Early-rearing Environment and Mate Choice in Chinook Salmon (*Oncorhynchus tshawytscha*) Aquaculture: Effects on the Immune System

by

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A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Doctor of Philosophy

in

Biology

Waterloo, Ontario, Canada, 2011

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Canada is the fourth largest producer of farmed salmon in the world, with Atlantic salmon being the major species cultivated. Paradoxically British Columbia (BC), which borders the Pacific Ocean, is the major producer province where Atlantic salmon was introduced in the mid-80's. Escaped salmon may constitute a threat to natural populations of Pacific salmon as they compete for the same resources such as food and spawning territory. A potential solution to the aquaculture industry would be to further develop the aquaculture of native species in the region.

The work presented here used semi-natural spawning channels to evaluate the effects of breeding strategies and early-rearing environments on the immune performance of Chinook salmon. Breeding strategy was tested analyzing artificial hatchery practices versus semi-natural propagation in spawning channels. Early-rearing environmental assessment contrasted indoor plastic hatchery tanks with outdoor gravelled-bottom spawning channels. A disease challenge involving over 1400 fish showed interaction effects between breeding strategy and rearing environment. Fish artificially mated presented a disease susceptibility influenced by the rearing environment. The contrary occurred in the offspring of self-breeding brood stock in the spawning channels, as no differences were observed in their susceptibility to the disease regardless of rearing environment. Monitoring of anti-*Vibrio anguillarum* antibodies during the disease challenge and a follow up of the survivors in sea net pens further confirmed the interaction between breeding strategy and rearing environment. Gene expression in pre- and post-infected artificially propagated fish showed differential gene expression when

analyzed with a 695-gene cDNA microarray for Chinook salmon. Genotyping of major histocompatibility (MH) class II β1 alleles showed a tendency of a higher heterozygosity in survivors as expected, as well as a general tendency of a higher heterozygosity in semi-naturally propagated fish. The latter is likely a direct consequence of MH-linked mate choice, which was recently described in Chinook salmon (Neff et al., 2008). To further characterize the mating system of Chinook salmon in the spawning channels, brood stock were genotyped at 12 microsatellite loci. Females and males were found to mate randomly with regards to genetic pairwise relatedness, but they tended to mate with fish of similar condition as revealed by their pairwise differences in Fulton's condition factor.

This work demonstrated that genotype-by-environment interactions can modify the disease resistance of Chinook salmon. More importantly, these effects were seen after just one round of semi-natural spawning of domesticated hatchery fish, suggesting that further studies on spawning channels may highlight other hidden benefits. Therefore, breeding strategy and early-rearing environment should be considered when propagating cultured stocks. The use of more natural propagation methods such as spawning channels could improve the immune performance of Chinook salmon and help to expand the aquaculture of this native species in BC.

Acknowledgments

I would like to start by thanking my supervisor, Dr. Brian Dixon, and my co-supervisor, Dr. Daniel Heath, who have supported me and encouraged me through this entire project. I would also like to thank Dr. Jonathan Witt for the many enlightening chats and his advice on this work.

My experience in YIAL will always be unforgettable because of its exceptional people. I don't have enough words to thanks Ann and John Heath for their enormous hospitality and support during my long stays in Quadra Island. I am in debt to Kieran Jones for his dedication and his caring for my little guys. I also thank Keith, Jamie, and Shon for their help in pulling all those nets out of the water.

I would also like to thank Dr. Simon Jones for receiving me at the Pacific Biological Station and William Bennett for training me in histological techniques. To Dr. Gina Prosperi-Porta for her advice on bacteria cultures and Dr. Catherine Young for lending me some of her valuable antibodies.

I want to thank Dr. Michael Power, Dr. Julio Lancelotti, and MSc. Terin Robinson for their help in getting me out of the bootstrap trap.

The past and present members of the Dixon Lab: Dr. Pablo Conejeros, Dr. Steve Kales, MSc. Darah Christie, MSc. Shati Eshaque, MSc. Cynthia Tang, MSc. Colleen Ryan, MSc. Samantha Hodgins, MSc. Johny Bozdarov, MSc. Lital Sever, Kevin Robinson, Liisa Kadlec and Alex Raben.

