2010

The Major Histocompatibility (MH) Genes: Somatic Mutation and Mate Choice in Chinook Salmon (Oncorhynchus Tshawytscha)

Leila Helou
University of Windsor

THE MAJOR HISTOCOMPATIBILITY (MH) GENES: SOMATIC MUTATION AND MATE CHOICE IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

by

Leila Helou

A Thesis
Submitted to the Faculty of Graduate Studies
through Great Lakes Institute for Environmental Research (GLIER)
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2010

THE MAJOR HISTOCOMPATIBILITY (MH) GENES: SOMATIC MUTATION AND MATE CHOICE IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

by

Leila Helou

APPROVED BY:

_____

Dr. T. Pitcher
Department of Biological Sciences

_____

Dr. M. Cristescu
Department of Biological Sciences
Great Lakes Institute for Environmental Research

_____

Dr. D. Heath, Advisor
Department of Biological Sciences
Great Lakes Institute for Environmental Research

_____

Dr. D. Haffner, Chair of Defense
Great Lakes Institute for Environmental Research

May 18, 2010

STATEMENT OF ORIGINALITY

Chapter 1 of this thesis incorporates the outcome of research undertaken in collaboration with D.D. Heath, and M. Docker; however, the analyses used are novel.

I declare that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

An analysis of a library consisting of major histocompatibility (MH) class I α_1 and class II β_1 sequences for Chinook salmon (*Oncorhynchus tshawytscha*) identified mutation clusters or "hotspots" in the sequence, as well as identical mutations occurring independently. This suggests that the MH genes in Chinook salmon undergo somatic mutation. In sperm competition experiments jacks sired a higher proportion of eggs than hooknose males. Furthermore, an analysis of variance indicated that cryptic female choice had occurred. To further investigate this, I genotyped parents and offspring at the MH class I and class II peptide binding region to determine whether the differential fertilization success is driven by variation in genetic compatibility between the ova and sperm. I found that mate choice for the MH class I gene had not occurred; however, jack sperm that were more similar at the MH class II gene to the female had higher fertilization success.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Daniel Heath for his patience, encouragement, and for always steering me in the right direction. I would also like to thank my committee members for their knowledgeable input, and taking the time to read my thesis. My two chapters were supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant, and Canada Research Chair (CRC) funding to Dr. D. Heath. I would also like to thank Sara Jamieson for revising my thesis and Farrah Chan for sharing her valuable computer skills. Special thanks to my student colleagues for their advice and good humour, which helped make this experience an enjoyable one. Finally, I extend heartfelt thanks to my family for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER TWO

CHAPTER THREE

LIST OF FIGURES

CHAPTER TWO

CHAPTER THREE

TABLE OF ABBREVIATIONS

Average Amino Acid Dissimilarity Index	AADI
Basepairs	bp
Cryptic Female Choice	CFC
Major Histocompatibility Complex	MHC
Major Histocompatibility	MH
Peptide Binding Region	PBR
Polymerase Chain Reaction	PCR
Single Nucleotide Polymorphism	SNP
Yellow Island Aquaculture Ltd.	YIAL

CHAPTER ONE
GENERAL INTRODUCTION

The major histocompatibility complex (MHC) and mate choice

Major histocompatibility complex (MHC) molecules are cell-surface glycoproteins encoded by a large cluster of genes commonly known as the MHC (Lawlor et al., 1990). MHC molecules bind foreign and self peptides in specialized grooves and present them to T cells, thereby triggering the appropriate immune response (Nei and Hughes, 1991). The MHC genes code for two categories of MHC peptide-binding molecules: class I and class II. The MHC class I genes are expressed in all nucleated cells, and these molecules present peptides derived from intracellular pathogens, such as those derived from the degradation of viral proteins, to CD8+ T cells (Nei and Hughes, 1991). MHC class II molecules are constitutively expressed on antigen-presenting cells of the immune system such as macrophages, B-cells, monocytes, and dendritic cells (Ting and Trowsdale, 2002). These molecules present peptides derived from extracellular pathogens that are internalized by phagocytosis or endocytosis, to CD4+ T cells (Nei and Hughes 1991). Both MHC molecules are composed of immunoglobulin-like domains (α_3 and β_2-m in class I, α_2 and β_2 in class II), which interact with T cells, peptide binding regions (PBR; α_1 and α_2 in class I, α_1 and β_1 in class II), and a hydrophobic trans-membrane domain (Lawlor et al., 1990). The genes encoding the PBR are typically highly polymorphic, and characterized by numerous loci and a high degree of allelic diversity in most species (Nei and Hughes, 1991). Genetic variation at the PBR is beneficial since it is thought to enhance immunocompetence, as a diverse repertoire of MHC molecules would be able to recognize and present a wider range of pathogens. There are examples in nature of species lacking MHC polymorphism, and consequently having greater susceptibility to pathogens. For instance, the cheetah and the cottontop tamarin are species lacking MHC allelic diversity at all loci, and as a result are highly susceptible to certain pathogens (O'Brian et al., 1985; Watkins et al., 1988).

Teleost fish represent approximately half of the extant fish species, and 99.8% of the ray-finned fishes (actinopterygians) belong to the teleosts (Volff, 2005). Teleosts provide a unique opportunity for studying the evolution of the MHC genes because,

unlike mammals, the genes encoding the α chains of MHC class I, and the α and β chains of MHC class II molecules are not linked, thus allowing independent evolution to occur (Grimholt et al., 2002). Since teleost class I and class II genes are unlinked, and because they do not form a complex they are more correctly referred to as MH genes (Stet et al., 2002). Teleost MH genes are studied extensively in an attempt to pinpoint genes responsible for disease resistance in economically important fishes, and to determine how MH polymorphisms are maintained through evolutionary mechanisms. In order to explain the maintenance of MH polymorphism, researchers have developed two disease-based models: the *overdominant (heterozygote advantage) hypothesis*, and/or *negative frequency-dependant selection hypothesis*. The *overdominant hypothesis* states that heterozygous individuals have an immunological advantage as they can present a wider range of pathogen-derived antigens due to a larger number of different MH molecules; thus, selection for heterozygous individuals maintains MH polymorphism in a population (Langefors et al., 2001). There is evidence supporting this theory, for example, one study examined the resistance of winter-run Chinook salmon (*Oncorhynchus tshawytscha*) to infectious hematopoietic necrosis virus (IHNV), and found that MH heterozygotes had a higher survival than MH homozygotes (Arkush et al., 2002). The alternative mechanism is *frequency-dependent selection*, where rare alleles are more advantageous than common alleles because parasites would have developed resistance to the more commonly encountered molecule over time (Langefors et al., 1998). Selection for the rare allele would increase its frequency in a population, thereby maintaining MH polymorphism. Although balancing selection for particular alleles associated with disease resistance has been demonstrated in teleosts (Langefors, 1998), frequency-dependant selection is problematic to prove empirically as allele frequencies would need to be monitored over time to show cycling dynamics between alleles and parasites.

Sexual selection is an evolutionary mechanism which favours selection of traits valuable for increasing reproductive success, and can also function to maintain MHC polymorphism in a population. The process of MHC-dependant mate preference involves female choice for more genetically dissimilar males in an attempt to produce offspring with maximized immunocompetence (Penn and Potts, 1999). The hypothesized mechanism is that MHC associated molecules serve as olfactory cues, which females

utilize when choosing a mate. For instance, studies on mice have shown that females use urinary odour cues to choose mates, and prefer MHC-dissimilar males, thus contributing to diversity at the MHC in offspring (Potts et al., 1991; Roberts and Gosling, 2003). MH dependant mate choice has also been demonstrated for the stickleback *Gasterosteus aculeatus*, and contrary to findings that promote MH diversity, mate choice provided offspring with an optimal number of MH alleles (Aeschlimann et al., 2003). A recent study on Chinook salmon (*Oncorhynchus tshawytscha*) also reported surprising results, and in this study mate choice occurred for more genetically similar males, resulting in offspring with more similar MH alleles (Yeates, 2009).

Female mate choice is typically pre-copulatory, and exercised behaviourally by the female, which will usually choose a mate on the basis of material benefits or physical characteristics that are indicators of high genetic quality (Tregenza and Wedell, 2000). There is also evidence for mate choice occurring at the sperm-egg level, without the involvement of a female pre-copulatory behavioural choice. One example for a mechanism of post-copulatory mate choice at the sperm-egg level is demonstrated by the comb jelly *Beroë ovata*. In this species, multiple sperm fertilize the oocyte cytoplasm, and the female pronucleus inspects and evaluates each of the male pronuclei before choosing to fuse with one of them (Ziegler et al., 2005). In external fertilizers such as teleosts, there is evidence for sperm selection by eggs based on the MH genes (e.g. Yeates et al., 2009; Turner et al., 2009; Wedekind et al., 2001); however, the exact mechanism has yet to be determined.

Chinook salmon, *Oncorhynchus tshawytscha*

Chinook salmon are the largest of the Pacific salmon, and in North America their distribution ranges from the Ventura River in California to Point Hope, Alaska, as well as the Great Lakes (Myers et al., 1998; Kocik and Jones, 1999). Chinook salmon have also been introduced to other parts of the world including New Zealand, Chile, and Argentina (Ciancio et al., 2005). Chinook salmon are anadromous, meaning they live in fresh water as juveniles, migrate to sea water where they sexually mature, then return to fresh water to spawn (Quinn, 2005). Chinook salmon are also semelparous, meaning they die shortly after spawning (Quinn, 2005). Characterization of Chinook salmon life histories is often based on the timing of the downstream migration of juveniles, and the upstream

migration of adults; however, it is important to note that there is much variation in timing of migration, and length of residencies (Quinn, 2005). In general, Chinook salmon juveniles, which overwinter in fresh water and emigrate to salt water in early spring, are referred to as "stream-type" with adult runs occurring in spring and summer (Schaffter, 1980). "Ocean-type" salmon emigrate to salt water within the first 3 months of life, and predominately make up the fall-run and summer-run populations (Reimers, 1973). Also, there is variation in age and size at reproductive maturity in male Chinook salmon. At the spawning site, male Chinook salmon compete for access to females' eggs aggressively (hooknose) or through a sneaking tactic (jack). Which male phenotype a fish adopts is genetically and environmentally determined, and the incidence of jacking varies from 10% to over 90% in some populations (Healey, 1991; Heath et al., 1994; Heath et al., 1991).

The impact of salmon fisheries as well as environment degradation has led to a decline in natural spawning populations of Pacific salmon to the extent that several populations have been placed under the U.S. Endangered Species Act. As a result, a large number of Pacific Northwest hatcheries have been propagating fish in order to supplement wild populations. For instance, over 200 million juvenile anadromous Pacific salmon are released into the Columbia River annually, and hatchery fish represent almost 85% of returning salmonids (Campton, 2004). Whether salmon hatcheries are augmenting wild populations successfully is controversial, and commonly used fertilization protocols have been criticized for causing artificial selection and inbreeding (Campton, 2004). Thus, the aim of several hatcheries has been to employ breeding methods to minimize genetic change as well as maintain genetic diversity to ensure the program's success.

Due to the commercial importance of salmon, the MH genes of salmonids are well characterized and available for 16 different species (Wegner, 2008). Although salmonids underwent an entire genome duplication approximately 100 million years ago, they have very few MH loci in comparison to other fish species (Miller et al., 2002). For instance, Atlantic cod may have as many as 42 MH class I loci (Miller et al., 2002), while only three class I loci in Pacific (*Oncorhynchus* spp.) and Atlantic salmon (*Salmo salar*) have been identified: A, B, and UA, and none are the result of the gene duplication

(Grimholt et al., 1993; Miller and Withler, 1998). Furthermore, failure to isolate cDNA from the B locus, and its substantial divergence from the other loci has led researchers to believe that the B locus is a pseudogene (Grimholt et al., 1993; Miller and Withler, 1998). In addition, only one MH class II gene has been identified in salmonids, and it is substantially less polymorphic than class I genes (Miller and Withler, 1996).

Somatic mutation

Somatic mutation has been extensively studied in the variable region of immunoglobulins (Ig) as it is believed to be the mechanism for the generation of new antibodies during the primary immune response (Yang et al., 2006). In this process, mutated B cells expressing higher affinity antibodies on the cell surface are selected for by antigens, while B cells expressing lower affinity receptors presumably undergo apoptosis (Han et al., 1995). Several features of the somatic mutation mechanism in Ig has been described (reviewed in Denépoux et al., 1997), yet the possibility of somatic mutations occurring in the peptide binding region of the MHC has not yet been proposed or investigated.

The mechanism for the generation of novel MHC alleles is yet to be proven; however, research shows that recombination and gene conversion may be important processes for the generation of MHC allelic diversity (Martinsohn et al., 1999). Alternately, the MH genes may be diversifying through point mutations occurring in somatic cells. Differences in the MH alleles of Chinook salmon are produced by relatively small differences in large numbers of nucleotides. For example, an analysis of MH class II β_1 gene in Chinook salmon revealed three alleles differing only in 3-7 nucleotides; therefore, point mutations rather than recombination or gene conversion, is more likely the mechanism for diversifying alleles at the MH genes (Miller et al., 1997). For species that lack MH diversity, such as Chinook salmon, somatic point mutation would be an important mechanism for the creation of new MH alleles and would explain how the fish maintain immunological competence in two very different aquatic environments (fresh and salt water) despite having a limited number of functional MH loci. It is important to note that in order for the new alleles to be heritable, mutation would have to occur in the germ cells.

STUDY OBJECTIVES

Chapter two analyzes a large MH class I and II sequence library in order to quantify and characterize base pair substitutions, and investigates the effects of PCR conditions on the frequency of such mutations. In the MH sequence library, regions of the MH gene containing a high number of mutations were investigated in order to determine whether mutations were occurring in clusters at "hotspots" or spread randomly along the gene. A series of PCR-based experiments were conducted to determine how polymerase type and the number PCR cycles affect the frequency of mutations observed. From these analyses I provide valuable insight into the nature of PCR induced MH artifacts versus genuine somatic mutations.

Chapter three investigates the outcome of sperm competition between jack and hooknose Chinook salmon and the role of MH variation in that process. An artificial breeding experiment was performed to produce 25 full- and half-sib families with jack and hooknose male parent sperm competing for fertilization, and microsatellite analysis was used to determine parentage of offspring. From the microsatellite analysis I determine which male (jack versus hooknose) sired the majority of offspring, and whether there are any significant sire, dam, and sire-dam interaction effects. In Chapter one I used cloning and sequencing to accurately genotype parents and offspring for the MH gene in order to determine whether the outcome of sperm competition was associated with the MH genotype in parents. From this I was able to investigate whether female choice is occurring for sperm expressing more similar/dissimilar MH alleles.

Results from chapter two and three help us understand the mechanisms for MH evolution, and the source of MH polymorphism in Chinook salmon, allowing us to gain insight on key evolutionary processes, and enabling us to utilize this information to develop successful breeding protocols in hatcheries.

LITERATURE CITED

Aeschlimann, P.B., Häberli, M.A., Reusch, T.B.H., Boehm, T., Milinski, M., 2003. Female sticklebacks Gasterosteus aculeatus use self-reference to optimize MHC allele number during mate selection. Behavioral Ecology and Sociobiology 54, 119–126.

Aguilar, A., Garza, J.C., 2007. Patterns of historical selection on the salmonid major histocompatibility complex class II β gene. Journal of Molecular Evolution 65, 34-43.

Arkush, K.D., Giese, A.R., Mendonca, H.L., McBride, A.M., Marty, G.D., Hedrick, P.W., 2002. Resistance to three pathogens in the endangered winter-run Chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. Canadian Journal of Fisheries and Aquatic Sciences 59, 966-975.

Campton, D.E., 2004. Sperm competition in salmon hatcheries: the need to institutionalize genetically benign spawning protocols. Transactions of the American Fisheries Society 133, 1277-1289.

Ciancio, J.E., Pascual, M.A., Lancelotti, J., Rossi, C.M.R., Botto, F., 2005. Natural colonization and establishment of a Chinook salmon, *Oncorhynchus tshawytscha*, population in the Santa Cruz River, an Atlantic Basin of Patagoni. Environmental Biology of Fishes 2, 219-227.

Denépoux, S., Razanajaona, D., Blanchard, D., Meffre, G., Capra, J.D., Banchereau, J., Lebecque, S., 1997. Induction of somatic mutation in a human B cell line in vitro. Immunity 6, 35-46.

Grimholt, U., Hordvik, I., Fosse, V.M., Olsaker, I., Endresen, C., Lie, O., 1993. Molecular cloning of major histocompatibility complex class I cDNAs in Atlantic salmon (Salmo salar). Immunogenetics 37, 469-473.

Grimholt, U., Drablos, F., Jorgensen, S.M., Hoyheim, B., Stet, R.J.M., 2002. The major histocompatibility class I locus in Atlantic salmon (Salmo salar L.): polymorphism, linkage analysis and protein modeling. Immunogenetics 54, 570-581.

