to the food source. Therefore we considered time required to consume or attempt to consume an egg (hereafter referred to as consume) to represent the same predation event in our study. First, chi-square tests were used to determine whether there was a significant difference between number of times red versus white eggs were consumed first by rainbow trout within each population. Trials were excluded if both eggs were consumed at the same time (n = 4 out of 278 trials in total) or if neither egg was consumed within two minutes of being dropped into the tank (n = 24 trials). Next, using linear mixed models with Gaussian error distribution in the R software (R Core Team 2014) package lme4 (Bates et al. 2009), we determined whether egg colour and trout population had a significant effect on the time required to consume an egg. Thus, the model included egg colour and population as fixed factors with random factors of date, trial number (1 to 5), tank, time of day and side of tank from which the egg was released (left/right). The interaction between egg colour and population was also tested in the model, and if the interaction was not significant it was removed from the model. Eggs were removed from the analysis if they went out of the video frame within two minutes of being released (n = 10 eggs) or if the egg was not consumed within two minutes (n = 97 eggs), therefore our analysis involved a total of 446 eggs (data points). Time required to consume the egg (dependent variable) was inverse transformed (i.e., 1/time) to meet assumptions of homogeneity and improve normality of model residuals. Given that the inverse transformation results in the reverse order of data values, the inverse transformed data were reflected and reversed by multiplying the inverse by -1 then adding a constant of 2 to return data to positive values in their original order. Date was removed from the model to avoid over-parameterization, as date did not contribute to the variance observed
for time required to consume an egg (p = 0.99). Using log-likelihood ratio tests, models were compared with and without each factor to determine their effect in the predictive capability. Finally, we also examined colour bias over time, where we first used a general linear model in R with logit link function for binary data where each egg was coded as 1 if it was consumed first or 0 if it was not consumed first (n = 500 eggs). To test the colour bias over time, we tested the interaction of colour and date. If the interaction was significant signifying that egg colour bias changed over time, we then used chi-square tests to compare the number of red versus white eggs consumed first on each day of the experiment.

Results

During choice trials, we found that twice as many red eggs as white eggs were selected first by rainbow trout from both populations within trials (Figure 3.1C; $\chi^2$ tests; Blackwater: $\chi^2 = 15.23$; p < 0.001; Pennask: $\chi^2 = 17.31$; p < 0.001; overall: $\chi^2 = 32.40$; p < 0.001). Although we found a significant colour effect, we found no effect of side of tank from which the egg was released ($\chi^2$ tests, all p-values > 0.78). Given that red and white eggs were dropped from alternating sides of the tank, it is possible that fish could predict the side from which the red egg would be released next after the first trial.

Therefore, we also compared the difference between the number of red and white eggs selected first during only trial 1 for each set of predation trials. Although these trials represent only one fifth of the data, we still found that significantly more red eggs were consumed first overall during the first trial (n = 35 red and 14 white; $\chi^2 = 9.00$, p = 0.003), where the difference was significant in the Blackwater population ($\chi^2 = 6.00$, p = 0.014) and the difference approached marginal significance in the Pennask population ($\chi^2 = 3.24$, p = 0.07). Next, we note that not all eggs were consumed during the trials, and we
found that overall trials significantly more white eggs (n = 66) than red egg (n = 31) were left unconsumed within two minutes of being released (i.e., within a trial) ($\chi^2$ test; $\chi^2 = 12.63$, $p < 0.001$). Of the eggs that were consumed (n = 446 eggs), red eggs were consumed significantly faster than white eggs (Figure 3.1D and Table 3.1; Linear mixed model $\chi^2 = 8.03$, $p = 0.005$). Although we found a significant tank effect (random effect) on time to consumption ($\chi^2 = 34.4$; $p < 0.001$), there were no population (fixed effect) differences in how quickly eggs were consumed ($\chi^2 = 2.18$, $p = 0.14$) (Table 3.1). We also found that the amount of time required to consume an egg upon release increased significantly ($\chi^2 = 16.1$, $p < 0.001$) from trial 1 to trial 5 within each round of egg choice (Table 3.1), consistent with partial satiation. Random effects including time of day and side of tank were not significant in the model (see Table 3.1). Additionally, we note that there was no significant interaction between egg colour and population on time to consumption ($\chi^2 = 0.30$, $p = 0.58$), therefore the interaction term was not included in the model. Next, we examined colour bias over time using a logistic regression. Based on the analysis of deviance from the logistic regression, we found a significant interaction between colour and date in the model ($p = 0.004$). Given the significant interaction, we used chi-square tests to compare the number of red versus white eggs consumed first on each day of the experiment. We found that the colour bias was greater at the beginning of the experiment (Figure 3.2). On the first and second day of the experiment, we found a significant difference between the number of red and white eggs that were consumed first (Figure 3.2; $p$ values < 0.001), however the difference was no longer significant by the third and later days of the experiment ($p$ values > 0.05).

**Discussion**


In our study, we found that increased redness (i.e., astaxanthin content) of salmon eggs can increase predation risk, thus demonstrating, for the first time, a cost of carotenoids in salmon. The observed behavioural bias for red egg predation demonstrated by both trout populations in our study seems most likely to be a consequence of differences in their detection ability for the two egg colours, which may be a result of differences in colour (red versus white) or luminosity (dark versus light). Rainbow trout are visual predators and a previous study found that rainbow trout food colour preference was often dependent on contrast with background colour (Ginetz and Larkin 1973), and in our study, conspicuous red eggs had higher contrast with tank background (light blue) compared to white eggs. Alternatively, the behaviour may be driven by a pre-existing sensory bias for red colouration (as documented in fishes (Smith et al. 2004; Spence and Smith 2008), including salmon (Clarke and Sutterlin 1985)) given that rainbow trout have their own carotenoid requirements to fulfill. This hypothesis may be supported by the fact that both trout populations demonstrated a similar bias, even though only the Blackwater population (and not the Pennask population) overlaps with spawning Chinook salmon. Additionally, bias may also be motivated by preference for red eggs and/or avoidance of white egg. Although odor was not measured here, it is possible that the different chemical composition or concentration of red and white eggs could influence their odor and therefore their detection. Salmon egg predators, such as sculpin (Cottus sp.), have previously been demonstrated to rely on chemical cues from eggs for detection (Dittman et al. 1998). However, trout often approached eggs quite rapidly without much time to assess the odor. Therefore, the difference in visibility (due to colour or luminosity) of red and white eggs is most likely responsible for the bias because we found that the colour bias was greatest at the beginning of the experiment. The decrease in colour bias over
time may indicate that experience can alter the ability of trout to detect both egg colours and indicate that negative frequency-dependent selection may be operating to maintain the polymorphism, where the rare morph experiences a fitness advantage due to an inability of predators to recognize their unfamiliar eggs (Oldendorf et al. 2006). Alternatively, changes over time may represent a decrease in carotenoid requirements by rainbow trout if enough carotenoids have been ingested and the resource has become less valuable; however, this scenario seems unlikely given the short amount of time. Additionally, there is no evidence to suggest a detrimental effect of excess carotenoid supplementation in salmonids (Torrissen and Christiansen 1995; Amar et al. 2004; Page et al. 2005; Bazyar Lakeh et al. 2010; but see Costantini et al. 2007; Huggins et al. 2010 for evidence in other taxa) or that fishes know when they have attained their requirement.

Our documented advantage for white Chinook salmon may not only be important during the egg stage, but also during the critical life stage following hatch (alevin stage) as visible colour (i.e., carotenoid) differences still exist in alevin yolk sacs. Although the percentage of salmon eggs (and alevins) that are lost due to predation is not well documented, previous studies indicate that in some rivers during spawning, salmon eggs can represent 84% of the rainbow trout diet (Idyll 1942) and >90% of the diet of other juvenile salmonids (Johnson and Ringler 1979). In sockeye salmon (O. nerka), it was estimated that up to 16% of spawned eggs may be consumed by sculpin predators (Foote and Brown 1998). Therefore, we estimated relative fitness for Chinook salmon during the egg stage, where we used egg incubation survival for red and white eggs from a previous study (Tyndale 2005) and we estimated predator avoidance based on our study and assuming varying levels (low to high) of egg predation (see Table 3.2). Using these measures, we estimate that relative fitness for white Chinook salmon eggs is 0.84 under
low (5%) predation rate and 0.92 under medium (25%) predation rate (Table 3.2). Under a high (50%) predation rate scenario, white Chinook salmon would have a fitness advantage, where the relative fitness of red Chinook salmon eggs would be 0.95. Thus, when considering the increased predation risk due to carotenoids, the relative fitness of white eggs increases with increasing predation rate, where fitness of both morphs is equal when predation rate is approximately 41.5%. Although the advantage of reduced predation risk does not outweigh the cost of reduced incubation survival in white eggs under rates of predation that are likely ecologically relevant (low to medium risk), differences in fitness during later life stages may further minimize fitness differences between morphs. Specifically, if white Chinook salmon have evolved compensatory mechanisms to deal with their lack of carotenoids throughout life stages the relative fitness of the red and white morphs may be further modified. In this case, lifetime fitness of morphs could be balanced and thus maintain the persistence of the white morph.

Salmon egg predation can occur when eggs are released by spawning females or when eggs are mobilized from the gravel by nest building activity or hydraulic actions of the river, and our study design can be argued to represent both of these situations when eggs are present in the water column. In our experiment trout experienced no fitness costs for their choices; however, in nature, both red and white eggs will not be available from a single female at the same time. Trout may thus need to invest energetically in moving among spawning females, and thus would need to assess costs and benefits of movement decisions. If the bias in our study is driven by a preference for red eggs, trout may be willing to accept certain costs to obtain these preferred red eggs (i.e., choosiness). Under certain natural conditions, the bias for red eggs detected in our study under experimental conditions may be amplified or reduced depending on the mechanism for the bias and the
environmental conditions found in the river. For example, if the mechanism driving the bias is due to colour-based detection differences, then the spectrum of light entering the water column may influence the bias, where under high water clarity and light intensity, red eggs may be more detectable than white eggs thus increasing predation risk on red eggs. Whereas, under low-light conditions, predators may rely on other cues such as odor to detect eggs that may minimize predation differences between colours (unless egg colours differ in odor cues). Alternatively, if predation bias differences in our study are a result of egg luminosity, predation bias could increase under low light conditions when visual predators could more readily detect darker (red) eggs. In our study, artificial light conditions may not be representative of the conditions found in the wild, however in nature these conditions are not static, as light will change with time of day, amount of coverage, siltation, water depth and other environmental parameters. Thus although our predation rates observed in experimental tanks may not be directly representative of predation under natural conditions, salmon bearing rivers throughout the Pacific coast can vary in environmental conditions and predator communities. Such potential sources of variation in predator bias, as well as predator density, among different rivers may explain why the white phenotype only persists in certain populations of Chinook salmon.

Nevertheless, our study demonstrates a clear bias by rainbow trout egg predators for red Chinook salmon eggs; thus, a trade-off between red and white Chinook salmon in egg survival (Tyndale et al. 2008) and predation may provide an evolutionary mechanism responsible for the maintenance of this colour polymorphism in nature and explain why not all fish are red.

References


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Kolluru GR, Grether GF, South SH, Dunlop E, Cardinali A, Liu L, Carapiet A. 2006. The effects of carotenoid and food availability on resistance to a naturally occurring
parasite (*Gyrodactylus turnbulli*) in guppies (*Poecilia reticulata*). Biol J Linnean Soc. 89:301-309.


Table 3.1. Estimates (± standard error) and variance components (± standard deviations) of fixed and random effects produced from linear mixed effect models with results of log-likelihood ratio tests ($\chi^2$ and p value) from model comparisons. The full model included the effects listed with the response variable of time (in seconds) required for the egg to be consumed (or attempted to be consumed). A total of 446 Chinook salmon (*Oncorhynchus tshawytscha*) eggs were consumed (or attempted to be) by rainbow trout (*O. mykiss*) during experimental predation trials.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Estimate ± S.E.</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.501 ± 0.085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg colour</td>
<td>0.068 ± 0.024</td>
<td>8.03</td>
<td>0.005*</td>
</tr>
<tr>
<td>Population</td>
<td>0.138 ± 0.107</td>
<td>2.18</td>
<td>0.14</td>
</tr>
<tr>
<td>Random</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial</td>
<td>0.004 ± 0.065</td>
<td>16.1</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Time of day</td>
<td>0.000 ± 0.028</td>
<td>1.61</td>
<td>0.20</td>
</tr>
<tr>
<td>Tank</td>
<td>0.011 ± 0.105</td>
<td>34.4</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Side of tank</td>
<td>0.001 ± 0.025</td>
<td>1.04</td>
<td>0.31</td>
</tr>
<tr>
<td>Error</td>
<td>0.062 ± 0.249</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Relative fitness estimates of red and white Chinook salmon (*Oncorhynchus tshawytscha*) during the egg stage based on estimates of incubation survival and predation avoidance (proportions) for red and white eggs under different levels of predation.

<table>
<thead>
<tr>
<th>Fitness trait</th>
<th>Predation rate</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (5%)</td>
<td>Medium (25%)</td>
<td>High (50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>White</td>
<td>Red</td>
<td>White</td>
</tr>
<tr>
<td>Incubation survival$^\S$</td>
<td>0.921</td>
<td>0.762</td>
<td>0.921</td>
<td>0.762</td>
</tr>
<tr>
<td>Predation avoidance$^\S$</td>
<td>0.966</td>
<td>0.984</td>
<td>0.830</td>
<td>0.920</td>
</tr>
<tr>
<td>Overall egg survival$^\S$</td>
<td>0.890</td>
<td>0.750</td>
<td>0.764</td>
<td>0.701</td>
</tr>
<tr>
<td>Relative fitness</td>
<td>1</td>
<td>0.843</td>
<td>1</td>
<td>0.917</td>
</tr>
</tbody>
</table>

$^\S$Based on results obtained from Tyndale (2005) from wild populations of red and white Chinook salmon.

$^\S$Based on number of red versus white eggs consumed first overall eggs consumed and multiplied by predation rate then subtracted from 1.

$^\S$Incubation survival multiplied by predation avoidance.
Figure 3.1. Typical red and white eggs from red and white Chinook salmon (Oncorhynchus tshawytscha) females from the Quesnel River, British Columbia, Canada, where A: shows red and white fertilized eggs in an incubation tray and B: shows unfertilized red and white eggs with scale (one centimeter). C: Number of red and white Chinook salmon eggs consumed first by rainbow trout (O. mykiss) from two trout populations (Blackwater and Pennask) during egg predation experiments, where significant differences based on chi-square tests are indicated by asterisks (*) and mean ± standard error (m) M means based on chi-square tests are indicated by asterisks (*). D: Mean (± standard error) time required by rainbow trout from both populations (Blackwater and Pennask) to consume (or attempt to consume) red and white Chinook salmon eggs overall trials (n = 446 eggs), where asterisk (*) indicates significant differences between egg colours based on linear mixed models (see Table 3.1 for full model results).
Figure 3.2. Proportion of red and white Chinook salmon (*Oncorhynchus tshawytscha*) eggs consumed (or attempted to be consumed) first by rainbow trout (*O. mykiss*) overall tanks during each day of the predation experiment from October 15 to 19, 2015. Asterisk (*) over bar indicates significant difference between the number of red and white eggs consumed first by rainbow trout (Days 1-2: p values < 0.006; Days 3-5: p values ≥ 0.052). Dotted line represents equal consumption of red and white eggs.
CHAPTER 4: VARIATION IN MATERNAL PROVISIONING OF CAROTENOIDS IN CHINOOK SALMON EGGS: EFFECTS ON OFFSPRING PERFORMANCE

Introduction

During early life, maternal effects play an important role in determining offspring phenotype, where both the mother’s genotype and environmental experiences can have substantial impacts on offspring fitness (Mousseau and Fox 1998). In oviparous species, an important maternal effect is egg quality, which can be determined by egg size as well as the provisioning of maternally derived compounds into the egg such as lipids, antioxidants, antibodies and hormones (Williams 1999; Groothuis et al. 2005; Hasselquist and Nilsson 2009; Deeming and Pike 2013). One group of antioxidants that are considered important for determining egg quality includes carotenoid pigments, which have been widely-studied in the context of maternal effects in birds (Blount et al. 2002; McGraw et al. 2005; Biard et al. 2005; Ewen et al. 2009; Marri and Richner 2014) and fishes (Svensson et al. 2006; Grether et al. 2008; Tyndale et al. 2008; Bazyar Lakeh et al. 2010; Brown et al. 2014).

Carotenoids are produced by photosynthetic organisms and microorganisms; therefore, animals cannot synthesize carotenoids de novo but must acquire these pigments through their diet (Goodwin 1986). The maternal provisioning of carotenoids to eggs can provide offspring with benefits due to their role as antioxidants (Krinsky 2001), where carotenoids can shield the offspring from oxidative stress. Oxidative stress occurs when reactive oxygen species (ROS) accumulate and lead to damage of proteins, lipids and DNA. Carotenoids neutralize ROS produced during normal cell metabolism (Chen et al. 2010). This manuscript is part of a collaborative research project. Co-authors on the manuscript (in prep) include Lehnert SJ, Devlin RH, Pitcher TE, Garver KA, Richard J, Heath DD.
2003), and, during embryonic development, egg carotenoids may be important for quenching the high levels of ROS that are produced during this period of rapid growth (Deeming and Pike 2013). ROS are also generated during the immune response (Nathan and Cunningham-Bussel 2013). Therefore the antioxidant role of carotenoids may also have indirect effects on several components of the immune system (reviewed in Chew and Park 2004), where increased carotenoids could allow an individual to mount a greater immune response. The benefits of egg carotenoids can therefore influence offspring fitness in diverse ways, where, across taxa, the link between maternal carotenoids and offspring performance includes increased egg and early life survival (McGraw et al. 2005; Tyndale et al. 2008), enhanced immune function (Biard et al. 2005; Saino et al. 2003; Ewen et al. 2009), increased size (Marri and Richner 2014), higher growth rate (Bazyar Lakeh et al. 2010) and greater antioxidant status (McGraw et al. 2005).

Despite the demonstrated benefits of maternal carotenoids, large variation can still exist within and among species in egg carotenoid content (Withler 1986; Svensson et al. 2006; Svensson et al. 2009; Deeming and Pike 2013). Chinook salmon (Oncorhynchus tshawytscha) can exhibit such variation, where differences in egg carotenoid content exist due to genetic polymorphisms that affect carotenoid deposition into tissues including eggs, skin and flesh (Withler 1986; Tyndale et al. 2008), resulting in two colour morphs: red and white. Unlike the more abundant and widespread red Chinook salmon, white Chinook salmon have white (or pale) eggs and flesh, and they appear grey in external spawning colouration. Frequencies of red and white morphs vary throughout western North American Chinook salmon populations (Hard et al. 1989), with each morph ranging from 0-100%. The many benefits of carotenoids on early life stages in salmon have been demonstrated, where for example, increased egg carotenoid content can
increase egg survival, offspring disease resistance (Tyndale et al. 2008) and offspring growth rate (Bazyar Lakeh et al. 2010). Additionally, studies that use carotenoid supplementation in salmon fry have shown that increased dietary carotenoids can lead to increased survival and growth (Christiansen et al. 1995b), as well as resistance to bacterial (Christiansen et al. 1995a) and viral pathogens (Amar et al. 2012). Carotenoid supplementation in salmon fry has also been demonstrated to enhance non-specific immunity (Amar et al. 2004) and improve antioxidant status of fry (Christiansen et al. 1995a). Given the many benefits of carotenoids, it is perhaps surprising that white Chinook salmon can continue to persist in nature. However, it is possible that the white morph has evolved mechanisms to compensate in the absence of carotenoids. For example, although carotenoids may increase immune function, white Chinook salmon may compensate through functional genetic mechanisms (Lehnert et al. 2016). Lehnert et al. (2016) found that white Chinook salmon were more heterozygous at an important immune function gene (major histocompatibility complex; MHC II B1) which could indicate that white Chinook salmon compensate for their lack of carotenoids through increased MHC diversity that allows them to deal with a wider range of pathogens. Additionally, the reduced carotenoid content of white Chinook salmon eggs may lead to benefits in terms of lower predation risk (Lehnert et al. submitted). Therefore, despite the difference in carotenoid utilization between red and white Chinook salmon, both morphs may gain advantages by adopting different strategies to improve offspring fitness.

In our study, we investigate a population of red and white Chinook salmon from the Quesnel River, British Columbia where the frequency of each morph is relatively equal (Withler 1986). Spawning red and white Chinook salmon were crossed to create four 4x4 full factorial crosses, resulting in 64 families that included pure red (red eggs
sired by red males), pure white (white eggs sired by white males) and reciprocal red/white crosses (white eggs sired by red males and red eggs sired by white males). Using a common garden approach, we measured fitness related traits in offspring to assess the effect of dam colour (i.e., egg carotenoid content) during early life stages. Although we were mainly interested in the effect of dam colour on offspring performance, our design also allowed us to test differences in offspring sired by red and white males, however we expect male effects to be minimal during early life when maternal effects are strong. Fitness related traits included egg and fry survival, as well as fry size and condition. Additionally, given that carotenoids have been correlated to immune function in salmon (Christiansen et al. 1995a; Amar et al 2004; Tyndale et al. 2008), including resistance to an endemic fish pathogen, infectious hematopoietic necrosis virus (IHNV) (Amar et al 2012), Chinook fry herein were subjected to a live IHNV challenge to assess susceptibility differences between colours under laboratory challenge conditions. Survival and gene expression of immune, stress and oxidative stress genes were measured to determine the response of fry to the virus challenge. If maternal carotenoids influence early life fitness in Chinook salmon, we would expect to detect differences in fitness traits between crosses involving red versus white dams. If differences are not detected here, given demonstrated effects in many other studies, our results may indicate that white Chinook salmon have evolved compensatory mechanisms to deal with their presumed carotenoid handicap and thus explain in part how the stable polymorphism is maintained in nature.

Methods
**Fish and gamete collection**

In fall 2015, spawning Chinook salmon were collected by seine from the Quesnel River, Likely, BC, Canada. Fish collection occurred from September 18 to 30. Fish were captured by net then held in the river temporarily before being transported to the Quesnel River Research Center (QRRC). During transport, fish were held in tanks with aerated river water for approximately 15 minutes (5 km). At QRRC, fish were transferred to semi-natural spawning channels at 10°C until sampling.

All fish were characterized as “red” (pigmented) or “white” (non-pigmented) based on external spawning colouration (Withler 1986; Lehnert et al. 2016). Individuals were assigned as red when they displayed external red skin pigmentation and individuals were assigned as white when they showed no external red pigmentation and were grey in colouration. Colour assignment of females was also confirmed by egg colour, where egg colour always agreed with skin colour assignment. Following colour assignment, fish were fin clipped for genetic analysis and then gametes were collected. For males, milt was collected through either live spawning or humane euthanization, where males were wiped dry to remove excess water and then gentle pressure was applied to the abdomen to express milt for collection. Milt was collected into a plastic bag, then sealed and kept cool at approximately 4°C. For females, eggs were stripped after the fish was humanely euthanized and eggs were kept covered in plastic bags at approximately 4°C until fertilization.

**Fertilizations**

The breeding design included four 4x4 full factorial crosses, where each 4x4 included two red and two white males crossed with two red and two white females. The breeding design resulted in 64 families, which included 16 pure red families, 16 red-white
(red dam x white sire) families, 16 white-red (white dam x red sire) families and 16 pure white families. The full factorial design allowed us to determine the main effect of dam colour and sire colour on offspring performance while accounting for effects on variation caused by individual dam, sire and their interaction. Gamete collection and fertilizations of crosses occurred on September 21, 26 and 29, and all fertilizations were performed within 24 hours of gamete collection. Fertilized eggs were moved into a vertical stack incubation tray system. Each incubation tray was divided into 16 cells; therefore, eggs from each cross were divided between two replicate cells in the incubation tray. Upon placement into the incubation tray, eggs were subjected to a 100 ppm free iodine disinfectant solution (Ovadine; DynamicAqua Supply, Canada). Eggs were incubated in hatchery (well) water at 10°C until the eyed egg stage (250-500 accumulated thermal units, ATU).