I would like to thank Farzana Premji and Stephanie Prins for their help with my lab work, but more especially to Megan Kirkland, who suffered me most.

I am grateful to the people at GLIER, to MSc. Russell Hepburn and especially Tutku Aykanat, to whom I owe the craziest and funniest but very fruitful research days. I would also like to thank Dr. Nick Bernier (U. Guelph) for his help on qPCR matters.

Of course, I would not forget to thank my partners of so many months in BC Barry Madison, William Callery, Romina Bortoluzzi and Shawn Garner who helped me collecting endless samples and preparing experiments.

Finally, the most important person without whom this work would not have been possible: my beloved Rita. Her unconditional love and support throughout the course of this thesis were unmeasurable.

Dedication

To mamá.

To Adrián.

To Rita and Iñaki.

In the loving memory of my dad.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AA	amino acid
APC	antigen presenting cell
β_2m	beta-2-microglobulin
BC	British Columbia
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eEF1α	elongation factor alpha
ELISA	enzyme-linked immunosorbent assay
gDNA	genomic DNA
kb	kilobase
IgM	immunoglobulin M
IL	interleukin
IP	intra-peritoneal
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani media
LOD	logarithm (base 10) of the odds ratio
LPS	lipopolysaccharide
MH	major histocompatibility
MHC	major histocompatibility complex

MH I	class I major histocompatibility receptor
MH II	class II major histocompatbility receptor
mRNA	messenger RNA
NK	natural killer
PBR	peptide binding region
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIT	passive integrated transponder
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription-PCR
SAM	Significance analysis of microarray
SAS	Statistical analysis system
SD	standard deviation
SEP	Salmonid enhancement program
TCR	T-cell receptor
TNF	tumour necrosis factor
VNTR	Variable number of tandem repeats
YIAL	Yellow Island Aquaculture Ltd.

Chapter 1. General introduction

General Introduction

The research developed in this thesis has been characterized by laboratory work in several research institutions and by fieldwork carried out in a research-oriented and family-owned salmon farm in British Columbia (BC), Canada. It is the aim of this work to contribute to the knowledge of the immune system of the Chinook salmon (*Oncorhynchus tshawytscha*) as well as the factors affecting its performance so as to improve aquaculture conditions. It is hoped that this will help to expand the aquaculture of this native species on the West Coast of Canada in a sustainable manner.

Aquaculture in Canada

The world's growing population is steadily increasing the demand for seafood products. Aquaculture has become, over the last four decades, an indispensable contributor to responding to this demand. Fish and shellfish wild stocks around the world have become threatened for several reasons, such as overfishing and habitat deterioration. The global aquaculture industry currently contributes almost half of the fish consumed by the human population worldwide, and will likely exceed capture fisheries in supplying food in the near future (FAO 2009). Particularly, global salmon aquaculture overcame the worldwide salmon capture fisheries as early as 1996, exceeding wild harvests by more than a million metric tons in 2004 (Knapp et al., 2007).

Although Canada ranks 23rd among aquaculture producing nations in terms of volume (DFO 2008a), it is the fourth largest producer of farmed salmon in the world following Norway, Chile and the United Kingdom (DFO 2008b). As in those countries,

Atlantic salmon (*Salmo salar*) is the most popular salmon species farmed in Canada, where the main provincial producer is BC in the Pacific Coast, followed by New Brunswick. Two Pacific salmon species – Chinook and Coho (*O. kisutch*) – are raised on a smaller scale solely in BC. For comparison, 72,140 tonnes of salmonids were produced in BC in 2003, of which 55,000 tonnes (76%) were Atlantic salmon (DFO 2006a), 15,719 tonnes (22%) corresponded to Chinook salmon (DFO 2006b), and 1,421 tonnes (2%) to Coho salmon (DFO 2006c). Therefore paradoxically, salmon aquaculture in the Pacific Coast of Canada is based mainly on the exotic Atlantic salmon species.