Han, S., Hathcock, K., Zheng, B., Kepler, T.B., Hodes, R., Kelsoe, G., 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. Journal of Immunology 155, 556-567.

Healey, M.C., 1991. Life history of chinook salmon (*Oncorhynchus tshawytscha*). In: Groot C (ed) *Pacific Salmon Life Histories*. UBC Press, Vancouver. pp. 311–394.

Heath, D.D., Heath, J.W., Iwama, G.K., 1991. Maturation in chinook salmon, *Oncorhynchus tshawytscha* (Walbaum): early identification based on the development of a bimodal weight frequency distribution. Journal of Fish Biology 39, 565–575.

Heath, D.D., Devlin, R.H., Heath, J.W., Iwama, G.K., 1994. Genetic, environmental and interaction effects on the incidence of jacking in *Oncorhynchus tshawytscha* (Chinook salmon). Heredity 72, 146-154.

Klein, J., 1986. Natural history of the major histocompatibility complex. Wiley, New York.

Kocik, J. F., Jones, M.L., 1999. Pacific salmonines in the Great Lakes basin. Great Lakes fishery policy and management: a binational perspective. Taylor, W.W.,

Ferreri, C.P. (ed) Michigan State University Press pp. 455–488
Langefors, A., Schantz, T., Widegren, B., 1998. Allelic variation of MHC class II in Atlantic salmon: a population genetic analysis. Heredity 80, 568-575.
Langefors, K., Lohm, J., von Schantz, T., 2001. Allelic polymorphism in MHC class IIB in four populations of Atlantic salmon (Salmo salar). Immunogenetics 53, 329–336.
Lawlor, D.A., Zemmour, J., Ennis, P.D., Parham, P., 1990. EVOLUTION OF CLASS I MHC GENES AND PROTEINS: from natural selection to thymic selection. Annual Review of Immunology 8, 23-63.
Lee, S.S., Tranchina, D., Ohta, Y., Flajnok, M.F., Hsu, E., 2002. Hypermutation in shark immunoglobulin light chain genes results in contiguous substitutions. Immunity 16, 571-582.
Martinsohn, J.T., Sousa, A.B., Guethlein, L.A., Howard, J.C., 1999. The gene conversion hypothesis of MHC evolution: a review. Immunogenetics 50, 168-200.
Miller, K.M., Kaukinen, K.H., Schulze, A.D., 2002. Expansion and contraction of major histocompatibility complex genes: a teleostean example. Immunogenetics 53, 941-963.
Miller, K.M., Withler, R.E., 1996. Sequence analysis of a polymorphic MHC class II gene in Pacific salmon. Immunogenetics 43, 337-351.
Miller, K.M., Withler, R.E., Beacham, T.D., 1997. Molecular evolution at MHC genes in two populations of Chinook salmon *Oncorhynchus tshawytscha*. Molecular Ecology 6, 937-954.
Miller, K.M., Withler, R.E., 1998. The salmonids class I MHC: limited diversity in a primitive teleosts. Immunological Reviews 166, 279-293.
Myers, J.M., Kope, R.G., Bryant, G.J., Teel, D., Lierheimer, L.J., Wainwright, T.C., Grant, W.S., Waknitz, W., Neely, K., Lindley, S.T., Waples, R.S., 1998. Status review of Chinook salmon from Washington, Idaho, Oregon, and California. NOAA Technical Memorandum.
Nei, M., Hughes, A.L., 1991. Polymorphism and evolution of the major histocompatibility complex loci in mammals. Evolution at the Molecular Level 87, 222-247.
Penn, D.J., Potts, W.K., 1999. The evolution of mating preferences and major histocompatibility complex genes. The American Naturalist 153, 145-164.
Potts, W.K., Manning, C.J., Wakeland, E.K., 1991. Mating patterns in seminatural populations of mice influences by MHC genotype. Nature 352, 619-621.
Quinn, T.P., 2005. The behavior and ecology of Pacific salmon and trout. University of Washington Press, Seattle, WA, 328 pp.
Reimers, P.E., 1973. The length of residence of juvenile fall Chinook salmon in Sixes River, Oregon. Research Report of the Fish Commission of Oregon 4, 1-43.
Roberts, S.C., Gosling, M., 2003. Genetic similarity and quality interact in mate choice decisions by female mice. Nature Genetics 35, 103-106.
Schaffter, R.G., 1980. Fish occurrence, size, and distribution in the Sacramento River near Hood, California during 1973 and 1974. California Department of Fish & Game Fisheries Administration Report 76, 80-83.
Stet, R.J.M., deVries, B., Mudde, K., Hermsen, T., van Heerwaarden, J., Shum, B.P., Grimholt, U., 2002. Unique haplotypes of co-segregating major histocompatibility

class II A and class II B alleles in Atlantic salmon (salmo salar) give rise to diverse class II genotypes. Immunogenetics 54, 320-331.
Ting, J.P.Y., Trowsdale, J., 2002. Genetic control of MHC class II expression. Cell 2, S21- S33.
Tregenza, T., Wedell, N., 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. Molecular Ecology 9, 1013-1027.
Turner, S.M., Chaves-Campos, J., 2009. Parental relatedness and major histocompatibility effects on early embryo survivorship in Atlantic salmon. Genetica 137, 99-109.
Volff, J-N., 2005. Genome evolution and biodiversity in teleost fish. Heredity 94, 280-294.
Watkins, D.I., Hodi, F.S., Letvin, N.L., 1988. A primate species with limited major histocompatibility complex class I polymorphism. Proceedings of the National Academy of Sciences of the United Sates of America 85, 7714-7718.
Wedekind, C., Müller, R., Spicher, H., 2001. Potential genetic benefits of mate selection in whitefish. Journal of Evolutionary Biology 14, 980-986.
Wegner, K.M., 2008. Historical and contemporary selection of teleosts MHC genes: did we leave the past behind? Journal of Fish Biology 73, 2110-2132.
Yang, F., Waldbieser, G.C., Lobb, C.J., 2006. The nucleotide targets of somatic mutation and the role of selection in immunoglobulin heavy chains of a teleost fish. The Journal of Immunology 176, 1655-1667.
Yeats, S.E., Einum, S., Fleming, I.A., Megens, H.J., Stet, R.J.M., Hindar, K., Holt, W.V., Van Look, K.J.W., Gage, M.J.G., 2009. Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles. Proceeding of the Royal Society B 276, 559-566.
Ziegler, A., Kentenich, H., Uchanska-Ziegler, B., 2005. Female choice and the MHC. Trends in Immunology 26, 496-500.

CHAPTER TWO

Mutation analysis of the Major Histocompatibility (MH) genes in Chinook salmon (*Oncorhynchus tshawytscha*)

INTRODUCTION

The major histocompatibility complex (MHC) includes genes of special interest in evolutionary biology due to their roles in disease resistance, mate choice, and kin recognition (Bernatchez and Landry, 2003). The MHC genes code for membrane-spanning glycoprotein heterodimers that bind both self and foreign antigens. At the cell surface, the MHC-antigen complex interacts with T cells, which recognize an antigen only when combined with an MHC molecule (Cuesta et al., 2005). There are two types of MHC peptide-binding molecules (class I and class II) differing in structure and function: In the cell, foreign, endogenously synthesized proteins, such as those derived from a virus, are degraded within the cytoplasm, and are presented by MHC class I molecules to CD8+ T cells (Cuesta et al., 2005). Pathogens and proteins that are exogenously derived are internalized within the cell by phagocytosis or endocytosis, and are presented by MHC class II molecules to CD4+ T cells (Cuesta et al., 2005). Both types of MHC molecules are composed of immunoglobulin-like domains (which interact with T cells), peptide binding regions (PBR), and a hydrophobic trans-membrane domain (Lawlor et al., 1990). The highest level of polymorphism observed in the MHC genes is concentrated within the sequences encoding the PBR, and this variability is correlated to breadth of pathogen recognition and disease resistance (Klein and Figueroa, 1986). Although the class I and class II genes form a linkage group in mammals, the genes encoding the α chains of MHC class I and the α and β chains of MHC class II molecules are not linked in teleost fishes; as they do not form a single complex, they are more correctly referred to as MH genes in teleosts (Stet et al., 2002). Furthermore, since the class I and class II loci are not physically linked in teleosts, independent evolution can occur, making the MH

genes in teleosts a good model for molecular evolution studies (Grimholt et al., 2002).

The degree of polymorphism in the MHC genes varies greatly by species and by population within species (Miller et al., 1997). For instance, the MHC in humans is estimated to contain 421 loci; whereas, salmonids are estimated to have only 3 class I genes (designated A, UA, and B), and only 1 class II gene (Horton et al., 2004; Miller and Withler, 1998). Furthermore, the MH class I B locus in salmonids is likely a non-classical MH locus, due to the failure to isolate cDNA, and its reduced α2 variability compared to those of MH class I A (Grimholt et al., 1994). Salmonids underwent a whole-genome duplication 25-100 million years ago resulting in the current pseudotetraploidy state, yet this event was not followed by a corresponding increase in the number of functional MH loci (Allendorf and Thorgaard, 1984).

MHC polymorphism can be attributed to a variety of mutagenic mechanisms such as gene duplications, deletions, point mutations, recombination, and gene conversion; however, such novel mutations must occur in the germ-line for selection to act on them (Lawlor et al., 1990). A single nucleotide substitution resulting in an amino acid change in the PBR sequences can create a new/rare allele, having important fitness consequences for the organism (Langefors et al., 1998). Miller et al. (1997) examined MH evolution in Chinook salmon (*Oncorhynchus tshawytscha*) and found that much of the variability in MH class I α1 alleles were produced by point mutations, as well as inter-allelic exchange or conversion. Furthermore, Miller et al. (1997) discovered that most of the point mutations found in the PBR genes were nonsynonymous indicating that selection is responsible for maintaining the polymorphisms.

The MH genes have been used widely for behavioural, population genetic, and

survival-related evolutionary analyses in salmon (e.g. Landry et al., 2001; Evans et al., 2009; Pitcher and Neff, 2006). The likelihood of MH genotyping error is high, yet only one systematic assessment of MH genotyping error or mutation in teleosts has been published (Lenz and Becker, 2008). *In vitro* PCR conditions are known to significantly influence the mutation rate, and numerous methods have been proposed to eliminate artificial amplicons during and after the PCR (Borriello and Krauter, 1990; Eckert and Kunkel, 1991; Zylstra et al., 1998; Thompson et al., 2005). Several population genetic studies have discovered singleton mutants in individuals, and dismissed these as likely PCR artefacts (Alcaide et al., 2008; Grimholt et al., 2002; Grimholt et al., 1994). Alternatively, somatic hypermutation occurring *in vivo*, an important mechanism for maintaining variation in immune genes, may be responsible for some of those single-copy mutants. Due to the relatively low number of functional MH loci in salmonids, it would be beneficial for the salmon to have alternative mechanisms of allele diversification such as somatic mutation. Somatic hypermutation is seen in the variable regions of the immunoglobulin (Ig) genes (Lee et al., 2002; Yang et al., 2005); however, there have been no studies to date on the potential for such mutations to occur in the peptide binding region of the MH genes.

In this study we conduct a large-scale survey of rare single-copy MH PBR sequences in 67 Chinook salmon and report a very high frequency of such alleles. Detailed characterization of those mutations identified potential somatic mutations as well as mutation hotspots in the MH class I and II PBR. We also use a series of PCR-based experiments to investigate how PCR conditions affect mutation frequencies, and show that the polymerase used can significantly increase base pair substitutions (BPS)

and chimera mutations and increasing the number of PCR cycles increases chimera mutations. This analysis, coupled with the sequence survey, provides insight on the relative occurrence of PCR artefacts and somatic mutations in Chinook salmon.

METHODS AND MATERIALS

Our analyses consisted of two separate approaches: 1) a detailed analysis of an MH sequence library consisting of 396 MH class I α1 sequences (n=38 fish), and 389 MH class II β_1 sequences (n= 29 fish); and 2) an experimental evaluation of the contribution of PCR artefacts to the observed frequency of rare MH sequence mutants.

Sequence Library

Two sequence libraries were used to characterize and quantify mutations occurring in Chinook salmon MH genes across multiple individuals. The fish were all from Yellow Island Aquaculture in British Columbia, and DNA was extracted either from fin tissue or blood using a standard plate-based extraction method (Ephinstone et al. 2003). The MH class I α1 sequence library (396 sequences) was constructed using primers previously developed (Miller et al. 1997). The sequence of the sense primer was 5'-TGA CTC ACG CCC TGA AGT A-3', and the anti-sense primer was 5'-CTC CAC TTT GGT TAA AAC G-3' producing either a 228bp and/or a 222bp fragment(s). The PCR consisted of: 1μL of extracted DNA, 0.5 μL of each primer (100ng/μL), 2.5 μL 10X PCR buffer (10mM Tris-HCl (pH-8.4) 50mM KCl), 3.0μL of $MgCl_2$ (25mM), 1.0μL of dNTP's (200μM), and 0.5 units of DNA *Taq* polymerase (Invitrogen), and ddH_20 to make a 25μL reaction. The reaction profile consisted of: 2 min initial denaturation (95°C), 30 cycles of 30 sec denaturation (95°C), 30 sec annealing (52°C), 1 min extension (72°C), and 10 min extension (72°C). The MH class II β_1 sequence library (389

sequences) was constructed using primers previously developed by Docker and Heath (2002), and conditions identical to those used to amplify the α_1 gene. The sequence of the sense primer was 5'-CCG ATA CTC CTC AAA GGA CCT GCA-3', and the anti-sense primer was 5'-GGT CTT GAC TTG MTC AGT CA-3' producing a 294bp fragment. PCR products were ligated into a TA cloning vector, and inserts were amplified using the M13 forward primer (5'-GTA AAA CGA CGG CCA GT-3') and M13 reverse primer (5'-AAA CAG CTA TGA CCA TG-3'). The reactions were comprised of: 50-100 ng of template, 0.5μL of each primer, 2.5 μL of 10X PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 units DNA *Taq* polymerase (Sigma), and ddH_2O to adjust final volume to 25 μL. On average 10 sub-clones were used for sequencing for each fish. Inserts were sequenced using a $1/8^{th}$ reaction of a CEQ DTCS Quick Start Kit and analyzed on a CEQ 800 automated DNA sequencer (Beckman Coulter, Fullerton, CA). Sequence variants were confirmed in 15 sub-clones by reverse sequencing; therefore, we rejected sequencing error as the source of the sequence variation.

Analysis of Sequence Library

Alignments were constructed for all the MH class I α_1 sequences, and all class II β_1 sequences separately using Geneious Pro (Biomatters Limited) software. Because fish samples originated from a single captive population, there were relatively few alleles, and their identification was possible even with as few as 4 clones per fish. Once alleles were identified, base pair substitutions were recorded, and mutation frequency was calculated for each gene. To allow visualization of the relationships between the sequence variants, TCS version 1.21 was used to create a haplotype network with a subset of alleles and mutants obtained from the α_1 gene (Clement et al., 2000). To identify alleles versus

mutant sequences, a frequency distribution histogram was created for the MH class I α_1 sequences, and alleles were identified by a sequence frequency greater than four. The MH class II β_1 gene is substantially less polymorphic and detection of alleles was made directly by viewing alignments, and determining which sequences had occurred at a high frequency.

The number of nonsynonymous and synonymous base pair substitutions was determined for both α_1 and β_1 alleles and base pair substitution (BPS) mutants. Cross tabulation was used to determine whether the number of nonsynonymous and synonymous substitutions in the true alleles differed significantly from the substitutions found in the BPS mutants.

Chimera mutants were identified as having sequence polymorphisms from the two recognized alleles recombined to generate an apparently novel allele sequence. Chimera mutations were excluded from the mutation rate calculation, as studies have suggested these are likely PCR artefacts (Judo et al., 1998; Lenz and Becker, 2008; see below).

A 20-base-pair sliding window analysis was used to determine whether specific sequence regions had higher (or lower) substitution rates than expected. For each 20-base-sliding window, the number of mutations was counted and an average across all sliding windows was calculated. Mutation rate estimates obtained from individual sliding windows were used in a Z-score test to determine the probability of observing that value by chance.

PCR Treatments

To investigate the effect of PCR conditions on mutation frequency in the MH II β_1 gene, we ran a series of PCR treatments using DNA from two fish (Table 1). Each

Table 1. The experimental design used to determine the effect of PCR conditions on mutation frequency in the MH class II β_1 region. AmpliTaq® polymerase and Phusion® polymerase were used to PCR amplify the MH class II β_1 region in Chinook salmon. All treatments include the 30 PCR cycles conducted after cloning in the total number of PCR cycles.