**Egg survival and transport**

Egg survival to the eyed-egg stage was determined by counting all live and dead eggs at the eyed-egg stage. Given that we could not discriminate between dead fertilized and dead unfertilized eggs, eyed-egg survival may be an underestimate of actual survival if not all eggs were fertilized. On November 4, 2015, live eyed-eggs were transported approximately 8 hours from QRRC to the Fisheries and Oceans Canada Center for Aquaculture and Environmental Research (CAER) in West Vancouver, Canada. During transport, eggs were kept cool and moist in insulated boxes designed for salmonid egg transport (Troutlodge Inc.). Upon arrival at CAER, approximately 200 eggs (based on weight) from each cross (i.e., family) were placed in Whitlock-Vibert boxes in a stacked incubation tray system in 10°C well water. Due to mortalities within families, some boxes contained fewer than 200 eggs and some families were lost due to low viability. At this
stage, there were 56 (of the 64) families remaining, and 10 families had fewer than 200 eggs (range 87-177 eggs). In the Whitlock-Vibert boxes, eyed-egg survival was recorded as well as subsequent alevin survival after hatching until offspring reached the exogenous feeding stage (1000 ATU).

**Fry growth and survival**

When offspring reached the exogenous feeding stage (i.e., fry), 120 fish from each family (n = 56 families) were moved into individual 19-L buckets. Five families had fewer than 120 fish, where offspring remaining in these families ranged from 73 to 106. Fish were moved to buckets on either December 22 or 29, 2015 depending on days post-fertilization. On the day following transfer, a total of 15 fish per family were weighed and measured for fork length. Fish were fed a diet of low-pigment feed (Taplow Feeds Ltd.), which contained no added carotenoids and only natural pigments. Fish were fed daily and all mortalities were recorded. To determine fry growth, weight and fork length of 15 fish per family were recorded approximately every 8 to 10 weeks at three more sampling dates including February 17, April 25 and July 7, 2016. Prior to the February measurement, fish from some families were sampled and transported for an immune challenge (see below), thus fish were culled across families in February to equalize densities. At the February sampling, mean fish density was 34 fish/family with 54 families remaining. No fish were culled after this date, therefore we had two estimates of fry survival: 1) early fry survival (approximately 4 weeks from freshwater entry to late January) and 2) late fry survival (approximately 20 weeks from February to July).

**Immune challenge**

On January 27, 2016, a subsample of fry (n = 1952) from 32 families (two full 4x4 crosses) were moved to the Pacific Biological Station (PBS) in Nanaimo, BC for an
immune challenge. At time of virus exposure, fish were approximately 1 gram and were
chosen as the earliest life stage to likely retain maternal carotenoids in the body, yet were
of suitable size to allow sufficient RNA isolation from tissues and be immunocompetent.
Additionally, an early life stage was chosen as younger fish are typically considered more
susceptible to infectious hematopoietic necrosis virus (IHNV) infection (LaPatra 1998).
For the immune challenge, salmon fry were exposed to live IHNV, which is a well
studied fish pathogen belonging to the family Rhabdoviridae (Bootland and Leong 2011).
IHNV is a negative-sense single-stranded RNA virus that infects both farmed and wild
salmonids globally and it can lead to significant mortality in these species (LaPatra 1998;
Saksida 2006; Bootland and Leong 2011). When fish are infected with the virus some of
the symptoms they may exhibit include darkening body colouration, lethargy, swollen
abdomen, abnormal bulging of the eye (exophthalmia), paleness of gills, hemorrhaging and
mortality (Bootland and Leong 2011). It is suggested that sockeye salmon and kokanee
(O. nerka) are natural hosts of IHNV (LaPatra 1998); therefore, IHNV prevalence may be
higher in rivers with abundant sockeye and kokanee populations. We thus chose IHNV
due to its ecological relevance given that the Quesnel River maintains both sockeye and
kokanee populations with close to one million sockeye moving through the Quesnel
system during a dominant spawning year (Fisheries and Oceans Canada 2014). IHNV
was also chosen because carotenoid supplementation was previously correlated to IHNV
resistance in rainbow trout (Amar et al 2012). In our study, to measure the immune
response following viral exposure, we used two approaches: 1) post-challenge survival
and 2) post-challenge gene expression. The IHNV strain used in our study was isolated
from Chinook salmon from Robertson Creek and belonged to the endemic U genogroup
(one of the 5 major genogroups of IHNV).
Post-challenge survival

The survival challenge was started one week after fish arrived at PBS in order to provide an acclimation period. Three 50-L tanks each with 480 fish (15 fish/family) were used to measure survival following viral exposure. Two tanks were subjected to the live IHNV and one tank was a ‘mock’ (control) tank that was subjected to a buffer solution (Hank’s balance saline solution, HBSS). On February 4, fry were exposed to IHNV or HBSS via one-hour immersion in an aerated static bath. After one-hour exposure in static water, water flow was resumed in each tank. Viral titers of water were determined at one-hour post challenge where the control tanks was negative for the virus and the two challenge tanks had viral concentrations of $1.14 \times 10^5$ pfu/ml and $1.10 \times 10^5$ pfu/ml. Throughout the pre- and post-challenge period, fish were fed and mortalities were recorded three times per day. Mortalities were monitored and recorded for 35-days following the challenge. A fin clip from dead fry was preserved in high salt preservative buffer ($3.5 \text{ M ammonium sulfate; } 15 \text{ mM EDTA; } 15 \text{ mM sodium citrate; pH 5.2}$) to assign the fish to a family using genetic analyses. DNA was extracted from fin clips of dead offspring and parents using a plate-based extraction method (Elphinstone et al. 2003). Six microsatellite loci (OtsG68, OtsG432 (Williamson et al. 2002), Ots211 (Greig et al. 2003), Omy325 (O’Connell et al. 1997), Ots107 (Nelson and Beacham 1999) and Ots1 (Banks et al. 1999)) were chosen to discriminate between families for parentage assignment. PCR conditions included: a 5 minute denaturation step ($94^\circ \text{C}$), followed by 33-38 cycles of a 20 second denaturation step ($94^\circ \text{C}$), a 20 second annealing step ($53^\circ \text{C}$ – OtsG68, Ots1; $54^\circ \text{C}$ – Omy325; $56^\circ \text{C}$ – OtsG432; $58^\circ \text{C}$ – Ots107; $60^\circ \text{C}$ – Ots211) and a 30 second extension step ($72^\circ \text{C}$), followed by a final extension of 3 minutes. PCRs were performed with fluorescently dye-labeled forward primers and products were visualized.
using a LiCor 4300 DNA analyzer (LiCor Biosciences, Inc.). Alleles were scored using GENE IMAGIR 4.05 software (Scanalytics Inc.) to determine fragment sizes. CERVUS version 3.0 (Kalinowski et al. 2007) was used to assign parentage to all mortalities using a 1% genotyping error rate and a strict 95% confidence level.

*Post-challenge gene expression*

In addition to survival post challenge, we also quantified the expression of immune, stress and oxidative stress genes (see Table 4.1 for list of genes). To measure gene expression following viral exposure, four 15-L tanks each with 128 fish (4 fish/family) were used to measure the response, where two tanks were used as ‘mock’ controls and two tanks were subjected to the live virus. The challenge occurred on February 15, 2016, where two challenge tanks received live IHNV and two mock tanks received buffer solution (HBSS). The exposure conditions were the same as those described above in the survival challenge (see above), where water flow was stopped for one hour during the viral or HBSS exposure. Viral titers of water were determined at one-hour post challenge where control tanks were negative for the virus and the two challenge tanks had viral concentrations of $1.74 \times 10^4$ pfu/ml and $1.11 \times 10^4$ pfu/ml. Fish were lethally sampled 72-hours post exposure, where fish were captured and euthanized in MS-222. Fish were cut along the ventral side to open the abdominal cavity and allow proper preservation of tissues. Each fish was placed in individual tubes with high salt preservative buffer for later RNA and DNA analysis. Fish were weighed post-preservation to determine relative weight and a fin clip was taken for genetic analysis. Given that all 32 families were combined into tanks during experiments, individuals were genotyped at six microsatellite markers (as described above) to assign fish to their individual family.
RNA extraction and cDNA synthesis

Total RNA was extracted using mechanical homogenization of gill tissue in 0.5 mL of TRIzol (Invitrogen). We chose to extract RNA from the gill tissue, as the gills are one site of viral entry for IHNV (Bootland and Leong 2011), and given the small size of fry, gill tissue was large enough to acquire sufficient RNA. Isolation of total RNA followed the manufacturer’s protocol (http://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf). Quality of total RNA was visually assessed using gel electrophoresis where well-defined 28S and 18S rRNA bands with minimal low molecular weight smear indicated high-quality RNA. Additionally, purity and quantity of RNA were assessed using spectrophotometry on a NanoVue spectrophotometer (GE Healthcare Bio-Science Corp), where good RNA samples were those that had concentrations >125ng/uL and purity values that ranged from 1.8-2.0 (A260/280). After quality checking, a total of 462 samples (out of 493) were deemed to be of high quality and quantity and thus used for subsequent analysis. Total RNA was diluted to a concentration of 125 ng/uL, and diluted RNA (0.5 ug) was then treated with DNase I (Promega Corp) to remove any genomic DNA contamination. Next, total RNA was converted to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), with an amount of 0.5 ug of total RNA was used in each reaction.

Quantitative real-time PCR

Primers and TaqMan MGB probes of selected genes (see Table 4.1 and Appendix 2) were synthesized and spotted onto OpenArray chips prepared by Applied Biosystems. OpenArray chips were designed using the 56x48 format, which allowed us to measure gene expression for 48 individual cDNA samples at 28 target genes in duplicate on a
single chip, and therefore a total of 10 chips were used in our study. QuantStudio 12K Flex Real-Time PCR System was used for OpenArray quantitative real-time PCR (qRT-PCR) following instructions from the manufacturer. Briefly, in a 384-well plate, a 5 uL mixture was prepared for each sample which contained 2.5 uL of TaqMan® OpenArray® Real-Time PCR Master Mix (Applied Biosystems) and 2.5 uL of diluted cDNA (1.2 uL cDNA with 1.3 uL of ddH2O). The prepared samples were loaded onto the OpenArray chips with the OpenArray AccuFill System where the qRT-PCR reaction volume was 33 nL.

Selection of candidate genes

All genes used in our study and their accession numbers are provided in Table 4.1. Primers and TaqMan MGB probes were previously designed in other salmonid studies, and a list of these studies along with the primer and probe sequences are provided in Appendix 2. First, we selected genes to act as endogenous controls during qRT-PCR. Selected endogenous controls included elongation factor 1-alpha (EF1A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and acidic ribosomal phosphoprotein P0 (ARP). All three genes have been previously used as endogenous controls in various studies on salmonids (Purcell et al. 2004; Purcell et al. 2006; Miller et al. 2007; Cuesta and Tafalla 2009; Chaves-Pozo et al. 2010; Ching et al. 2010; Wellband and Heath 2013; Henriksen et al. 2015). We also included a gene from the virus which encodes the IHNV nucleocapsid (N) protein to determine presence and absence of virus within each sample (Purcell et al. 2013).

We selected immune genes that were demonstrated to be upregulated following viral exposure in other salmonid studies (Hansen and LaPatra 2002; Purcell et al. 2004; Purcell et al. 2006; Eder et al. 2008; Bootland and Leong 2011; Herath et al. 2012). Genes
involved in the early anti-viral response include interferon regulatory factors (interferon regulatory factor 3, IFR3), type I interferons (interferon alpha 1, IFNA1) and interferon inducible genes such as Mx protein (MX1) and VHSH induced gene 1 (VIG1) (Purcell et al. 2004; Purcell et al. 2006; Bootland and Leong 2011). The early response will also involve upregulation of pro-inflammatory cytokines such as interleukin 1 beta (IL1B), tumour necrosis factor alpha (TNFA) and the upregulation of the chemokine interleukin 8 (IL8), serum amyloid A (SAA) and heat shock protein 70 (HSP70) (Purcell et al. 2004; Purcell et al. 2006; Bootland and Leong 2011; Herath et al. 2012). Production of a type II interferon (interferon gamma, IFNG) will also be activated during the early viral response (Purcell et al. 2006). Upon activation of the innate immune response, components of the adaptive immune response may be recruited to the infected cells including CD8 alpha chain (CD8), major histocompatibility complex genes (MHC I and II), T cell receptor beta chain (TCRB), immunoglobulin Mu membrane form heavy chain (IGM) and immunoglobulin Tau heavy chain (IGT) (Purcell et al. 2006; Bootland and Leong 2011; Herath et al. 2012).

Next, we selected genes to assess physiological stress and oxidative stress in salmon fry. The stress response and oxidative stress response are not independent processes, as stress can induce oxidative stress. Selected genes involved in the stress response included heat shock proteins (HSP70 and HSP90A), which are chaperone proteins (Iwama et al. 1991; Roberts et al. 2010), and glucocorticoid receptor 2 (GR2), which binds to cortisol released upon stress exposure (Iwama et al. 1999). HSP70 is also upregulated during the immune response (as indicated above). Selected oxidative stress genes include antioxidant enzyme genes: superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST) and thioredoxin reductase (TRDNX)
These genes have been associated with oxidative status in fishes (Hansen et al. 2006; Jin et al. 2010; Wacyk et al. 2012; Solberg et al. 2012). Metallothioneins (metallothionein A, META) also play a role in protection from reactive oxygen species and META expression can also be induced by glucocorticoids (Sato and Bremner 1993; Olsson et al. 1995).

**Normalizing gene expression and calculating ΔCt**

Relative cycle threshold (C<sub>RT</sub>) values were obtained for each reaction using ExpressionSuite Software version 1.0 (Applied Biosystems). Reactions were filtered to remove those that showed no amplification, had an undetermined C<sub>RT</sub> value or those where the C<sub>RT</sub> value was greater than 32 cycles. Next, mean C<sub>RT</sub> was calculated for each sample using technical replicates. In some cases, only one technical replicate was usable. Next, using mean C<sub>RT</sub> values, we calculated the theoretical starting concentration (N<sub>0</sub>) of cDNA in each sample. Starting concentration (N<sub>0</sub>) was calculated following Ramakers et al. (2003), where mean threshold (N<sub>CRT</sub>) was divided by mean PCR efficiency (E) to the power of C<sub>RT</sub> (i.e., N<sub>0</sub> = N<sub>CRT</sub>/E<sup>CRT</sup>). Mean PCR efficiency and threshold for each gene were determined using LinRegPCR (Ramakers et al. 2003). Starting concentration (N<sub>0</sub>) was also calculated for three endogenous controls (EF1A, GAPDH, ARP), and the mean of the three N<sub>0</sub> values was calculated to determine a reference starting concentration. Relative expression was then calculated as N<sub>0</sub> target divided by N<sub>0</sub> reference (Relative expression = N<sub>0</sub> target/N<sub>0</sub> reference) for each sample. Relative expression values were used for subsequent analyses.

**Statistical analyses**

All statistical analyses were carried out in R statistical software v3.3.1 (R Core Development Team 2016). Alpha levels were adjusted using Bonferroni correction for
statistical analyses. For performance traits (survival, size and condition), we used an adjusted alpha level of 0.003 (0.05/16) given that 4 measures of performance were measured at 4 time points. For gene expression analyses, we used an adjusted alpha level of 0.002 (0.05/21) given that 21 genes were compared in our analyses. We note that statistical significance of main effects (fixed factors, described below) did not change depending on the type of correction used, as less conservative correction methods (false discovery rate, FDR) showed qualitatively similar results.

Egg survival measures

Prior to egg survival analyses, we removed crosses (n = 8) that had very low egg survival as these crosses were likely not biologically relevant. Four of the families that were removed from the analyses were sired by a single white male, which likely indicate a sperm viability issue for that male. The additional four families were from one pure red, one pure white and two red-white crosses. The total number of families included in our analyses was thus 56 families, where 15 were pure red (red dam x red sire), 12 were red-white (red dam x white sire), 16 were white-red (white dam x red sire) and 13 were pure white (white dam x white sire). We measured survival at two time points during the egg stages: 1) survival to the eyed-egg survival and 2) eyed-egg to fry survival. At the eyed-egg stage all eggs (dead and alive) were counted, and all dead eggs were discarded. Eyed-eggs were then transported to CAER (see above for details) where egg number was equalized among crosses (approximately 200 eggs per cross if possible; see above) and survival was recorded until the fry stage. For each measure of survival, dead eggs/offspring were coded as 0 and live eggs/offspring were coded as 1. Generalized linear mixed models (GLMMs) with logit link function for binary data were run using the glmer function in lme4 package (Bates et al. 2014) in R, where dam colour, sire colour,
egg density (within the cell) and egg size (weight) were fixed factors in the model. We also tested the interaction of dam colour and sire colour, and if the interaction was significant it was included in the models. GLMMs also included random factors of dam, sire, dam x sire interaction, fertilization date, tray position and cell position. Log-likelihood ratio tests were used to determine the significance of each factor in the model, where models were compared with and without each factor. The total numbers of offspring used in our analyses were 41,244 eggs for eyed-egg survival and 10,549 offspring for eyed-egg to fry survival. GLMMs were tested for overdispersion, where overdispersion was determined by dividing residual deviation (rdev) by residual degrees of freedom (rdf) for the model, and if the ratio value was less than 1 then we concluded that the model was not overdispersed. GLMM for eyed-egg survival was slightly overdispersed (rdev/rdf = 1.12), however GLMM for eyed-egg to fry survival was not overdispersed.

**Fry survival measures**

Fry survival was measured at two time points: 1) early fry survival (approximately 4 weeks after freshwater entry) and 2) late fry survival (approximately 20 weeks from February to July), where culling occurred once in between these time points (described above). Analysis of fry survival was conducted using GLMMs in the same way as egg survival analyses, where dead fry were coded as 0 and live fry were coded as 1. GLMMs for fry survival included dam colour, sire colour and density as fixed factors. We also tested the interaction of dam colour and sire colour, and if the interaction was significant it was included in the models. Models included the random effects of dam, sire, dam x sire interaction and date of freshwater entry. Given that fry from a single family were reared in a single bucket (i.e., one bucket per family), we acknowledge that the effect of
the interaction between dam x sire (i.e., family) cannot be separated from the effect of environment (i.e., bucket). Models did not violate the assumption for GLMM of overdispersion (rdev/rdf < 1).

**Fry size and condition**

Fry weight and fork length were measured at four sampling points, which included December 2015, February, April and July 2016. Additionally, we used weight (wt) and fork length (FL) measures to calculate Fulton’s condition factor (K) for fry at each time point using the equation: \( K = 100 \times (\text{wt} \cdot \text{FL}^{-3}) \). The three measures of fish size and condition were used as response variables in linear mixed models (LMMs). LMMs were run using the *lmer* function in *lme4* package (Bates et al. 2014) in R. Models included the fixed factors of dam colour, sire colour and fry density with random effects of dam, sire, dam x sire interaction and date of freshwater entry. We also tested the interaction of dam colour and sire colour, and if the interaction was significant it was included in the models. To determine the significance of each factor in the model, we used log-likelihood ratio tests, where models were tested with and without each factor. Model residuals were assessed for conformation to normality. Generally model residuals followed a normal distribution but in some cases (early freshwater measurements) the distribution was slightly skewed due to a few individuals with higher or lower values. However, in these cases, no transformation (i.e., log, inverse, square root) could improve normality distribution and thus the analyses were run with non-transformed data.

**Immune challenge survival and gene expression**

First, we examined whether there was a significant effect of treatment (mock challenge versus IHNV challenge) on post-challenge survival. We used GLMMs with logit link function for binary data, where offspring that died post-challenge were coded as
0 and offspring that survived were coded as 1. Any offspring that died pre-challenge were removed from the analysis. GLMMs included treatment (challenge versus control) as a fixed effect, as well as dam colour and sire colour. Random effects in the model included dam, sire, dam x sire interaction and tank. If treatment had no effect in the model, then we determined viral exposure had no influence on mortality.

Next, linear mixed effects models with relative gene expression as the response variable were used for gene expression analyses. Linear mixed effect models included treatment (IHNV challenge versus mock challenge) as a fixed effect, as well as dam colour and sire colour. The interaction of sire colour and dam colour was also tested, but only included in the model if it was significant. Fish weight was included as a fixed effect if it was significantly correlated to relative expression (p < 0.05). Random effects in the model included dam, sire, dam x sire interaction, OpenArray chip and tank. Models were compared in the same way as described above, where log likelihood ratio tests were used to determine significance of each factor in the model. We first were interested in whether treatment resulted in a significant effect on relative expression, thus indicating that fish were responding to the viral challenge. Dam colour and sire colour were also included in the model to determine their effect on relative gene expression of offspring. Residuals of models were inspected for deviations from normality. If necessary, data were transformed (natural log transformed, negative inverse or square root transformed) to improve normality of model residuals.

**Results**

*Egg and fry survival*

All survival results are presented in Table 4.2. There was no significant effect of dam colour (i.e., egg colour) on survival to the eyed-egg stage or survival from the eyed-
egg to fry stage (see Figure 4.1; p values > 0.06). Mean (± standard error) family eyed-egg survival for red (n = 26 families) and white (n = 29 families) dams was 44.5% (± 3.7) and 50.3% (± 4.8), respectively. We note that eyed-egg survival may be an underestimate of survival if not all eggs were fertilized, as we could not discriminate between dead fertilized and dead unfertilized eggs. Additionally, mean (± S.E) for eyed-egg to fry survival for red and white dams was 94.6% (± 1.0) and 96.1% (± 1.3), respectively. Sire colour also had no effect on measures of egg survival (p values > 0.53). For survival to the eyed-egg stage, we found a significant effect of egg density (p < 0.001) but no effect of egg mass (p = 0.36). Random effects of dam x sire interaction and cell position significantly contributed to variance observed for eyed-egg survival (see Table 4.2). For eyed-egg to fry survival, there was no significant effect of density on survival (p = 0.98), and random effect dam x sire interaction (p < 0.001) significantly contributed to the variation in survival. For early and late fry survival, we found no significant effect of dam or sire colour (p values > 0.46). For early fry and late fry survival, rearing density did not have a significant effect on survival (p values > 0.07) and no random effects had a significant influence on variation in survival (p values > 0.04).

**Body size and condition**

All results for fork length, wet weight and condition factor are presented in Table 4.2. Sampling was conducted at four time points, and density (fixed factor) had no significant effect on size or condition at any of the time points (see Table 4.2; p values > 0.03). Dam colour had no significant effect on any trait across all points (Table 4.2; p values > 0.17). The same was true for sire colour (Table 4.2), however condition factor in February approach statistical significance (p = 0.005), where offspring sired by red males had significantly higher condition compared to offspring sired by white males. Dam
effects (random effect) significantly contributed to the variation of traits at several time points representing 4-76% of the variation observed for the trait (Table 4.2). Dam effects were strongest at the first sampling point (Table 4.2). Sire effects were present but did not significantly contribute to the variation in phenotype for most traits, where sire effects represented 0-13% of the variation observed for the trait. The interaction of dam x sire significantly influenced all traits at many sampling points, where the interaction effect accounted for 3-15% of the observed trait variation (Table 4.2).

**Immune function**

*Post-challenge survival*

All survival challenge tanks started with 480 individuals. Mortalities that occurred before and after the challenge (mock HBSS or IHNV exposure) were recorded, genotyped and assigned to parents. Three post-challenge mortalities could not be assigned to parents and therefore could not be included in the analysis as their family identity was unknown. One of the unassigned mortalities was from the control tank and two unassigned mortalities were from one of the challenge tanks. Mortalities occurring prior to the challenge (n = 5-9 fish per tank) were removed from the analysis and starting number of individuals in the tank at the time of the challenge was calculated. Mortality was low in all tanks over the 35-day period following the challenge, where survival in the mock control tank was 97.9% and survival was equally high in the two tanks subjected to IHNV (97.3% and 97.9%). Using GLMM we found that none of the variance could be explained by the random effects in the model, which included dam, sire, dam x sire interaction and tank; therefore, we used generalized linear model (GLM) to test the main effects in the model. Given that only one analysis was performed on survival post-challenge data, no alpha level adjustment was performed (alpha level = 0.05). Using GLM, we found no
significant effect of treatment (p = 0.69) or dam colour (p = 0.08) on survival, however sire colour did have a significant effect on survival (p = 0.014). We further investigated the effect of sire colour on survival within each tank. We found that the difference in survival between offspring of red and white sires was only marginally statistically significant in the control tank (p = 0.049), where survival of red sired and white sired offspring were 99.5% and 96.6%, respectively. No difference in survival of red and white sired offspring were found in the either of the challenge tanks (p values > 0.18), therefore the small difference in survival between offspring of red and white sires is not the result of differences in viral susceptibility.

Post-challenge gene expression

In total 493 offspring were sampled for RNA. However, samples were excluded if RNA quality/quantity was low (n = 31 out of 493) and samples were excluded if parentage could not be assigned with 95% confidence (n = 26 out of 493). Therefore, after the removal these samples, our analysis of relative gene expression included a total of 436 individuals. For some genes, all individuals were analyzed, however as described in the methods, C_{RT} values were filtered and screened, therefore some genes had lower sample sizes if samples did not meet the criteria. Additionally, four genes, including CD8, IFNA, SAA and IHNV-N showed limited or no amplification in our qRT-PCR reactions, therefore they were not analyzed. IHNV-N gene represented the gene used to detect viral RNA in our sample. The lack of amplification of IHNV-N likely indicates that the virus was absent in the sample as there did not appear to be any amplification during the 40 cycles in all samples.