Introduction of Atlantic salmon in the West Coast

Salmon farming on the West Coast started with the native species Chinook, Coho and sockeye (*O. nerka*) on the Sunshine Coast (BC) in the early 1970s, but the industry found problems with water temperature and algae blooms and had to relocate to remote, northern sites on Vancouver Island by mid 1980s (DFO 2006a). At about the same time, Norwegian salmon farmers were seeking new locations to expand their business and found both the East and West coasts of Canada very suitable places for salmon farming. At the beginning BC did not allow the introduction of the exotic Atlantic salmon in their waters and proposed these investors to expand the already established, albeit small, aquaculture of Pacific salmonids. Since the biological knowledge of these species was much more limited than that for Atlantic salmon, attempts to cultivate native species on a large scale were not successful. The main impediment at that time was that Pacific salmonids were not domesticated while Atlantic salmon had been in intensive breeding programs since 1971 (Gjedrem 2000). Important commercial traits such as growth rate

and age at maturity in Atlantic salmon were already under study with good response to genetic selection (Gjerde 1986). On the other hand, native species were less tolerant of the crowding and stresses impose by farming conditions having a higher incidence of diseases. Therefore the entrepreneurs finally gained permission from the provincial government to introduce Atlantic salmon in 1984 (DFO 2010a). At present time there are some 130 farming sites in BC, most of them dedicated to the production of Atlantic salmon (BC Aquaculture Statistics 2010).

Farming the natives

Despite the fact that the native species Chinook, and to a lower extent Coho, continued to be cultured by farmers at a small scale in BC therefore domesticating some strains and increasing the knowledge for its cultivation, the reasons for the preference for Atlantic salmon continue to be the same as in the early 1980s. These are its better growth and survival rates than Pacific salmonids, the strong international market for Atlantic salmon and its meat yield (DFO 2005). However, all these reasons may be argued against when considering Chinook salmon, for which at least four domesticated strains have been identified in BC (Kim et al., 2004; Withler et al., 2007). First, this Pacific species has good survival rates when cultured properly in net pens that are not overcrowded. Second, the Atlantic salmon demand was actually created by the aquaculture industry after many years of marketing, which could also be done for other species (Harris 1995). Finally, Chinook salmon has a good meat yield in comparison to Atlantic salmon. Therefore Chinook salmon, having the most developed aquaculture among the Pacific salmonids, represents an alternative farmed species to the exotic Atlantic salmon. Moreover, recent

increases in energy and food prices as well as the threat of climate change are challenging the conditions for aquaculture making production more expensive for the high-intensity salmon farming industry, whereas the low-intensity and small-scale aquaculture may benefit from the increase in salmon prices and could expand production (FAO 2009). This would be another motive and an opportunity to stimulate and promote the low-intensity farming of Chinook salmon already in place in BC.

Brief description of the Chinook salmon's life cycle

Chinook salmon is an anadromous species, meaning it has fresh and saltwater stages in its life cycle, and it is a semelparous species, i.e. dies after spawning once (Healey 1991). The freshwater stage occurs at the beginning and at the end of their life cycle. Sexually matured salmon spawn in the upper shallow waters of streams or lakes in which females bury the eggs under gravel. Eggs hatch becoming alevins carrying a yolk sac that nourishes them until the beginning of the following fry stage in which they start eating zooplankton (Healey 1991). Juvenile salmon remain in the river or lake until they undergo smoltification, a process of physiological and behavioral change (Hoar 1976; Folmar and Dickhoff 1980) that prepares them for the saltwater stage. There are two ecotypes of Chinook salmon depending on how much time they spend in freshwater before migrating to the ocean. Some migrate to sea within their first months of life and are known as the ocean type. Those remaining in freshwater a considerable longer period of time up to a year are known as the stream type (Healey 1991). Chinook salmon spend several years in the ocean before sexually mature fish initiate their migration towards freshwater usually returning to the same original river they left as smolts, a phenomenon

known as homing (Healey 1991). Upstream migration may take weeks or even months depending the distance they need to swim to reach their natal stream, in which they will spawn and dye.