Treatment	DNA polymerase	Total No. of PCR cycles
1	AmpliTaq®	60
2	AmpliTaq®	90
3	AmpliTaq®	120
4	AmpliTaq®	150
5	Phusion®	60
6	Phusion®	90
7	Phusion®	120
8	Phusion®	150

treatment differed in the number of total PCR amplification cycles (60, 90, 120, and 150 cycles), and each reaction was conducted with either AmpliTaq® (Applied Biosystems), or Phusion® High Fidelity polymerase (Finnzymes). Phusion®, unlike AmpliTaq®, has proof-reading ability, and is a Pyrococcus (*Pfu*)-like enzyme with a double-stranded DNA binding domain, which reportedly increases processivity. The expectation is that the observed frequency of mutations should be greater in the treatments with AmpliTaq® polymerase and with greater number of PCR cycles, if the mutations are resulting from PCR error. Furthermore, mutations should occur randomly across the PBR sequence if they are indeed artefacts, so identical mutations should not be observed in separate reactions. The conserved MH class II β_2 region was used as a control, since it was found to be highly conserved in comparison to the PBR region (Miller and Withler, 1996). If most of the mutations were generated by PCR error, we would predict similar mutation rates in both β_1 and β_2 regions.

DNA extraction and amplification

Chinook salmon collected from Yellow Island Aquaculture Ltd. (YIAL) in British Columbia, Canada were used for this experiment. DNA was extracted from fin tissue using a standard plate based extraction method (Elphinstone et al., 2003). The PBR β_1 of MH class II was PCR amplified with primers developed by Docker and Heath (2002) to produce a 294 bp fragment. The PCR conditions used are identical to those used for the sequence library except 1.0 units of AmpliTaq® polymerase (Applied Biosystems) was used. For treatments consisting of reactions greater than 60 total PCR cycles, 1μL of PCR product (obtained using the previous PCR) was added to a fresh master mix, and run for an additional 30 cycles. This was repeated until the number of desired PCR cycles was

achieved. For PCRs utilizing Phusion®, the reagent concentrations consisted of 1μL of extracted DNA, 0.7μL of each primer (100ng/μL), 4μL of 5x Phusion® HF buffer, 0.4μL of dNTPs (200μM), 0.2μL (0.02U/μL) of Phusion® DNA polymerase (Finnzymes), and ddH_20 to make a 20μL reaction. The thermal cycler profiles used were identical to those used to amplify the α_1 and β_1 gene, described above.

The conserved β_2 region (control) was PCR amplified in two fish using AmpliTaq® polymerase for 120 cycles. The sense primer (5'-CCG ATA CTC CTC AAA GGA CCT GCA-3'), is situated at the N-terminal region of the β_1 domain (Miller and Withler, 1996), and the antisense primer (5'-GGT CTT GAC TTG MTC AGT CA-3') is located at the end of the β_2 domain. The PCR conditions used were the same as those used to amplify the β1 domain. These primers amplified a single fragment approximately 290 bp in length.

Following PCR amplification, the products were cloned into the pGEM®-T vector (Promega) following the manufacturer's protocol. The insert was amplified using the M13 forward and reverse primers under the following conditions: 2 min initial denaturation (94°); then 35 cycles of 1 min denaturation (94°), 1 min annealing (55°), 1 min extension (72°); a final 3 min extension cycle (72°). Each 25μl reaction consisted of: 50-100ng of template DNA, 0.05μg of each primer, 0.5 units of DNA Taq polymerase, 25 mM $MgCl_2$, and 200 μM dNTPs. Approximately 29-65 sub-clones containing inserts were cleaned for each PCR-cycle treatment using AMPure (Agencourt) purification system, and sequenced using the M13 forward primer along with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification using CLEANseq (Agencourt), sequencing was performed using ABI BigDye Terminator version 3.1 on an

ABI 3130xl sequencer.

Data Analysis

Alignments were constructed for all β_1 sub-clone sequences (N=397), and β_2 sequences (N=58) with Geneious Pro (Biomatters Limited) software. Alleles were identified as the sequences occurring at the greatest frequency, and remaining sequences were characterized as either chimera or BPS mutants. Chimera and BPS mutation frequency was determined separately for each treatment by counting the number of sequences containing the mutation divided by the total number of sequences in that treatment. Mutations were pooled across all treatments for each polymerase type, and cross tabulation was used to determine whether there were significant differences between the total number of chimera and BPS mutants using AmpliTaq® versus Phusion®. A log-linear analysis was conducted to determine whether there was a significant effect of polymerase type on the frequency of BPS and chimeras produced, and whether the number of PCR amplification cycles (when combining all treatments) had a significant effect on the frequency of BPS and chimeras produced (SYSTAT version 12). Finally, cross-tabulation was used to determine whether the number of mutations observed in the β_2 exon (n= 58) was significantly different from the mutation count observed in the β_1 exon.

RESULTS

Sequence Library

MH class I α_1: Analysis of 396 MH class I α_1 sequences (n=38 fish) revealed 86 distinct sequences. Fifteen alleles were identified, with seven of those previously identified (GenBank accession numbers: DQ647923, DQ647922, U80293, U80273,

U80284, AF362114, and AF162868). BPS in the MH class I α_1 sequences occurred at a frequency of 15.2%, and chimeras occurred at a frequency of 4.8%. For this study we further analyze BPS, but excluded chimeras from our analyses since they are known to be common PCR artefacts. Sequences with only one BPS represented 88.3% of the BPS mutants, and sequences having two BSP represented 11.7% of the BPS mutations. The haplotype network for alleles DQ647922 and U80284 shows a typical BPS mutation pattern dominated by single BPS mutations (Figure 1a). Of the sequences with mutations a large proportion of the sequences 72% were singletons i.e. were found only once in the sequence library (Figure 1b). Across all sequences and both genes, the majority of BPS (88.1%) occurred in only a single clone sequence (Figure 2a). Identical BPS occurring twice (in separate PCRs) represented 6.8% of mutations, and identical BPS occurring three times represented 5.1% of mutations (Figure 2a). As expected, most of the BPS in both the true MH PBR α_1 alleles and the mutations were nonsynonymous (87% and 60% respectively). However, the frequency of nonsynonymous substitutions in the α_1 alleles was significantly greater than in the BPS mutations (χ^2=14.83; p<0.0010).

MH class I α_1 transitions made up 75% of the observed BPS, while transversions represented 25% of mutations. Furthermore, A→G mutations occurred at the highest frequency (33.3%; Table 2), followed by T→C mutations (20%; Table 2). The frequency of BPS mutations found in the 37-61bp (z score=2.39; p=0.0082 and z score=1.88; p=0.03), and 113-132bp (z score=1.88; p=0.03) region of the α_1 gene was found to be significantly higher than background (Figure 3a). Also, in the MH class I α_1 gene the 154-194bp region had significantly lower levels of base-pair substitution than background (z score = -2.25; p=0.012 and z score=-1.73; p=0.04; Figure 3a).

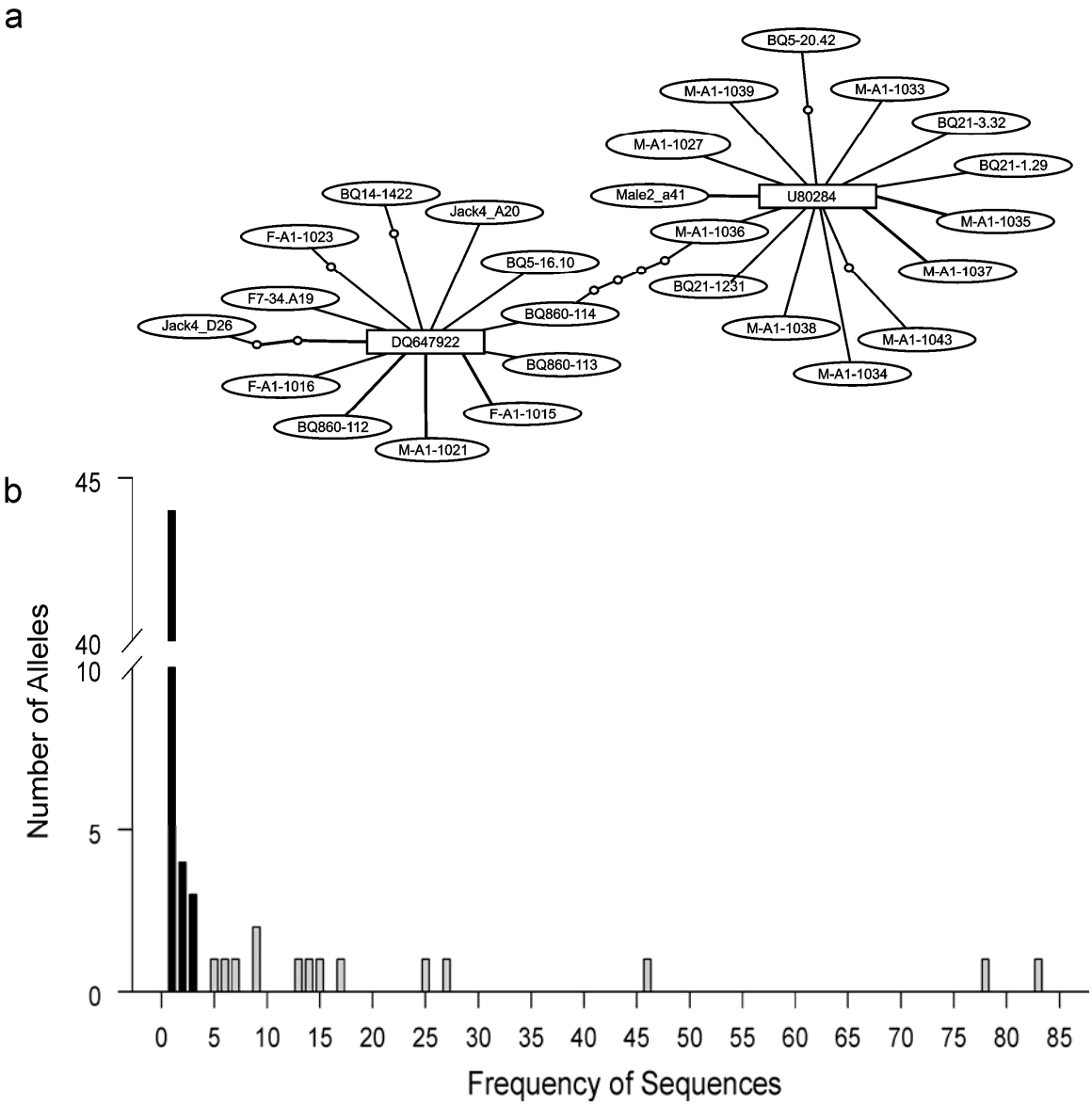

Figure 1. Panel a: Haplotype network of a subset of sequences (n=31) obtained from the MH class I α_1 gene library for Chinook salmon. Allele labels are enclosed by rectangles and mutants by ovals. The number of connecting branches represents the number of base pair differences between sequences. Panel b: The number of alleles and their respective sequence frequencies for the MH class I α_1 gene library (N=396) for Chinook salmon. Sequence frequencies greater than five (in grey) are the alleles.

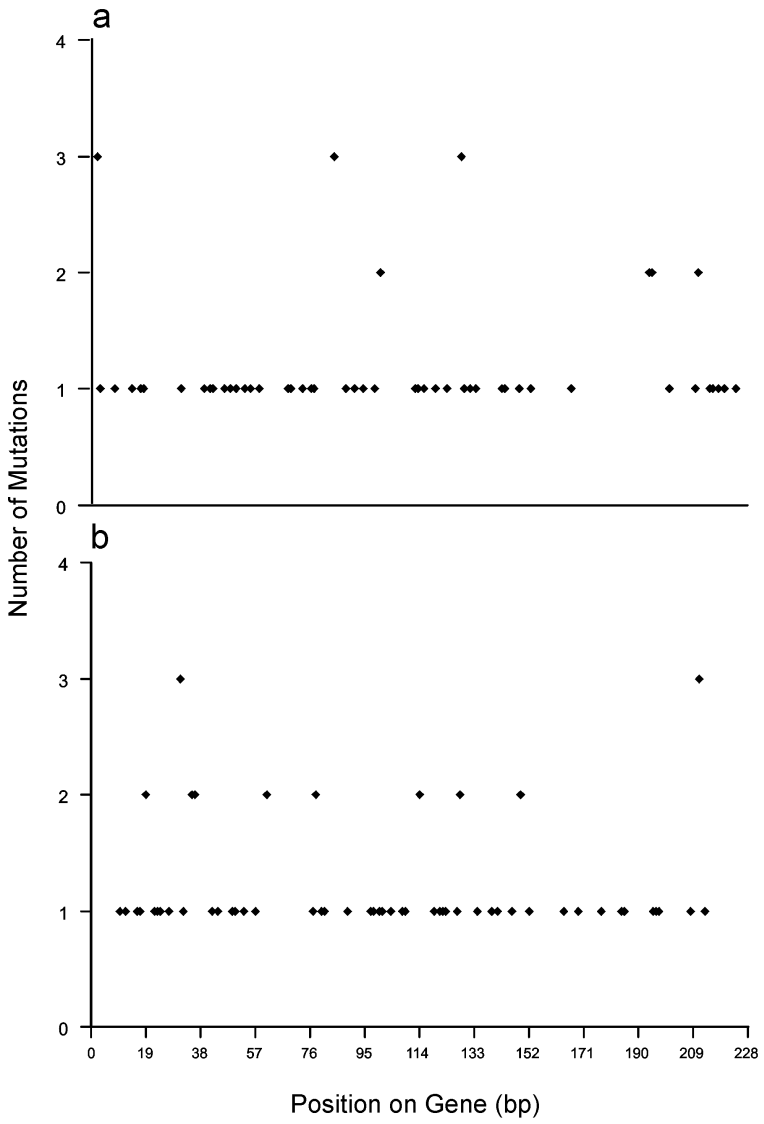

Figure 2. The position and frequency of single base pair substitutions (BPS) along the gene in the 3' to 5' direction (complementary to the sense primer) resulting from separate PCR reactions. Panel a: MH class I α_1 peptide binding region (N=61 BPS; 222-228 bp), and Panel b: MH class II β_1 peptide binding region (N=68 BPS; 213 bp).

Table 2. Distribution of nucleotide substitutions in the MH class I α_1 (n=61mutations) and MH class II β_1 (n=68 mutations) sequence libraries from Chinook salmon. Bolded frequencies represent the two highest values.

			Original Base				
			A	**T**	**G**	**C**	**Total**
Substitution	**MH class I α1**	**A**		0.050	0.100	0.017	0.230
		T	0.067		0.050	0.117	0.475
		G	**0.333**	0.000		0.017	0.131
		C	0.050	**0.200**	0.000		0.164
		Total	0.450	0.250	0.150	0.150	1.000
	MH class II β1	**A**		0.015	0.044	0.000	0.059
		T	0.029		0.044	0.162	0.235
		G	**0.412**	0.000		0.000	0.412
		C	0.029	**0.265**	0.000		0.294
		Total	0.471	0.279	0.088	0.162	1.000

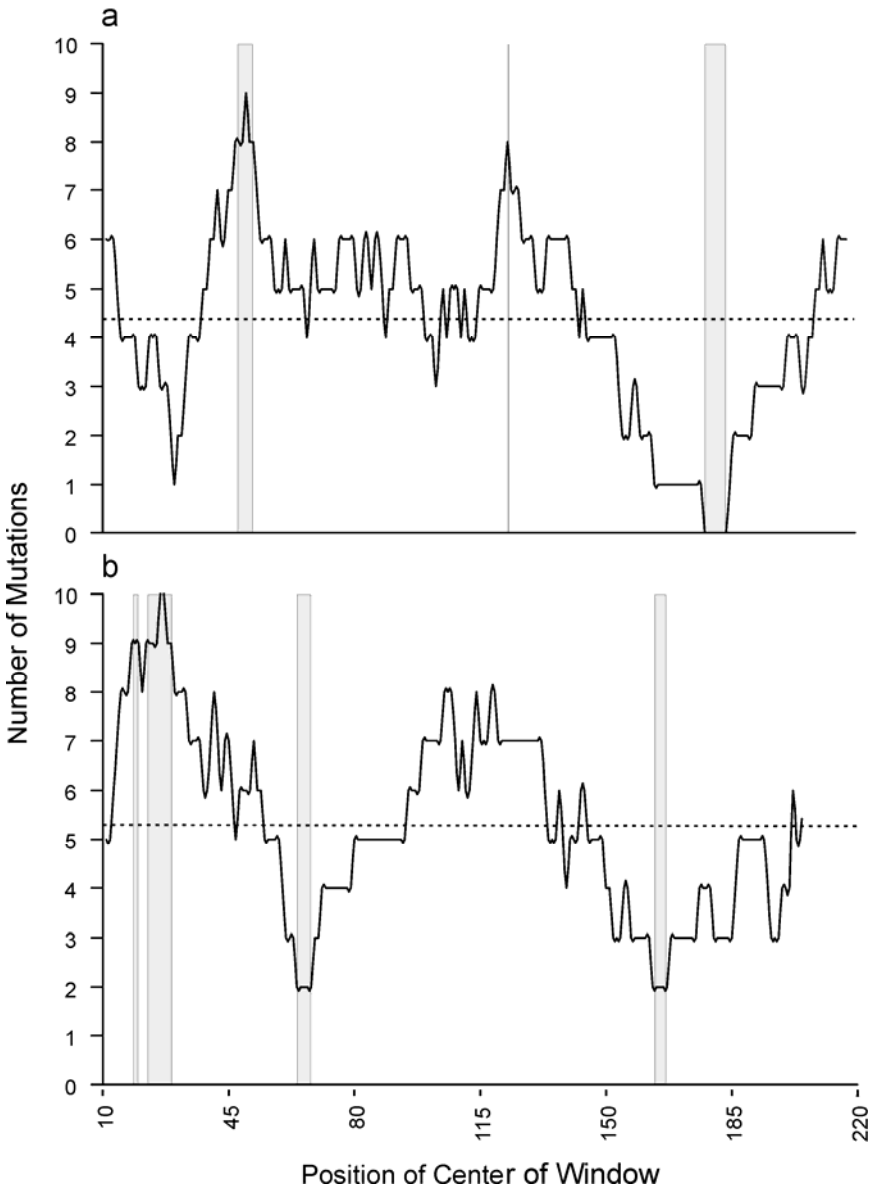

Figure 3. Line graphs showing the number of mutant base pairs in a sliding 20-base-pair window analysis of the MH sequence library data. Panel a: MH class I α_1 PBR, and Panel b: MH class II β_1 PBR. The dashed lines indicate the mean mutation value across the entire region, and grey shaded areas represent mutation counts in the sliding window found outside the 95% confidence interval.