We analyzed relative gene expression for 21 genes, where 15 genes showed high amplification (sample size > 393 individuals/gene) and the remaining 6 genes showed
more variable amplification where individuals were excluded due to lack of or low amplification (sample size = 215-355 individuals/gene). Using linear mixed effect models, we first examined relative expression of genes between treatments (challenge and mock control). All results of linear mixed effect models are presented in Table 4.3. Treatment had no significant effect on relative expression of immune genes (Figure 4.2), stress and oxidative stress genes (Figure 4.3) (all p values > 0.07; see Table 4.3), thus indicating that virus exposure elicited no response in the fry at 72-hours post-exposure.

Treatment was removed from the model, and we tested the effect of dam colour on offspring gene expression, where the difference in gene expression in the offspring of red and white dams was not significant (Table 4.3, p values > 0.02). However, for two genes (IFR3 and MX1), the difference in gene expression in the offspring of red and white dams approached statistical significance (p = 0.02 IFR3; p = 0.02 MX1). For both genes, offspring of red dams showed lower relative expression. Additionally, these two genes also showed a significant effect of fish weight on relative expression (Table 4.3, p values < 0.001). We found no effect of sire colour on relative gene expression across all genes (Table 4.3; p values > 0.05). The random effects of dam, sire and OpenArray chip significantly contributed to the variation in relative expression for some genes, however tank and the interaction of dam x sire had no significant effect on expression (see Table 4.3).

To examine the transcriptional profiles of red and white dams across all genes, we generated a heat map, where normalized values (Z-scores) of family means for relative expression at each gene are shown Figure 4.4. One gene was removed (IL1B), as two families were missing for this gene. The heat map was created using the heatmap.2 command in the R software (R Core Development Team 2016) package gplots (Warnes et
al. 2016), where default methods were used for hierarchical clustering of expression data. The heat map shows the relationship in transcriptional profiles among the 32 families and 20 genes (Figure 4.4). Transcriptional profiles did not cluster by dam colour, thus further demonstrating no clear differentiation in expression profiles of offspring from red and white dams. The clustering of genes based on transcriptional profiles showed multiple gene clusters with some genes of similar function demonstrating similar responses. For example, genes involved in anti-viral response including VIG1, MX1, IFR3 and MHCI clustered together along with an antioxidant enzyme gene TRDNX. Additional immune genes clustered together, including IGM, TCRB and TGF1 along with the stress gene, HSP90A. The transcriptional profiles of additional immune genes including IGT, IL8, TNFA, IFG were similar, and clustered together with the antioxidant enzyme gene, GPX. Stress genes including META and HSP70 clustered together, and antioxidant enzyme genes (GST, SOD) and glucocorticoid receptor-2 gene (GR2) clustered together, along with the MHCIIIB1 gene.

Discussion

In this study, we aimed to determine performance differences in offspring derived from colour polymorphic Chinook salmon that exhibit visible differences in egg carotenoid content. Maternal carotenoids have been demonstrated to provide important benefits to offspring in oviparous species, where the maternal provisioning of carotenoids can impact egg and early life survival (McGraw et al. 2005; Tyndale et al. 2008), offspring immune function (Saino et al. 2003), offspring body size (Marri and Richner 2014), growth rate (Bazyar Lakeh et al. 2010) and antioxidant status (Christiansen et al. 1995a; McGraw et al. 2005). The effect of maternal carotenoids can persist for just a short period (Marri and Richner 2014) or throughout the entire life of the animal (McGaw
et al. 2005). In our study, we examined a wide range of fitness related measures including egg and juvenile survival, offspring size and condition and offspring response to a viral challenge. Although our study incorporated a robust design with a large number of families and individuals, we found no significant differences in any measured phenotype between offspring derived from red (carotenoid rich eggs) and white (carotenoid poor eggs) dams.

Maternal carotenoids have previously been demonstrated to influence egg and early life survival in both fishes (Tyndale et al. 2008) and birds (McGraw et al. 2005). In Chinook salmon, Tyndale et al. (2008) found a significant relationship between egg carotenoid content and egg incubation survival using eggs derived from populations of red and white Chinook salmon. Conversely, Torrissen (1984) and Christiansen and Torrissen (1997) found no significant relationship between egg carotenoid content and egg or alevin survival in Atlantic salmon (*Salmo salar*). However, Craik (1985) found that the relationship between egg carotenoid content and egg survival in rainbow trout (*O. mykiss*) was not linear, and proposed that a critical threshold for egg carotenoid content may exist (ranging between 1-3 ug/g) above which egg survival will be high. Indeed, the relationship between egg survival and carotenoids found in Tyndale et al. (2008) supported the threshold effect proposed by Craik (1985), where in Chinook salmon the threshold level was found to be approximately 2 ug/g. Eggs from white females in the Quesnel River contain visibly less carotenoids than eggs of red females, however white eggs are not devoid of carotenoids (personal observation). White Chinook salmon provision less carotenoids to their eggs, however the levels of carotenoids may be near or above the proposed 2 ug/g threshold thus not impacting early life survival. Although, egg carotenoid content was not directly measured in our study, white Chinook salmon females
from the Chehalis (BC) population had eggs with carotenoid concentration that were near or greater than 2 μg/g (Tyndale et al. 2008).

In addition to egg survival, we also measured size, condition and survival of salmon fry from freshwater entry to a later juvenile stage when salmon are expected to undergo the transition into salt water (i.e., smolting). Throughout these stages, although we found significant maternal (dam identity) effects on performance traits, we found no significant difference between offspring of red and white dams. Similarly, Tyndale et al. (2008) found no significant relationship between egg carotenoid content and offspring growth in red and white Chinook salmon. In the two-spotted goby (*Gobiusculus flavescens*), natural variation in egg carotenoid content (1.1 – 7.5 μg/g) showed no relationship with measures of offspring quality including offspring length (Svensson et al. 2006). Additionally, in birds, Saino et al. (2003) found no effect of yolk carotenoids on offspring tarsus size in barn swallows (*Hirundo rustica*). Nonetheless, studies that manipulate carotenoids in the maternal diet have found evidence that maternal carotenoid supplementation can influence offspring survival, size and growth (McGraw et al. 2005; Bazyar Lakeh et al. 2010; Brown et al. 2014; Ewen et al. 2009; but see Grether et al. 2008). In rainbow trout, Bazyar Lakeh et al. (2010) showed that a higher carotenoid diet in mothers resulted in increased egg carotenoid content and consequently higher offspring growth rates. However, Brown et al. (2014) found that increasing maternal dietary carotenoids in convict cichlids (*Amatitlania siquia*) did not influence maternal provisioning of carotenoids to eggs, but did increase offspring growth and survival. Indeed many studies supporting the benefits of increased carotenoids on offspring performance involve diet manipulation rather than natural variation in carotenoids such as in our study (Bazyar Lakeh et al. 2010; McGraw et al. 2005; Baird et al. 2005; Ewen et al.
2009; Brown et al. 2014). These studies may not be able to separate the effects of maternal diet quality and egg carotenoid content on subsequent offspring performance. White Chinook salmon females consume and metabolize carotenoids, however they have a limited ability to deposit these pigments into their eggs and tissues. If maternal dietary carotenoid availability and thus quality of the maternal diet (and not egg carotenoid content) influence offspring performance (see Brown et al. 2014) then this may explain why the offspring of red and white Chinook salmon females exhibit similar performance. Alternatively, the influence of maternal carotenoids on offspring performance may be species or population specific, as the carotenoid effects on offspring size, growth or survival are present in some studies (rainbow trout; Bazyar Lakeh et al. 2010; zebra finch (Taeniopygia guttata), McGraw et al. 2005; blue tit (Parus caeruleus), Baird et al. 2005) but not others (Poecilia reticulata, Grether et al. 2008; barn swallow, Saino et al. 2003; Two-spotted goby; Svensson et al. 2006; Chinook salmon, Tyndale et al. 2008).

In addition to the above offspring performance metrics, we also examined offspring performance after exposure to infectious hematopoietic necrosis virus (IHNV): a pathogen that has been detected in the Quesnel River. We first investigated susceptibility of Chinook fry to IHNV and revealed a low susceptibility as evident by the lack of viral induced mortality over a 35-day period, as there was no significant difference in mortality between the control and challenge fish. Second, we measured the expression of immune, oxidative stress and stress genes at 72-hours post-IHNV exposure. In our study, it is evident that Chinook salmon fry did not respond to the viral challenge, as we found no difference between the mock control and the challenge in relative expression of all genes. It is possible that 72-hours post-challenge may not have been the optimal time to detect differential expression; however this seems unlikely as other
studies in salmonid fry have demonstrated the upregulation of immune genes at 72 hours after IHNV injection of fry (Purcell et al. 2004). In rainbow trout fry exposed to waterborne IHNV, infected fish began shedding the virus 2-3 days after exposure (Ogut and Reno 2003) and in another study on rainbow trout, viral replication was found to be occurring in gill tissue within 2 days of waterborne exposure (Yamamoto and Clermont 1990). Alternatively, Quesnel River Chinook salmon fry may be resistant to the specific strain of the virus used in our study, as none of the fry in our gene expression study showed evidence of the virus in their gills based on the qRT-PCR assay that targeted the IHNV nucleocapsid N gene. Viral susceptibility can depend on several factors including environmental condition, age, population, species and viral strain (LaPatra 1998; Garver et al. 2006; Hernandez et al. 2016). For example, susceptibility of 1 gram Chinook salmon fry (same size as our study) to IHNV can depend on the life-history strategy of the population as well as the virus strain (genogroup) (Hernandez et al. 2016). Recently, Hernandez et al. (2016) found that survival (over 30 days) following IHNV exposure could range from 51-100% in Chinook salmon fry, where fry appeared most susceptible to the L genogroup of IHNV and stream-type fry were more susceptible to infection relative to ocean-type. The Chinook salmon from the Quesnel River are considered stream-type (Clarke et al. 1994) and in our study the fry were exposed to the U genogroup of IHNV.

Although fish did not respond to the virus in our study, we were also interested in examining transcriptional differences between red and white Chinook salmon, as we might still expect to observe resting differences in relative gene expression if functional differences exist due to variation in carotenoid concentration. In many taxa, carotenoids have been associated with immune function (Christiansen et al. 1995a; Blount et al. 2002;
Saino et al. 2003; Amar et al. 2004; Tyndale et al. 2008; Amar et al 2012) and oxidative or stress status (Christiansen et al. 1995a; Surai and Speake 1998; Wang et al. 2006; Hõrak et al. 2007; Pike et al. 2007; Fitze et al. 2009; Mougeot et al. 2010). We examined differences in offspring of the colour morphs across treatments, and we found no significant difference in relative expression for any immune, stress or oxidative stress genes. The lack of differences in gene expression in the offspring of red and white dams is perhaps surprising, however the expected difference is based on the assumption that the only difference between red and white eggs is carotenoid (astaxanthin) concentration. Yet, these colour phenotypes have been shaped by natural selection over evolutionary time, therefore it seems likely that white Chinook salmon may have evolved compensatory mechanisms to counteract the lack of carotenoids in their eggs. White Chinook salmon may therefore have evolved to increase the concentration of alternative antioxidants into their eggs, such as vitamin E, vitamin C or retinoids, which are found in salmon eggs (Cowey et al. 1985; Garner et al. 2010). Additionally, white females may increase the concentration of maternal antibodies in eggs (Blount et al. 2002), as a previous study found that eggs from white Chinook salmon females had higher and more variable antibody levels relative to eggs from red females (Tyndale 2005). Differences in maternal provisioning of antioxidant and/or antibodies to eggs between morphs could thus explain why red and white Chinook salmon display similar transcription levels for immune, stress and oxidative stress genes.

Although the main effect that we were interested in for our study was the effect of dam colour (i.e., egg carotenoid content) on offspring performance, we also designed our study to examine the effect of sire colour type on offspring performance. Salmon have a non-resource based mating system where males provide no parental care, and in these
systems, male traits may signal genetic quality and females may select males in order to obtain superior offspring (Neff and Pitcher 2005). However, evidence that carotenoids signal male genetic quality and contribute to offspring fitness is scarce in non-resource based mating systems (but see Evans et al. 2004). We found no difference between offspring of red and white sires in terms of egg and offspring survival, offspring size and offspring gene expression. Although difference between red and white sired offspring condition approached statistical significance in February (early juvenile period), where red sired offspring were 3.2% greater in condition compared to white sired offspring. The difference detected here was not present upon initial freshwater entry and disappeared by later juvenile stages, and did not translate into higher survival in our study. Nevertheless, it is possible that offspring in greater condition could gain an advantage under natural conditions.

In conclusion, we measured a suite of performance traits in offspring derived from red and white Chinook salmon. Red and white Chinook salmon eggs differ visibly in egg carotenoid content, and thus we hypothesized that this would have measurable effects on offspring performance as demonstrated in previous studies (McGraw et al. 2005; Tyndale et al. 2008; Ewen et al. 2009; Bazyar Lakeh et al. 2010; Marri and Richner 2014). However, we found no detectable differences in offspring of red and white eggs for any trait measured here between the egg and later juvenile stages. We have provided four potential explanations for the lack of observable differences: 1) white Chinook salmon egg carotenoid content is low but at or above a critical threshold required for adequate offspring performance, 2) maternal dietary carotenoid availability and hence diet quality (and not egg carotenoids) are predictors of offspring performance, 3) effects of maternal carotenoids on offspring performance are species or population specific and 4) white
Chinook salmon have evolved to compensate for the lack of carotenoids by increasing the concentration of alternative antioxidants and/or antibodies into their eggs. These four mechanisms are not mutually exclusive. Moreover, we acknowledge that our study was conducted under experimental conditions where rearing environment was held constant among family. Therefore, the results of our study may differ under more natural conditions where selection pressures are greater. Nevertheless, if carotenoids provide offspring with direct fitness benefits, our study design should allow for the detection of these benefits on traits such as size, survival, immune function and antioxidant status.

White Chinook salmon continue to persist in nature despite the lower levels of carotenoids found in their tissues. It is clear from the present study that offspring performance in Chinook salmon is not influenced by egg carotenoid concentration (i.e., dam colour). The paradigm that increased maternal provisioning of carotenoids provides increased benefits to offspring may be driven by research focused on particular species and studies that employ dietary carotenoid supplementation rather than examine resulting fitness from natural variation in carotenoids. Indeed, in nature, variation in carotenoid utilization exists and this variation may be shaped through evolutionary processes that allow animals like white Chinook salmon to incur their own benefits by employing different strategies and thus may explain in part how this unique polymorphism can be maintained in nature.

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Table 4.1. List of genes used to measure gene expression in red and white Chinook salmon (*Oncorhynchus tshawytscha*) fry. Gene types included reference genes used as endogenous controls, immune genes, and stress/oxidative stress genes. Viral IHNV-N protein gene was also included to score presence/absence of virus in the sample. Primer and probe sequences along with the original reference for each gene are provided in the Appendix 2.

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Primer</th>
<th>Gene</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>NM_001124246.1</td>
</tr>
<tr>
<td></td>
<td>ARP</td>
<td>Acidic ribosomal phosphoprotein P0</td>
<td>AY685220</td>
</tr>
<tr>
<td></td>
<td>EF1A</td>
<td>Elongation factor 1-alpha</td>
<td>AF498320.1</td>
</tr>
<tr>
<td>Immune</td>
<td>IL1B</td>
<td>Interleukin 1 beta</td>
<td>DQ778946.1</td>
</tr>
<tr>
<td></td>
<td>IL8</td>
<td>Interleukin 8</td>
<td>DQ778949.1</td>
</tr>
<tr>
<td></td>
<td>TNFA</td>
<td>Tumour necrosis factor alpha</td>
<td>DQ778945.1</td>
</tr>
<tr>
<td></td>
<td>IFNA1</td>
<td>Interferon alpha 1</td>
<td>AY788890</td>
</tr>
<tr>
<td></td>
<td>MX1</td>
<td>Mx protein</td>
<td>GT897808</td>
</tr>
<tr>
<td></td>
<td>VIG1</td>
<td>VHSV induced gene 1</td>
<td>AF076620/CA058263</td>
</tr>
<tr>
<td></td>
<td>IFNG</td>
<td>Interferon gamma</td>
<td>GT897806</td>
</tr>
<tr>
<td></td>
<td>IFR3</td>
<td>Interferon regulatory factor 3</td>
<td>CB515644</td>
</tr>
<tr>
<td></td>
<td>MHCIIIB</td>
<td>Major histocompatibility complex class 2</td>
<td>U34718.1</td>
</tr>
<tr>
<td></td>
<td>MHC1</td>
<td>Major histocompatibility complex 1</td>
<td>AY523661</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>CD8 alpha chain</td>
<td>AF178053</td>
</tr>
<tr>
<td></td>
<td>IGM</td>
<td>Immunoglobulin Mu membrane</td>
<td>X65263/CB506793</td>
</tr>
<tr>
<td></td>
<td>IGT</td>
<td>Immunoglobulin Tau heavy chain</td>
<td>AY870265</td>
</tr>
<tr>
<td></td>
<td>TGFB1</td>
<td>Transforming growth factor beta 1</td>
<td>X99303</td>
</tr>
<tr>
<td></td>
<td>TCRB</td>
<td>T cell receptor beta chain</td>
<td>AF29700/CB498619</td>
</tr>
<tr>
<td></td>
<td>SAA</td>
<td>Serum amyloid A</td>
<td>NM_001124436.1</td>
</tr>
<tr>
<td>Stress/ Oxidative stress</td>
<td>GR2</td>
<td>Glucocorticoid receptor 2</td>
<td>AY495372.1</td>
</tr>
<tr>
<td></td>
<td>HSP70</td>
<td>Heat shock protein 70</td>
<td>U35064.1</td>
</tr>
<tr>
<td></td>
<td>HSP90A</td>
<td>Heat shock protein 90a</td>
<td>U89945.1</td>
</tr>
<tr>
<td></td>
<td>META</td>
<td>Metallothionein A</td>
<td>DQ139342.1</td>
</tr>
<tr>
<td></td>
<td>SOD</td>
<td>Superoxide dismutase</td>
<td>AF469663.1</td>
</tr>
<tr>
<td></td>
<td>GPX</td>
<td>Glutathione peroxidase</td>
<td>AF281338.1</td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>Glutathione S-transferase</td>
<td>NM_0011605591</td>
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<tr>
<td></td>
<td>TRDNX</td>
<td>Thioredoxin reductase</td>
<td>CA057296</td>
</tr>
<tr>
<td>Viral protein</td>
<td>IHNV N</td>
<td>IHNV nucleocapsid (N) gene</td>
<td>FJ265710/FJ265715</td>
</tr>
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</table>
Table 4. Results of linear mixed effect models examining fitness-related traits (survival, size, and condition) in Chinook salmon (Oncorhynchus tshawytscha) fry derived from red and white males and females. Significance (p-values) of fixed factors are provided. The percentage of the observed variation in gene expression accounted for by each random factor are provided, and significant effects are indicated in bold with asterisk (adjusted alpha level = 0.003).

<table>
<thead>
<tr>
<th>Trait (sample size)</th>
<th>Dam colour</th>
<th>Sire colour</th>
<th>Sire x Dam</th>
<th>Date</th>
<th>Tray</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (GLMM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyed-egg (n = 41,244)</td>
<td>0.39</td>
<td>0.53</td>
<td>&lt;0.001*</td>
<td>0.36</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Eyed-egg to fry (n = 10,549)</td>
<td>0.06</td>
<td>0.80</td>
<td>0.98</td>
<td>0.66</td>
<td>0.3</td>
<td>11.1</td>
</tr>
<tr>
<td>Early fry (n = 6,546)</td>
<td>0.60</td>
<td>0.46</td>
<td>0.07</td>
<td>4.4</td>
<td>3.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Late fry (n = 1,836)</td>
<td>0.82</td>
<td>0.73</td>
<td>0.48</td>
<td>0.0</td>
<td>6.2</td>
<td>16.4</td>
</tr>
<tr>
<td>Fork length (LMM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>December (n = 840)</td>
<td>0.66</td>
<td>0.46</td>
<td>0.19</td>
<td>33.0*</td>
<td>5.3</td>
<td>6.2*</td>
</tr>
<tr>
<td>February (n = 804)</td>
<td>0.21</td>
<td>0.04</td>
<td>0.15</td>
<td>6.1</td>
<td>4.5</td>
<td>13.4</td>
</tr>
<tr>
<td>April (n = 803)</td>
<td>0.41</td>
<td>0.19</td>
<td>0.65</td>
<td>21.5*</td>
<td>4.3</td>
<td>3.1</td>
</tr>
<tr>
<td>July (n = 803)</td>
<td>0.49</td>
<td>0.76</td>
<td>0.08</td>
<td>19.1*</td>
<td>9.0</td>
<td>12.1*</td>
</tr>
<tr>
<td>Wet weight (LMM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>December (n = 840)</td>
<td>0.64</td>
<td>0.92</td>
<td>0.72</td>
<td>75.6*</td>
<td>1.9</td>
<td>10.3</td>
</tr>
<tr>
<td>February (n = 804)</td>
<td>0.23</td>
<td>0.02</td>
<td>0.14</td>
<td>10.4</td>
<td>6.2</td>
<td>11.7*</td>
</tr>
<tr>
<td>April (n = 804)</td>
<td>0.44</td>
<td>0.09</td>
<td>0.93</td>
<td>18.8*</td>
<td>4.6</td>
<td>6.7*</td>
</tr>
<tr>
<td>July (n = 803)</td>
<td>0.61</td>
<td>0.75</td>
<td>0.08</td>
<td>12.6</td>
<td>12.7*</td>
<td>15.1*</td>
</tr>
<tr>
<td>Condition (LMM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>December (n = 840)</td>
<td>0.17</td>
<td>0.52</td>
<td>0.03</td>
<td>18.4*</td>
<td>3.7</td>
<td>9.3*</td>
</tr>
<tr>
<td>February (n = 804)</td>
<td>0.60</td>
<td>0.005</td>
<td>0.34</td>
<td>6.8*</td>
<td>0.0</td>
<td>7.2</td>
</tr>
<tr>
<td>April (n = 803)</td>
<td>0.82</td>
<td>0.03</td>
<td>0.21</td>
<td>4.0</td>
<td>0.0</td>
<td>5.1</td>
</tr>
<tr>
<td>July (n = 803)</td>
<td>0.18</td>
<td>0.05</td>
<td>0.35</td>
<td>4.5</td>
<td>4.0</td>
<td>3.9</td>
</tr>
</tbody>
</table>

ns: indicates that the term accounted for no variance in the model thus it was not included in the model.
Table 4. Results of linear mixed effect models examining relative gene expression (N0 target/N0 reference) of immune genes, stress and oxidative stress genes in Chinook salmon (*Oncorhynchus tshawytscha*) fry after exposure to control or challenge (infectious hematopoietic necrosis virus; IHNV) conditions (treatment). Treatment was not included in the final model. Significance (p-values) of fixed factors in the model are provided with the percentage of the observed variation in gene expression accounted for each random factor. Significant effects are indicated in bold with asterisk (alpha level = 0.002).

<table>
<thead>
<tr>
<th>Gene (sample size)</th>
<th>Significance of fixed effects</th>
<th>% Variance of random effects</th>
</tr>
</thead>
</table>
| GPX (n = 434)     | Treatment (not in model) 
                  | Dam colour                      |
|                  | Sire colour                      |
|                  | Sire x Dam (n = 32)          |
|                  | Array chip (n = 10)           |
|                  | Tank (n= 4)                    |
| GPX               | 0.73                          | 0.74                        |
| GPX               | 0.34                          | 3.5                         |
| GPX               | 3.0                           | 23.4*                       |
| GPX               | 0.0                           | 0.0                         |

Note: Significance values for treatment are reported however it was not included in the final model as treatment was not significant. Weight was included in the model if weight was significantly correlated with relative expression (p < 0.05).
Figure 4.1. Box plot of mean family survival (proportion) derived from red and white Chinook salmon (Oncorhynchus tshawytscha) females, where A represents survival from fertilization to the eyed-egg stage and B represents survival from eyed-egg to fry stage.
Relative gene expression values (except MHCI which was square root transformed) to meet model assumptions. Relative expression was measured in all fish at 72 hours post challenge. Relative gene expression values are presented as natural log transformed values (except MHCI which was square root transformed) to meet model assumptions. Figure 4.2. Boxplots of relative gene expression (N/0 target/N/0 reference) of immune genes in Chinook salmon (Oncorhynchus tshawytscha) after exposure to control (buffer solution HBSS) and challenge (infectious hematopoietic necrosis virus; IHNV) conditions. Relative expression was measured in all fish at 72 hours post challenge. Relative gene expression values are presented as natural log transformed values (except MHCI which was square root transformed) to meet model assumptions.
Figure 4.3: Boxplots of relative gene expression (N_0 target/N_0 reference) of stress and oxidative stress genes in Chinook salmon (Oncorhynchus tshawytscha) fry after exposure to control (buffer solution HBSS) and challenge (infectious hematopoietic necrosis virus, IHNV) conditions. Relative expression was measured in all fish at 72 hours post challenge. Relative gene expression values are presented for GPX and GPS, whereas transformed values are presented for GR2, HSP90A, HSP70, TRDNX (natural log), SOD (square root) and META (negative inverse) to meet model assumptions.