Chinook salmon present a huge variability of life history patterns and the plasticity of their life cycle may have facilitated the successful introductions in the South hemisphere in New Zealand and Chilean Patagonia (Pascual and Ciancio 2007), as well as its recent colonisations of Atlantic flow-out rivers in Argentinean Patagonia (Ciancio et al., 2005; Becker et al., 2007). This intrinsic plasticity may as well be a good quality of the species when considering it for aquaculture. Environmental change is likely to bring both ecological and evolutionary changes to populations (Reed et al., 2010). Farmed salmonids, which spend about two-thirds of their life in sea cages, may then be subject to changes beyond the ones produced by domestication in order to adapt to the new conditions. The acclimatizations experienced by Chinook salmon in different parts of the world highlight its ability to adapt to and cope with change quickly (Kinnison et al., 2008). This may be an important characteristic of the species that deserves to be considered when discussing the further aquaculture development in BC.

Common procedures in salmon farming and domestication

In farming facilities the adult sexually mature fish are seined from the net pens and moved into freshwater-running hatchery troughs. Females are killed by a blunt trauma and eggs are removed into a plastic bowl, to which the sperm of one or more males is added and mixed with freshwater to complete the fertilization process. Fertilized eggs are placed in wire mesh stacked incubation trays with a constant freshwater supply

in dark room incubators. After a period of about two months the embryos hatch into alevins carrying a yolk sac. Once the yolk sac has been consumed they develop into fry and are removed to fiberglass or aluminum indoor freshwater throughs to be fed on commercial food pellets. Fry are reared under artificial light with controlled photoperiod until they reach the smolt stage and are ready to be moved into sea cages in the ocean. Most commercial hatcheries vaccinate their smolts especially for vibriosis before transferring them to seawater, either by intraperitoneal injection or immersion. Fish are kept in net pens and fed regularly until they reach market size, which can take two years for Atlantic salmon and three to four years for Chinook salmon. Male salmonids may mature earlier and at a smaller size than females, which has led the establishment of all-female stocks to be a common practice in aquaculture (Benfey 1996). By treating newly hatched larvae with androgens, genotypic females are converted into functional males that once mature are used for breeding purposes (Benfey 1996).

The hatchery techniques described above lead stocks to diverge phenotypically and genetically from their wild relatives, a process known as domestication. Domestication of wild species for food production, the genetic modification of plant and animal populations born and reared in captivity, is an ancient practice of civilization (Diamond 2002). Ninety percent of species cultivated on land today were already domesticated 2000 years ago, whereas 97% of the cultured aquatic species went through domestication in the last century (Duarte et al., 2007). Norway started selective breeding programs for Atlantic salmon and rainbow trout in 1971 focusing in important commercial traits such as growth rate, age at sexual maturation, disease resistance, and meat quality (Gjedrem 2000). There are now several species already domesticated such as

Atlantic salmon, Chinook salmon (Kim et al., 2004) and sea brook charr *Salvenilus fontinalis* (Sauvage et al., 2010). Other marine fish species are also in the process of domestication as is the case with the striped bass (Woods 2001). The expansion of aquaculture has raised concerns regarding introgression of escaped farmed fish into wild populations, which is a highly debate topic in the Pacifc Northwest communities.

Salmon escapes: the threatening byproduct of aquaculture

Escapes of Atlantic salmon from ocean net pens and their straying to rivers to spawn have been reported in all areas having farming activities worldwide including the East and West coasts of Canada (reviewed by Naylor et al., 2005). The first reported escape occureed in 1988 involving 2000 individuals (McKinnell et al., 1997). A joint project run by DFO and the BC government, the Atlantic Salmon Watch Program (ASWP), has been actively collecting information from a wide range of sources since 1991 (McKinnell and Thomson 1997). According to compiled data from the ASWP and the Washington Department of Fish and Wildlife by the BC Salmon Farmers Association (Ginetz 2002), a total of 421,030 and 613,639 Atlantic salmon escaped from production facilities in BC and Washington State respectively for the period 1991 to April 1 2002. These data included two important incidents occurred in 1997, one in Puget Sound with 369,661 escapees and an accidental spilled in Lois Lake (BC) releasing 10,464 fry (Clarke 1998). On average, 46,255 Atlantic salmon escaped from BC farming sites between 1991 and 2001 (Morton and Volpe 2002), though the number is likely to be higher due to unreported escapes (McKinnell et al., 1997) and chronic “leakage” product of daily operations (Morton and Volpe 2002).