MH class II β_1: The 389 MH class II β_1 sequence library (n= 29 fish) revealed 73 distinct sequences. Four alleles were identified in our MH class II β_1 sequence library and all have been reported previously (GenBank accession numbers: DQ450874, AY100006, AY100007, and AY100008). The BPS frequency in the MH class II β_1 sequence library was 17.5%, and the frequency of chimeras was 3.3%. Sequences with only one BPS accounted for 82.9% of the total identified mutations; two BPS sequences represented 15.8% of mutations, and one sequence was found to have 3 BPS representing 1.3% of mutations. Most mutations (84%; Figure 2b) were unique, occurring only once in the sequence library; however, 13% of mutations (Figure 2b) were found occurring in another sequence generated from a separate PCR reaction, and two mutations occurred in three separate reactions (3%; Figure 2b). Similar to the α_1 gene, the number of nonsynonymous and synonymous substitutions in β_1 alleles differed significantly from the number of substitutions in the sequences with BPS mutations (χ^2=5.59; p=0.018) with the alleles having a larger proportion of nonsynonymous mutations than the mutants (88.8% and 74% respectively).

Analysis of nucleotide substitutions at MH class II β_1 revealed that transitions occurred at a frequency of 88%, with transversions occurring at a frequency of 13%. There was a strong bias for A→G substitutions (41.2%), followed by T→C substitutions (26.5%; Table 2). The 20 base sliding window analysis revealed that the 17-36bp region had a significantly elevated frequency of BPS (χ2=5.062; p=0.024) (Figure 3b).

Analysis of PCR Effects on Mutations

The possibility of somatic mutations in the MH PBR has been masked by a perception of high levels of PCR error, and researchers consequently dismissed all “rare”

MH PBR sequences as probable PCR artefacts. Thus the purpose of our PCR experiments was to determine the effect of PCR conditions on MH PBR mutations, as well as to characterize those mutations. The number of clones sequenced for each treatment ranged from 29 to 65, giving a total of 397 sequences from two fish for the MH class II β_1 gene: two alleles were identified differing by six base pair substitutions. The first allele (GenBank accession: AY100008) represented 5.4% of the sequences, the second allele (GenBank accession: DQ450874) represented 53.0% of the sequences, and the remaining sequences exhibited mutations and were placed in either the BPS or chimera category. Of the mutant sequences, three were found in GenBank (accessions AY100009, EF432121, and EF432117), and all were chimeras.

Of the mutations produced with AmpliTaq® polymerase, 50% were chimeras, while the remaining mutant sequences were BPS. Of the mutations produced by Phusion® polymerase, 94% were chimeras, while only 6% were BPS. Pooling sequences across all treatments, there is a significant difference (χ^2=39.96; $p<0.001$) in the total number of chimeras produced by the two polymerases, with Phusion® producing a greater frequency (41.0%) than AmpliTaq® (13.1%). A log-linear analysis used to test for the effect of polymerase and PCR cycle number revealed polymerase type significantly affected chimera production (χ^2=23.15; $p<0.001$), as did the number of PCR amplification cycles (χ^2=73.13; $p<0.001$; Figure 4). AmpliTaq® produced a greater frequency of BPS (13.1%) than Phusion® polymerase (2.7%), and the effect of polymerase type was significant (χ^2=16.16; $p<0.001$). The number of PCR amplification cycles did not have a significant effect on the frequency of BPS across all treatments (χ^2=2.731; $p=0.44$).

An alignment of all of the MH class II β_2 (trans-membrane) sequences (N = 58)

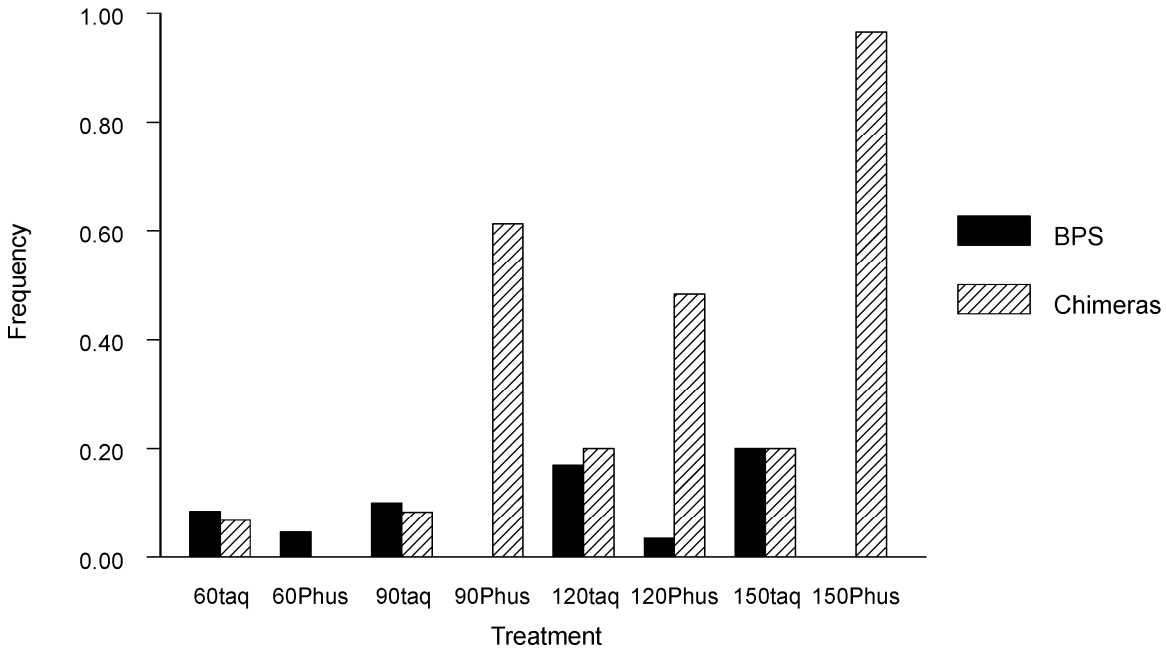

Figure 4. The frequency of chimeras and base-pair substitutions (BPS) with various PCR cycle numbers and two polymerase enzymes AmpliTaq® and Phusion®. The number of PCR amplification cycles significantly affected chimera frequency (χ^2=73.13; p<0.001), as did polymerase type (χ^2=23.15; p<0.001). Polymerase type had a significant effect on BPS frequency (χ^2=16.16; p<0.001); however, the number of PCR cycles did not have a significant effect on BPS frequency (χ^2=2.731; p=0.435).

included as a conserved region, revealed two alleles differing by a single synonymous base pair substitution. These two sequences had both been previously identified (GenBank accession numbers U34719 and U34718). Seven mutant sequences (12.1%) were identified; none were found on GenBank. Since this gene contained two sequences differing by a single BPS, we could not reliably identify chimeras. As a result, only the frequency of BPS in β_1 and β_2 genes were compared. The BPS frequency in the β2 exon (12.1%) was not significantly different from the BPS frequency in the β_1 exon of (16.9%) at 120 PCR cycles (χ^2=0.5780; p=0.45).

DISCUSSION

In this study we analyzed MH class II β_1 and MH class I α_1 sequences to provide insight into the nature of MH mutations. In both sequence libraries we discovered BPS occurring at identical sites but in different reactions. The probability of this occurring by chance is very low; therefore, it is more likely that these mutants represent somatic mutations. The criteria for acceptance of new human MHC alleles is that the sequence must be represented by two or more clones, preferably from separate reactions (Marsh et al., 2005). Applying this rule, the mutations we identified are likely somatic mutations, and their low occurrence is reflective of somatic mutations being rare events. We also found that A→G substitutions predominated over other mutations, followed by T→C mutations, in both the α_1 and β_1 genes, with equal substitution rates in C and G in the α_1 gene. A study on somatic mutation in immunoglobulin genes of a teleost fish revealed a predominant accumulation of G·C→A·T substitutions, and this has also been shown in humans to occur as a result of the spontaneous deamination of DNA bases (Yang et al., 2006; Cooper and Youssoufian, 1988). In this study we found an accumulation of A→G

and T→C substitutions because coding DNA sequences (complementary to template) were analyzed; however, if cDNA were used we would observe the Watson-Crick complement and G→A and C→T mutations would predominate. Furthermore, there are general patterns associated with mammalian somatic mutation in immunoglobulin genes such as the approximately equal substitution rates in C and G, and a greater number of mutations in A versus T, which is what we also found in the MH genes (Smith et al., 1996; Milstein et al., 1998; Foster et al., 1999). Our analysis of the sequence library data revealed mutation clusters or "hotspots" in both the β_1 and α_1 gene; although *Taq* error may be responsible for the majority of BPS, it is plausible that the MH sequence characteristics increases the likelihood of polymerase error in particular areas of the gene, and the same mechanism could be occurring *in vivo* and *in vitro*.

There are no existing studies on somatic mutations in the MH genes; however, somatic mutations in the immunoglobulin genes are well characterized (e.g. Lee et al., 2002; Rogozin et al., 2001; Shapiro et al., 2002; Yang et al., 2006). One study found that the complementarity-determining regions (CDRs) in immunoglobulins, which form antigen binding sites, have a greater frequency of somatic mutations than the framework regions (Rogozin et al., 2001). Similarly, increased somatic mutation rates in the PBR of MH would be advantageous as it would increase the MH repertoire, and could potentially be beneficial when encountering novel pathogens. However, there exists a limit to the advantages of MH allele diversity because adding new MH molecules would increase the potential for destruction of T cells that are activated by MH molecules complexed with self peptides in the thymus (Matzinger et al., 1984). There are published examples where a lack of MHC PBR polymorphism has had deleterious effects on an organism through

increasing susceptibility to pathogens (O'Brian et al., 1985; Siddle et al., 2007). Chinook salmon are anadromous and as a result they encounter different pathogen communities as they migrate from fresh water to their ocean rearing environment at about eight months of age. Allelic diversity at the MH genes may play an important role in their survival and fitness, and several studies have linked MH allelic characteristics with increased survival when encountering specific pathogens in salmonids (Arkush et al., 2002; Langefors et al., 2001). Chinook salmon have a limited number of MH loci, and we suggest that somatic mutation may be an important mechanism for increasing allelic diversity at the MH loci in Chinook salmon.

In this study, we analyzed mutation rates when amplifying the MH class II β_1 gene in Chinook salmon under various PCR cycle numbers, and with proof-reading (Phusion®) versus non proof-reading (AmpliTaq®) polymerase. We found that the polymerase used had a significant effect on the type of mutations produced, with AmpliTaq® producing a greater frequency of BPS, and Phusion® producing a greater frequency of chimeras. It is not surprising that AmpliTaq® produced a greater frequency of BPS since it has no proof-reading capabilities. Furthermore proof-reading polymerases have been shown to create chimera sequences at a high rate (Zylstra et al., 2008), and we found that higher numbers of PCR cycles exacerbated this problem. A study by Zylstra and colleagues (1998) showed that *Pfu*, another proof-reading polymerase, increased the formation of recombination artefacts, and they suggest reducing PCR cycle number to minimize or eliminate those artefacts (Judo et al., 1998). Additionally, Borriello and Krauter (1990) significantly reduced chimera sequence frequency using "PCR+1", which effectively eliminated/reduced PCR heteroduplexes,

and in turn sequence scrambling in *E. coli*. Although the most frequently occurring artifact mutations formed during PCR are chimeras, they are generally easily identified and eliminated. However, three chimera sequences identified in this study were found in GenBank, thereby providing evidence that undetected PCR artefacts have been mistaken for true MH PBR alleles in past studies, and more caution should be taken, particularly for highly polymorphic genes. We also found that the number of PCR cycles did not have a significant effect on the frequency of BPS. This is surprising since the proportion of mutant sequences produced is a function of the number of PCR cycles assuming that all PCR amplicons will be amplified at an equal rate. We acknowledge that PCR bias occurs, and somatic mutations likely represented a portion of mutations; nevertheless, new mutations are created in each cycle, and further amplified in subsequent cycles so we expected to observe an increase in the frequency of mutations with PCR cycle number.

The mechanism of allele diversification of the MH genes is largely unknown, and we propose that somatic mutation is a plausible mechanism for the diversification of MH alleles. This is the first study to analyze MH sequence variants in Chinook salmon for somatic mutations. In previous MH studies, sequence mutants were eliminated from the analyses unless they were common, and thus were deemed a true allele. This practice may have masked the presence of unexpected allele diversifying mechanisms such as somatic mutations. On the other hand, it is important that sequences are examined thoroughly before including them as possible alleles, since including artefacts in genetic analysis will inflate allele diversity estimates, as possibly bias estimates of other effects such as recombination (Lenz and Becker, 2008). In order to identify true alleles reliably and the most likely candidates for somatic mutation, we recommend using a proof-

reading polymerase, the lowest number of PCR cycles possible, and, ideally, conducting multiple independent PCRs for each sample. Future planned studies will determine whether the putative somatic mutations are expressed. Our findings provide insight into a possible novel mechanism acting to enhance the immune function of Chinook salmon through the creation of somatic MH diversity.

LITERATURE CITED

Alcaide, M., Edwards, S.V., Negro, J.J., Serrano, D., Tella, J.L., 2008. Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (*Falco naumanni*). Molecular Ecology 17, 2652-2665.

Allendorf, F.W., Thorgaard, G.H., 1984. Tetraploidy and the evolution of salmonid fishes. In: Evolutionary genetics of fishes (Turner BJ, ed). New York: Plenum, 1-53.

Arkush, K.D., Giese, A.R., Mendonca, H.L., McBride, A.M., Marty, G.D., Hedrick, P.W., 2002. Resistance to three pathogens in the endangered winter-run Chinook salmon (Oncorhynchus tshawytscha): effects of inbreeding and major histocompatibility complex genotypes. Canadian Journal of Fisheries and Aquatic Sciences 59 (6), 966-975.

Bernatchez, L., Landry, C., 2003. MHC studies in non model vertebrates: what have we learned about natural selection in 15 years? Journal of Evolutionary Biology 16, 363-377.

Borriello, F., Krauter, K.S., 1990. Reactive site polymorphism in the murine protease inhibitor gene family is delineated using a modification of the PCR reaction (PCR+1). Nucleic Acids Research18 (8), 5481-5487.

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9 (10), 1657-1660.

Cooper, D.N., Youssoufian, H., 1988. The CpG dinucleotide and human genetic disease. Human Genetics 78, 151-155.

Cuesta, A., Esteban, A.M., Meseguer, J., 2005. Cloning, distribution, and up- regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells. Molecular Immunology 43, 1275-1285.

Docker, M.F., Heath, D.D., 2002. PCR-based markers detect genetic variation at growth and immune function-related loci in Chinook salmon (*Oncorhynchus tshawytscha*). Molecular Ecology 2, 606- 609.

Eckert, K.A., Kunkel, T.A., 1991. DNA polymerase fidelity and the polymerase chain reaction. Genome Research 1, 17-24.

Eckert, K.A., Kunkel, T.A., 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. Nucleic Acids Research18, 3739-3744.