Control
IHNV

TRDNX

SOD

GST

GPX

META

HSP70

HSP90A

GR2

Relative gene expression
Relative expression is indicated by yellow, whereas lower relative expression is indicated by blue. Higher relative expression is indicated along the bottom of the heat map, with families derived from red and white dams indicated above the heat map. Hierarchical clustering of rows and columns represent genes and families, respectively, of similar transcriptional profiles. Families (dam ID x sire ID) are indicated along the bottom of the heat map, with families derived from red and white dams indicated above the heat map. Higher relative expression is indicated by yellow, whereas lower relative expression is indicated by blue.

**Figure 4.** Heat map showing normalized (Z-score) values of mean relative expression of Chinook salmon (*Oncorhynchus tshawytscha*) fry by family (n = 32) for 20 genes involved in immune, stress and oxidative stress responses. Hierarchical clustering of rows and columns represent genes and families, respectively, of similar transcriptional profiles.
CHAPTER 5: RED AND WHITE CHINOOK SALMON: GENETIC DIVERGENCE AND MATE CHOICE

Introduction

Understanding the mechanisms promoting the maintenance of variation within and among natural populations is a major goal in the field of evolutionary biology. Specifically, colour polymorphisms are widespread in many taxa and have garnered considerable research interest, as colour traits are often heritable, easily discernable by humans and subject to forces of both sexual and natural selection (Maan and Seehausen 2011; Wellenreuther et al. 2014). Colour polymorphisms within species can be observed as discrete colour morphs as well as colour variation that exist across a continuous spectrum. Although many studies have focused on the evolution of colour polymorphisms (see Gray and McKinnon 2007), there is limited empirical data related to the processes that maintain colour polymorphisms in wild populations.

Within populations, the persistence of colour polymorphisms may result from various mechanisms including disruptive selection, frequency-dependent selection and/or mate choice (Greene et al. 2000; Galeotti et al. 2003; Gray and McKinnon 2007; Wellenreuther et al. 2014). Disruptive selection may occur if different colour morphs occupy different niches within which fitness can be maximized (Green et al. 2000; Galeotti et al. 2003; Munday et al. 2003; Hugall and Stuart-Fox 2012). Negative frequency-dependent selection (NFDS) has also long been hypothesized as a mechanism maintaining polymorphisms in nature (Clarke 1962), whereby rare morphs experience a fitness advantage (Sinervo and Lively 1996; Olendorf et al. 2006; Takahashi and Kawata

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Maintenance of colour polymorphisms may also occur through non-random mate choice (Roulin and Bize 2007; Wellenreuther et al. 2014), where sexual selection, colour assortative or colour disassortative mating can maintain colour variation within a population (Tuttle 2003; Pryke and Griffith 2007; Elmer et al. 2009). For example, Pryke and Griffith (2007) found that in the Gouldian finch (Erythrura gouldiae), mates paired assortatively based on head colouration in both wild and captive populations. Assortative mate choice may not by itself maintain polymorphisms; nevertheless, it can act in combination with other selective pressures to retain polymorphisms (Wellenreuther et al. 2014).

In nature, Chinook salmon (Oncorhynchus tshawytscha) exhibit extreme variation in flesh colouration, where red-coloured individuals co-exist with white-coloured individuals with intermediate variants existing across the spectrum. The difference in colouration does not only appear in flesh colour, but also translates into differences in egg and external spawning colouration (Withler 1986). White Chinook salmon do not occur in all rivers, and the percentage of white Chinook salmon within a river can range from 0 to 100% (Hard et al. 1989). The difference between red and white Chinook salmon does not appear to reflect diet (Lehnert et al. unpublished stable isotope data), but instead results from genetic polymorphisms (Withler 1986). White Chinook salmon have reduced ability to deposit carotenoids into the flesh, eggs and skin, and thus white individuals have white (or pale) eggs and appear uncharacteristically grey in colour during spawning (see Figure 5.1A; Withler 1986; Rajasingh et al. 2007). Currently, little is known about the evolutionary mechanisms that maintain the red and white phenotypes in nature.

The maintenance of the colour morphs could be explained if morphs are genetically isolated and undergoing ecological speciation. Reproductive isolation between
morphs can be tested using neutral markers (i.e., microsatellites), as genetic divergence between individuals at such loci is expected to occur through reduced gene flow and neutral processes, such as genetic drift, rather than selection (Selkoe and Toonen 2006). In a previous study, genetic isolation of sympatric gold and dark colour morphs of Midas cichlid (Amphilophus citrinellus) was revealed by significant genetic divergence between morphs using microsatellite markers (Elmer et al. 2009). Additionally, colour-based assortative mate choice could provide another evolutionary mechanism responsible for the persistence of the morphs. Controlled mating experiments are often employed as a first step in testing for mate choice in colour polymorphic species. For example, in cichlid species, laboratory studies have demonstrated that female choice for male colouration plays an important role in speciation (Knight and Turner 2004; Maan and Sefc 2013; Selz et al. 2014). In a colour polymorphic population of poison dart frogs (Dendrobates pumilio), experiments have shown that red and yellow female frogs show a strong preference for males with the same colour phenotype as their own (Richards-Zawacki and Cummings 2011).

If morphs are not reproductively isolated and mating occurs randomly with respect to colour, mate choice and/or natural selection acting on functional genes could be responsible for the co-existence of red and white individuals. Provided that carotenoids act as immunostimulants and antioxidants (Nakano et al. 1995; Blount et al. 2003; Faivre et al. 2003; Amar et al. 2004; but see Costantini and Møller 2008), natural selection may operate differentially on immune genes in red and white individuals. For example, in rainbow trout (O. mykiss), circulating levels of carotenoids can be correlated to immunoenhancement (Amar et al. 2004; Amar et al. 2012) and protection from oxidative damage (Nakano et al. 1995). Similarly, carotenoid supplementation reduces production
of endogenous antioxidant enzymes in fishes including olive flounder (*Paralichthys olivaceus*; Pham et al. 2014), characins (*Hyphessobrycon callistus*; Wang et al. 2006) and yellow croaker (*Pseudosciaena crocea*; Li et al. 2014). In Chinook salmon, reduced carotenoid storage in muscle tissue prior to maturation limits the ability of females to move pigments into plasma, eggs and skin (Garner et al. 2010b). Thus, white Chinook salmon may experience greater oxidative stress and be immune compromised relative to red conspecifics, unless white individuals have evolved means to counteract these handicaps. One possibility is that white Chinook salmon may benefit from differences at major histocompatibility complex (MHC) immune loci. Two distinct classes (class I and class II) of MHC genes are found in salmon (Miller et al. 1997). Class I molecules are typically associated with response to viruses, as the molecules bind peptides derived from intracellular antigens, whereas class II molecules bind peptides derived from extracellular antigens and thus are more commonly associated with bacterial response (Klein 1986). It is possible that mate choice and/or selection operates to increase heterozygosity or to favour specific MHC alleles, both mechanisms having been demonstrated to confer fitness advantages (McClelland et al. 2003; Kjøglum et al. 2006; Evans and Neff 2009; Evans et al. 2010). Given that salmon mate non-randomly relative to MHC genotype (Landry et al. 2001; Neff et al. 2008; Yeates et al. 2009), we test the hypotheses that mate choice and natural selection contribute to the persistence of the colour phenotypes.

In this study, we investigate a population of colour polymorphic Chinook salmon in the Quesnel River, within which red and white individuals co-exist in relatively equal proportions (Withler 1986). First, we test for genetic divergence between red and white individuals at microsatellite marker loci to determine whether reproductive isolation exists between the morphs. Next, we examine mate choice in the system to test whether
colour assortative mate choice could be a mechanism acting to maintain the polymorphism in nature. Mate choice is examined under experimental conditions in semi-natural spawning channels and determined using behavioural analyses as well as genetic parentage assignment of offspring. Subsequently, genetic differences between morphs at two functional immune genes (MHC class I and II) were assessed to evaluate the possibility of differential selection pressure operating on immune genes between morphs. Finally, given the importance of MHC genes to mate choice in salmonids (Landry et al. 2001; Neff et al. 2008; Yeates et al. 2009), we test whether non-random mate choice at the MHC class II gene can explain the persistence of the two morphs. The co-existence of red and white Chinook salmon in the Quesnel River system provides a unique opportunity to examine the fitness consequences of carotenoids in a controlled and quantitative fashion, thus allowing us to test the long-standing hypotheses about why salmon are red.

**Methods**

**Field collection**

Adult Chinook salmon were netted from the Quesnel River in Likely, British Columbia, Canada during two spawning seasons. Fish were collected from September 13 to 30 in 2013 and September 18 to October 1 in 2014. Fish that were in good condition and that did not exhibit signs of having already spawned were transported in holding tanks with aerated river water approximately 15 minutes (5 km) to the Quesnel River Research Center (QRRC). Fish were then held in 3000-L freshwater tanks until sampling. Fish were sampled for weight, length and colour, and a fin clip was collected and placed in a high salt preservative buffer (3.5 M ammonium sulfate; 15 mM EDTA; 15 mM sodium citrate; pH 5.2). Colour was assessed visually where individuals were characterized as “red” or “white” based on external spawning colouration (Figure 5.1A;
Withler 1986). Individuals were characterized as red when they showed external red pigmentation and individuals were characterized as white when they exhibited no external red pigmentation and were grey in colouration. In fall 2014, spawning colour was also assessed post-mortem using a Jaz spectrophotometer (Ocean Optics) with triplicate readings obtained at three locations along the lateral body following a similar protocol to Pitcher et al. (2009). The nine spectral readings were averaged and smoothed per individual using the R package pavo (Maia et al. 2013) to generate the average reflectance spectra for red (n = 19) and white (n = 18) salmon (Figure 5.1B). In addition to live adults captured, fin clips for genetic analyses were also collected from carcasses along the shore of the Quesnel River at QRRC. All carcasses were assessed for red or white phenotype by dissection and visual identification.

Neutral genetic divergence and diversity

DNA extraction

Fin clips from adult fish collected in both the fall of 2013 (n = 73) and 2014 (n = 51) were used to extract DNA following either an automated plate based extraction protocol (Elphinstone et al. 2003) or the Wizard genomic DNA purification kit (Promega Corp.).

Microsatellite genotyping

Spawning salmon from the fall 2013 and 2014 were genotyped at 14 previously described microsatellite loci, specifically OtsG68, OtsG78b, OtsG432 (Williamson et al. 2002), RT212, RT36 (Spies et al., 2005), Ots204, Ots209, Ots211, Ots213 (Greig et al. 2003), Omm1053 (Rexroad et al. 2002), Ots1 (Banks et al. 1999), Ots107 (Nelson and Beacham 1999), Omy325 (O’Connell et al. 1997) and Oneµ13 (Scribner et al. 1999). Polymerase chain reaction (PCR) conditions included: a 5-minute denaturation step
(94°C), followed by 33-38 cycles of a 20-second denaturation step (94°C), a 20-second annealing step (51°C – Ots213; 52.5°C – OtsG68, Ots1, OtsG78b; 54°C – Omy325, Omm1053, Ots209; 56°C – OtsG432, RT212, RT36; 58°C – Onem13, Ots107; 60°C – Ots211; 62°C – Ots204) and a 30-second extension step (72°C), followed by a final extension step of 3 minutes. Amplicons were run on LiCor 4300 DNA analyzer (LiCor Biosciences, Inc.) and fragment sizes were scored using GENE IMAGIR 4.05 software (Scanalytics Inc.). DNA extraction and PCR amplification were performed for all fin clips, but only the final numbers of individuals included in the analyses during each year are presented in the results.

**Behavioural mate choice experiment**

**Spawning channels**

Salmon captured through netting in September 2013 (as described above) were sampled and subsequently used for a mate choice experiment. Fish were externally tagged with a white Petersen disc (Floy Tag Inc.) that was 3 cm in diameter and numbered on both sides of the body. Tagged fish were then moved onto a spawning channel, which was 70 m long and was divided into multiple sections that were each 9.5 m long by 2 m wide. Water depth was approximately 0.5 – 0.75 m and water velocity was approximately 0.06-0.1 m/s. Gravel depth was 0.4 m and was composed of gravel ranging from 0.02-0.1 m in diameter. Six sections of the spawning channel were used where six fish were placed within each section, which included two females (1 red and 1 white) and four males (2 red and 2 white). The section provided 9.5 m² of spawning area per female, which is greater than previous successful experiments with Chinook salmon (Neff et al. 2008; Lehnert et al. 2013). When possible, we attempted to size match red and white males within a section so that males of similar sizes were represented by each colour. However,
given that our fish were captured from a wild population, it was difficult to select for matched sizes in all cases. Among all sections, there was no significant difference in fork length between red and white males \((p = 0.56)\), where the mean \((\pm \text{ standard error})\) fork length for red and white males was 86.8 \((\pm 3.32)\) cm and 89.5 \((\pm 3.32)\) cm, respectively. The range in fork length for red and white males was 70-107 cm and 75-110 cm, respectively. Females were limited in number and thus were not matched for size. Care was taken to utilize only fish that appeared to be in good reproductive condition based on visual inspection, as we chose fish with limited fin deterioration and scale loss and no evidence of prior spawning.

*Video recordings and behaviour*

Fish were left to spawn and video recordings were taken haphazardly during daylight hours throughout the experimental period (September 19 – October 11). GoPro cameras were placed underwater at the downstream end of the spawning channel section, and video recordings occurred over 30 to 98 minute intervals between 08:00 – 19:00. The hours of videos recorded varied between channel sections, as this was dependent on the number of days where possible spawning activity (both males and females present) could occur in the channel section, as well as the number of channel sections with possible spawning activity as only two GoPros were used and were thus rotated among spawning sections throughout the day. Approximately 68 hours of video were recorded and analyzed, where the mean \((\pm \text{ standard error})\) number of hours per channel section was 11.4 \((\pm 0.68)\) hours. All video recordings were analyzed for three aggressive behaviours (similar to Garner et al. 2010a), which included biting, chasing (pursuit of another individual) and lunging (rapid increase in swimming speed towards another individual). Two observers recorded behaviours, where each observer recorded behaviour from three
of the channel sections. Each time a behaviour was observed in a video, the fish displaying the behaviour and the fish receiving the behaviour was recorded, and the number of each aggressive behaviour by a female towards a male was calculated for each female and male pair.

**Offspring collection**

After spawning, fish were removed and sampled post-mortem for body weight and gonad weight. Fertilized eggs were left to incubate in the gravel and offspring were collected at two sampling periods. Eggs were collected at the eyed stage (250-500 accumulated thermal units, ATU) by hydraulic sampling (see Lehnert et al. 2013) from November 11 to 14, 2013. Sampling was conducted across the entire area of the spawning channel section. Both eyed eggs and newly hatched alevins were collected from the spawning channels with a total of 2039 live offspring (1373 eyed eggs and 666 alevins). Dead eggs were counted and discarded, but we could not differentiate between dead fertilized and unfertilized eggs. Live eyed eggs and alevins were preserved for genetic analysis. Eggs collected were assumed to be a subsample of the total eggs in the substrate, thus offspring were also collected at the emerging fry stage (approximately 1000 ATU). Fry were captured using a minnow seine net from January 11 to 12, 2014. Total number of fry collected was 2030, where a subsample of 100 fry were kept per spawning section for genetic analysis with the exception of one section that had fewer than 100 fry (n = 13, Section 1 excluded from analyses).

**Genotyping and parentage assignment**

Parent and offspring DNA was extracted using an automated plate-based extraction protocol (Elphinstone et al. 2003). Parents and offspring were PCR amplified at 6 previously described microsatellite loci: Ots107 (Nelson and Beacham 1999), Ots211
(Greig et al. 2003), Omy325 (O’Connell et al. 1997), OtsG432, OtsG68 (Williamson et al. 2002) and Oneμ13 (Scribner et al. 1999). PCR conditions and allele scoring followed the same methods as described for adult spawners (described above). Parentage was assigned using CERVUS version 3.0 (Kalinowski et al. 2007) at an 85-95% confidence range with 1% genotyping error. The stated parameters resulted in assignment rates between 95.4 and 100% per spawning section for all eggs and alevins genotyped at 2 or more loci (see Table 5.1). After initial parentage analysis of fry, it was evident that movement had occurred between channels following fry emergence. To assign parentage to fry, parents that successfully contributed to eggs and alevins in surrounding channels were also included in the assignment analysis when assignment rates were low. Upon inclusion of a greater pool of potential parents, fry assignment rates increased and were between 82 and 98% per spawning section (see Table 5.1). All offspring (eggs, alevins and fry) were assigned at the strict 95% confidence, with the exception of only 4 fry that were assigned at the relaxed confidence of 85%. For microsatellite loci genotyped, the mean (± standard error) probability of exclusion of a parent pair per locus was 0.82 (± 0.05) and ranged from 0.68 – 0.95.

**MHC genetic divergence and mate choice**

**MHC genotyping**

In addition to microsatellite loci, DNA extracted from spawning salmon from the fall 2013 (n = 73) were also genotyped at two MHC loci including the class II beta 1 gene (MHC II-B1) and class I alpha 1 gene (MHC I-A1) using primers designed by Miller et al. (1997). Both primers amplify the peptide-binding regions of the molecules. We performed the following steps in replicate. PCRs were performed using the following: a 5-minute denaturation step (94°C), followed by 30 cycles of a 20-second denaturation
step (94°C), a 20-second annealing step (52.5°C – MHC II-B1; 63.5°C – MHC I-A1) and a 30-second extension step (72°C), followed by a final extension step of 3 minutes. PCR products from both loci were pooled by individual (5uL of each locus/individual), cleaned by precipitation with isopropanol, re-suspended in ddH₂O then individually barcoded. Barcoding PCR included: a 2-minute denaturation step (94°C), followed by 7 cycles of a 30-second denaturation step (94°C), a 30-second annealing step (60°C) and a 1-minute extension step (72°C), followed by a final extension of 5 minutes. Next, Agencourt AMPure XP (Beckman Coulter, Inc.) was used to purify barcoded amplicons, and amplicons were pooled then gel extracted using GenCatch Gel Extraction Kit (Epoch Life Science Inc.). The library was run on a 2100 Bioanalyzer (Agilent Technologies) to assess size distribution and concentration of DNA fragments in preparation for dilution. Replicate libraries were combined, and then emulsion PCR was performed using an Ion OneTouch System (Life Technologies) with an Ion OneTouch 400 bp template kit. An Ion Torrent Personalized Genome Machine (Life Technologies) was used to sequence the library with a 318™ chip (Life Technologies).

MHC genotypes were determined using jMHC software (Stuglik et al. 2011) and followed a similar protocol to that described by Lighten et al. (2014). Briefly, jMHC was used to identify all sequence variants (i.e., potential alleles) for each gene. Given that PCRs were performed in duplicate for each locus, each individual was genotyped twice and thus assigned two different barcodes. Therefore, for the remaining analyses, we separated barcodes from the two replicate PCRs and analyzed the replicates independently to generate replicate genotypes for each individual. For all individuals within a replicate, sequence variants occurring at high frequency (these comprised approximately 26-30 sequence variants containing >65% of the total read number once
singletons were removed) were compared using a neighbor joining tree in CodonCode Aligner (CodonCode, Dedham, MA, USA). Based on the neighbor joining tree, we identified putative true alleles versus alleles that were likely sequencing errors/artifacts (mismatched at 1-3 bp). Sequence reads from a putative true allele and its artifacts were combined to increase sequencing depth (Lighten et al. 2014). Next, following Lighten et al. (2014), and assuming that an individual could have either one or two alleles, individual genotypes were estimated by model fitting. Model fitting is described in Lighten et al. (2014) and uses two approaches: 1) copy number variation (CNV) model and 2) degree of change (DOC) model. Briefly, the CNV approach considers the number of reads for a maximum of 10 sequence variants (“alleles”) per individuals (Lighten et al. 2014). Assuming a one-locus model, approximately equal number of reads should be observed for both alleles if an individual is considered heterozygous (Lighten et al. 2014). An excel macro fits the data to possible genotype models (in our case heterozygous or homozygous) based on the observed number of reads for the potential alleles, then compares the fit of the possible models (Lighten et al. 2014). The two models that fit the data best are compared, and if there is a significant difference between the two models, then the best-fit model is used to assign the genotype (Lighten et al. 2014). In cases, where there was no significant difference between the two models, we also considered the second approach (DOC) offered by Lighten et al. (2014), where the model considers the change in number of reads between each allele from the most abundant to the least abundant allele. In this approach, the allele with the greatest DOC value is considered the last true allele in the genotype (Lighten et al. 2014). We considered the genotype to be true if the DOC and best-fit CNV model agreed. Generally, genotypes were easily assigned using the CNV approach. A few individuals had ambiguous genotypes, and
therefore individuals were compared with their replicate genotype to ensure accurate genotyping.

**Statistical analyses**

*Neutral genetic divergence and diversity*

First, microsatellite loci were tested for linkage disequilibrium (LD) to assess suitability of markers for further analyses. Loci were tested for conformity to Hardy-Weinberg Equilibrium (HWE) using GenePop version 4.2 (Rousset 2008). ARLEQUIN version 3.5 was used to determine genetic divergence ($F_{ST}$) between red and white individuals in the population (Excoffier and Lischer 2010). Population differentiation based on allele frequency distributions was also tested using Fisher’s exact test in GenePop version 4.2 (Rousset 2008). Genetic diversity estimates, including heterozygosity observed ($H_O$) and expected ($H_E$), were calculated using GenAlEx version 6.5 (Peakall and Smouse 2012), and FSTAT was used to estimate allelic richness (Goudet 2001). Estimates of genetic diversity were compared between red and white groups using a Mann-Whitney test.

*Female aggression towards males*

Aggressive behaviour by females towards males was compared between colour-matched and colour-mismatched pairs. For all females that performed at least one aggressive act, we summed the number of each behaviour (lunging, biting and chasing) for each male-female pair in the channel. Next, given that the three behaviours were significantly correlated (all $r > 0.70$, $p < 0.01$), we ran a principle component analysis (PCA) using the three aggressive behaviours using JMP version 12 (SAS Institute Inc.). Any informative PC axis (Eigen value > 1) was used as a response variable in our model. We used a linear mixed model in the lme4 package (Bates et al. 2009) with R software (R
Core Team 2014). The model included a fixed factor as pair type (colour-matched versus colour-mismatched), with random effect of channel section, and the model was weighted by the amount of time a female was observed on video. Two observers recorded behaviours for different channel sections (G.F. observed sections 1, 2 and 5; M.L. observed sections 3, 4 and 6). To compare scoring between observers, both observers recorded behaviours for the same video. We calculated two measures of reliability in scoring, including Cohen’s kappa for inter-rater reliability (IRR; Cohen 1960) and intra-class correlation (ICC, Shrout and Fleiss 1979). IRR was 0.88, suggesting that behaviours were interpreted in the same way by each observer and ICC was 0.82, indicating consistency in scoring between observers.

**Colour assortative mate choice**

All analyses were conducted in R software (R Core Team 2014), unless otherwise stated. Parentage of eyed eggs and alevins was considered a quantification of mate choice. First, a chi-square test was used to compare the number of offspring (eggs and alevins) produced by colour assortative and disassortative mating. The difference in number of offspring produced by colour assortative and disassortative mating between red and white females was also tested with a chi-square test, as well as differences between individual females. Next, number of colour assortative and disassortative mating events were compared using a chi-square test for both the number of primary mating events (i.e., based on a female’s primary mate) and for all mating events combined (i.e., all mating pairs detected). Using Fisher’s exact test, we also compared the number of colour assortative and disassortative mating events between red and white females to determine if female preference for colour differed between female phenotypes.
Offspring survival (fry)

In addition to parentage assignment of eggs and alevins, fry were also parentally assigned, and the parentage of fry was considered as a measure of offspring survival. Because fry appeared to have moved between sections of the spawning channel (described above), channels could no longer be considered as independent replicates. However, we chose to consider the overall proportions of each mating pair within the fry sample and compare those to the proportions for the same mating pair at the egg/alevin stage. The proportions at both early life stages were calculated as the proportion of offspring from the total genotyped offspring population (egg/alevins n = 1885, fry n = 458). To test whether offspring from assortative and disassortative mating events experience differences in survival between the egg and fry stages, we calculated the change in proportion from the egg stage to the fry stage. Change in offspring proportion between assortative and disassortative mating pairs was compared using a Mann-Whitney test, as sample sizes were not equal between groups.