The number of recoveries and sightseeings has been more eratically reported. The first recovery of a cultured Atlantic salmon in coastal BC waters happened in 1987 (McKinnell et al., 1997). A DFO report for the year 1997 indicated 4,904 marine catches in BC, Washington and Alaska, of which 2,655 fish corresponded to BC coastal waters (Clarke 1998). Freshwater recoveries for the same year totalled 155 adult Atlantic salmon in BC and 53 in Washington State, with juvenile Atlantic salmon reported at three BC freshwater sites close to hatchery facilities (Clarke 1998). McKinnell and colleagues (1997) collected biological data from 478 and 82 Atlantic salmon caught between 1991 and 1995 in BC and Alaska respectively. The study also included 20 adult recovered from freshwater systems of BC. Most marine captured adults were immature whereas most freshwater captured males and females were maturing (McKinnell et al., 1997). A stomach content inspection of 133 and 73 marine recoveries in BC and Alaska revealed that only 5,8% and 13.1% of individuals presented preys in their stomach respectively, suggesting a low food intake by the escapees which could be in part due to their inability to capture fish (McKinnell and Thomson 1997). In another study, Morton and Volpe (2002) recorded 10,826 Atlantic salmon caught by commercial fisheries during a 17-day period in the fall of 2000 in an intensive farm area of BC coastal waters. Their stomach content results were similar to those of the ASWP in 1997 with only 3.9% of caught fish having preys in their stomachs, though in a particular area it reached to 24% of the sample (Morton and Volpe 2002). Sexually matured Atlantic salmon represented a small percentage (2.3%) of the total capture (Morton and Volpe 2002).

Succesful spawning of escapees has been scarcely reported. One dead, matured unspawned female was found in the Harrison River, whereas one matured female was

captured alive in the Glen Lyon Creek, Port Hardy (BC), the latter not known to support any Pacific salmon population (McKinnell et al., 1997). The first evidence of Atlantic salmon spawning in BC freshwater was reported by Volpe and colleagues (2000) who observed two year-classes of juveniles in the Tsitika River in 1998. The fish were found upstream of a barrier, for which the authors concluded that these individuals were likely the offspring of feral adults that cleared the barrier (Volpe et al., 2000). Two other reports of single year-classes of juvenile Atlantic salmon occurred in 1999 in Amor de Cosmos River and Adam River (Ginetz 2002), further confirming the capacity of escaped farmed Atlantic salmon to spawn in the wild. Experiments conducted with Atlantic salmon showed these fish to perform well in underoccupied habitats (Volpe et al., 2001). Taken together, these observations suggest that if native species continue to decline in BC freshwater systems, Atlantic salmon might occupy these spawning grounds.

Despite the huge number of escapees that totalled more than a million individuals in about a decade, no self-sustained populations have been observed in the West Coast of Canada. This agrees with the fact that no populations were established after 13.5 million eggs, alevins and fry were deliberately released in BC freshwaters between 1905 and 1935 (MacCrimmon and Gots 1979). Moreover, all attempts to establish Atlantic salmon outside its natural range have failed whereas the only transplanted population that succeeded was in the Faroe Islands on the Northeast Atlantic Ocean (MacCrimmon and Gots 1979). Considering the lack of success in introducing Atlantic salmon in BC during the early 20th century, one could assume that the potential risk for an Atlantic salmon population to be established in BC is unlikely. However, eventhough the risk for colonization may be low, it can never be ruled out completely since environmental

conditions may change with time and the uncontrollable accidental escapes are constantly introducing farmed individuals to the environment. Therefore, it seems appropriate to consider the main ecological and genetic impacts farmed fish in general may potentially have on the wild populations and look for alternatives that may help to mitigate them.