Elphinstone, M.S., Hinten, G.N., Anderson, M.J., Nock, C.J., 2003. An inexpensive and high-throughput procedure to extract and purify total genomic DNA for population studies. Molecular Ecology 2, 317-320.

Ennis, P.D., Zemmour, J., Salter, R.D., Parham, P., 1990. Rapid cloning of HLA-A,B cDNA by using the polymerase chain reaction: frequency and nature of errors produced in amplification. Proceedings of the National Academy of Sciences of the United States of America 87, 2833-2837.

Evans, M.L., Neff, B.D., Heath, D.D., 2009. MHC genetic structure and divergence across populations of Chinook salmon (Oncorhynchus tshawytscha). Heredity, 1-11.

Foster, S.J., Dorner, T., Lipsky, P.E., 1999. Targeting and subsequent selection of somatic hypermutations in the human V_K repertoire. European Journal of Immunology 29, 3122- 3132.

Grimholt, U., Drablos, F., Jorgensen, S.M., Hoyheim, B., Stet, R.J.M., 2002. The major histocompatibility class I locus in Atlantic salmon (Salmo salar L.): polymorphism, linkage analysis and protein modeling. Immunogenetics 54, 570-581.

Grimholt, U., Olsaker, I., Lindstrøm, C.V., Lie, Ø., 1994. A study of variability in the MHC class II β1 and class I α2 domain exons of Atlantic salmon, Salmo salar L. Animal Genetics 25, 147-153.

Horton, R., Wilming, L., Rand, V., Lovering, R.C., Bruford, E.A., Khodiyar, V.K., Lush, M.J., Povey, S., Talbot Jr, C.C., Wright, M.W., Wain, H.M., Trowsdale, J., Zielger, A., Beck, S., 2004. Gene map of the extended human MHC. Nature Reviews Genetics 5, 889-899.

Judo, M.S.B., Wedel, A.B., Wilson, C., 1998. Stimulation and suppression of PCR-mediated recombination. Nucleic Acids Research26, 1819-1825.405.Kanagawa, T., 2003. Bias and artefacts in multitemplate polymerase chain reactions (PCR). Journal of Bioscience and Bioengineering 96, 317-323.

Klein, J., Figueroa, F., 1986. Evolution of the major histocompatibility complex. CRC Critical Review in Immunology 6, 295-386.

Landry, C., Garant, D., Duchesne, P., Bernatchez, L., 2001. 'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (Salmo salar). Proceedings of the Royal Society of London, Series B 268, 1279-1285.

Langefors, A., Schantz, T., Widegren, B., 1998. Allelic variation of MHC class II in Atlantic salmon; a population genetic analysis. Heredity 80, 568-575.

Langefors, A., Lohm, J., Grahn, M., Andersen, O., Shanz, T., 2001. Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. Proceedings of the Royal Society of London, Series B 268, 479-485.

L'Abbé, D., Belmaaza, A., Decary, F., Chartrand, P., 1992. Elimination of heteroduplex artefacts when sequencing HLA genes amplified by polymerase chain-reaction (PCR). Immunogenetics 35, 395-397.

Lawlor, D.A., Zemmour, J., Ennis, P.D., Parham, P., 1990. EVOLUTION OF CLASS-I MHC GENES AND PROTEINS: from natural selection to thymic selection. Annual Review in Immunology 8, 23-63.

Lee, S.S., Tranchina, D., Ohta, Y., Flajnok, M.F., Hsu, E., 2002. Hypermutation in shark immunoglobulin light chain genes results in contiguous substitutions. Immunity 16, 571-582.

Lenz. T.L., Becker, S., 2008. Simple approach to reduce PCR artefact formation leads to reliable genotyping of MHC and other highly polymorphic loci – Implications for evolutionary analysis. Gene 427, 117-123.

Longeri, M., Zanotti, M., Damiani, G., 2002. Recombinant DRB sequences produced by mismatch repair of heteroduplexes during cloning in *Escherichia coli*. European Journal of Immunogenetics 29, 517-523.

Marsh, S.G.E., WHO Nomenclature Committee for Factors of the HLA System., 2006. Nomenclature for factors of the HLA system, update December 2005. Tissue Antigens 67, 262-264.

Matzinger, P., Zamoyska, R., Waldman, H., 1984. Self tolerance is H-2 restricted. Nature

308, 738-741.
Miller, K.M., Withler, R.E., 1996. Sequence analysis of a polymorphic MHC class II gene in Pacific salmon. Immunogenetics 43, 337-351.
Miller, K.M., Withler, R.E., Beacham, T.D., 1997. Molecular evolution at MHC genes in two populations of Chinook salmon *Oncorhynchus tshawytscha*. Molecular Ecology 6, 937-954.
Miller, K.M., Withler, R.E., 1998. The salmonids class I MHC: limited diversity in a primitive teleosts. Immunological Reviews 166, 279-293.
Milstein, C., Neuberger, M.S., Staden, R., 1998. Both DNA strands of antibody genes are hypermutation targets. Proceedings of the National Academy of Sciences of the United States of America 95, 8791-8794.
Modrich, P., 1987. DNA mismatch correction. Annu. Rev. Biochem. 56, 435-466.
Moxon, E.R., Rainey, P.B., Nowak, M.A., Lenski, R.E, 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. Current Biology 4, 24-33.
Nei, M, Hughes, A.L., 1991. Polymorphism and evolution of the major histocompatibility complex loci in mammals. Evolution at the Molecular Level 87, 222-247.
O'Brien, S.J., Roelke, M.E., Marker, L., Newman, A., Winkler, C.A., Meltzer, D., Colly, L., Evermann, J.F., Bush, M., Wildt, D.E., 1985. Genetic basis for species vunerability in the cheetah. Science 227, 1428-1434.
Pavlov, A.R., Pavlova, N.V., Kozyavkin, S.A., Slesarev, A.I., 2004. Recent developments in the optimization of thermostable DNA polymerases for efficient applications. Trends in Biotechnology 22 (5), 253-260.
Pitcher, T.E., Neff, B.D., 2006. MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in Chinook salmon. Molecular Ecology 15, 2357-2365.
Pompanon, F., Bonin, A., Bellemain, E, Taberlet, R., 2005. Genotyping errors: causes, consequences and solutions. Nature Reviews Genetics 11, 847-859.
Qiu, X., Wu, L., Huang, H., McDonel, P.E., Palumbo, A.V., Tiedje, J.M., Zhou, J., 2001. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Applied Environmental Microbiology 67 (2), 880-887.
Rogozin, I.B., Pavlov, Y.I., Bebenek, K., Matsuda, T., Kunkel, T.A., 2001. Somatic mutation hotspots correlate with DNA polymerase η error spectrum. Nature 2, 530-536.
Shapiro, G.S., Aviszus, K., Murphy, J., Wysocki, L.J., 2002. Evolution of Ig DNA sequence to target specific ase positions within codons for somatic hypermutation. Journal of Immunology 168, 2302-2306.
Siddle, H.V., Kreiss, A., Eldridge, M.D.B., Noonan, E., Clarke, C.J., Pyecroft, S., Woods, G.M., Belov, K., 2007. Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. Proceedings of the National Academy of Sciences of the United States of America 104, 16221-16226.
Smith, D.S., Creadon, G., Jena, P.K., Portanova, J.P., Kotzin, B.L., Wysocki, L.J., 1996. Di- and trinucleotide target preferences of somatic mutagenesis in normal and autoreactive B cells. Journal of Immunology 156, 2642-2652.
Stet, R.J.M., Vries, B., Mudde, K., Hermsen, T., Heerwaarden, J., Shum, B.P., Grimholt, U., 2002.Unique haplotypes of co-segregating major histocompatility class II A

and class II B alleles in Atlantic salmon (*Salmo salar*) give rise to diverse class II genotypes. Immunogenetics 54, 320-331.

Yang, F., Waldbieser, G.C., Lobb, C.J., 2006. The nucleotide targets of somatic mutation and the role of selection in immunoglobulin heavy chains of a teleost fish. Journal of Immunology 176, 1655-1667.

Zylstra, P., Rothenfluh, H.S., Weiller, G.F., Blanden, R.V., Steele, E.J., 1998. PCR amplification of murine immunoglobulin germline V genes: strategies for minimization of recombination artefacts. Immunology and Cell Biology 76, 395-405.

CHAPTER THREE

Sperm competition and MH-mediated mate choice in jack versus hooknose Chinook salmon (*Oncorhynchus tshawytscha*)

INTRODUCTION

Sexual selection is widely recognized as a powerful evolutionary process having important consequences for reproductive behaviour, morphology, and fitness (Birkhead and Pizzari, 2002). The majority of research on sexual selection is focused on pre-copulatory mate choice; however, the discovery of female promiscuity and polyandry indicated that mate choice also persists after copulation (Birkhead and Pizzari, 2002). For males, it is well-known that promiscuity has evolved to increase reproductive fitness; however, the female benefits are less pronounced, and researchers predict that females copulate with multiple males to increase genetic diversity and fitness in offspring (Birkhead and Pizzari, 2002; Dixson and Anderson, 2001). Sexual selection occurring post-copulation is the result of two possible mechanisms: sperm competition and/or cryptic female choice (CFC). Sperm competition is competition between the sperm of multiple males to fertilize ova (Parker, 1984), and CFC occurs when the female influences the outcome of sperm competition (Eberhard, 1996). Although the mechanisms for sperm competition vary across taxa, the outcome can typically be predicted based on the knowledge of relative sperm numbers, sperm quality, the timing of inseminations, and the timing of female ovulation (Birkhead and Pizzari, 2002). In contrast, identifying CFC is challenging as it is often subtle, and masked by male-driven components (Birkhead and Pizzari, 2002).

The genes most extensively studied in mate choice research are the major histocompatibility complex (MHC) genes, presumably as a result of its crucial function in the immune system as well as its role in behavioural contexts. The MHC genes code for cell-surface receptors which bind self and non-self peptides, and present them to T cells (Lawlor et al., 1990). There are two functionally distinct MHC molecules, class I and class II, and they present peptides derived from intracellular and extracellular antigens respectively. The genes encoding the peptide binding region (PBR) of the MHC molecule are highly polymorphic and the focus of all MHC research. In mammals the MHC class I and class II genes are located in close proximity forming a linkage group or "complex" (Klein, 1986). Interestingly, in teleosts, the genes encoding the class I and class II genes are unlinked, and since they do not form a single complex they are more correctly referred to as MH genes (Stet et al., 2002). Because of the highly polymorphic nature of

the MHC PBR genes, they have been candidate genes in numerous mate choice studies. Researchers postulate that the mechanism for MHC-mediated pre-copulatory mate choice involves individual MHC-influenced body odours (in the form of soluble MHC molecules, fragments, or ligands), which females use as a cue to assess her potential partner's MHC genotype (Brown et al., 1989; Singh et al., 1987). While there have been several studies exploring the effect of MHC on pre-copulatory mate choice (reviewed in Tregenza and Wedell, 2000), there is evidence the MHC may also play a role in post-copulatory sexual selection, presumably through egg-sperm interactions (Wedekind et al., 1996; Rülicke et al., 1998). Salmonid species are a good model system for the study of post-copulatory sexual selection, and specifically for investigating sperm competition and CFC because fertilization is external, making in-vitro fertilizations possible under controlled settings. The structure of the teleosts egg consists of an outer envelope (the chorion), and unlike mammals, only a single location for sperm entry into the egg (the micropyle; Kamler, 1992). The teleost egg offers a unique opportunity for investigating sperm competition because having only a single entrance available for fertilization should dramatically elevate sperm competition, and result in the evolution of characteristics that increase sperm competitiveness. The micropyle may also facilitate CFC. During sperm attraction and movement through the funnel-shaped micropyle, the potential exists for the egg to favor certain sperm over others, and this should lead to mate choice based on genetic compatibility at loci such as the MH genes (Skarstein et al., 2005).

Male Chinook salmon compete aggressively for spawning access to females to ensure that their sperm will fertilize the eggs (Esteve, 2005). Male Chinook salmon that mature precociously and return to freshwater at least one year earlier than other fish in the same cohort are referred to as "jacks," while males with later maturation are referred to as "hooknose" males (Heath et al., 1994). Usually, the larger hooknose males with well-developed secondary sexual characteristics have an advantage over the smaller jacks, since the larger males guard females to prevent other males from fertilizing the eggs (Esteve, 2005). However, the smaller jacks have developed an alternative strategy to obtain fertilizations using a "sneaking" tactic (Esteve, 2005). In this tactic, jacks remain strategically hidden from the hooknose males and the females until female oviposition, when the jacks emerge from hiding, and use their small body size and cryptic colouration

to gain a position closer to the female's vent (Esteve, 2005). Research has shown that with this reproductive strategy jacks can achieve comparable paternity to that of hooknose males (Fleming and Reynolds, 2004). Although it is generally thought that jacks are the inferior male phenotype (Gross, 1984), studies have suggested that jacks have a survivorship advantage over hooknose males since exposure to ocean factors such as predators and parasites is reduced by at least one year (Gross, 1991). Because the quality of available refuges for jacks may not always be favourable for reproductive success, it is plausible that the jack phenotype would be under strong selection to evolve a strategy to enhance fertilization success through sexual selection for genetic and physical traits increasing jack sperm competitiveness.

In this study, I investigated the outcome of sperm competition between jack and hooknose male Chinook salmon in a 5x5 in-vitro fertilization experiment. Microsatellite paternity analysis revealed that jacks out-competed hooknose males, and fertilized the majority of eggs. An analysis of variance (ANOVA) conducted on these results revealed that the majority of the variance was attributed to differences in sperm traits between jack and hooknose males; however, significant interaction effects indicate a sperm-egg compatibility mechanism was also playing a role. In this study I further investigate whether genetic compatibility at the MH genes had influenced the outcome of sperm competition. I show that jacks fertilized eggs at the highest frequency when their MH class II β_1 alleles were more similar to the female's alleles than the hooknose males. This study provides the first evidence for a substantial sperm advantage, and a novel role for the MH genotype in sperm-egg interactions during fertilization resulting from jack Chinook salmon. These results have important implications in fundamental evolutionary biology, and practical implications for the successful propagation and management of the species.

METHODS AND MATERIALS

Sperm Competition

The Chinook salmon used in this study were from the Quinsam River, British Columbia. Gametes were collected from a total of ten males and five females in reproductive condition. The males consisted of five mature males ("hooknose males") and five jacks. Jacks were distinguished from hooknose males based on their lack of

secondary sexual characteristics, gonad inspection, and body size (Heath et al. 2002). Males were dried around the vent and stripped of sperm by applying gentle abdominal pressure. Care was taken to avoid contamination with urine, mucous, and water during stripping and storage. Spermatocrit (the percentage of the milt volume occupied by the spermatozoa) was measured (Skarstein et al. 2005) for all males, and sperm density was corrected so that eggs were fertilized with the same number of sperm cells from each male. Eggs were collected by humanely euthanizing the fish and cutting the abdominal wall to release the eggs. Eggs from each female were divided into five equal groups, and sperm from a sire pair (consisting of the mixture of sperm from one jack and one hooknose male) was added to one group of eggs from each dam. This was repeated so that each sire pair fertilized one group of eggs for each dam to make a 5 x 5 factorial cross with a total of 25 maternal half-sib families. Each egg/sperm mixture was then activated by adding approximately 3X its volume of fresh water, and eggs were then incubated separately in a vertical stack incubator. The experiment was terminated when the eggs developed eyespots, and 48 eggs were selected from each cross for genetic parentage assignment and MH genotyping.

Parentage Assignment

DNA was extracted from parental fin clips, as well as from embryos dissected from the eyed eggs, using a standard plate-based extraction method (Elphinstone et al., 2003). Parentage was determined for 48 offspring per cross using microsatellite markers: Ots107 (Nelson and Beacham, 1999), OtsG83b (Williamson et al., 2002), and Omy1191UW (Spies et al., 2005). Parental and offspring DNA were amplified in polymerase chain reactions (PCR) with solutions comprised of: 1.0 μL of extracted DNA, 0.50 μL of each primer (100 ng/μL), 2.5 μL 10x reaction buffer, 3.0 μl $MgCl_2$ (25mM), 1.0 μl dNTP's (200 μM), 0.20 U AmpliTaq® polymerase (Applied Biosystems, Foster City, CA), and ddH_20 to make a 25 μl reaction. The reaction profile consisted of: 2 min. initial denaturation (95°C), 30 cycles of 30 sec. denaturation (95°C), 30 sec. annealing (52-60 °C), 1 min. extension (72°C), followed by a final 10 min. extension (72°C). The forward primers for all PCRs were dye-labeled, and products were analyzed on a LI-COR 4300 DNA analyzer. Gene ImagIR software was used to visually score and determine

fragment sizes which were then assigned to the dam and either the jack or hooknose males.