MHC genetic divergence and mate choice

First, genetic divergence and diversity at MHC genes between red and white spawning salmon in the population were analyzed in the same way as neutral loci (described above). Next, we tested whether mate choice occurred randomly based on MHC II-B1 divergence (genotyping analyses described above). MHC II-B1 alleles were compared using MEGA version 5 (Tamura et al. 2011) to determine the number of amino acid (AA) differences between male and female genotypes. The average divergence between potential mates was calculated similar to Landry et al. (2001), where the number of AA differences between each male and female allele combination was calculated to determine the average number of AA differences between mates. For each mating pair,
the expected proportion of heterozygote offspring based on parental genotypes was calculated to determine an index of mate choice based on heterozygosity. Following Neff et al. (2008), we used a Monte Carlo simulation in Excel (Microsoft Corporation) to randomly assign males to female mates, and we included all males however we used only females that were observed to have successfully reproduced based on genetic data. In our simulation, females were randomly assigned to males in their spawning section based on their number of observed mates in the experiment, and the MHC divergence and heterozygosity value for each female was calculated as a weighted average based on the percent offspring sired by different males. We generated a distribution for red and white females separately based on 5000 simulations of random mating. Observed MHC divergence and heterozygosity indices among mates were compared against the estimated distributions to determine p-values (one-tailed).

Results

Neutral genetic divergence and diversity

For fall 2013, we included only individuals that were genotyped at 7 or more of the 14 microsatellite loci in our analyses. Three loci (Ots209, OtsG78 and Ots204) showed highly significant deviations from HWE in both red and white groups, thus these loci were removed from further analyses and were not genotyped for fall 2014 samples. Next, linkage disequilibrium (LD) was tested and an adjusted p-value of 0.0045 (0.05/11) was used to account for multiple comparisons among the 11 loci. No pairs of loci showed evidence of significant LD in either sampling year (all p values > 0.005) or overall (all p values > 0.02), thus all further analysis included 11 loci with 69 individuals in 2013 (n = 33 red; 36 white) and 45 individuals in 2014 (n = 22 red; 23 white). Genetic divergence was not significant between red and white groupings in either sampling year (2013: $F_{ST} =$
0.002, p = 0.16; 2014: $F_{ST} = -0.0003$, p = 0.37; see Table 5.2). Fisher’s exact tests also showed no significant population differentiation based on allele frequencies over all loci between red and white groups in both 2013 ($\chi^2 = 21.2; p = 0.51$) and 2014 ($\chi^2 = 23.8; p = 0.36$). In both sampling years, genetic diversity estimates did not differ significantly between red and white, including heterozygosity observed (p values > 0.90) and expected (p values > 0.74) (see Table 5.2). Estimates of allelic richness were also similar between red and white individuals in both 2013 (p = 0.84) and 2014 (p = 0.95) (Table 5.2). Fisher’s exact tests were used to assess genetic differentiation between the sampling years, and we found only 1 out of 11 loci showed a significant difference in allele frequencies between 2013 and 2014 samples. Therefore, samples were combined to assess genetic divergence and differentiation between red and white over both sampling years, and we found no significant genetic divergence between red and white ($F_{ST} = 0.0005$, p = 0.25) and no significant population differentiation between red and white based on allele frequencies ($\chi^2 = 25.91$, p = 0.26).

**Behavioural mate choice experiment**

**Female aggression towards males**

A total of 275 aggressive acts by females towards males were observed in the videos. Both reproductively successful and unsuccessful females exhibited aggressive acts. Females in four of the six channel sections (no female aggression towards males was observed in Section 3 and 6) exhibited aggression towards at least one male within her section. Thus, our analyses included 8 females and 16 males from four channel sections, which resulted in 32 pair combinations (18 colour-matched and 18 colour-mismatched). Of the 32 pair combinations, at least 1 aggressive act was observed for 25 pairs, and 9 of these 25 pairs had reproductive events (i.e., produced offspring). PCA revealed that one
PC axis (PC1 herein referred to as aggression score) could explain 85.4% of the variation (Eigen value = 2.56). Aggression scores were log-transformed for linear mixed modelling to meet assumption of normality for model residuals (Shapiro-Wilks test, p = 0.08). We found no significant difference in female aggression towards males when comparing between colour-matched and colour-mismatched males (LMM, $\chi^2 = 0.039; p = 0.84$)

**Colour assortative mate choice**

In the spawning channels, three of the 12 females failed to reproduce, and 50% of the males ($n = 12$) did not successfully contribute to the parentage of eyed eggs and alevins (hereafter referred to as offspring). All three females that did not spawn were white females, and males that did not spawn included 5 red and 7 white males. Successful males and females showed variation in mating success, as the number of offspring assigned to females ranged from 40 to 424 offspring, whereas the variation in males ranged from 19 to 599 offspring.

Based on parentage assignment of offspring, there was a significant difference in the number offspring produced by colour assortative and disassortative mating ($\chi^2 = 342.07, p < 0.001$; Table 5.3). Furthermore, there was a significant difference between red and white females in the number of offspring produced by assortative and disassortative mating ($\chi^2 = 66.50, p < 0.001$; Table 5.3), where approximately 75.9% and 55.3% of offspring from red and white females, respectively, were produced through assortative mating (Figure 5.2). When comparing individual females, we also found a significant difference between females in the number of offspring produced by colour assortative and disassortative mating ($\chi^2 = 1582.9, p < 0.001$; Table 5.3). The number of primary mating pairs (i.e., mate that sired the majority of a female’s eggs, which was >70% of a female’s offspring in our study) that were colour assortative was greater than, but not significantly
different from, the number of mating pairs that occurred through colour disassortative mating ($\chi^2 = 1.00, p = 0.32$; Table 5.4), and qualitatively similar results were found when considering all mating events ($\chi^2 = 2.57, p = 0.11$; Table 5.4). Furthermore, the number of colour assortative versus disassortative primary mating events was not significantly different between red and white females (Fisher’s exact test, $p = 0.99$; Table 5.4), and the same was true when considering all mating events (i.e., primary and secondary mates) (Fisher’s exact test, $p = 0.58$; Table 5.4).

*Offspring survival (fry)*

There was no significant difference in offspring survival between assortative and disassortative mating pairs (Mann-Whitney, $p = 0.65$). The mean (± standard error) change in proportion of offspring between the egg and fry stage for colour assortative and disassortative mating pairs were -0.62 (± 2.60) % and 1.38 (± 4.22) %, respectively.

*MHC genetic divergence and mate choice*

Genotypes for four individuals at MHC I-A1 and six individuals at MHC II-B1 were not identical between replicate samples and thus genotypes were discarded. For MHC II-B1 analyses, we removed individuals that were not replicated ($n = 7$). Therefore our analyses included 30 red and 31 white individuals. Because the sample size was lower for MHC I-A1 (due to individuals that did not amplify), all individuals with genotypes were analyzed for divergence and diversity estimates including non-replicated individuals ($n = 26$ red; 30 white).

Analyses of MHC I-A1 and MHC II-B1 genes identified 8 and 6 alleles, respectively, in the Quesnel River population (Figure 5.3; see Appendix 3 and Appendix 4). The MHC I-A1 locus exhibited significant deviations from HWE in both red and white groups ($p < 0.001$) due to a significant heterozygote deficiency. MHC II-B1 did not
deviate significantly from HWE in either red or white groups (p values > 0.06). Both MHC I-A1 and MHC II-B1 loci showed significant genetic divergence between red and white individuals in the population, where $F_{ST}$ values were 0.0495 ($p = 0.028$) at MHC I-A1 and 0.0346 ($p = 0.039$) at MHC II-B1 (Table 5.2). At MHC II-B1, both red and white individuals shared the same three major alleles and there was no significant difference in the frequency of these alleles between red and white fish (Fisher’s exact test, $p = 0.06$; Figure 3B). In contrast, for MHC I-A1, the allele frequency distributions differed significantly between red and white groups (Fisher’s exact test, $p = 0.01$; Figure 5.3A). Heterozygosity estimates ($H_O$ and $H_E$) were greater for white than red individuals at both loci (Table 5.2), where the observed percentage of heterozygotes at MHC II-B1 was 47% and 77% for red and white, respectively, and for MHC I-A1 heterozygosity was 35% and 43% for red and white, respectively.

The estimated distribution of random mating based on average MHC II-B1 divergence and heterozygosity between mates are provided in Figure 5.4. Red and white females mated randomly based on MHC II-B1 amino acid divergence (all $p$ values > 0.10; Figure 5.4A,B). Furthermore, red and white females mated randomly based on heterozygosity at the MHC II-B1 gene (all $p$ values > 0.27; Figure 5.4C,D).

**Discussion**

In our study, we examined how mate choice, as well as neutral and functional genetic processes can shape phenotypic variation in a population of colour polymorphic Chinook salmon. Based on neutral microsatellite markers, we found no genetic differentiation between red and white phenotypes over multiple years (overall $F_{ST} = 0.0005$), suggesting that there are no barriers to gene flow between the phenotypes. Furthermore, in experimental spawning channels, female aggression towards males was
not dependent on the colour of the male relative to the female. Specifically, females
displayed the same amount of aggression towards colour-matched and colour-mismatched
males thus indicating no evidence of a behavioural bias by females based on male colour.
However, we found significantly more offspring were produced through colour
assortative than disassortative mating under experimental conditions, although the
difference was primarily driven by red females producing more offspring with red males
than white males. Nevertheless, we found no difference in the number of mating pairs
occuring through colour assortative and disassortative mating. We also found no
difference in offspring survival between colour assortative and disassortative mating,
indicative of no negative consequences on early life survival of interbreeding between red
and white individuals. Therefore, although we found some degree of assortative mating
(71%) in our experiment, substantial gene flow between the morphs makes it unlikely that
colour-based assortative mating is a primary mechanism underlying the continued
presence of the polymorphism. Furthermore, the persistence of white individuals as well
as the interbreeding of red and white morphs suggests that red spawning colouration
previously shown to be important to sexual selection in salmon (Fleming and Gross 1994;
Skarstein and Folstad 1996; Craig and Foote 2001) is not universally so, at least not in
populations where red and white individuals exist in sympatry. Mate choice in red and
white Chinook salmon in the Quesnel River may be driven by other factors (discussed
below).

Previous studies on colour polymorphisms and genetic divergence have found
that, similar to our study, sympatric colour morphs exhibited weak or no genetic
divergence at neutral markers (Walter et al. 2009; Huyghe et al. 2010). For example, in
Dalmatian wall lizards (Podarcis melisellensis), there was evidence of weak genetic
divergence between three colour morphs where overall $F_{ST}$ between morphs was -0.001 (Huyghe et al. 2010). Although Walter et al. (2009) observed significantly more colour assortative than disassortative mating among colour polymorphic sailfin silversides, genetic data revealed no significant differentiation between the blue and yellow colour morphs ($F_{ST} = 0.003$). Given that the flesh colour phenotype in Chinook salmon is controlled by few loci, it is possible that if these alleles do not affect fitness that the polymorphism would be maintained in nature despite interbreeding between red and white individuals. Withler (1986) examined inheritance patterns in offspring from red and white Chinook salmon parents. Based on offspring phenotypes, Withler (1986) proposed that flesh colour may be controlled by two loci, each with two alleles, where one red determining allele (dominant) must be present at both loci to exhibit red colouration. Thus crosses between red and white parents can produce both red and white offspring, where the ratio of red:white offspring will depend on the genotype of the parents (Withler 1986).

In the Quesnel River, assuming the Withler (1986) two-locus model and equal frequencies of red and white alleles in the population, we would expect 56% of the population to have the red phenotype (9:7 ratio of red:white individuals). The frequency of red and white individuals in the Quesnel River population is estimated to be approximately 50% red and 50% white (Withler 1986). Therefore, even some bias for assortative mating, as we found in our study, could lead to equal proportions of red and white individuals that are found in the Quesnel River population assuming equal fitness among colour genotypes. In this case, an increase in assortative mating by red females (as demonstrated in our study) could consequently result in an increase in the proportion of white individuals in the population due to more opportunities for white-white mating events. Thus interbreeding between red and white Chinook salmon could occur, yet
colour diversity in the population could also be maintained through mechanisms such as balancing selection acting on the red and white alleles.

While we found no evidence of neutral genetic divergence, we did find significant functional genetic differences between red and white individuals at two MHC genes. Therefore, if mating occurred non-randomly at the MHC genes in the present study, this would contribute to the maintenance of the polymorphism. However, when we examined mate choice based on MHC II-B1, which is the gene class commonly found to be associated with mate choice in salmon (Landry et al. 2001; Skarstein et al. 2005; Neff et al. 2008), we found no evidence for non-random mating. Thus our results indicate that natural selection, rather than mate choice, is likely shaping the differences between the morphs at MHC II-B1. Mate choice based on MHC I-A1 was not assessed, as not all individuals in the experiment were successfully genotyped. Non-random mate choice at MHC I-A1 may explain the significant difference in allele frequencies detected in our study. Although few studies have examined mate choice in relation to MHC class I genes in salmonids, Yeates et al. (2009) found that Atlantic salmon (Salmo salar) males with more similar MHC class I genotypes to the female had greater competitive fertilization success relative to males with dissimilar genotypes. Therefore, if red and white Chinook salmon are choosing mates with more similar MHC I-A1 alleles, this could provide a mechanism for maintenance of the two colour morphs in the system, a hypothesis that warrants further investigation.

Carotenoids have been found to enhance immune response across a broad range of taxa including salmon (Blount et al. 2003; Grether et al. 2004; Butler and McGraw 2013; Amar et al. 2012), thus we speculate that white Chinook salmon may be immune compromised relative to red individuals unless they have evolved compensatory
mechanisms to deal with this handicap (Tyndale et al. 2008). In our study, white individuals were 30% more heterozygous than red individuals at the MHC II-B1 gene. However, given that no evidence for non-random mate choice based on MHC II-B1 was identified, diversity at MHC genes may be explained through balancing selection, where individuals that are heterozygous at the MHC gene exhibit a fitness advantage over homozygotes (see Bernatchez and Landry 2001). Therefore, it is possible that white Chinook salmon may enhance immunity through increased heterozygosity at the MHC gene (McClelland et al. 2003; Evan and Neff 2009). Indeed, in Chinook salmon, evidence of a heterozygote advantage at MHC II-B1 gene has been demonstrated for resistance to bacterial (Evans and Neff 2009) and viral pathogens (Arkush et al. 2002). Although there is some evidence that carotenoids can also improve specific (adaptive) immunity (Grether et al. 2004), the majority of research indicates that carotenoids improve non-specific (innate) immunity (Blount et al. 2003; Butler and McGraw 2013; Amar et al. 2012).

Given that heterozygosity at the MHC II-B1 gene can be beneficial, why do red individuals not increase both their non-specific immunity (via carotenoids) and specific immunity (via MHC diversity)? One possibility is that excessive MHC diversity within an individual can result in autoimmune issues (Penn and Potts 1999), thus there may be an optimal number of MHC alleles (Milinski et al. 2005), which may vary depending on the level of other immune response capability (e.g., carotenoids present or not).

In addition to differences found at MHC II-B1, we found a significant difference in allele frequencies between red and white groups and a significant heterozygote deficiency overall at the MHC I-A1 gene. Previous studies have also found evidence for heterozygote deficiency at MHC class I genes in Chinook salmon populations (Miller et al. 1997; Heath et al. 2006). Although we cannot rule out mate choice for shaping
differences in MHC I-A1 gene, in salmon, specific alleles can infer fitness advantages as local adaptation may drive directional selection for certain alleles that enhance immunity when encountering common pathogens. In Atlantic salmon, specific alleles at MHC I-A1 have been demonstrated to confer higher fitness when exposed to infectious salmon anemia (ISA) virus (Kjøglum et al. 2006). Although red and white Chinook salmon are thought to utilize the same environments, the extreme difference between the morphs in carotenoid assimilation may result in deviation in susceptibility to different viruses. For example, in rainbow trout, fish fed a carotenoid (astaxanthin) diet had significantly higher survival after exposure to a virus than fish fed a control diet (Amar et al. 2012).

Moreover, two alleles in white individuals that occur at a high frequency (33.3% for OTS A-5 and 13.3% for OTS A-4) lacked a two codon-insertion, which were termed motif 2 alleles by Miller et al. (1997) (Figure 5.3A; for DNA sequences see Appendix 4). The two alleles each occurred at less than 10% frequency in red individuals (Figure 5.3A).

Interestingly, motif 2 alleles have been found at high frequency in another white Chinook salmon population (Harrison River), and these alleles could be used to differentiate the Harrison population from the Nechako River population (Miller et al. 1997). Thus the insertion may be selected against in white populations, and alleles lacking the insertion (motif 2) at MHC I-A1 locus may infer some fitness advantage in white individuals. Nonetheless, studies designed to test for differences in susceptibility between morphs to both viral and bacterial pathogens are required to further characterize the MHC allelic differences detected in our study.

There is little known about the evolutionary processes maintaining the sympatric red and white Chinook salmon phenotypes and our study is the first to quantify neutral and functional genetic differences between the phenotypes, as well as examine mate
choice in this system. We demonstrate that gene flow is occurring between red and white individuals, although some bias for colour assortative mating was detected which may explain in part the relatively equal proportions of red and white individuals in the Quesnel River population. Nevertheless, colour assortative mating is likely not the primary mechanism operating to maintain the polymorphism. However, our study highlights that natural selection is likely operating differentially on red and white Chinook salmon at two immune genes, as we found that white individuals were more heterozygous at the MHC II-B1 gene and there was a significant difference in allele frequencies at the MHC I-A1 gene between red and white conspecifics. The possibility of non-random mating at MHC I-A1 may explain differences in MHC detected here, but no evidence for mate choice based on MHC II-B1 was observed. If the limited ability to store carotenoids in tissues subsequently impacts the immune response in white Chinook salmon, our research provides a possible compensatory genetic mechanism that may allow white Chinook salmon to deal with an immune handicap. No studies to date have specifically tested whether white Chinook salmon are actually immunocompromised relative to the red morph, and thus the present interpretation is based on current knowledge of carotenoids, MHC and immune function in fishes. Our study contributes to our understanding of the evolutionary factors that maintain the red/white colour polymorphism in Chinook salmon and also adds to our growing understanding of the more general evolutionary question of: why are salmon red?

References


Bates D, Maechler M, Bolker B. 2009. lme4: linear mixed-effects model using S4 classes. R packaged version 0.999375-31. See http://lme4.r-forge.r-project.org


Table 5.1: Number of Chinook salmon (*Oncorhynchus tshawytscha*) eggs, alevins and fry assigned parentage from experimental spawning channels where parentage assignment rates were based on the number of offspring genotyped at 2 or more microsatellite loci (see text for details).

<table>
<thead>
<tr>
<th>Section</th>
<th>Spawning Channel</th>
<th>Eggs</th>
<th>Alevins</th>
<th>Fry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Section 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Section 2</td>
<td>85</td>
<td>82</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Section 3</td>
<td>96</td>
<td>92</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Section 4</td>
<td>116</td>
<td>114</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Section 5</td>
<td>141</td>
<td>138</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Section 6</td>
<td>276</td>
<td>274</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Assignment</th>
<th>Fry</th>
<th>% Assignment</th>
<th>Fry</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>16</td>
<td>95.4%</td>
<td>599</td>
</tr>
<tr>
<td>95.7%</td>
<td>176</td>
<td>96.3%</td>
<td>261</td>
</tr>
<tr>
<td>98.3%</td>
<td>-</td>
<td>100%</td>
<td>431</td>
</tr>
<tr>
<td>95%</td>
<td>76</td>
<td>98%</td>
<td>276</td>
</tr>
<tr>
<td>92.9%</td>
<td>58</td>
<td>92%</td>
<td>276</td>
</tr>
<tr>
<td>95%</td>
<td>85</td>
<td>95%</td>
<td>276</td>
</tr>
<tr>
<td>97.8%</td>
<td>18</td>
<td>95%</td>
<td>599</td>
</tr>
<tr>
<td>82%</td>
<td>76</td>
<td>82%</td>
<td>599</td>
</tr>
</tbody>
</table>
Table 5. Estimates of neutral (microsatellites) and functional (major histocompatibility complex genes) genetic diversity and genetic divergence ($F_{ST}$) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) from the Quesnel River over multiple sampling years. Genetic diversity estimates include mean allelic richness ($A_R$) for microsatellites or number of alleles ($A_N$) for functional genes, and heterozygosity (observed $H_O$ and expected $H_E$).

<table>
<thead>
<tr>
<th>Loci/Genes</th>
<th>Year</th>
<th>Colour</th>
<th>No. fish</th>
<th>$A_R$ (or $A_N$)</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>Divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsatellites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHC-I-A1</td>
<td>2013</td>
<td>Red</td>
<td>33</td>
<td>0.433</td>
<td>0.788</td>
<td>1.79</td>
<td>$F_{ST}$ = 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>39</td>
<td>0.436</td>
<td>0.774</td>
<td>0.74</td>
<td>$F_{ST}$ = 0.002</td>
</tr>
<tr>
<td>MHC-II-B1</td>
<td>2013</td>
<td>Red</td>
<td>36</td>
<td>0.824</td>
<td>0.805</td>
<td>0.805</td>
<td>$F_{ST}$ = 0.0346*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>31</td>
<td>0.843</td>
<td>0.874</td>
<td>0.874</td>
<td>$F_{ST}$ = 0.0346*</td>
</tr>
<tr>
<td>Microsatellites</td>
<td>2014</td>
<td>Red</td>
<td>22</td>
<td>0.832</td>
<td>0.819</td>
<td>0.819</td>
<td>$F_{ST}$ = 0.0495*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>White</td>
<td>23</td>
<td>0.843</td>
<td>0.824</td>
<td>0.824</td>
<td>$F_{ST}$ = 0.0495*</td>
</tr>
</tbody>
</table>

* Asterisks (*) indicate significant genetic divergence between red and white groups ($p < 0.05$).
Table 5.3. Number of offspring (eggs and alevins) produced by red and white Chinook salmon (*Oncorhynchus tshawytscha*) females through colour assortative and disassortative mating in experimental spawning channel sections. Significant differences in number of offspring produced by colour assortative and disassortative mating are indicated in footnote.

<table>
<thead>
<tr>
<th>Female ID</th>
<th>Section</th>
<th>Number of offspring</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Assortative</td>
<td>Disassortative</td>
<td></td>
</tr>
<tr>
<td>Red 1</td>
<td>1</td>
<td>81</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Red 12</td>
<td>2</td>
<td>424</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Red 26</td>
<td>3</td>
<td>21</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>Red 27</td>
<td>4</td>
<td>42</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td>Red 28</td>
<td>5</td>
<td>391</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Red 36</td>
<td>6</td>
<td>154</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total red</strong></td>
<td></td>
<td>1113</td>
<td>354</td>
<td></td>
</tr>
<tr>
<td>White 11</td>
<td>2</td>
<td>0</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>White 33</td>
<td>5</td>
<td>28</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>White 48</td>
<td>6</td>
<td>203</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total white</strong></td>
<td></td>
<td>231</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td><strong>Total overall</strong></td>
<td></td>
<td><strong>1344</strong></td>
<td><strong>541</strong></td>
<td></td>
</tr>
</tbody>
</table>

Between individual females: $\chi^2 = 1582.9$, $p < 0.001$
Between red and white females: $\chi^2 = 66.50$, $p < 0.001$
Overall: $\chi^2 = 342.07$, $p < 0.001$
Table 5.4. Number (and percentage) of mating events for colour assortative and disassortative mating in red and white Chinook salmon (*Oncorhynchus tshawytscha*) females in experimental spawning channels when considering only primary mating events, as well as all mating events.