Ecological and genetic potential consequences of farmed escapees

There are different ecological and genetic risks inherent to the fish farms that use net pens in marine waters. Ecological risks do not discriminate between exotic or endemic farmed species as they are mainly related to disease transfer and direct competition for food and spawning ground access. Diseases that affect farmed fish may potentially spread to sympatric populations (Costello 2009; Frazer 2009; Krkošek et al., 2006). Recently, sea lice has attracted much attention due to their high presence in individuals in proximity to salmon farms in BC (Krkošek and Hilborn 2011) as well as in Europe (Heuch et al., 2005). However, the association between farm sites and the incidence of the disease on wild populations continues to be under debate (e.g. Krkošek et al., 2007; Brooks and Jones 2008; Riddell et al., 2008) and some wild stocks located far from farms have also experienced epizootics (Beamish et al., 2009). Another major ecological risk that is imposed by farming exotic species is the potential for establishing self-sustaining populations outside their natural range of distribution (Naylor et al., 2001).

Genetic risks, on the other hand, must be analyzed separately for exotic and native farmed species, as the effects of each one of these is very different on wild populations. Escapees belonging to exotic species could potentially hybridize with native species

though hybrids are usually not adapted to local conditions and do not pose a serious problem. On the other hand, escapees belonging to species with natural occurrence in the farm area present a different problem as farmed stocks quickly diverge from wild ones due to the use of a small number of breeders and intentional and unintentional selection through domestication (Naylor et al., 2005). Domestication implies changes in genotypic frequencies that may be detrimental to the wild populations upon hybridizations as has been demonstrated with F1, F2 and backcrosses to wild and farmed Atlantic salmon (McGinnity et al., 2003). Recently, trancriptomic differences have also been observed between farmed and wild conspecifics suggesting that genetic divergence may affect negatively wild populations that are adapted to local habitats (Roberge et al., 2008).

There are concerns that escaped farmed salmon could potentially alter local adaptation of wild populations (Fraser et al., 2008) and also that they could induce outbreeding depression (McClelland et al., 2005) the further the generational distance is between the domesticated and the wild populations. A recent review of the interactions between farmed and wild Atlantic salmon by Jonsson and Jonsson (2006) may apply to other species that are cultivated side by side with their conspecifics. The risk of displacing wild popualtions or degrading their genetic pool through introgression is of great concern. Concerns about the effect hatchery fish could have on wild populations were raised by many researchers since declines started in early 1990s (e.g. Meffe et al., 1992; Beamish et al., 1997; Lichatowich et al., 1999). This papers indicate that in 1989-1990 the Pacific regime changed to a lower productivity system, which would naturally support less salmonids. Under this paradigm, the critical effect is the relative survival of hatchery and wild stocks and how this would affect wild populations. Flagg et al. (1995)

proposed to evaluate hatchery programs to focuse them for conservation than merely supplementation for fisheries goal. The use of sterile triploids in aquaculture has been largely explored as it may help to mitigate the effects of potential spawners in the wild, but it is not extensively applied yet due to the large variability within the stocks, casual presence of morphological abnormalities and poor performances compared with diplod fish (Benfey 1999).

History of salmon enhancement in BC

Adult salmonids formed the basis for survival of native people in BC throughout history, as they made an excellent food resource when smoked and stored for the winter (Childerhose and Trim 1979). After Europeans arrived, the incredible abundance of fish produced a large industry for catching salmon and smoking or salting it for export, which was later perfected with canning (Childerhose and Trim 1979). Successive improvements of catching and processing methods resulted in more fish taken out of the natural ecosystem, which started the depletion of wild salmon populations. Since 1848 with the discovery of gold in California, salmon runs have dramatically declined across the region due to several causes: water pollution; loss of spawning, rearing, and riparian habitat from a multitude of human actions such as extensive logging in the 1960s (DFO 2010b); a history of over-fishing; dam construction and operation; water withdrawal for irrigation and industrial cooling; competition with hatchery-produced salmon; competition with various non-indigenous fish species (i.e. yellow perch). Many current runs showing high density in the Pacific Northwest are composed in part of fish produced in hatchery

enhancement programs and several runs depend mainly or entirely on hatcheries (National Research Council 1996).