MH genotyping

The PBR of the MH class II β_1 gene was PCR amplified from parental samples with primers developed by Docker and Heath (2002). The sequence of the sense primer was 5'-CCG ATA CTC CTC AAA GGA CCT GCA-3', and the anti-sense primer was 5'-GGT CTT GAC TTG MTC AGT CA-3', and amplified a 294 bp fragment.. The PCR consisted of: 1.0 μL extracted DNA (50-100 ng), 0.50 μL of each primer (100 ng/μL), 2.5 μL 10x reaction buffer, 3.0 μl $MgCl_2$ (25mM), 1.0 μl dNTP's (200 μM), and 0.20 U AmpliTaq® polymerase (Applied Biosystems), and ddH_20 to make a 25 μl reaction. The reaction profile consisted of: 2 min. initial denaturation (95°C), 30 cycles of 30 sec. denaturation (95°C), 30 sec. annealing (52°C), 1 min. extension (72°C), followed by a final 10 min. extension (72°C). The MH class II β_1 gene was not genotyped in the offspring due to limited parental allele variation (see Results section, below).

The MH class I α_1 gene was PCR amplified from parental and offspring samples using primers previously developed (Miller et al., 1997). The sequence of the sense primer was 5'-TGA CTC ACG CCC TGA AGT A-3', and the anti-sense primer was 5'-CTC CAC TTT GGT TAA AAC G-3'. The reaction and cycling conditions were identical to those used for amplification of the β_1 gene (see above).

MH PCR products (both MH class I and II) from parental samples were cloned into a pGEM®-T vector following the manufacturer's protocol (Promega). White colonies were selected, boiled in ddH_2O, and then used for insert verification PCR. The insert was amplified using the M13 forward primer (5'-GTA AAA CGA CGG CCA GT-3') and M13 reverse primer (5'-AAA CAG CTA TGA CCA TG-3') under the following conditions: 2 min. initial denaturation (94°); 35 cycles of 1 min. denaturation (94°), 1 min. annealing (55°), 1 min. extension (72°); and a final 3 min. extension cycle (72°). Each 25 μl reaction consisted of: 50-100 ng plasmid DNA, 0.50 μL of each primer (100 ng/μL), 2.5 μL 10x reaction buffer, 25 mM $MgCl_2$, 200 μM dNTPs, and 0.50 U AmpliTaq® polymerase (Applied Biosystems). Eight sub-clones containing appropriately- sized inserts were sequenced from each parent. PCR products were purified using AMPure (Agencourt) purification system, and sequencing reactions were

performed using the M13 forward primer along with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification using CLEANseq (Agencourt), sequencing was performed using the M13 forward primer and ABI's BigDye Terminator version 3.1 on an ABI 3130xl sequencer.

To evaluate the inheritance of the MH alleles in the F1 generation, a subset of the offspring from each of the 25 maternal half-sib families were genotyped at the MH class I α_1 gene. Offspring MH class I α_1 PCR products were directly sequenced, and allelic variation was read from the chromatogram. The MH class II β_1 gene lacked sufficient polymorphism amongst the parents to allow meaningful segregation analysis, and, therefore, offspring were not genotyped at the MH class II β_1 locus.

DATA ANALYSIS

Analysis of reproductive success

The frequency of jack-sired and hooknose-sired offspring was determined for each family, and cross tabulation (SYSTAT, version 12) was used to determine whether the number of jack sired offspring differed significantly from the number of hooknose sired offspring. An ANOVA was used to partition the variance in male mating success into dam, sire-pair, and dam-sire interaction effects.

Analysis of MH sequences and segregation

Parental genotypes: Geneious Pro software (Biomatters) was used to create alignments to identify alleles. Two alignments, one each for the α_1 and β_1 gene, were built using all sequences from parental samples. Actual alleles occurred at a greater frequency than PCR artifacts, and sequences were verified by comparison to alleles identified in the previous project. Identical sequences were grouped and assigned an allele name.

Offspring genotypes: Each maternal half-sib family consisted of a dam and two possible sires (either the jack or hooknose male); fifteen to 34 offspring were genotyped from each maternal half-sib family for the α_1 gene. Sequence scanner version 1.0 (Applied Biosystems) was used to view overlapping base peaks in sequences obtained from offspring PCR products for the α1 gene. The overlapping peaks identified in offspring sequences were examined, and compared to the nucleotide polymorphisms identified in parental sequences to deduce offspring genotype.

Segregation analysis: Parental alleles are expected to occur at 50% frequency in the offspring, based on Mendel's principle of segregation. To determine whether offspring were inheriting maternal and paternal α_1 alleles as expected by Mendel's principle, a chi-square test for each family was conducted to detect significant departures from the 1:1 ratio. Because of low sample size, a chi-square analysis could not be used to establish whether offspring genotype frequencies departed significantly from the expected Mendelian ratios.

Analysis of offspring genotypes

The purpose of the following analyses is to determine whether the egg-sperm compatibility is affected by whether the sperm MH genotypes are more similar or dissimilar to the egg.

Expected offspring α_1 and β_1 genotypes were determined for all parental crosses based on Mendelian segregation. An allele amino acid dissimilarity index (AADI) was estimated for expected offspring genotypes as the number of amino acid differences between the two allele sequences. Thus, a higher number represents greater dissimilarity between alleles, and homozygotes would have a dissimilarity of zero. For each maternal half-sib family, the four AADI values were used to determine an average value. For each maternal half-sib family the AADI average obtained from jack-sired offspring genotypes was subtracted from the AADI average from hook-nose sired offspring, and this value was used to determine whether AADI was associated with higher/lower jack fertilization success. A scatter-plot was created by plotting the frequency of jack-sired offspring against the difference in the average AADI for each family; regression was used to determine whether the former was dependant on the latter. This analysis was conducted for both the $\alpha 1$ and $\beta 1$ gene.

RESULTS

Reproductive success

Parentage analysis revealed that jacks had sired the majority of offspring in all half-sib families, and in male pair 5, jacks had sired all offspring (Figure 1). The

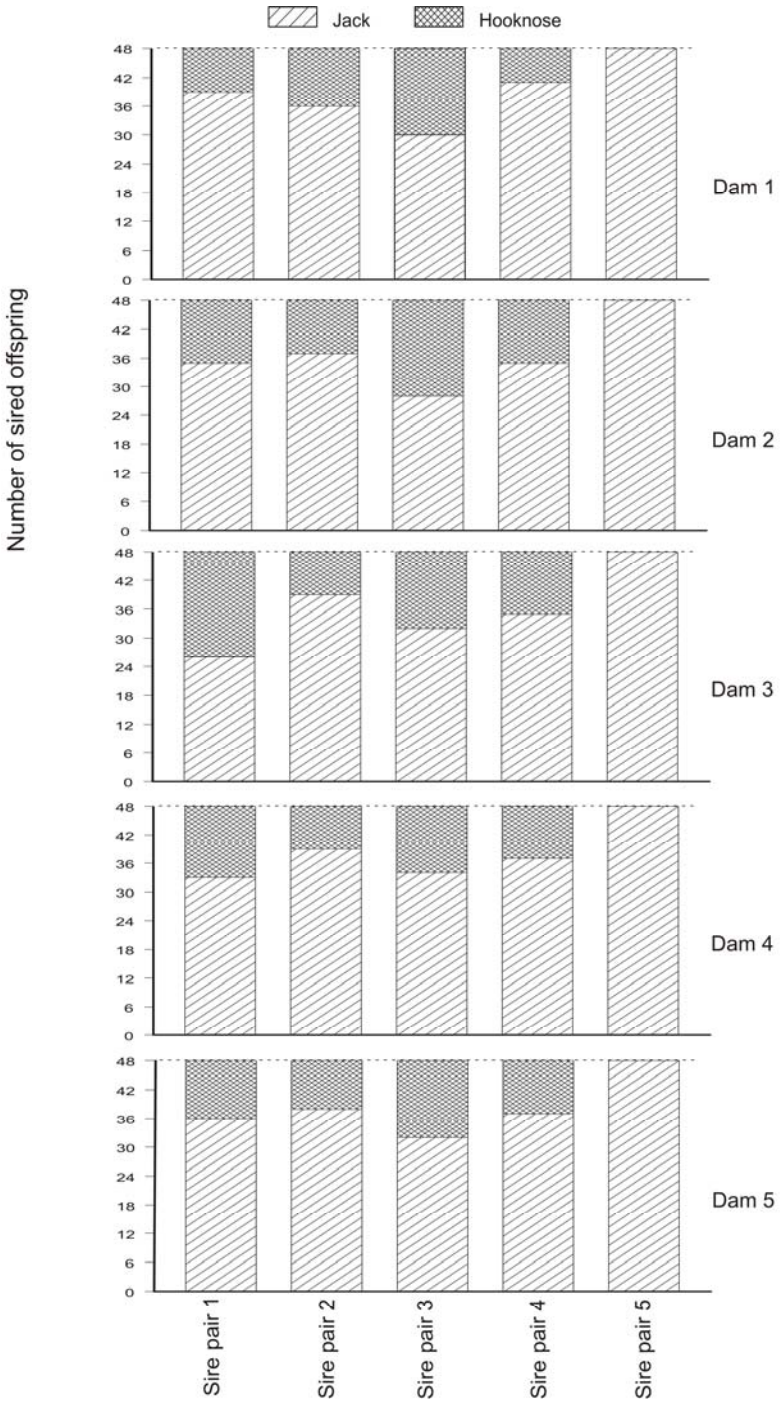

Figure 1. Microsatellite analysis was used to determine parentage of 48 Chinook salmon offspring per maternal half-sib family. Each bar displays the number of jack-sired and hooknose-sired offspring in each half-sib family. The difference between the number of jack-sired offspring, and the number of hooknose-sired offspring across all families was highly significant (χ^2=132.6; p<0.0010).

difference between the number of jack-sired offspring, and the number of hooknose-sired offspring was highly significant (χ^2=133; p<0.0010). An ANOVA revealed a significant dam effect (F=3.18; p=0.013), a significant sire-pair effect (F=23.2; p<0.0010), and a significant dam-sire pair interaction (F=2.53; p=0.0010).

MH sequence and segregation

A total of four MH II β_1 alleles were identified in parental samples, B1-Ots-A, B1-Ots-B, B1-Ots-C, and B1-Ots-D, and all nucleotide sequences are found in GenBank (accession numbers: AY100006, EF432120, DQ450874, and AY100008 respectively; Table 1). Two sire pairs had identical genotypes, and four of five females were homozygous for the U80299 allele. Due to the lack of allelic diversity at this gene in the parental samples, offspring were genotyped for the MH I α_1 gene only, which was found to be more polymorphic, thus making it possible to determine inheritance of maternal and paternal alleles.

A total of 13 MH I α_1 alleles were identified in parental samples, and of these only four were found in the GenBank database (Table 1). A1-Ots-N, A1-Ots-T, A1-Ots-H, and A1-Ots-L are found in GenBank under the accession numbers DQ647922, U80293, DQ647923, and U80284 respectively. Translation of the 13 nucleotide sequences revealed that all base substitutions are non-synonymous, and thus form distinct amino acid sequences (Table 1). A1-Ots-N, A1-Ots-E, and A1-Ots-T are alleles found only in jacks, and A1-Ots-D, A1-Ots-C, and A1-Ots-I were found only in hooknose males (Table 1). The alleles occurring at the greatest frequency were A1-Ots-P (17.6%), followed by A1-Ots-R (15.7%), A1-Ots-A (12.6%), and A1-Ots-M (12.4%; see Table 1).

Analysis of the segregation of paternal and maternal α_1 alleles in jack-sired offspring revealed no departure from the 1:1 ratio (too few offspring were sired by hooknose males to allow an assessment of segregation in the hooknose offspring).

Offspring Genotypes

To determine whether jack sperm were more successful at fertilization when more genetically similar/dissimilar to the ovum at the MH gene than the hooknose male, a regression analysis was performed. The frequency of jack-sired offspring was not dependant upon the difference in average AADI between jack and hooknose offspring expected genotypes for the α_1 gene (F = 0.0129; p = 0.911; Figure 2a). For the β_1 gene,

Table 1. Alleles identified in the Chinook salmon study population for the MH class I α_1 and MH class II β_1 gene. J denotes the jack male, H the hooknose male, and D the dam, and indicates in which parental sample the alleles were identified. The frequency was determined by counting the occurrence of that allele in the entire population, and dividing by 2N (N=483). Since offspring were not genotyped for the β_1 gene, a frequency is not available.

Allele	Fish	Frequency	Amino Acid Sequence	GenBank No.
A1-Ots_A	D1,D3,H4	0.126	FYTASSEVPNFPEFVIVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWESETGNFKGAQQTFKANIDIVKQ	NA
A1-Ots-R	D2,D5,H4	0.157	FYTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWVNKAAD--PQYWERNTGNFKGDQQTFKANIDIAKQ	NA
A1-Ots-I	H1	0.034	FYTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWVNKAAD--PQYWERNTGIFKGNQQTFKANIDIAKQ	NA
A1-Ots-C	H2	0.023	FYTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWERETGIFKGDQQTFKANIDIAKQ	NA
A1-Ots-E	J2	0.034	FYTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWERNTGNFKGAQQTFKANIDIAKQ	NA
A1-Ots-N	J3,J4	0.062	FFTASSEVPNFPEFVVVGTVDGVQMFHYDSNSQRAVPKQDWINKAAETLPQYWERETGICKGTQQTFKANIDIVKQ	DQ647922
A1-Ots-T	J5	0.034	FYTASSEVPNFPEFVVVGTVDGVQMFHYDSNSQRAVPKQDWMNKAAD--PQYWERNTGNCKGTQQTFKANIDIVKQ	U80293
A1-Ots-L	D5,H5	0.046	FYTASSEVPNFPEFVVVGTVDGVQMFHYDSNSQRAVPKQDWMNKAAETLPQYWERETGIDKGAQQTFKANIDIVKQ	U80284
A1-Ots-P	D4,J3,J5,H2, H3,H5	0.176	FYTTSSEVPNFPEFVVVGMVDGVQMFHYDSNSQGAVPKQDWMNKAAETLPQYWERETGNCKGDQQTFKANIDIAKQ	NA
A1-Ots-D	H1	0.032	FFTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWVNKAAD--PQYWERNTGNCKGDQQTFKANIDIVKQ	NA
A1-Ots-H	D3	0.057	FFTASSEVPNFPEFVVVGMVDGVQMFHYDSNSQRAVPKQDWMNKAAETLPQYWERNTGNCKGDQQTFKANIDIVKQ	DQ647923
A1-Ots-M	D4,J1,J4	0.124	FFTASSEVPNFPEFVIVGMVDGVQMVHYDSNSQRAVPKQDWVNKAAD--PQYWERNTGNGKGAQQTFKANIDIAKQ	NA
A1-Ots-B	D1,J2	0.085	FYTASSEVPNFPEFVVVGMVDGVQMVHYDSNSQRAVPKQDWINKAAETLPQYWESETGNFKGAQQTFKANIDIVKQ	NA
B1-Ots-A	D1-5, H1-5, J1-5	NA	GIEFIDSYVFNKAEYIRFNSTVGRYVGYTELGVKNAEAWNKGPQLGQEQAELERFCKPNAALHYRAILDK	AY100006
B1-Ots-B	D4	NA	GIEFIDSYVFNKVEHIRFNSTVGRYVGYTELGLKNAEAWNKGPQLGQEQAELERFCKPNAALHYRAILDK	EF432120
B1-Ots-C	H2	NA	GIEFIDSYVFNKVENIRFNSTVGRYVGYTELGVKNAEAWNKGPQLGQEQAELERFCKPNAALHYRAILDK	DQ450874
B1-Ots-D	H5, J4	NA	GIEFIHSYVFNKVEHIRFNSTVGRYVGYTELGLKNAEAWNKGPQLGQEQAELERVCKPNAALEYRAILDK	AY100008