<table>
<thead>
<tr>
<th>Colour</th>
<th>Assortative</th>
<th>Disassortative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary Mating Events</td>
<td>All Mating Events</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Red</td>
<td>4 (22%)</td>
<td>10 (71%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>White</td>
<td>2 (12%)</td>
<td>3 (21%)</td>
<td>1 (6%)</td>
</tr>
</tbody>
</table>
Figure 5.1. Photograph (A) of white (top) and red (bottom) Chinook salmon (Oncorhynchus tshawytscha) males from the Quesnel River in the fall of 2014, and mean and standard error reflectance spectra (B) for spawning red (n = 19) and white (n = 18) Chinook salmon, including both males and females, captured from the Quesnel River in the fall of 2013.
Figure 5.2. Percentage of offspring (including eggs and alevins) from red (n = 1467 offspring) and white (n = 418 offspring) Chinook salmon (*Oncorhynchus tshawytscha*) females that were sired by red and white males in experimental spawning channels.
Figure 5.3. Allele frequency distributions for spawning red and white Chinook salmon (*Oncorhynchus tshawytscha*) from the Quesnel River, British Columbia at two major histocompatibility complex (MHC) genes, including (A) MHC class I alpha 1 and (B) MHC class II beta 1. MHC I-A1 alleles are referred to as OTS A-1 to 8 and MHC II-B1 alleles are referred to as OTS B-1 to 6.
Figure 5.4. Distribution of average MHC II-B1 amino acid divergence (A, B) and MHC II-B1 expected offspring heterozygosity (C, D) between Chinook salmon (Oncorhynchus tshawytscha) mates based on 5000 simulations of random mating in experimental spawning channels. White (A, C) and red (B, D) female distributions are represented with the average observed value indicated by a solid line.
CHAPTER 6: POST-SPAWNING SEXUAL SELECTION IN RED AND WHITE CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

Introduction

Sexual selection is an important evolutionary process that acts both before and after copulation (Birkhead and Pizzari 2002; Andersson and Simmons 2006). Although early sexual selection research focused on pre-copulatory mechanisms, researchers have recently shown the critical role of post-copulatory sexual selection in diverse evolutionary processes, such as speciation (Mendelson et al. 2007; Yeates et al. 2013), local adaptation (Palumbi 1999; Yeates et al. 2009) and the maintenance of genetic variation (Birkhead and Pizzari 2002; Gasparini and Pilastro 2011; Løvlie et al. 2013). Post-copulatory sexual selection includes both sperm competition and cryptic female choice (CFC). Sperm competition arises when sperm from more than one male compete to fertilize the eggs of a female (Parker 1970), and the outcome can depend on sperm quality (Gage et al. 2004; García-González and Simmons 2005; Snook 2005; Gasparini et al. 2010; Beausoleil et al. 2012). Although sperm quality traits can be good predictors of fertilization success, CFC can also influence the outcome of sperm competition where females may bias fertilization in favour of a specific male that will confer the greatest fitness benefit for her offspring (Eberhard 1996; Birkhead 1998; Neff and Pitcher 2005). Potential mechanisms for CFC include egg-sperm recognition (Palumbi 1999; Yeates et al. 2009) and female-related chemical processes that mediate sperm performance or fertilization success (Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013).

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Evidence for CFC has been demonstrated in many taxa and has been shown to discriminate among conspecifics to skew fertilization success in favour of males with specific genotypes (Palumbi 1999; Yeates et al. 2009; Butts et al. 2012; Løvlie et al. 2013) or phenotypes (Evans et al. 2003; Bussière et al. 2006). Additionally, CFC may operate as a form of reinforcement to reduce the risk of hybridization between closely related species (Yeates et al. 2013). While empirical evidence indicates that CFC is found in many species, the specific mechanisms by which CFC is achieved are less understood. Studies on CFC can be confounded by male effects, such as differences between males in sperm performance (Birkhead and Pizzari 2002; Evans et al. 2013). Although both sperm competition and CFC are important to our understanding of sexual selection, partitioning the relative effects of female, male and their interaction on post-copulatory success is often difficult (Birkhead and Pizzari 2002; Evans et al. 2013). However, studies designed to evaluate CFC and sperm competition simultaneously can properly quantify post-copulatory success attributed to each process.

While post-copulatory sexual selection has many important evolutionary consequences, one of particular interest is its role in maintaining genetic variation in nature (Birkhead and Pizzari 2002). Chinook salmon (Oncorhynchus tshawytscha) display remarkable variation in flesh colouration, resulting from genetic polymorphisms that affect their ability to deposit dietary carotenoid pigments into their tissues (Withler 1986; Lehnert et al. 2016). Consequently, within some populations of Chinook salmon, individuals exhibit flesh colour that is white (unpigmented) or red (pigmented) (Withler 1986), and the percentage of white individuals within a population can range from 0 to 100% of the population (Hard et al. 1989). Differences in flesh pigmentation also
translate into differences in egg and spawning colouration (Withler 1986; Rajasingh et al. 2007; Lehnert et al. 2016). Carotenoid pigments play an important role in salmonid fitness (Rajasingh et al. 2007) as carotenoids have been linked to salmon immune function (Amar et al. 2012), egg survival (Tyndale et al. 2008), mate choice (Fleming and Gross 1994; Skarstein and Folstad 1996; Craig and Foote 2001) and sperm quality (Ahmadi et al. 2006; Janhunen et al. 2009; Pitcher et al. 2009). However, despite this potential handicap to white Chinook salmon, both phenotypes persist in mixed populations in nature. Thus, in our study, we investigate post-spawning sexual selection as a possible mechanism that contributes to the maintenance of the colour polymorphism.

While sperm performance has been shown to be influenced by carotenoid pigments (Evans et al. 2003; Locatello et al. 2006; Pike et al. 2010; Tizkar et al. 2015), differences in sperm performance between red and white Chinook salmon males have yet to be evaluated. The persistence of the white phenotype in nature may indicate that white Chinook salmon have evolved compensatory mechanisms to increase their relative fitness despite lacking carotenoids. Therefore, by assessing sperm performance differences between red and white males, we can determine whether competitive differences exist between the phenotypes during reproduction that may contribute to the maintenance of the two morphs in nature. In addition to sperm performance, CFC may also influence fertilization success, and in salmon, CFC may be mediated by ovarian fluid (a viscous liquid that is expelled with the eggs during spawning) (Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013) and/or egg-sperm recognition (Yeates et al. 2009). Although CFC has not been examined in red and white Chinook salmon, a recent study showed that the red and white phenotypes do successfully interbreed in one
population; however, under semi-natural conditions, 71% of mating events were found to be colour assortative (Lehnert et al. 2016). The potential fitness consequences of interbreeding between colour morphs have not been established. CFC may act to reinforce pre-copulatory choice (Evans et al. 2003; Parker 2009), although studies have also found that CFC can act antagonistically with pre-copulatory sexual selection (Danielsson 2001; Bussière et al. 2006). If there is a cost to interbreeding between morphs (i.e., hybrid breakdown) then CFC may bias paternity in favour of a male that has the same phenotype as the female, thus offering an evolutionary mechanism contributing to the maintenance of the morphs.

Chinook salmon are external fertilizers, thus sperm competition and CFC can be studied using in vitro experiments where sperm, eggs and other reproductive components (i.e., ovarian fluid) can be easily manipulated. Furthermore, Chinook salmon have high fecundity and high volume of semen allowing factorial mating designs that allow the partitioning of male, female and interaction effects on fertilization success (Evans et al. 2013). In this study, we first quantify differences between red and white Chinook salmon males in sperm velocity upon activation in fresh water (sperm quality). Next, given that the ovarian fluid of Chinook salmon females may mediate CFC (Rosengrave et al. 2016), we test for colour-based CFC in red and white Chinook salmon females by quantifying relative sperm velocity in ovarian fluid (using microscopy). In addition, although sperm velocity in ovarian fluid may determine the outcome of post-copulatory sexual selection, to ultimately partition the relative contribution of each process (i.e., sperm competition and cryptic female choice) to fertilization success, we use in vitro competitive fertilization trials. Our study evaluates how post-spawning sexual selection operates in a
species that exhibits genetic polymorphisms for carotenoid pigmentation, where the relative contribution of both sperm competition and CFC to post-spawning success is quantified. The results of our study will determine whether post-spawning sexual selection may be a mechanism contributing to the maintenance of the colour polymorphism in nature.

**Methods**

**Fish collection**

During the fall of 2013 and 2014, adult Chinook salmon were caught by seine from the Quesnel River, Likely, British Columbia, Canada (GPS coordinates 52°36’28”N and 121°32’57”W). During both sampling seasons, fish collection occurred from September 13 to October 1. In 2013, we collected only male fish to measure sperm velocity in water, and in 2014, we collected male and female fish to measure sperm velocity and to evaluate CFC. After capture, fish were held in the river temporarily before being transported to the Quesnel River Research Center (QRRC). During transport, fish were placed in holding tanks with aerated river water and transported for approximately 15 minutes (5 km). Fish were then held in 3000 L freshwater tanks or semi-natural spawning channels (see Lehnert et al. 2016 for description) at 10°C until sampling. All fish were sampled within 2 weeks of capture.

**Gamete collection**

In both the fall of 2013 and 2014, live males were anesthetized in clove oil and sampled for semen, weight, colour score (see below) and a fin clip for DNA extraction. The age of the fish was unknown, however it is expected that males and females were approximately 4-5 years of age based on their size. Weights and lengths did not differ for
males between sampling years (n = 48; t tests, p values > 0.29). Thus, across both years, red and white males did not differ significantly in weight or length (t tests, p values > 0.64), as the mean (± standard error) weights of red and white males were 7.2 (± 0.57) and 7.1 (± 0.66) kg, respectively, and weights ranged from 3.3 – 13.3 kg for red males and 3.0 – 13.9 kg for white males. Additionally, mean (± SE) fork lengths for red and white males were 88.3 (± 2.2) and 86.7 (± 2.5) cm, respectively, where red males ranged in length from 70-107 cm and white males ranged from 71-110 cm. Semen samples were collected from males by first drying the fish and then applying gentle pressure to the abdomen. Semen was collected into a plastic bag, then sealed and kept at approximately 4°C. In the fall of 2014, females were also sampled for eggs, ovarian fluid, colour score and a fin clip. Females were euthanized then wiped dry to removed excess water, and gametes were collected by applying pressure to the abdomen to remove both eggs and ovarian fluid. Eggs and ovarian fluid were kept covered in plastic containers at approximately 10°C until fertilizations were performed (< 6 hours). For both males and females, colour was determined visually such that individuals were categorized as “red” (pigmented) or “white” (non-pigmented) based on external spawning colouration (Withler 1986; Lehnert et al. 2016). Individuals were assigned as red when they exhibited external red skin pigmentation and individuals were assigned as white when they showed no external red pigmentation and were gray in colour. Colour assignment of females was also confirmed by egg colour, and there were no cases where external colour did not correspond to egg colour. Additionally, in 2014, spectral readings of external spawning colour were taken from fish post-mortem using a Jaz Spectrophotometer (Ocean Optics), where readings were taken in triplicate at three positions along the lateral body of the fish.
(see Lehnert et al. 2016 for details). Given that some fish had been deceased for several hours, we only analyzed individuals that showed no evidence of discolouration due to their mortality. Using the pavo package (Maia et al. 2013) in R software (R Core Team 2016), we calculated chroma (red saturation; S1.red colour variable in pavo) (see Montgomerie 2006) for each male. Red and white males (n = 9 red and 10 white) differed significantly in chroma (t = 5.39; df = 17; p < 0.001), where mean (± standard error) chroma was 0.286 (± 0.007) and 0.247 (± 0.003) for red and white males, respectively.

**Sperm velocity in red and white Chinook salmon**

Sperm velocity was chosen as a measure of sperm quality as sperm velocity is the primary predictor of competitive fertilization success in salmonids (Lahnsteiner et al. 1998; Gage et al. 2004; Liljedal et al. 2008), including Chinook salmon (Flannery 2011). In the fall of 2013 and 2014, sperm velocity was assessed upon activation in fresh water (river water in 2013 and hatchery water (well water) in 2014) using video recordings (see Lehnert et al. 2012) of sperm recorded under a negative phase-contrast microscope (CX41 Olympus) with 10x magnification and a CCD B/W video camera (at 50 Hz vertical frequency). Using HTM-CEROS sperm analysis system (CEROS version 12, Hamilton Thorne Research, Beverly, MA, USA), the following parameters were used to assess sperm velocity: number of frames = 60, minimum contrast = 11 and minimum cell size = 3 pixels. Sperm velocity estimates were represented by the mean velocity of all individual motile sperm cells in the video. Sperm velocity was measured as average path velocity (VAP), which describes a smoothed path of the sperm cell’s trajectory (Rurangwa et al. 2004). We chose VAP as our measure of sperm velocity because it is often used in other Chinook salmon studies (Rosengrave et al. 2008; Lehnert et al. 2012;
Evans et al. 2013; Rosengrave et al. 2016) as well as studies on other salmonids (Lahnsteiner et al. 1998). Additionally, two other measures of sperm velocity, curvilinear velocity (VCL, defined as the average velocity along the actual path of the sperm cell’s trajectory) and straight line velocity (VSL, defined as the average velocity along a straight line connecting the start and end points of the sperm cell’s path) were highly correlated with VAP when we examined their relationship for videos of sperm velocity measures in water (Pearson correlation, p values < 0.001; n = 95 videos) thus we present only VAP in our results. In 2013, two video recordings were taken for each male, and in 2014, two video recordings were taken if time permitted where 60% of data points were from replicated videos (replication addressed in statistical analyses). Sperm velocity was evaluated at 5 seconds post-activation in fresh water, as a previous study found that the majority (80%) of fertilization occurs within 5 seconds of sperm and egg association in salmon (Hoysak and Liley 2001). Sperm velocity was measured for different males over multiple days during the spawning season in both sampling years. In 2013, sperm velocity was recorded on 8 dates between September 18 and 30, and different males were sampled on each of these dates (n = 28 males in total). In 2014, sperm velocity was recorded on three dates between September 18 and October 2, where the same males were sampled on multiple dates if possible (n = 20 males in total). In this case, one male was sampled on September 18, a total of 15 males were sampled on September 27, and 12 males were sampled on October 2. In total, 20 different males were sampled in 2014, as 8 of the same males were sampled on both September 27 and October 2.
Cryptic female choice in red and white Chinook salmon

Sperm velocity in ovarian fluid

In the fall of 2014, sperm velocity (VAP) was assessed in both fresh water (described above) and in diluted ovarian fluid of eight females (4 red and 4 white) on two sampling dates: September 27 and October 2. Ovarian fluid was collected from females through lethal sampling (as described above), where four different females were sampled on each date. If possible, sperm from all males was collected on both dates; however, in some cases, males could not be sampled on both dates due to differences in ripening (i.e., reproductive status). Ovarian fluid was diluted to 20% in hatchery water, and used to activate sperm under the microscope. Although the concentration of ovarian fluid in wild spawning events is unknown, ovarian fluid represents < 30% of the combined mass of eggs and fluid in salmonids (Lahnsteiner et al. 1995), thus it is likely that the concentration of ovarian fluid in nature would be low. Therefore, we chose a dilution of 20%, which has been used in a previous study in salmonids (Butts et al. 2012). Sperm velocity in ovarian fluid was measured using the same protocol and same males (from 2014) as described above. A total of 19 males were used, where each male was activated in the ovarian fluid from a minimum of 4 females. In total, our analysis involved 104 male x female ovarian fluid combinations, where sperm from 10 to 15 males was activated in the ovarian fluid of each female (n = 8 females). Again, we chose to only present VAP as a sperm velocity metric, as VAP was highly correlated with VCL and VSL (Pearson correlation, p values < 0.001) when using all 104 data points (male x female combinations) for sperm velocity in ovarian fluid. However, given that Yeates et al. (2013) found that ovarian fluid can have a strong effect on the straightness of sperm
trajectory, we have included analyses for VSL and sperm path straightness (STR, calculated from VSL/VAP) in Appendix 5.

**Competitive fertilizations**

In addition to sperm velocity in ovarian fluid, we also examined CFC through competitive fertilization success of males under *in vitro* sperm competition in 2014. Eight males were used to create four male pairs where one male was red and one male was white within each pair. The male pairs included 8 of the same males that were tested for sperm velocity in water and ovarian fluid as described above. Although a greater number of males would be ideal, given that fish were captured from a wild population during a low escapement year (28% lower than in 2013; R. Bailey DFO Stock Assessment, personal communication), logistical and biological constraints (i.e., low population density, equal colour and sex ratios, maturation stage and holding space) reduced our ability to incorporate more individuals within the short time frame necessary to have all gametes for testing available simultaneously (i.e., within a 4 day period). Each male pair competed to fertilize the eggs of eight females (four red and four white females), resulting in a total of 32 competitions (4 male pairs x 8 females). Eggs were separated from ovarian fluid using a sieve and each female’s eggs were divided into batches for fertilization. The number of eggs per batch depended on the number and size of a female’s eggs, where the mean (± standard error) number of eggs per batch was 135.1 (± 5.98) eggs. After the eggs were separated, ovarian fluid was measured then divided and poured onto egg batches. Given that different females had different volumes of ovarian fluid, different volumes of water were added to activate sperm and eggs (ranging from 20 - 100 mL) to ensure that
ovarian fluid represented 20% of the total volume added for each competitive fertilization.

All fertilizations trials were performed using 50 uL of semen from each male within the male pair. We measured sperm density in all males during competitive fertilizations using the same protocol described in Lehnert et al. (2012). Based on sperm density estimates, the volume of semen used in the fertilization should be high enough to ensure fertilization as the sperm:egg ratio for all crosses ranged for 16,526 to 54,311 sperm cells per egg. Although sperm densities were not controlled for in competition, we consider sperm density effects in our analysis of competitive fertilization below. During fertilizations, sperm from paired males were pipetted simultaneously with hatchery water onto the eggs and ovarian fluid. Eggs, sperm, ovarian fluid and water were mixed, and left undisturbed for two minutes. Water was poured off the eggs, and eggs were transferred to vertical incubation trays. Eggs were left to incubate at 10°C until the eyed-egg stage (250-500 accumulated thermal units), and on November 5 and 6, 2014, all eyed-eggs were counted and preserved in high salt preservative buffer (3.5 M ammonium sulfate; 15 mM EDTA; 15 mM sodium citrate; pH 5.2) for DNA-based paternity analyses. Percent survival to the eyed-egg stage was calculated as the number of live eyed-eggs divided by the total number of eggs (dead and live). However, our survival estimates may be underestimates of actual survival because it is possible that some dead eggs were not fertilized, as we could not discriminate between unfertilized and fertilized dead eggs.
Paternity analysis

DNA was extracted from parental fin clips and eyed-egg samples using a plate-based extraction method (Elphinstone et al. 2003). DNA was extracted from a total of 21 to 24 eggs per competition experiment, with the exception of one of the total 32 competitions where only four eggs survived; we thus excluded that competition from the analyses. Three microsatellite loci were genotyped to accurately differentiate between paired males for paternity assignment. In total, six microsatellite loci were chosen: OtsG68, OtsG78b, OtsG432 (Williamson et al. 2002), Ots211 (Greig et al. 2003), Omy325 (O’Connell et al. 1997) and Ots107 (Nelson and Beacham 1999). PCR conditions included: a 5 minute denaturation step (94°C), followed by 35-38 cycles of a 20 second denaturation step (94°C), a 20 second annealing step (52.5°C – OtsG68, OtsG78b; 54°C – Omy325; 56°C – OtsG432; 58°C – Ots107; 60°C – Ots211) and a 30 second extension step (72°C), followed by a final extension of 3 minutes. All forward primers were a fluorescently dye-labeled and PCR products were visualized using a LiCor 4300 DNA analyzer (LiCor Biosciences, Inc.). Fragment sizes (alleles) were scored using GENE IMAGIR 4.05 software (Scanalytics Inc.). Allele scores were used to determine paternity by exclusion of one male and positive inclusion of the other male at genotyped loci.

Statistical analyses

Sperm velocity in fresh water

All statistical analyses were performed in R software version 3.3.1 (R Core Team 2016) unless otherwise stated. Sperm velocity in water data for 2013 and 2014 were combined and red and white males (n = 48 males) were compared using a linear mixed
effects model in the *lme4* package (Bates et al. 2009). Linear models were compared using log-likelihood ratio test for random effects, whereas Kenward-Roger approximation test was used to examine the effect of the fixed factor. The model included the fixed factor of male colour, with random factors of year, sampling date and male ID. Male ID was included as a random factor because sperm velocity in water was recorded for two videos per male (if possible). Additionally, some of the same males were sampled over two of the sampling dates in 2014 (see above for description), where a total of 8 males were sampled on both dates and 12 males were sampled on only one date (n = 20 different males in total). Therefore, all replicate videos were used as data points in the analysis. In 2013, sperm velocity measurements were replicated (i.e., two videos) for each male (n = 28); however not all males were replicated in 2014. In 2014, videos were recorded on three sampling dates (see above), and the percentage of males (with total males sampled) that were replicated on each date was 100% (n = 1 total), 87% (n = 15 total) and 25% (n = 12 total) on September 18, 27 and October 2, respectively. We assessed repeatability of video replicates for sperm velocity using Pearson’s correlation. Using 45 videos and their replicates, we found that replicates were highly correlated (r = 0.81; p < 0.001), therefore we do not expect the lack of replication for some individuals to influence the overall results of our study. Assumptions of linear mixed models were assessed using diagnostic checks. Model residuals were assessed for normality using Shapiro-Wilk test and by examining Q-Q plots. Additionally, residual homogeneity was evaluated by plotting model residuals and fitted values. Assumption of linear mixed effect models were met, as model residuals met assumption of normality (Shapiro-Wilk test, W = 0.99, p-value = 0.95) and homogeneity.
**Sperm velocity in ovarian fluid**

Analyses were conducted using linear mixed effect models with male colour, female colour and their interaction as fixed factors and sampling date, male ID and female ID as random factors. When replicate videos were recorded for a male (within a female’s ovarian fluid), velocities were averaged over replicate videos. Our analysis therefore involved a total of 104 male x female combinations (data points). Diagnostic checks for linear mixed model revealed that model residuals met assumption of normality (Shapiro-Wilk test, W = 0.99, p-value = 0.45) and homogeneity. In addition to average path sperm velocity (VAP), we conducted the same analyses for straight line velocity (VSL) and sperm path straightness (STR) and these results are provided in Appendix 5.

**Competitive fertilization success**

After paternity of offspring from competitive fertilizations was determined for 31 crosses, offspring from each cross were coded as 1 if sired by a red male and 0 if sired by a white male (n = 727 offspring). Using generalized linear mixed effects model (GLMM) with binomial distribution and logit link function, we used female colour, male pair and their interaction as fixed factors with sampling date and female ID as random factors. Using the GLMM, we could determine the main effect of female colour, male pair and their interaction on fertilization success, while controlling for confounding effects of date and female ID. The main effect that we were interested in was the interaction of female colour and male pair, as a significant interaction effect would indicate colour-based cryptic female choice where red and white male success within the pair differed by female colour.
Given that the results of competitive fertilization can be influenced by sperm velocity differences between males (Gage et al. 2004), we also calculated the difference in sperm velocity between paired males in both water and ovarian fluid. Therefore, within a male pair, velocity of the white male was subtracted from the velocity of the red male. Additionally, we considered that sperm density could also be important for fertilization success, therefore we calculated the ratio of red:white sperm cells within each male pair during fertilizations. We used three GLMMs that included the fixed effects of male sperm differences (differential sperm velocity measures or density ratio) and female colour in the model with the random effect of sampling date, male pair, female ID and the interaction of male pair and female ID. We thus used three different GLMMs, where each model included a different measure (fixed effect) of male sperm differences. Therefore, one model included differential sperm velocity in ovarian fluid, the next model included differential sperm velocity in water and the final model included sperm density ratio within the male pair. In this way, we could determine whether sperm differences between paired males significantly contributed to fertilization success. Next, if sperm differences (velocity or density) were a significant predictor of success in the model, we partitioned the variance in success attributed to random effects in the model that were associated with both post-spawning processes (CFC and male competitiveness). In our model, the variance associated with the interaction of male pair x female ID represents the variation due to CFC at the individual level. The variance associated with male competition is represented by the variation caused by male pair. In some cases, sperm velocity was not recorded for all males due to unusable videos when flow affected velocity measures; nevertheless, the models for sperm velocity in ovarian fluid and water included 586 and
634 data points, respectively. For all GLMMs in our study, we used log-likelihood ratio tests to examine the effects of both fixed and random factors in the models. Additionally, all GLMMs were examined for overdispersion. Overdispersion was assessed by dividing residual deviation (rdev) by residual degrees of freedom (rdf) for the model, and if the ratio value was less than 1 then we concluded that the model was not overdispersed. None of the full GLMMs were overdispersed (rdev/rdf < 0.85).