Therefore stock enhancement, stocking cultured organisms to replenish or increase abundance of wild stocks (Leber et al., 2004), has a long history in BC. There have been three different enhancement technologies used by different governmental agencies since enhancement started in Canada: hatcheries, lake enrichment and spawning channels. Hatcheries operate in a similar way as those used for aquaculture but their involvement in the fish cycle is just in egg fertilization and early rearing to the smolt stage before release to local rivers. Lake enrichment was used mostly to increase juvenile growth in some coastal lakes. Spawning channels are artificial streams with regulated flow and particular gravel size which augment the surface of spawning grounds for wild brood stock while allowing control of the density of spawners. By having stable water flow, eggs incubating in the spawning channel gravel are not affected by flooding, preventing losses due to dislodging.

By the late 1800s gold mining and logging, which affected much of the freshwater habitat of Sockeye salmon by disturbing spawning gravel and stream stability (Hartman and Scrivener 1990), together with an intense commercial gillnet fishery at the mouth of the Fraser River were rapidly depleting wild salmon populations. In the fall of 1885, eggs obtained from Sockeye salmon in Weaver Creek were transplanted to other streams and lakes in the province (DFO 2010b). By the early 1900s large hatchery programs planted eyed Sockeye eggs or fry into lakes but as Foerster (1954) indicated there were no improvements in the number of seaward-migrating smolts, suggesting the use of gravel beds instead of troughs. Thus by 1938, the scarce results of the hatchery technology for

artificial Sockeye propagation plus the lack of funding due to the Great Depression motivated the closure of the hatcheries (Ross 1991).

In the following years, the federal government adhered to Foerster's recommendations and started to improve natural spawning grounds and to use spawning channels for stock enhancement (Wilks 2004). Spawning channels were constructed for pink (*O. gorbuscha*) and chum (*O. keta*) salmon, which migrate to sea within days after swim-up, and for sockeye salmon due to low survival in the hatchery environment (DFO 2010b). Hatcheries for Coho and Chinook salmon were also established (MacKinlay et al., 2004). By the same time, an influential essay written by a senior fisheries scientist, Peter Larkin (1974), recognized the necessity of a salmon enhancement program to improve the freshwater survival of salmon. In 1979 the Salmonid Enhancement Program (SEP) was established with the long-term goal of doubling salmon catches in BC through the use of hatcheries and spawning channels. The SEP also promoted the involvement of local communities that developed and operated other spawning channels and on-site river hatcheries thus increasing the artificial propagation of salmonid species even more.

The use of hatcheries in stock enhancement programs

Salmon hatcheries represent the most extended human intervention in the Pacific Northwest where hatchery fish make up the majority of salmon in rivers and streams (National Research Council 1996). In Canada, as in much of the United States, hatcheries are supported by government funding and are socially accepted in the hope of mitigating the impact of numerous human activities that have dramatically altered the habitats of Pacific salmon. Although these artificial propagation programs are usually claimed as

successes, researchers have called them into question (Riddell 1993; Bowles 1995; Snyder et al., 1996) as they address the symptoms but not the causes of the declines (Meffe 1992). In particular, investigations have shown that captive breeding programs succeed at maintaining genetic diversity, but they can cause rapid loss of fitness (Fraser 2008).

One notorious characteristic of artificial propagation programs has been the absence or insufficiency of monitoring and evaluating the effects hatcheries may have on wild populations. For instance, the Canadian SEP has not gathered enough information to critically evaluate the benefits of its enhancement programs, neither to estimate the potential risks to which wild populations could be exposed (Winton and Hilborn 1994). Scientific literature recognizes the adverse effects many hatcheries have imposed on natural populations, such as reduction of genetic diversity and domestication, which tends to lower fitness in natural environments (National Research Council 1996).