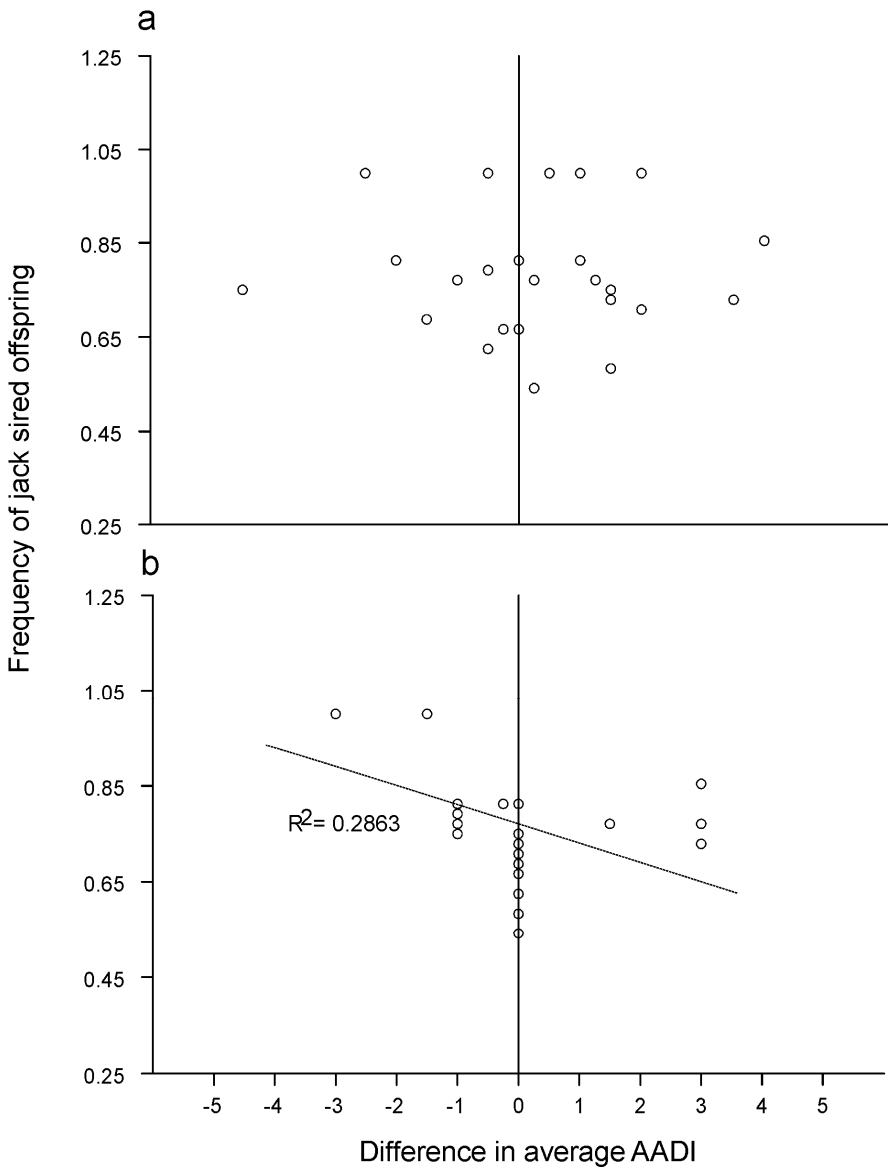

Figure 2. The frequency of jack sired offspring versus the difference in the average amino acid dissimilarity index (AADI) in jack and hooknose offspring per maternal half-sib family. The average AADI was determined from the individual AADI values obtained from expected genotypes in offspring in each maternal half-sib cross. The frequency of jack-sired offspring was not dependant upon the difference in average AADI between jack and hooknose offspring for a) the α_1 gene ($F = 0.0129$; $p = 0.911$); however, this relationship was significant for b) the β_1 gene ($F = 9.23$; $p = 0.006$).

when jack sperm were more genetically similar to the female ovum than the hooknose sperm, they sired offspring at a higher frequency, and this relationship was significant ($F = 9.23$; $r^2 = 0.2863$; $p = 0.006$; Figure 2b).

DISCUSSION

In this study jack sperm out-competed hooknose sperm, and fertilized the majority of eggs in 25 maternal half-sib families. The elevated jack fertilization success is not surprising since studies on other salmonid species have shown that sperm from precociously maturing males is higher in quality than the sperm from the dominate male phenotype. For instance, Gage and colleagues (1995) investigated sperm characteristics in alternative male reproductive strategies in Atlantic salmon (*Salmo salar*), and discovered that precociously maturing parr produced more motile and long-lived sperm than anadromous males. Similar findings were observed in rainbow trout (Salmo gairdneri), sticklebacks (Gasterosteidae), and in other Atlantic salmon studies (Linhart, 1984; de Fralpont, 1993; Vladic and Jarvi, 2001; Stoltz and Neff 2006). My ANOVA revealed a highly significant male pair effect which likely reflects differences in sperm swimming velocity between jack and hooknose males. Jack fertilization success did vary by female; nevertheless, jack sperm sired the majority of offspring in all families indicating that on average jack sperm was more effective than hooknose sperm, likely due to sperm velocity. In Chinook salmon, preliminary data has shown that jack sperm swimming velocity did not differ significantly from hooknose sperm swimming velocity five seconds after activation (T. Pitcher, University of Windsor, unpublished data). This indicates that there may be unexplored factors associated with increased sperm quality in jacks apart from swimming speed.

I found that the dam component in the ANOVA had a significant effect on the relative success of the jack sire in the jack/hooknose pair. The dam component of the variance in jack fertilization success reflects differences between the eggs of each female that affect jack fertilization success, such as the size of the eggs, the amount of ovarian fluid surrounding the eggs, or even the chemical composition of the egg and/or ovarian fluid. Female cryptic choice is female choice exerted post-copulation (Eberhard 1996). In salmonids, researchers have discovered that ovarian fluid released with the female's eggs

can influence sperm behaviour, and is a putative mechanism for CFC (Lahnsteiner 2002; Gage et al. 2004; Rosengrave et al. 2009). Rosengrave et al. (2008) analyzed CFC in Chinook salmon, and found that the composition of female ovarian fluid differentially enhanced sperm swimming speed among males. Although ovarian fluid was not controlled for between females in this study, the fertilization medium consisted of mostly water, and ovarian fluid would have been too dilute to contribute significantly to sperm swimming speed. Furthermore, the female variance only represented 0.96% of the total variance; thus, although interesting, ovarian fluid as a mechanism for CFC is likely not a major component of the observed variance in fertilization success in this study.

In this study I found a significant dam x sire-pair interaction that reflects egg-sperm compatibility variation among families. Analysis of the class I α_1 gene revealed high allelic polymorphism; however, mate choice was not occurring for this gene. However, for the β_1 gene, greater fertilization success occurred when jack sperm carried alleles that were more similar to the female's allele than the hooknose sperm. Contrary to studies that demonstrate mate choice promoting genetic diversity, in this study mate choice resulted in a decline in polymorphism at the β_1, which is already considerably less polymorphic than the α_1 gene in Chinook salmon (Miller and Withler 1998). The class I and class II MH genes are unlinked in salmon, and thus can evolve independently, perhaps explaining the difference in polymorphism at the two genes. Mate choice for higher genetic similarity is a mechanism for hybridization avoidance or for limiting outbreeding depression. Limiting hybridization may be adaptive as several studies have shown that interbreeding between hatchery and wild fish significantly reduces productivity and viability of the naturally spawning population (reviewed in Reisenbichler and Rubin 1999). Alternatively, local adaptation may drive selection for specific alleles rather than heterozygosity to enhance immunity when encountering common pathogens. My finding is similar to the results of another study on Atlantic salmon (*Salmo salar*), which also found that males won more fertilizations when they more genetically similar at the MH gene to females; however, this was discovered for the MH class I locus (Yeates et al. 2009). Alternatively, another non-MH based mechanism may be driving the significant interaction such as a receptor-ligand binding interaction at the egg and sperm cell surface. In the sea urchin, genus *Echinometra*, the protein bindin

mediates sperm attachment to eggs, and, similar to the MH molecules, bindin is highly polymorphic (Palumbi 1999). One study demonstrated that sea urchin eggs discriminated amongst different bindin molecules, and chose sperm from the male with which they shared the largest number of bindin alleles (Palumbi 1999). Teleosts lack the protein bindin; however, it is plausible that other unidentified polymorphic molecules play a similar function in promoting assortative mating in salmon.

The outcome of sperm competition in salmonids is largely dependant on sperm traits such as sperm velocity and longevity (Gage et al. 2004), and here I provide evidence for a bias towards fertilization success in a specific male life history in *in vitro* sperm competition. Such factors are critically important in artificial fertilizations where natural pre-zygotic male-male competition and behavioural female choice are eliminated. Wedekind et al. (2007) argued that mixed-milt fertilizations can result in artificial selection for life history traits in salmonids due to variance in reproductive success between male life histories. In this study I showed that jacks had significantly greater reproductive success than hooknose males. Since there is evidence for a strong additive genetic component to jacking rates (Heath et al. 1994), one would expect that selection for jacks would occur over a relatively short period of time under hatchery conditions. Furthermore, the increased variance in male reproductive success in artificial fertilizations can increase probability of inbreeding and increase loss of genetic variation (Wedekind et al. 2007). Although CFC may operate to negate the above mentioned genetic consequences, my study showed that male sperm quality was the main determinate of post-copulatory reproductive success. In order to reduce genetic change in future populations, supportive breeding programs need to implement fertilization protocols that mimic reproduction occurring in the wild; however, this would be near impossible and the population genetics of eggs fertilized artificially will always differ from the one of eggs fertilized naturally in the wild (Campton 2005; Quinn 2005; Wedekind et al. 2007). Nevertheless, hatcheries play a crucial role in management and conservation of salmon, as human demand far exceeds the number of fish natural habitats alone can supply; therefore, it is of great importance that more genetically benign management strategies are implemented (Campton 2005).

In this study jacks had higher reproductive output than hooknose males in all breeding experiments, and this result is attributed to differences in sperm quality, such as sperm swimming velocity and longevity, between jack and hooknose males. I also found subtle differences in ovarian fluid or egg composition between the females; however, the significant interaction effect can only be explained by CFC mediated by the egg itself. Analysis for genetic compatibility revealed that CFC for MH α_1 gene had not occurred; however, when jacks were more similar at the β_1 gene to the female in comparison to the hooknose male, jack fertilization success increased. These results demonstrate that mixed-milt fertilizations lead to artificial selection for the jack life history, and mate choice occurring at the sperm-egg level can also contribute to male reproductive success. This is the first study to have analyzed post-copulatory sexual selection in jack and hooknose Chinook salmon, and my results provide novel insight into the evolutionary mechanisms of sperm competition and CFC occurring in the alternate male phenotypes. These mechanisms have important evolutionary as well as management implications, which with further research are valuable tools for predicting population dynamics and implementing management strategies.

LITERATURE CITED

Aeschlimann, P.B., Häberli, M.A., Reusch, T.B.H., Boehm, T., Milinski, M., 2003. Female sticklebacks Gasterosteus aculeatus use self-reference to optimize MHC allele number during mate selection. Behavioral Ecology and Sociobiology 54, 119–126.

Andersson, M.B., 1994. Sexual selection. Princeton University Press, Princeton.

Arcaro, K.F., Eklund, A., 1999. A review of MHC-based mate preferences and fostering experiments in two congenic strains of mice. Genetica 104, 241-244.

Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3, 262-273.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., Wiley, D.C., 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature 329, 512-518.

Brown, R.E., Roser, B., Singh, P.B., 1989. Class I and class II regions of the major histocompatibility complex both contribute to individual odor in congenic inbred strain of rats. Behavioral Genetics 19, 659-674.

Campton, D.E., 2004. Sperm competition in salmon hatcheries, the need to institutionalize genetically benign spawning protocols. Transactions of the American Fisheries Society 133, 1277-1289.

Campton, D.E., 2005. Sperm competition in salmon hatcheries: the need to institutionalize genetically benign spawning protocols: Response to comment. Transactions of the American Fisheries Society 134, 1495-1498.

Carré, D., Sardet, C., 1984. Fertilization and early development in *Beroë ovata*. Developmental Biology 105, 188-195.

de Fraipont, M., FitzGerald, G.J., Guderley, H., 1993. Age-related differences in reproductive tactics in the three-spined stickleback, Gasterosteus aculeatus. Animal Behavior 46, 961-968.

Dixson, A., Anderson, M., 2001. Sexual selection and the comparative anatomy of reproduction in monkeys, apes, and human beings. Annual Review of Sex Research 12, 121-144.

Eberhard, W. G., 1996. Female Control: Sexual Selection by Cryptic Female Choice Princeton Univ. Press, Princeton, New Jersey.

Esteve, M., 2005. Observations of spawning behaviour in Salmoninae: *Salmo, Oncorhynchus and Salvelinus*. Reviews in Fish Biology and Fisheries 15, 1-21.

Garner, S.R., Bortoluzzi, R.N., Heath, D.D., Neff, B.D., 2009. Sexual conflict inhibits female mate choice for major histocompatibility complex. Proceedings of the Royal Society B Downloaded from rspb.royalsocietypublishing.org on April 22, 2010

Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Current Biology 14, 44-47.

Grimholt, U., Drablos, F., Jorgensen, S.M., Hoyheim, B., Stet, R.J.M., 2002. The major histocompatibility class I locus in Atlantic salmon (Salmo salar L.): polymorphism, linkage analysis and protein modeling. Immunogenetics 54, 570-

581.
Grimholt, U., Hordvik, I., Fosse, V.M., Olsaker, I., Endresen, C., Lie, Ø., 1993. Molecular cloning of major histocompatibility complex class I cDNAs from Atlantic salmon (*Salmo salar*). Immunogenetics 37, 469-473.
Gross, M.R., 1984. Sunfish, salmon, and the evolution of alternate reproductive strategies and tactics in fishes. Wootton, R., and Potts, G., eds. Academia Press, London, 55-75.
Gross, M.R., 1991. Salmon breeding behavior and life history evolution in changing environments. Ecology 72, 1179-1186.
Heath, D.D., Devlin, R.H., Heath, J.W., Iwama, G.K., 1994. Genetic, environmental and interaction effects on the incidence of jacking in *Oncorhynchus tshawytscha* (Chinook salmon). Heredity 72, 146-154.
Kamler, E., 1992. Early life history of fish: an energetics approach. Chapman & Hall, London.
Keller, L., Reeve, H.K., 1995. Why do females mate with multiple males? The sexually selected sperm hypothesis. Advances in the Study of Behavior 24, 291-315.
Klein, J., 1986. Natural history of the major histocompatibility complex. Wiley, New York.
Knapp, L.A., Ha, J.C., Sackett, G.P., 1996. Parental MHC antigen sharing and pregnancy wastage in captive pigtailed macaques. Journal of Reproductive Immunology 32, 73-88.
Landry C, Garant D, Duchesne P, Bernatchez L., 2001. 'Good genes as heterozygosity': the major histocompatibility complex and mate choice in Atlantic salmon (*Salmo salar*). Proceedings of the Royal Society of London, Series B 268, 1279–1285.
Lawlor, D.A., Zemmour, J., Ennis, P.D., Parham, P., 1990. EVOLUTION OF CLASS-I MHC GENES AND PROTEINS: from natural selection to thymic selection. Annual Review of Immunology 8, 23-63.
Linhart, O., 1984. Evaluation of sperm in some salmonids. Bull. Výzkummný Ústav Rybářský Hydrobiol. Vodnany 20, 20-34.
Lohm, J., Grahn, M., Langefors, A., Andersen, O., Storset, A., von Schantz, T., 2002. Experimental evidence for major histocompatibility complex-allele-specific resistance to a bacterial infection. Proceedings of the Royal Society of London, Series B 269, 2029-2033.
Neff, B.D., Garner, S.R., Heath, J.W., Heath, D.D., 2008. The MHC and non-random mating in a captive population of Chinook salmon. Heredity 101, 175-185.
Neff, B.D., Pitcher, T.E., 2005. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes [Invited Review]. Molecular Ecology, 14, 19-38.
Nelson, R.J., Beacham, T.D., 1999. Isolation and cross species amplification of microsatellite loci useful for study of Pacific salmon. Animal Genetics 30, 228-229.
Ober, C., 1999. Studies of HLA, fertility and mate choice in a human isolate. Human Reproduction Update 5, 103-107.
Palumbi, S.R., 1999. All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proceedings of the National