Results

Sperm velocity in red and white Chinook salmon

In 2013, we excluded data collected on two early sampling dates (September 18 and 19; n = 2 red males and 1 white male), as all males on these dates had low sperm velocity (all replicate videos ≤ 65 um/sec) and were likely not in full reproductive condition and thus not biologically relevant. Our analysis therefore involved a total of 45 males over the two years (n = 25 in 2013 and n = 20 in 2014). Linear mixed models revealed that red males had marginally faster sperm velocity in water relative to white males (see Table 6.1 and Figure 6.1; F = 4.06, p = 0.0506; n = 45 males). Sperm velocity did not differ significantly between years (Table 6.1; χ² = 0.47; p = 0.49), however random factors of male ID and date significantly contributed to the variance observed for sperm velocity (see Table 6.1; male ID: χ² = 21.6; p < 0.001; date: χ² = 13.4; p = 0.0003).

Cryptic female choice (CFC) in red and white Chinook salmon

Sperm velocity in ovarian fluid

Date did not contribute to the variance observed for sperm velocity (VAP) in ovarian fluid (p = 0.99), therefore it was excluded from the model to avoid overparameterization. For sperm velocity in ovarian fluid, we found a significant interaction
of male colour and female colour (see Table 6.1 and Figure 6.2; $F = 13.99; p = 0.0003; n = 104$ male x female combinations). Changes in mean sperm velocity for individual males when activated in the ovarian fluid of red versus white females are presented in Appendix 7. Sperm velocity for red males was higher when activated in the ovarian fluid of red females and sperm velocity of white males was higher when activated in the ovarian fluid of white females (Figure 6.2). Male ID (random factor) contributed significantly to the variance observed for sperm velocity (Table 6.1; $\chi^2 = 30.23; p < 0.001$). Female ID (random factor) was also significant in the model (Table 6.1; $\chi^2 = 4.18; p = 0.04$). The interaction detected between male colour and female colour (i.e., colour-based CFC) was also significant when assessing straight line sperm velocity ($VSL; F = 9.00; p = 0.004; n = 104$) in ovarian fluid but not for sperm path straightness ($STR; F = 1.97; p = 0.16; n = 104$) (see Appendix 5 for full results).

**Competitive fertilization success**

Mean (± standard error) eyed-egg survival from all 31 competitive fertilizations was 60.2% (± 4.35%) where survival ranged from 14.2 to 94.7%. Our estimates of survival may be underestimated, as we did not discriminate between dead eggs that were fertilized and those that were unfertilized. Nevertheless, the mean survival found in our study is similar to other studies on Chinook salmon, as Barnes et al. (2003) found mean survival to the eyed stage was 41-59% over different years and Pitcher and Neff (2006) found mean (± S.E.) egg survival to hatch was 71 ± 19% (range: 13-99%). All paternity calculations were based on 21 to 24 eggs per competition that were genotyped and assigned to one male within the male pair (all genotyped eggs were successfully assigned to one male parent) (see Appendix 6). We used a GLMM to assess paternity success as
our response variable was binary where eggs sired by red males were coded as 1 and eggs sired by white males were coded as 0. Using GLMM, we found a significant interaction between female colour and male pair (see Figure 6.3 and Table 6.2; \( \chi^2 = 26.0; p < 0.001; n = 726 \) eggs), indicating colour-based cryptic female choice on male competitive fertilization success, where the paternity success of red and white males within a pair differed between female colours. We found that red and white males sired a similar number of offspring when competing for fertilization in the eggs of red females (n = 172 red sired; 182 white sired), however white males had significantly higher paternity compared to red males when competing for fertilization of eggs from white females (n = 123 red sired; n = 250 white sired), thus suggesting that the strength of colour-based CFC is greater in white females compared to red females. Although we found no significant effect of female colour on fertilization success overall (\( \chi^2 = 1.01; p = 0.32 \)), we did find a significant effect of male pair (\( \chi^2 = 264.5; p < 0.001 \)) (see Table 6.2 for full results). Finally, we also found a significant random effect of female ID (\( \chi^2 = 25.3; p < 0.001 \)), but no effect of sampling date (\( \chi^2 = 1.42; p = 0.23 \)) (Table 6.2).

Next using GLMMs, we partitioned the effects of post-copulatory processes (i.e., male competitiveness and individual level CFC) on competitive fertilization success while accounting for sperm differences between paired males (sperm velocity and density). To account for sperm differences between paired males on fertilization success, we used three GLMMs, where each included a different fixed factor term representing sperm differences. The three measures of sperm differences used as fixed effects in the models included: 1) difference in sperm velocity in water, 2) difference in sperm velocity in ovarian fluid and 3) ratio of red:white sperm cells during competitive fertilization. If
the measure of sperm difference between males was a significant predictor of competitive fertilization success, we then extracted the variance associated with the random effects of male pair (male competitiveness) and the interaction of male pair x female ID (individual level CFC) in the model to assess the contribution of each post-copulatory process to fertilization success. We found that the difference in sperm velocity in ovarian fluid within a male pair was not significant for predicting fertilization success (Appendix 8; $\chi^2 < 0.001; p = 0.99; n = 586$ eggs). The same was true for sperm density, as the ratio of red:white sperm cells during fertilization did not predict fertilization success (Appendix 8; $\chi^2 = 0.08, p = 0.78, n = 726$ eggs). However, difference in sperm velocity in water was a significant predictor of fertilization success (Table 6.2; $\chi^2 = 8.28; p = 0.004; n = 634$ eggs). Date was removed from this analysis to avoid over parameterization as little variation in was explained by date in the model ($p = 0.99$). When we accounted for the effect of sperm velocity differences in water between paired males on fertilization success, we found that individual level CFC (male pair x female ID) was responsible for 43% of the total variance observed in fertilization success, whereas male competitiveness (male pair) accounted for 16% of the variance.

Discussion

In our study, we examine whether post-copulatory sexual selection processes, specifically sperm competition and cryptic female choice (CFC), play an important role in the maintenance of a colour polymorphism in Chinook salmon. Differences in carotenoid utilization are expected to influence sperm performance as suggested by studies in birds (Peters et al. 2004; Helfenstein et al. 2010) and fishes (Pike et al. 2009; Tizkar et al. 2015; but see Sullivan et al. 2014). For example, carotenoid levels have been positively
associated with sperm velocity in mallard ducks (Anas platyrhynchos; Peters et al. 2004) as well as fertilization success in sticklebacks (Gasterosteus aculeatus; Pike et al. 2009). Indeed, our experiment demonstrated that red Chinook salmon males had higher sperm velocity in water relative to white males, however we acknowledge that the difference only approached marginal significance (p = 0.0506). Carotenoids can act as antioxidants, and thus may protect metabolically active sperm cells against oxidative damage (Blount et al. 2001; Costantini et al. 2010). White Chinook salmon consume carotenoids and are capable of circulating the pigments; however, unlike red individuals, white Chinook salmon have a reduced ability to store carotenoids in their tissues (see Ando et al. 1994). Chinook salmon in the Quesnel River migrate more than 800 kilometers to spawning grounds and normally carotenoids are mobilized from muscle tissue into the bloodstream to preserve vital functions during migration (Rajasingh et al. 2007). Consequently, white Chinook salmon could be at greater risk of oxidative damage given their lack of carotenoid stores when undertaking migration. Assuming oxidative stress reduces sperm performance (reviewed in Velando et al. 2008; Costatini et al. 2010), it is plausible that the small difference observed in sperm velocity in our study is attributed to differences in carotenoid availability during migration. However, these conclusions are speculative, as we did not assay differences in antioxidant capacity or carotenoid content in semen in our study and other factors could contribute to differences between morphs such as genetic differences linked to the polymorphism. The degree to which red and white males are in sperm competition is unknown; however, under experimental conditions, 33% of Chinook salmon females (n = 3/9) spawned with both red and white males (Lehnert et al. 2016). Given that both morphs spawn at the same time and in the same area in the Quesnel
River, we would expect sperm competition to occur between morphs. Therefore, the lack of strong differences in sperm velocity between red and white males in water may suggest that both morphs can gain similar success under sperm competition, thus leading to the stable polymorphism seen in the natural population.

In addition to sperm velocity, cryptic female choice (CFC) can influence fertilization as demonstrated in a wide range of taxa including insects, fishes, birds and mammals (Eberhard 1996; Evans et al. 2003; Bussière et al. 2006; Yeates et al. 2009; Løvlie et al. 2013). In colour polymorphic systems, colour-based CFC has been demonstrated (Pryke et al. 2010). For example, in the Gouldian finch (*Erythrura gouldiae*), post-zygotic genetic incompatibilities have been detected when mating occurs between different head colour morphs (Pryke and Griffith 2009), and consequently, post-copulatory processes have evolved to skew paternity in favour of more genetically compatible mates (Pryke et al. 2010). In salmon, ovarian fluid has been shown to be a potential mechanism for cryptic female choice (Urbach et al. 2005; Rosengrave et al. 2008; Butts et al. 2012; Yeates et al. 2013; Rosengrave et al. 2016). In our study, we demonstrate that ovarian fluid affects sperm velocity based on the interaction of male colour and female colour, where red males had faster sperm in the ovarian fluid of red females relative to white females, and *vice versa* for white males. Therefore, our results show that ovarian fluid may be a mechanism for CFC in Chinook salmon and it operates based on male and female colour. However, higher sperm velocity in ovarian fluid was not predictive of higher competitive fertilization success (performed in 20% ovarian fluid). The results of our study indicate that although ovarian fluid (at least at concentrations of 20%) may level the playing field for red and white males overall, sperm
velocity changes via ovarian fluid may have limited influence on competitive fertilization success in Chinook salmon. Similar results have been demonstrated by Evans et al. (2013) in Chinook salmon, where they reported that sperm performance in ovarian fluid (10% dilution) differed from *in vitro* competitive fertilization success (but see Rosengrave et al. 2016). Sperm velocity differences in ovarian fluid mediated by female colour may relate to protein differences in ovarian fluid and semen between colour morphs, as protein composition of ovarian fluid is thought to be the primary mechanism for male-female interaction effects on sperm performance (Johnson et al. 2014).

Although sperm velocity in ovarian fluid has been demonstrated to be an important predictor of fertilization success in Chinook salmon (Rosengrave et al. 2016), in our study, we found that fertilization success was not correlated to sperm velocity in ovarian fluid. Nevertheless, under *in vitro* fertilization trials, we still found evidence of CFC based on male and female colouration (i.e., colour-based CFC). In this case, paternity of red and white males was similar for competitive fertilization in the eggs of red females; however, white males sired significantly more white female eggs compared to red males when in competition. Our results suggest that the strength of colour-based CFC may be greater for white females than red females. Previously, using semi-natural spawning channels, Lehnert et al. (2016) found that red females showed a stronger preference for red males compared to white males (i.e., colour assortative mating), whereas white females showed little preference for male colour. The study found that the percentage of offspring produced by colour assortative mating by red females was 75.9% whereas the percentage was only 55.3% for white females (Lehnert et al. 2016). Interestingly, under *in vitro* competitive fertilization, we found the opposite, where red
males sired 48.5% of red female offspring and white males sired 67.0% of white female offspring. Thus it is plausible that red females employ pre-spawning processes to bias offspring paternity in favour of red males; whereas, white females may use post-spawning processes to bias paternity in favour of white males. Differences between females in pre- and post-spawning decisions may in part provide a mechanism promoting the maintenance of the two morphs in nature.

In addition to colour-based CFC, results for competitive fertilization success were partly driven by differences between males in sperm velocity in water (but not in ovarian fluid), as sperm velocity in water was a significant predictor of fertilization success, thus suggesting that the effects of CFC via ovarian fluid may be lost due to sexual conflict, where sperm competitiveness counteracts female choice mediated by the ovarian fluid. Additionally, the significant relationship between sperm velocity in water and fertilization success found in our study is comparable to previous studies in salmonids: Chinook salmon (Flannery 2011), Atlantic salmon (Gage et al. 2004) and Arctic charr (*Salvelinus alpinus*; Liljedal et al. 2008). Given that sperm velocity is an important driver of fertilization success, we would have expected red males to sire more eggs under competition rather than white males given their marginally higher sperm velocity in water. However, other mechanisms are likely operating to influence fertilization success (i.e., egg-sperm interactions), as mechanisms of CFC (colour-based and individual-based) not mediated by ovarian fluid appear to also be driving fertilization success in our study.

When we accounted for differences in sperm velocity (in water) between paired males, individual level CFC (male pair x female ID) and male competitiveness (male pair) were attributed to 43% and 16%, respectively, of the total variance in *in vitro* competitive
fertilization success. While our study does not identify the specific mechanism for the identified individual level CFC, evidence of CFC in our study could be facilitated via egg-sperm recognition, which has been demonstrated in Atlantic salmon (Yeates et al. 2009). In teleosts, the egg has a single opening (micropyle) through which sperm must pass to achieve fertilization (Gilkey 1981). Little is known about a potential mechanism by which an egg could exert selection on sperm in salmon, but post-copulatory processes may continue to operate after entry of sperm into the egg (Yeates et al. 2009). One possibility is that egg-sperm level CFC may be driven by differences at major histocompatibility (MHC) genes (documented in Atlantic salmon (Yeates et al. 2009)), as red and white Chinook salmon differ significantly at MHC genes (Lehnert et al. 2016).

In conclusion, we found only marginal differences in sperm velocity in water between red and white males, which may in part be important for allowing both red and white males to gain success under sperm competition events. Additionally, we found that sperm velocity was influenced by ovarian fluid on the basis of colour suggesting a potential mechanism for CFC that could also contribute to the maintenance of the polymorphism. However, under in vitro sperm competition, we found that sperm velocity in ovarian fluid did not explain fertilization success, yet we still found evidence of colour-assortative CFC on competitive fertilization success. Sperm velocity in water was a significant predictor of competitive success, potentially indicating evidence of sexual conflict, where sperm competitiveness can reduce effectiveness of ovarian fluid mediated CFC. Nonetheless, CFC and male competitiveness explained variation in fertilization success even when accounting for differences in sperm velocity between paired males, where individual level CFC (at the male x female level) explained a greater proportion of
the variance (2.7 X more) relative to male competitiveness. Our results suggest that egg-sperm interactions may be important for determining post-spawning success, which could provide females with an advantage in the evolutionary arms race between sexes. Post-copulatory processes can have important evolutionary consequences and our results from sperm performance in ovarian fluid and competitive fertilization success suggest that, despite the marginally lower sperm velocity of white males, mechanisms of CFC can help white males gain similar success to red males overall. We can conclude that post-spawning sexual selection contributes in part to the maintenance of the colour polymorphism in the Quesnel River population.

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| Table 6.1. Results of linear mixed effect models for sperm velocity of red and white Chinook salmon (Oncorhynchus tshawytscha) males when activated in water and in ovarian fluid of red and white Chinook salmon females. Fixed effects are presented with estimate parameters including 95% confidence intervals (CI) and statistical results of Kenward-Roger approximation test. Random effects are presented with variance components with standard deviations and percentage of the total variance (% var) as well as statistical results of log-likelihood ratio tests. Significant effects are indicated by bold font and an asterisk. |
|-------------------------------------------------|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
Table 6.2. Results of generalized linear mixed effect models testing postcopulatory processes affecting fertilization success (paternity) in red and white Chinook salmon (Oncorhynchus tshawytscha) males under sperm competition in the eggs of red and white females. Fixed effects are presented with estimate parameters including 95% confidence intervals (CI). Random effects are presented with variance components with standard deviations and percentage of the total variance (% var). Significance of factors in models was determined by log-likelihood tests and significant effects are indicated by bold font and an asterisk.

<table>
<thead>
<tr>
<th>Model to test for</th>
<th>Fixed effects</th>
<th>Estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>male sperm velocity (VAP)</td>
<td>Intercept</td>
<td>-1.46</td>
<td>-3.24, 0.33</td>
</tr>
<tr>
<td></td>
<td>Female colo</td>
<td>1.08</td>
<td>-0.56, 2.66</td>
</tr>
<tr>
<td></td>
<td>Male pair</td>
<td>264.5</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td></td>
<td>Female colo x Male pair</td>
<td>26.03</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
<th>% var</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female ID</td>
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<td>0.71</td>
<td>11.7</td>
</tr>
<tr>
<td>Date</td>
<td>0.56</td>
<td>0.75</td>
<td>12.8</td>
</tr>
<tr>
<td>Error</td>
<td>3.29</td>
<td>1.81</td>
<td>75.5</td>
</tr>
</tbody>
</table>

Model to partition effects of postcopulatory processes on fertilization success while accounting for sperm velocity (VAP) differences between paired males in water.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Estimate</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>difference sperm velocity in water</td>
<td>0.07</td>
<td>0.03, 0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random effects</th>
<th>Variance</th>
<th>SD</th>
<th>% var</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female ID x Male ID</td>
<td>3.42</td>
<td>1.81</td>
<td>42.8</td>
</tr>
<tr>
<td>Male pair</td>
<td>1.28</td>
<td>1.13</td>
<td>16</td>
</tr>
<tr>
<td>Female ID</td>
<td>1.0</td>
<td>0.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Model 1: least for fertilization success based on sperm-based CFC on female gonads.
Figure 6.1. Box plot of sperm velocity (average path velocity, VAP) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in water. Males (n = 45) were sampled from the Quesnel River, British Columbia during two spawning seasons in 2013 (n = 13 red; 12 white) and 2014 (n = 10 red; 10 white). The difference between red and white males approached marginal significance (p = 0.0506) based on linear mixed models (see Methods and Table 6.1 for details).
Figure 6.2. Mean (± standard error) sperm velocity (average path velocity, VAP) of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in ovarian fluid (20% dilution) from red and white Chinook salmon females. Data represent sperm velocities from 104 male x female ovarian fluid combinations (n = 10 to 15 males x 8 females). Significance (p-values) for fixed factors (male colour, female colour and their interaction) in linear mixed effect models are presented in the right hand corner and full results are presented in Table 6.1.
Figure 6.3. Mean (± standard error) paternity (percentage of eyed-eggs) of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when in sperm competition to fertilize the eggs of red and white Chinook salmon females. Results are based on paternity of 727 genotyped eggs that were competitively fertilized in the presence of 20% ovarian fluid. Significance (p-values) for fixed factors (male pair, female colour and their interaction) in generalized linear mixed effect models are presented in the right hand corner with full results presented in Table 6.2.
CHAPTER 7: GENERAL DISCUSSION

Many animals have evolved to exploit carotenoid pigments from their environment and utilize them for different biological functions within the body, ranging from enhancing immune function and antioxidant capacity to sequestering the pigments into their integument to act as honest signals of quality during sexual selection (Svenssson and Wong 2011). It has been several decades since interest in carotenoid research began, and diverse and conflicting results have been found on the importance of carotenoids in sexual and natural selection processes (Olson and Owens 1998; Costantini and Møller 2008; Lorenzo 2009; Hill 2011; Svensson and Wong 2011). Nonetheless, today, evolutionary biologists are still invested in understanding the proximate and ultimate mechanisms of carotenoid colouration, and just recently genes associated with carotenoid colouration in birds have been identified (Lopes et al. 2016; Mundy et al. 2016).

Although it is widely accepted that carotenoids are important for animal fitness (Svensson and Wong 2011), variation in carotenoid utilization exists within and among species, thus highlighting our need for a better understanding of the mechanisms favouring carotenoid allocation and animal colouration.

Why are salmon red?

While much carotenoid research has focused on birds, fishes also represent an important group where carotenoid utilization is wide ranging: some fish not only provision carotenoids into eggs and skin, but others such as salmonids deposit carotenoids into their flesh (Rajasingh et al. 2007). Studies on carotenoid pigmentation in salmon have been helpful to our comprehension of the consequences of maternal carotenoids in eggs and the influence of dietary carotenoids on individual performance (Christiansen et al. 1995a; Christiansen et al. 1995b; Christiansen and Torrissen 1997; Amar et al. 2004;
Tyndale et al. 2008; Bazyar Lakeh et al. 2010). Additionally, studies have examined the potential role of red carotenoid colouration during mate choice in salmon (Fleming and Gross 1994; Craig and Foote 2001). Although the evolution of carotenoid pigmentation in salmonids has remained a mystery, perspectives on this topic have been discussed (Rajasingh et al. 2007). Rajasingh et al. (2007) proposed a framework describing the evolution of the carotenoid flesh pigmentation phenotype in salmonids, where they suggest that prior to the occurrence of the Salmonidae lineage, fish already had the required machinery for carotenoid uptake and metabolism. Rajasingh et al. (2007) speculated that the anadromous life history and nest making behaviour exhibited by salmonids evolved prior to the trait for carotenoid deposition. However, the advent of the carotenoid deposition trait in a common ancestor of Salmonidae allowed these fish to exploit carotenoid pigments from degrading tissue during reproduction thus resulting in positive selection for the trait, as carotenoids allowed these individuals to maintain vital functions during this arduous life stage (Rajasingh et al. 2007). Selection for carotenoids in eggs and skin likely evolved secondarily as a consequence of the increased carotenoids in the bloodstream during spawning due to their accumulation in the flesh (Rajasingh et al. 2007). However, as Rajasingh et al. (2007) indicate, the occurrence of white Chinook salmon (Oncorhynchus tshawytscha) living in a high carotenoid environment that have a limited ability to deposit carotenoids into their tissues presents a problem to this evolutionary framework.

White Chinook salmon consume, absorb and metabolize carotenoids, however due to genetic polymorphisms affecting carotenoid deposition, white Chinook salmon have limited ability to sequester these pigments into their skin, eggs and flesh (Withler 1986; Ando et al. 1994). It is thus expected that white Chinook salmon should be at a
disadvantage, however white morph Chinook salmon can co-exist at stable frequencies with red morph salmon within rivers (Withler 1986), or the white morph can completely dominate some river systems. Chinook salmon thus present a unique model system for the study of carotenoids in salmonids. My thesis therefore aimed to resolve the mystery of why salmon evolved to be red by examining the proximate (genetic) mechanisms resulting in the carotenoid phenotype, but also examining why the red phenotype (and also the white phenotype) has been positively selected for in nature by comparing fitness differences (ultimate mechanisms) between red and white Chinook salmon throughout their life. The synthesis of my thesis thus has allowed me to identify these mechanisms, and answer the question: why are salmon red?

The first question that I aimed to answer with my thesis was: are salmon red because of their genes? And the answer is yes, as I identified several loci that are significantly associated with carotenoid pigmentation in Chinook salmon (Chapter 2). Many of these loci mapped to positions in the Atlantic salmon (Salmo salar) genome that harbour candidate genes associated with carotenoid absorption, metabolism and deposition in other animals. Therefore, the results of my thesis indicate that salmon are red because of several genes involved in the carotenoid pigmentation process. Future work through genome and RNA sequencing will further confirm which of those genes are most important for the differences observed between red and white Chinook salmon. The identification of these genes will be valuable for creating genetic markers that can be used for selection programs in salmon aquaculture, but also these markers could be used to determine the proportion of red and white individuals in different populations to better characterize the distribution of the white phenotype in nature.
The next question that I aimed to answer with my thesis was: are salmon red because the maternal provisioning of high levels of carotenoids are important for early life fitness? The answer to this question appears to be no. During early life, I show that selection can actually act against carotenoid pigmentation in eggs, as increased carotenoids in the eggs can increase predation risk (Chapter 3). Furthermore, in terms of egg and offspring fitness, higher carotenoid content of eggs had no effect on survival or growth, as well as the relative expression of genes involved in immune function, stress and oxidative stress (Chapter 4). Therefore during early life, it appears that there is no strong selection for increased egg carotenoid content, so perhaps salmon eggs do not need high levels of carotenoids. However, they may require some level of carotenoids (i.e., threshold effect) or they may just require antioxidants in any form either as carotenoids or other antioxidants such as vitamin C, vitamin E or retinoids. Alternatively, Chinook salmon may be an exception to the rule for carotenoids in salmon eggs. This is because Chinook salmon may be able to reduce carotenoids in their eggs relative to other salmonids because Chinook salmon have large eggs and they exploit high quality spawning habitat with high levels of dissolved oxygen (Healy 1991), thus relaxing selection on egg carotenoid content and allowing the white morph to persist without consequence during early life (Einum et al. 2002; Rajasingh et al. 2007).

If the maternal provisioning of high levels of carotenoids is not necessary for egg and offspring performance, then the next question that my thesis asked was: are salmon red because carotenoids increase mating success? The answer to this question also appears to be no. To address this question, I examined colour assortative mating in a system where red and white Chinook salmon co-exist. First, I determined that red spawning colouration does not appear to be necessary for reproductive success, as I found
that red and white Chinook salmon were interbreeding and morphs were not genetically divergent in the population (Chapter 5). Under experimental conditions, I found colour assortative mating by red females; whereas, white females did not mate assortatively based on male colour. Although it is widely suggested that red spawning colouration is important in salmon, few studies have directly demonstrated this. Craig and Foote’s (2001) study is a strong example of where red carotenoid colouration does appear to be important for mating success in salmon (sockeye and kokanee; *O. nerka*). Although, it should be noted that Craig and Foote (2001) showed male mate choice for red female colouration, and female preference was not examined. However, aside from their study, few other studies have demonstrated any evidence for female mate choice for red colouration in salmon (Fleming and Gross 1994). In fact, in Chinook salmon under semi-natural spawning conditions, male mating success was positively correlated to brighter integument and more blue/green colouration (Neff et al. 2008). Although various studies in other systems show the importance of red colouration to mating success, many of these studies are in resource based mating systems, where carotenoid colouration may signal resource availability and male parental quality (Hill 1991; Houde and Torio 1992; Grether 2000; Barber et al. 2001; Blount et al. 2003; Pike et al. 2007). I would argue that the salmon mating system is very different from those examples, and this assumption that red spawning colouration is important in salmon needs to be revisited.

The next question that I addressed with my thesis was: **are salmon red because carotenoids increase sperm performance and fertilization success?** The answer to the first part of this question is yes, carotenoids may increase sperm performance in salmon. In my study, sperm performance (velocity) was marginally higher (*p = 0.0506*) in red males relative to white males indicating that carotenoid storage ability may affect sperm
performance. The relationship between higher carotenoid colouration and increased sperm performance has not been strongly demonstrated in salmonids (but see Janhunen et al. 2009), and may explain why we only observed marginal differences in sperm velocity between morphs. Additionally, my thesis goes beyond just examining sperm performance differences, as I also determined male fertilization success under in vitro competitive fertilization trials where I accounted for male and female effects. Although sperm velocity was an important predictor of fertilization success, cryptic female choice was also important for determining success in our study. Approximately 42% of the variation in fertilization success was attributed to individual level cryptic female choice, potentially operating through sperm and egg interactions during fertilization. Thus, in this chapter, white males sired more eggs than red males under competition. Therefore, the answer to the second part of the question is no, carotenoids do not improve fertilization success in salmon. Although, it should be noted that the overall results of Chapter 5 and 6 do suggest that colour is important during spawning, however it is not because mate choice favours red colour but rather mate choice may operate through colour assortative mating. Red and white females show assortative mate choice through different strategies, where, in my thesis, red females utilized pre-spawning selection and white females used post-spawning selection to bias paternity of their eggs in favour of males of their own colour morph. It makes sense that red and white females do not exclusively mate with their own colour morph, as this would immediately eliminate 50% of the males in the Quesnel River as potential mates, which undoubtedly would be a bad strategy. Additionally, female mate choice for specific males colours could also be influenced by male-male competition, where under a male biased operational sex ratio, female choice may be limited (Petersson et al. 1999; Garner et al. 2010).
Overall, my thesis suggests that increased carotenoids provide no benefit to Chinook salmon in the Quesnel River. Although it is widely suggested that carotenoids provide animals with many benefits, my thesis highlights that carotenoid research needs to be re-evaluated where we separate the studies that examine the effects of dietary carotenoids on performance and re-evaluate the carotenoid paradigms based on the studies that examine natural variation in carotenoid utilization and how that variation influences fitness. It is likely that if I had performed carotenoid supplementation experiments, I would have found effects of carotenoids on individual performance. However, supplementation studies do not necessarily reflect the genetic abilities of the individual, but rather how current environment can influence the expression of the phenotype. If animals have evolved in a high carotenoid environment (which both red and white Chinook salmon have) then those carotenoids may play an important role in determining the animal’s fitness even if individuals make use of those carotenoids in different ways. In my study, red and white Chinook salmon show discrete variation in carotenoid utilization, where these two phenotypes have been shaped over evolutionary time. Therefore, my study is stronger and very different from dietary supplementation studies, and perhaps the revision of current carotenoid research could help to clarify some of the conflicting results. Additionally, carotenoids studies should be re-assessed in the context of resource and non-resource based mating systems, as mate choice for carotenoid colouration may also differ between these systems.

**Mechanisms that maintain the colour polymorphism in Chinook salmon**

My thesis addressed the question of why salmon are red in several ways, where I have provided some new reasons and eliminated previously proposed ones, while also raising further questions about how these white Chinook salmon can thrive with limited
carotenoids. Thus my thesis centered on another important evolutionary question about how genetic variation is maintained in nature. Rajasingh et al. (2007) described some reasoning for why white Chinook salmon could exist in nature, with one reason being that the white phenotype may be possible because the white morph only exists in more coastal populations exhibiting the ocean-type life history as these populations only migrate short distances and reduce competition by spawning at different times than red Chinook salmon. However, this is wrong. In fact, white Chinook salmon from the Quesnel River, migrate considerable distances for spawning (more than 800 km up the Fraser River) which is not an easy feat, and certainly, other white Chinook salmon migrate even further such as the salmon of the Endako River. Furthermore, white Chinook salmon spawn at the same time and same location as red Chinook salmon, and as my thesis demonstrated they interbreed (Chapter 5). Therefore, it may be unlikely that carotenoids evolved for salmon to deal with long spawning migrations, or perhaps it was important to ancestral salmonids, but not in present day species or more specifically, not in Chinook salmon. In this case, the red phenotype may still be present if the cost involved in the deposition of carotenoids is minimal (Rajasingh et al. 2007), and indeed, no studies have demonstrated an energetic cost of carotenoids to salmonids.

Rajasingh et al. (2007) also suggested that red and white Chinook salmon may exhibit differences in size, where white Chinook salmon could benefit from a larger body size (Godfrey 1968) that could increase female fecundity (Barnes et al. 2000) and male reproductive success (Williamson et al. 2010). However, there is also no evidence to support this for red and white Chinook salmon in the Quesnel River. Mean values for body size as well as fecundity and egg size traits are summarized here in Table 7.1, and some of these traits are discussed and reported in my data chapters. Although, Rajasingh
et al. (2007) did not discuss the possibility of white Chinook salmon evolving mechanisms to counteract their carotenoid limitation, my thesis demonstrated that white Chinook salmon may have evolved to compensate through functional genetic mechanisms. I found significant differences between morphs at important immune genes known as the major histocompatibility complex (MHC) class I and II genes (Chapter 5). It is possible that genes associated with colour are linked to the MHC genes (Chapter 2), and this is another way that white Chinook salmon could evolve to improve immune function if indeed immunity was compromised by limited carotenoids. In my thesis however, red and white morphs did not show differences in viral susceptibility (Chapter 4), although perhaps other pathogens could affect red and white Chinook salmon differently. Lastly, another way that white Chinook salmon can benefit from limited carotenoids is through reduced predation pressure during the egg stage (Chapter 3).

**Why aren’t all salmon white?**

White Chinook salmon therefore seem to be quite successful with little or no carotenoids in their tissues. However, if there really is no negative fitness consequence of being a white Chinook salmon, why does the white morph not exist in all populations and why has this phenotype not arisen in other salmonid species? Although the distribution of white Chinook salmon within river systems is not well documented, the white flesh phenotype is mainly restricted to populations in the Fraser River, BC and in rivers in Southeast Alaska (Hard et al. 1989). During the last glaciation, Chinook salmon took refuge in the southern (Pacific coastal regions and Columbia River drainage) and northern (Bering) refugia (Hard et al. 199). Therefore, I think that the white flesh phenotype first arose within the southern refugium of Chinook salmon as a mutation prior to the postglacial colonization of these rivers approximately 10,000 years ago (Hard et al. 1989;
Beacham et al. 2003; Waples et al. 2008). However, it is also likely that not all rivers possess the characteristics necessary for the white morph to stably persist. It should be acknowledged that my thesis focused on a single population where red and white Chinook salmon morphs co-exist at relatively equal frequencies. Specific characteristics of the Quesnel River may allow the polymorphism to be stable, however, different environmental characteristics could lead to changes in the fitness of the morphs. For example, egg predation pressure may be one of the mechanisms that determines the success of the white phenotype, as under higher predation pressure, white Chinook salmon should have an advantage. Additionally, if carotenoids are necessary to some extent in salmon eggs, then the white morph may be restricted to rivers with cold temperatures and high water flows that help maintain high oxygen levels in reds during incubation (Hard et al. 1989). It is also possible that Chinook salmon are the only species capable of existing as a white flesh morph, perhaps due to their larger egg size, body size or other life history characteristics. However, this would be surprising as species that show freshwater resident morphs with limited carotenoids in their environment, such as rainbow trout (*O. mykiss*), could presumably be successful as a white phenotype. Instead, perhaps a flesh colour mutation has never occurred in other salmonid species potentially due to genetic constraints.

**Future directions**

Genetic and genomics research on the red and white morphs holds the greatest potential for future research programs. First, using transcriptomics and genome sequencing, we can identify whether differences in carotenoid deposition is caused by structural protein changes or changes in regulatory sequences. Furthermore, after candidate genes have been characterized, gene knockout could be conducted on red
individuals to confirm the role of these genes in carotenoid pigmentation. The characterization of colour genes would allow the quantification of allele frequencies at these genes for all Chinook salmon populations. This genetic work would allow us to determine whether the white phenotype arose multiple times independently (through the same or different mutations) or whether it arose once and subsequently spread to different rivers during colonization. The characterization of colour and genotype throughout Chinook salmon populations could also lend insight into additional mechanisms of selection that operate to maintain red and white. For example, the correlation of morph frequencies with abiotic and biotic factors could identify which factors limit the establishment of the white morph, and further explain the conditions that drive positive selection for carotenoids in salmon. Additionally, genetic data would allow fine scale quantification of genotype frequencies over time and allow us to determine whether morphs indeed represent stable evolutionary stable strategies and whether negative-frequency dependent selection is an important mechanism operating to maintain the two morphs. Finally, future research on carotenoids in salmon should also focus on characterizing the variation that exists within and among salmonid species, as variation in carotenoid colouration may be driven by adaptations to different environments and/or life history strategies. By characterizing carotenoid variation across species and populations and coupling this information with environmental and life history characteristics, we can determine why salmonids have evolved to differentially utilize carotenoid pigments.

Conclusions

The wide breadth of my PhD research has allowed me to provide extensive insight into the long-standing question of why salmon have evolved to be red. Although some of the results of my thesis have been contradictory to the expectation of carotenoids in
general, my results “make sense in the light of evolution”. Thanks to evolution, different phenotypes can employ different strategies to maximize fitness. White Chinook salmon have indeed evolved in many ways to succeed with limited carotenoids, which, as my thesis demonstrates, can include: taking advantage of reduced predation pressure (demonstrated in Chapter 3), potentially utilizing lower but adequate amounts of carotenoids or alternative antioxidants in their eggs (discussed in Chapter 4), compensating through functional genetic mechanisms (demonstrated in Chapter 5), and utilizing different means to achieve reproductive success (demonstrated in Chapter 5 and 6). These mechanisms of natural and sexual selection help explain the persistence of the red and white polymorphism in Chinook salmon, while also providing further insight into the evolution of carotenoid pigmentation in salmonids. In conclusion, my thesis demonstrates that not all salmon are required to deposit carotenoids into their tissues, thus further highlighting the degree of diversity that can flourish within Salmonidae.

References


dietary supplementation of astaxanthin to female rainbow trout (*Oncorhynchus mykiss*) broodstock. J Appl Ichthyol. 26:35-3


Table 7.1. Mean and range values of male and female spawning traits in red and white Chinook salmon (*Oncorhynchus tshawytscha*) over three sampling years in the Quesnel River, British Columbia population. Differences between red and white morphs were compared using t-tests, where significance (p-values) for each test is reported.

<table>
<thead>
<tr>
<th>Trait (units)</th>
<th>Year</th>
<th>Red</th>
<th>Mean</th>
<th>Range</th>
<th>White</th>
<th>Mean</th>
<th>Range</th>
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<tbody>
<tr>
<td>Egg size (g)</td>
<td>Year</td>
<td>2013-2015</td>
<td>0.62</td>
<td>3.0-13.3</td>
<td>0.88</td>
<td>0.199-0.348</td>
<td>0.201-0.357</td>
</tr>
<tr>
<td>Fecundity (egg number)</td>
<td>2013-2015</td>
<td>0.66</td>
<td>3.3-10.0</td>
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<td>2802-5092</td>
<td>3924</td>
<td>3044-5675</td>
</tr>
<tr>
<td>Female body weight (kg)</td>
<td>2013-2015</td>
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<td>3.924</td>
<td>0.199-0.348</td>
<td>0.279</td>
<td>0.199-0.348</td>
<td>0.88</td>
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</table>
## Appendix I

Significant and suggestive (see p-values) single nucleotide polymorphisms (SNPs) sequences identified for a F2 family derived from the backcrossing of two flesh colour populations of Chinook salmon (*Oncorhynchus tshawytscha*) that showed nucleotide diversity.

<table>
<thead>
<tr>
<th>SNP</th>
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<th>Score</th>
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<th>p-value</th>
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<tr>
<td>S1008_35574933</td>
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<td>7.00E-18</td>
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<td>S100_27149859</td>
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<td>5.00E-11</td>
<td>0.00288</td>
<td>99</td>
</tr>
</tbody>
</table>

**BLAST Results**

In the table, BLAST results including Max score, E-value and SNP description with accession number of the alignment hit are provided. Derived from the backcrossing of two flesh colour populations of Chinook salmon (*Oncorhynchus tshawytscha*), these sequences include those shown in the presence of significant and suggestive single nucleotide polymorphisms (SNPs) sequences identified for a F2 family.
Salmo salar clone 11F04_73D15 interferon alpha-1-like gene
Salmo salar IgH locus A genomic sequence
Salmo salar BAC CH214-397C7 (Children's Hospital Oakland Research Institute Atlantic Salmon BAC Library) complete sequence

Salmo salar clone BAC CHORI214-439H13 genomic sequence

Salmo salar clone 11F04_73D15 interferon alpha-1-like gene
Salmo salar IgH locus A genomic sequence
Salmo salar BAC CH214-397C7 (Children's Hospital Oakland Research Institute Atlantic Salmon BAC Library) complete sequence

Oncorhynchus mykiss genome, MHC class I region, complete sequence

Predicated: Salmo salar uncharacterized LOC106591335 RNA
Salmo salar clone BAC CHORI214-439H13 genomic sequence

Predicated: Salmo salar uncharacterized LOC106591335 RNA
Salmo salar clone BAC CHORI214-439H13 genomic sequence

Predicated: Salmo salar uncharacterized LOC106591335 RNA
Salmo salar clone BAC CHORI214-439H13 genomic sequence

Predicated: Salmo salar uncharacterized LOC106591335 RNA
Salmo salar clone BAC CHORI214-439H13 genomic sequence
### Appendix 2. Primer and Taqman probe sequences for all genes used in the study.

Primer/probe sequences were previously designed in other studies on salmonids and those references are provided for each gene.

<table>
<thead>
<tr>
<th>Assay Name</th>
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<th>Reverse Primer Sequence</th>
<th>Reference</th>
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</thead>
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<tr>
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<td>AATGCTTACGTC</td>
<td>Purcell et al.</td>
</tr>
<tr>
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<td>Purcell et al.</td>
</tr>
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<td>Purcell et al.</td>
</tr>
<tr>
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<td>Purcell et al.</td>
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<td>Purcell et al.</td>
</tr>
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<td>Purcell et al.</td>
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<tr>
<td>G3X</td>
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<td>Purcell et al.</td>
</tr>
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</tr>
<tr>
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<td>Purcell et al.</td>
</tr>
<tr>
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<td>CTGACATGGCACCCCTC</td>
<td>Purcell et al.</td>
</tr>
<tr>
<td>ARP</td>
<td>ATGCTTACGTC</td>
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<td>Purcell et al.</td>
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<td>CTGACATGGCACCCCTC</td>
<td>Purcell et al.</td>
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<td>Purcell et al.</td>
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<td>Purcell et al.</td>
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<td>IFN-γ</td>
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<td>CD8</td>
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<td>ARP</td>
<td>ATGCTTACGTC</td>
<td>CTGACATGGCACCCCTC</td>
<td>Purcell et al.</td>
</tr>
</tbody>
</table>
Appendix 3. MHC II-B1 and MHC I-A1 alleles and their corresponding allele frequencies, GenBank accession number (with original reference in footnote) for red and white Chinook salmon (*Oncorhynchus tshawytscha*) spawners in the Quesnel River (see text for details).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alleles</th>
<th>Allele frequencies</th>
<th>GenBank accession no.</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Red (n= 30)</td>
<td>White (n= 31)</td>
</tr>
<tr>
<td>MHC II-B1</td>
<td>OTS B-1</td>
<td>0.650</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>OTS B-2</td>
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<td>0.323</td>
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<tr>
<td></td>
<td>OTS B-3</td>
<td>0.117</td>
<td>0.177</td>
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<td></td>
<td>OTS B-4</td>
<td>0.017</td>
<td>0.000</td>
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<tr>
<td></td>
<td>OTS B-5</td>
<td>0.000</td>
<td>0.032</td>
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<tr>
<td></td>
<td>OTS B-6</td>
<td>0.000</td>
<td>0.032</td>
</tr>
<tr>
<td>MHC I-A1</td>
<td>OTS A-1</td>
<td>0.327</td>
<td>0.117</td>
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<tr>
<td></td>
<td>OTS A-2</td>
<td>0.250</td>
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<td></td>
<td>OTS A-3</td>
<td>0.135</td>
<td>0.033</td>
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<tr>
<td></td>
<td>OTS A-4</td>
<td>0.096</td>
<td>0.133</td>
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<td>OTS A-6</td>
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<td></td>
<td>OTS A-8</td>
<td>0.019</td>
<td>0.033</td>
</tr>
</tbody>
</table>


<sup>5</sup>Pitcher TE, Neff BD. 2006. MHC class IIB alleles contribute to both additive and non-additive genetic effects on survival in Chinook salmon. Mol Ecol. 15:2357-2365.
Appendix 4. DNA sequences for MHC I-A1 and MHC II-B1 alleles found in spawning Chinook salmon (*Oncorhynchus tshawytscha*) in the Quesnel River, British Columbia, Canada. Nucleotides that differ between alleles are highlighted. Allele frequencies and GenBank accession numbers are indicated in Table 5.5.

**MHC I–A1**

- **OTS-A1**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A2**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA
- **OTS-A3**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A4**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGAG
- **OTS-A5**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA
- **OTS-A6**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A7**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGAG
- **OTS-A8**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA

**MHC II–B1**

- **OTS-A1**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A2**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A3**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A4**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A5**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A6**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A7**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A8**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG

**MHC I–A1**

- **OTS-A1**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A2**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA
- **OTS-A3**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A4**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGAG
- **OTS-A5**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA
- **OTS-A6**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGACGG
- **OTS-A7**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGAG
- **OTS-A8**: TTCTACACCGCATCTTCAGATTTCCCAACTTCCCAAGGTCTGATTCGGTGTGGGGA

**MHC II–B1**

- **OTS-A1**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A2**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A3**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A4**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A5**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A6**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A7**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
- **OTS-A8**: GGACTGGATGAACAAGGAGCAGACAAACACTGCCCACTTGGGAGAGGGAGACAGGG
MHC II-B1

OTS-B1 - GGTATAGAGTTTATACACTCTTTATGTTTCTCAAATAGGTTGAACATATCACAGATTCAACA
OTS-B2 - GGTATAGAGTTTATAGACTCGTTTAGTTTCAATAAGGTTGAACATATCACAGATTCAACA
OTS-B3 - GGTATAGAGTTTATACACTCTTTATGTTTCTCAAATAGGTTGAACATATCACAGATTCAACA
OTS-B4 - GGTATAGAGTTTATAGACTCGTTTAGTTTCAATAAGGTTGAACATATCACAGATTCAACA
OTS-B5 - GGTATAGAGTTTATACACTCTTTATGTTTCTCAAATAGGTTGAACATATCACAGATTCAACA
OTS-B6 - GGTATAGAGTTTATACACTCTTTATGTTTCTCAAATAGGTTGAACATATCACAGATTCAACA

OTS-B1 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCA
OTS-B2 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B3 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAACATG
OTS-B4 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B5 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B6 - GCACGTGTTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG

OTS-B1 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B2 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B3 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B4 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B5 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG
OTS-B6 - GCACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATG

OTS-B1 - AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA
OTS-B2 - AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA
OTS-B3 -AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA
OTS-B4 - AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA
OTS-B5 - AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA
OTS-B6 - AACGCTGCTCTCGAGTACAGAGCCATACTGGACAAGACAGGTGAGCAGGGCTCCTTAA

OTS-B1 - CACCACTTACAGGACTC
OTS-B2 - CACCACTTACAGGACTC
OTS-B3 - CACCACTTACAGGACTC
OTS-B4 - CACCACTTACAGGACTC
OTS-B5 - CACCACTTACAGGACTC
OTS-B6 - CACCACTTACAGGACTC
## Appendix 5

### Results of linear mixed effect models for sperm path straightness (STR) and straight line sperm velocity (VSL) of red and white Chinook salmon (*Oncorhynchus tshawytscha*) males when activated in ovarian fluid of red and white Chinook salmon females.

**Fixed effects** are presented with estimate parameters including 95% confidence intervals (CI) and statistical results of Kenward-Roger approximation test. **Random effects** are presented with variance components with standard deviations and percentage of the total variance (% var) as well as significant results of log-likelihood ratio tests. Significant effects are indicated by bold font and an asterisk.

#### Sperm path straightness (STR) in ovarian fluid

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<th>Estimate</th>
<th>95% CI</th>
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</thead>
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<td>6.97</td>
<td>0.002</td>
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<tr>
<td>Male colour</td>
<td>0.08</td>
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<td>1.15</td>
<td>0.292</td>
</tr>
<tr>
<td>Female colour</td>
<td>0.18</td>
<td>-5.92, 6.29</td>
<td>1.93</td>
<td>0.167</td>
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<tr>
<td>Interaction</td>
<td>6.50</td>
<td>-2.47, 15.46</td>
<td>1.97</td>
<td>0.163</td>
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**Log-likelihood ratio test**

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#### Sperm straight line velocity (VSL) in ovarian fluid

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<tr>
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<td>63.90</td>
<td>55.28, 72.53</td>
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**Log-likelihood ratio test**

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**Variance (±SD) (%) var**

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**Log-likelihood ratio test**

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<td>77.3</td>
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</table>
Appendix 6. Percent fertilization success (and total number of eyed-eggs genotyped) calculated as the percentage of eyed-eggs sired by red Chinook salmon (*Oncorhynchus tshawytscha*) males when in sperm competition with white males (male pair, numbers indicate unique identities) to fertilize eggs of red and white Chinook salmon females. Eggs were competitively fertilized in the presence of 20% ovarian fluid with equal volumes of semen from each male within the pair and paternity was determined by genetic analyses using three polymorphic microsatellite loci (see Methods for details).

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<th>Male pair (Red-White)</th>
<th>Male pair</th>
<th>Female (Colour and ID)</th>
<th>Percent fertilization success (and total number of eyed-eggs genotyped)</th>
<th>Total number of eyed-eggs genotyped</th>
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<td>87.5 (24)</td>
<td>100 (24)</td>
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Appendix 7. Line interaction plot showing change in mean sperm velocity (average path velocity, VAP) of individual red and white Chinook salmon (*Oncorhynchus tshawytscha*) males (n = 19) when activated in the ovarian fluid of red and white females (n = 8). Data represent sperm velocities from 104 male x female ovarian fluid combinations, where a minimum of 10 males were activated in the ovarian fluid of each female.
Appendix G. Results of generalized linear mixed effect models testing post-copulatory processes affecting fertilization success (paternity) in red and white Chinook salmon (*Oncorhynchus tshawytscha*) males and females between paired red and white females.

### Log-likelihood tests

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<th>Log-likelihood test</th>
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### Random effects

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### Fixed effects

**Model 1:** Log-likelihood test

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<td>Difference sperm velocity in OF</td>
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**Model 2:** Log-likelihood test

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<td>-4.91, 2.73</td>
</tr>
<tr>
<td>Female colour</td>
<td>-0.67</td>
<td>-2.15, 0.81</td>
</tr>
<tr>
<td>Sperm density ratio (red:white)</td>
<td>0.41</td>
<td>-2.25, 3.08</td>
</tr>
</tbody>
</table>

### Notes

- Fixed effects are presented with estimate parameters including 95% confidence intervals (CI).
- Random effects are presented with variance components, including standard deviations and percentage of the total variance (% var).
- Significance of factors in models was determined by log-likelihood tests and significant effects are indicated by bold font and an asterisk.
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Nov 16, 2016

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