Academy of Sciences of the United States of America 22, 12632-12637.
Parker, G. A., 1984. Sperm Competition and the Evolution of Animal Mating Systems (ed. Smith, R. L.) Academic, London.
Pitcher, T.E., Neff, B.D., 2007. Genetic quality and offspring performance in Chinook salmon: implications for supportive breeding. Conservation Genetics 8, 607-616.
Pitcher, T.E., Neff, B.D., 2006. MHC class IIB alleles contribute to both additive and nonadditive genetic effects on survival in Chinook salmon. Molecular Ecology 15, 2357- 2365.
Quinn, T.P., 2005. Comment: Sperm competition in salmon hatcheries- The need to institutionalize genetically benign spawning protocols. Transactions of the American Fisheries Society 134, 1490-1494.
Reisenbichler, R.R., Rubin, S.P., 1999. Genetic changes from artificial propagation of Pacific salmon affect the productivity and viability of supplemented populations. ICES Journal of Marine Science 56, 459-466.
Reusch, T.B.H., Haberli, M.A., Aeschlimann, P.B., Milinski, M., 2001. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature 414, 300-302.
Rosengrave, P., Gemmell, N.J., Metcalf, V., McBride, K., Montgomerie, R., 2008. A mechanism for cryptic female choice in chinook salmon. Behavioral Ecology 19, 1179-1185.
Rülicke, T., Chapuisat, M., Homberger, F.R., Macas, E., Wedekind, C., 1998. MHC-genotype of progeny influenced by parental infection. Proceedings of the Royal Society of London Series B – Biological Sciences 265, 711-716.
Singh, P.B., Brown, R.E., Roser, B., 1987. MHC antigens in urine as olfactory recognition cues. Nature 327, 161-164.
Skarstein, F., Folstad, I., Liljedal, S., Grahn, M., 2005. MHC and fertilization success in the Arctic charr (*Salvelinus alpinus*) Behavioral Ecology and Sociobiology 57, 374-380.
Spies, I.B., Brasier, D.J., O'Reilly, P.T.L., Seamons,T.R., Bentzen, P., 2005. Development and characterization of novel tetra-, tri-, and dinucleotide microsatellite markers in rainbow trout (*Oncorhynchus mykiss*). Molecular Ecology Notes 5, 278-281.
Stoltz, J.A., Neff, B.D., 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. Journal of Evolutionary Biology 19, 1873-1881.
Tregenza, T., Wedell, N, 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. Molecular Ecology 9, 1013-1027.
Vladic, T.V., Jarvi, T., 2001. Sperm quality in the alternative reproductive tactic in Atlantic salmon: the importance of the loaded raffle mechanism. Proceedings of the Royal Society of London, Series B 268, 2375-2381.
Wedekind, C., Füri S., 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? Proceedings of the Royal Society of London, Series B 264, 1471-1479.
Wedekind, C., Chapuisat, M., Macas, E., Rülicke, T., 1996. Non-random fertilization in

mice correlates with the MHC and something else. Heredity 77, 400-409.
Wedekind, C., Rudolfsen, G., Jacob, A., Urbach, D., Müller, R., 2007. The genetic consequences of hatchery-induced perm competition in a salmonid. Biological Conservation 137, 180-188.
Williamson, K.S., Cordes, J.F., May, B., 2002. Characterization of microsatellite loci in chinook salmon (*Oncorhynchus tshawytscha*) and cross-species amplification in other salmonids. Molecular Ecology Notes 2, 17-19.
Wittzell, H., Madsen, H., Westerdahl, H., Shine, R., von Schantz, T., 1999. MHC variation in birds and reptiles. Genetica 104, 301-309.
Yamazaki, K., Beuchamp, G.K., Kupniewski, D., Bard, J., Thomas, L., Boyse, E.A., 1988. Familial imprinting determines H-2 selective mating preferences. Science 240, 1331-1332.
Yamazaki, K., Boyse, E.A., Mike, V., Thaler H.T., Abbott, J., Boyse, J., Zayas, Z.A., Thonas, L., 1976. Control of mating preference in mice by genes in the major histocompatibility complex. Journal of Experimental Medicine 144, 1324-1335.
Yeats, S.E., Einum, S., Fleming, I.A., Megens, H.J., Stet, R.J.M., Hindar, K., Holt, W.V., Van Look, K.J.W., Gage, M.J.G., 2009. Atlantic salmon eggs favour sperm in competition that have similar major histocompatibility alleles. Proceedings of the Royal Society of London, Series B 276, 559-566.
Ziegler, A., Kentenich, H., Uchanska-Ziegler, B., 2005. Female choice and the MHC. Trends in Immunology 26, 496-500.

CONCLUSIONS AND FUTURE DIRECTIONS

A library consisting of MH class I α_1 and class II β_1 sequences for Chinook salmon (*Oncorhynchus tshawytscha*) was surveyed in Chapter two, and analysis of the base pair substitutions revealed mutation clusters or "hotspots" on the MH gene. Also, identical mutations occurring in independent PCR reactions and fish were observed. This suggests that point mutation is occurring in somatic cells, and is the mechanism for the diversification of MH alleles in Chinook salmon. A series of PCR-based experiments were conducted to investigate the effects of PCR conditions on the observed mutation frequency. I found that chimera mutations were produced at the highest frequency, and were elevated with increased PCR cycles and when using proof-reading enzyme. Base pair substitutions also increased in frequency when using a non-proofreading polymerase; however, the number of PCR cycles did not affect the frequency of point mutations with this enzyme. To reduce the frequency of PCR artefacts I suggest that proof-reading enzyme is used along with the lowest number of PCR cycles necessary. The results of Chapter two provided the basis for proper MH allele identification, and were applied in Chapter three.

In order to provide more evidence for somatic mutation as a mechanism with which MH alleles diversify in Chinook salmon, it would be beneficial to detect the cDNA copies of the sequence variants identified in genomic DNA. This has already been attempted in the Heath lab; however, cDNA copies of the gDNA variants were not detected. Somatic mutation produces a genetic mosaic, meaning the mutation occurs in some cells but not others, and, as a result, detection of the somatic mutation is challenging especially when it is a rare event. I suggest using PCR product obtained with guidelines outlined in Chapter two to reduce PCR artefacts in a more accurate and high-throughput sequencing method for detection of mutants, such as pyrosequencing. Somatic mutation occurs in antibodies when B cells are stimulated by antigen and helper T cells, and this accumulation of point mutations is known as affinity maturation. It would be interesting to analyze MH sequences in fish subjected to a disease challenge to investigate whether antigens also stimulate point mutations in the MH genes.

The outcome of sperm competition between jack and hooknose males was analyzed in 25 maternal half-sib families, and I found that the number of jack-sired

offspring significantly exceeded the number of hooknose-sired offspring in all families. Analysis of the variance in male success revealed significant dam effects, sire pair effects, and sire pair-dam interaction effects. The sire variance represented the greatest portion of the total variance, and is most likely attributed to differences in jack sperm swimming velocity versus hooknose sperm swimming velocity. A significant sire pair-dam interaction effect indicated that cryptic female choice had occurred. Further investigation revealed that jacks sired a higher frequency of offspring when their MH class II β_1 alleles were more similar to the female's allele. This is contrary to the expectation of mate choice occurring for genetically dissimilar males; however, these results suggest that mate choice is occurring for particular alleles that provide an adaptive advantage to jacks.

In order to gain further insight on post-copulatory mate choice in Chinook salmon, it would be useful to repeat the experiment using wild fish. This would allow us to see whether mate choice is also occurring for more similar MH β_1 alleles in wild jacks. If so, it would strongly suggest that mate choice for more similar β_1 alleles is not only adaptive for hatchery fish, but also gives an adaptive advantage for jacks in their natural habitat. Also, it would be of interest to determine whether MH-related proteins or other polymorphic proteins are being expressed in the egg and sperm, and whether they function in mate choice. From a conservation standpoint, it is crucial that hatchery managers develop fertilization methods to reduce sperm competition as my results have indicated that a) there is high variance in reproductive success of males, and b) sperm competition can lead to a decrease in genetic diversity at crucial genes such as the MH genes.

The results of this work have important fundamental and applied implications to science. From an evolutionary standpoint, these results provide preliminary information on a key evolutionary process functioning at the MH genes. Somatic mutation at the MH genes would revolutionize our thinking on how the MH genes evolve in not only salmon, but in other species. From a conservation standpoint, improvement of existing fertilization protocols is necessary to maintain genetic diversity at the MH gene, and ensure the survival of fish in the wild. These results provide valid reason as to why more genetic insight is needed in hatcheries.

APPENDIX A

Mutations identified in the Chinook salmon MH sequence libraries for the MH I α_1 and MH II β_1 genes (Chapter two), their location on the gene (read complementary to sense primer i.e. in the 3' to 5' direction), the frequency observed, and the nucleotides flanking the base substituted.

MH Gene	Mutation Position	Base Substitution	Number of Independent PCR reactions	Flanking Sequence
class I α_1	84	T→C,A	3	TA<u>T</u>GA
	128	A→G,C	3	AA<u>A</u>CA
	100	A→G	2	AG<u>A</u>GA
	193	C→T	2	AG<u>C</u>AG
	194	A→G,T	2	GC<u>A</u>GA
	3	C→T	1	TT<u>C</u>TA
	9	C→T	1	AC<u>C</u>GC
	15	T→A	1	TC<u>T</u>TC
	18	T→C	1	TC<u>T</u>GA
	19	G→T	1	CT<u>G</u>AA
	31	T→C	1	AC<u>T</u>TC
	39	G→A	1	GA<u>G</u>TT
	41	A→G	1	GT<u>T</u>CG
	42	C→G	1	TT<u>C</u>GT
	46	G→T	1	TG<u>G</u>TT
	48	T→C	1	GT<u>T</u>GT
	50	T→C	1	TG<u>T</u>GG
	53	G→A	1	GG<u>A</u>GA
	55	A→G	1	GG<u>A</u>TG
	58	G→A	1	TG<u>G</u>TG
	68	T→C	1	TG<u>T</u>TC
	69	T→C	1	GT<u>T</u>CA
	73	A→G	1	AG<u>A</u>TG
	76	T→C	1	TG<u>T</u>TT
	77	T→C	1	GT<u>T</u>TC
	77	T→C	1	GT<u>T</u>TC
	88	A→G	1	AC<u>A</u>GC
	91	A→T	1	GC<u>A</u>AC
	94	A→G	1	AC<u>A</u>GC
	98	A→G	1	CC<u>A</u>GA
	112	A→C	1	CC<u>A</u>AA
	113	A→G	1	CA<u>A</u>AC
	115	C→T	1	AA<u>C</u>AG
	119	A→G	1	GG<u>A</u>CT
	123	G→A	1	TG<u>G</u>GT
	129	C→T	1	AA<u>C</u>AA
	131	A→G	1	CA<u>A</u>GG
	133	G→A	1	AG<u>G</u>CA
	142	A→C	1	AA<u>A</u>CA
	143	C→A	1	AA<u>C</u>AC
	148	C→T	1	TG<u>C</u>CA

	152	A→G	1	AC<u>A</u>GT
	152	A→G	1	AC<u>A</u>GT
	166	A→T	1	GG<u>A</u>AC
	200	T→C	1	TT<u>T</u>CA
	209	A→T	1	CA<u>A</u>CA
	214	G→T	1	TC<u>G</u>AT
	215	A→G	1	CG<u>A</u>TA
	217	A→G	1	AT<u>A</u>TT
	219	T→C	1	AT<u>T</u>GT
	223	A→C	1	TA<u>A</u>AG
class II β_1	211	A→G	3	AG<u>A</u>CA
	19	T→C	2	AC<u>T</u>CT
	31	A→G	2	TC<u>A</u>AT
	35	A→T,G	2	TA<u>A</u>GG
	36	G→T,A	2	AA<u>G</u>GC
	61	A→G	2	GC<u>A</u>CT
	78	T→C	2	GT<u>T</u>GG
	114	A→G	2	GC<u>A</u>TG
	128	C→T	2	TC<u>C</u>TC
	149	C→T	2	GG<u>C</u>GG
	10	T→C	1	AG<u>T</u>TT
	12	T→C	1	TT<u>T</u>AT
	16	C→T	1	TA<u>C</u>AC
	17	A→G	1	AG<u>A</u>CT
	22	T→C	1	CT<u>T</u>AT
	23	A→G	1	TT<u>A</u>TG
	24	T→C	1	TA<u>T</u>GT
	27	T→C	1	GT<u>T</u>TT
	32	A→G	1	CA<u>A</u>TA
	42	A→G	1	GA<u>A</u>TA
	44	A→G	1	ATATA
	49	A→G	1	TCAGA
	50	G→T	1	CAGAT
	53	T→C	1	ATTCA
	57	C→T	1	AACAG
	77	T→C	1	TGTTG
	80	G→T	1	TGGAT
	81	A→G	1	GGATA
	89	A→T	1	TGAGC
	97	T→C	1	GTGTG
	98	T→C	1	TGTGA
	100	A→G	1	TGAAG
	101	A→G	1	GAAGA
	104	A→G	1	GAATG
	108	A→G	1	GCAGA
	109	G→A	1	CAGAA
	119	A→G	1	GAACA
	121	A→G	1	ACAAA
	122	A→G	1	CAAAG
	123	A→C	1	GCACT
	127	C→T	1	GTCCT
	134	T→C	1	GCTGG
	139	T→C	1	GTCAA

141	A→G	1	CAAGA
146	A→C	1	GCAGG
152	A→G	1	GGAGC
163	T→C	1	GTTTC
164	T→C	1	TTTCT
169	T→A	1	GTAAG
177	C→T	1	AACGC
184	C→T	1	CTCTC
185	C→T	1	TCTCC
195	A→G	1	AGAGC
196	G→A	1	GAGCC
197	C→T	1	AGCCA
208	A→C	1	ACAAG
211	A→G	1	AGACA
213	A→G	1	ACA

APPENDIX B

The nucleotide sequences of the 13 MH class I α_1 alleles found in the study population of Chinook salmon from Chapter three. Nucleotide differences are in grey.

```
                1        10        20        30        40        50        60
                |        |         |         |         |         |         |
Consensus       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-M       TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGATTGTGGGGATGGTG
 A1-Ots-H       TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-D       TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-P       TTCTACACCACATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-L       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG
 A1-Ots-T       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG
 A1-Ots-N       TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGACGGTG
 A1-Ots-E       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-C       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-I       TTCTACACCGCATCTTCTGAAGTCCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-R       TTCTACACCGCATCTTCTGAAGTCCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG
 A1-Ots-A       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGATTGTGGGGATGGTG
 A1-Ots-B       TTCTACACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG

Consensus       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-M       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-H       GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-D       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-P       GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGGGAGCGGTGCCCAAACAGGAC
 A1-Ots-L       GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-T       GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-N       GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-E       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-C       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-I       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-R       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-A       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC
 A1-Ots-B       GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC

Consensus       TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTNC
 A1-Ots-M       TGGGTAAACAAGGCAGCAGA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGAATGGC
 A1-Ots-H       TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC
 A1-Ots-D       TGGGTAAACAAGGCAGCAGA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGAATTGC
 A1-Ots-P       TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGGAGACAGGGAATTGC
 A1-Ots-L       TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGGAGACAGGGATTGAC
 A1-Ots-T       TGGATGAACAAGGCAGCAGA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGAATTGC
 A1-Ots-N       TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGGAGACAGGGATTTGC
 A1-Ots-E       TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTTC
```

```
A1-Ots-C        TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGGAGACAGGGATTTTC
A1-Ots-I        TGGGTAAACAAGGCAGCAGA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGATTTTC
A1-Ots-R        TGGGTAAACAAGGCAGCAGA--C-C--C-ACAGTACTGGGAGAGGAACACTGGGAATTTC
A1-Ots-A        TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGTGAGACAGGGAATTTC
A1-Ots-B        TGGATAAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGTGAGACAGGGAATTTC

Consensus       AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-M        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-H        AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-D        AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-P        AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-L        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-T        AAGGGTACCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-N        AAGGGTACCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-E        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-C        AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-I        AAGGGTAACCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-R        AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG
A1-Ots-A        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
A1-Ots-B        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG
```

APPENDIX C

The nucleotide sequences of the 4 MH class II β_1 alleles found in the study population of Chinook salmon from Chapter three. Nucleotide differences are in grey.

```
            1        10        20        30        40        50        60
            |        |         |         |         |         |         |
Consensus   GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC
 B1-Ots-A   GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGCTGAATATATCAGATTCAACAGC
 B1-Ots-B   GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC
 B1-Ots-C   GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGTTGAAAATATCAGATTCAACAGC
 B1-Ots-D   GGTATAGAGTTTATACACTCTTATGTTTTCAATAAGGTTGAACATATCAGATTCAACAGC

Consensus   ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTNTGAAGAATGCAGAAGCATGGAAC
 B1-Ots-A   ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTGTGAAGAATGCAGAAGCATGGAAC
 B1-Ots-B   ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATGGAAC
 B1-Ots-C   ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTGTGAAGAATGCAGAAGCATGGAAC
 B1-Ots-D   ACTGTGGGGAGGTATGTTGGATACACTGAGCTGGGTCTGAAGAATGCAGAAGCATGGAAC

Consensus   AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT
 B1-Ots-A   AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT
 B1-Ots-B   AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT
 B1-Ots-C   AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCT
 B1-Ots-D   AAAGGTCCTCAGCTGGGTCAAGAGCAGGCGGAGCTGGAGCGTGTCTGTAAGCCTAACGCT

Consensus   GCTCTCCACTACAGAGCCATACTGGACAAGA
 B1-Ots-A   GCTCTCCACTACAGAGCCATACTGGACAAGA
 B1-Ots-B   GCTCTCCACTACAGAGCCATACTGGACAAGA
 B1-Ots-C   GCTCTCCACTACAGAGCCATACTGGACAAGA
 B1-Ots-D   GCTCTCGAGTACAGAGCCATACTGGACAAGA
```

VITA AUCTORIS

NAME:	Leila Helou
PLACE OF BIRTH:	Sydney, Australia
YEAR OF BIRTH:	1984
EDUCATION:	St. Thomas of Villanova Secondary School, Lasalle, Ontario 1998-2003 University of Windsor, Windsor, Ontario 2003-2008 B.Sc. 2008-2010 M.Sc.