Probably the most perilous practice of all hatchery procedures is artificial mating where the evolutionary process of sexual selection is overridden. This non-natural way of breeding eliminates differential reproduction based in male competition and female mate choice that has occurred for millions of years. Most of the consequences of altering the mating system are yet not clearly known, but among the potential outcomes are loss of general vigor and domestication which jeopardize the ability to mate in natural conditions. There are studies that have corroborated a genetic basis for some changes in reproductive behaviour resulting from artificial mating in hatcheries (Fleming and Gross 1993, 1994).

Another undesirable effect of hatcheries comes from the artificial rearing environment to which fish are subjected. This starts right from the fertilization process occurring in a plastic pot, passing through the egg incubation process in a wire mesh tray to alevin and fry rearing in aluminum or fibreglass troughs until smoltification; all these stages occur under artificial light and in a monotonous habitat with no selection pressures whatsoever causing the potential outcome of lower fitness of the fish in natural habitats. There is no certainty to what extent domestication has occurred due to hatchery enhancement programs, since only part of the salmon life cycle is artificially managed. A study performed by Nickelson and colleagues (1986) on the use of hatchery Coho salmon to stock populations showed that there was a decrease in the number of juveniles produced in stocked streams, which was likely a consequence of the earlier time of spawning in the hatchery stocked streams compared with unstocked streams.

Diseases are an integrative part of the evolutionary process of natural populations; hence immune defences have a strong genetic component. The loss of genetic diversity due to mixed hatchery-wild populations imperils the survival of current and future generations of Pacific salmon. For instance, natural populations in the Columbia River showed differences in transferrin genotypes which have been correlated with susceptibility to different pathogens such as bacterial kidney disease and vibriosis (Winter et al., 1980; Withler and Evelyn 1990). Furthermore, physiological stress due to overcrowding during rearing may also impinge on their eventual immune performance upon release to the natural environment (reviewed by Steward and Bjorn 1990).

Spawning channels in stock enhancement programs

In looking at the success of spawning channels, two goals need to be evaluated. First, their success at increasing the number of released fry and thus smolts that migrated to sea. Secondly, their success at increasing adult returns to a level comparable with the populations spawned in natural grounds. A research study comparing fry and smolt production before and after the construction of three spawning channels adjacent to the Skeena River near Babine Lake found egg-to-fry survival as expected, and that wild and channel-produced fry did not present differences in distribution, growth and survival in the lake (McDonald and Hume 1984). The increased smolt outputs allowed a large surplus of more than two to three times the pre-enhancement population level in the odd-numbered brood years (McDonald and Hume 1984), with the even-numbered brood years performing poorly. However, this low productivity was also seen in the decades before the installation of the channels and may have offset potential gains in adult returns. More studies are required to identify the causes of the even-year poor performance (McDonald and Hume 1984). West and Mason (1987) also examined smolt production in the Babine Lake project and found that increased fry into the lake did result in more smolts leaving the lake (Hilborn 1992). Another artificial spawning channel was constructed beside Weaver Creek in 1965 and it is still in operation (DFO 2010b). Therefore, the main goal of the artificial spawning channels which was to increase the number of fry and smolts released to local rivers did work as expected. The second goal, that of increasing adult returns, has generated some controversy as there have been no proper marking experiments with controls to distinguish returning adults of brood stock spawned in the channels from those spawned in natural streams (Winton and Hilborn 1994). The reasons

for potential low returning numbers are very complex and many variables are probably involved, such as oceanic predator pressure on the fish and food availability. Considering that hatcheries have also experienced lower than expected returns in the last few decades, a multidisciplinary approach may be needed to find the causes that are limiting adult returns.

Hatchery versus spawning channels

By comparing both hatchery and spawning channel enhancement technologies, it can be seen that both have increased the number of released fry and smolts. Therefore, since both are successful at accomplishing the first aspect of the enhancement, it is appropriate to evaluate the impact each has on the wild populations. Hatcheries, as was described above, involve a huge human intervention in the life cycle of the salmonids since individuals are spawned artificially by mixing eggs and milt in plastic buckets. Thus, one of the main components of the natural spawning process has been omitted and replaced by random mating. The existence of mechanisms of sexual selection is widely known in animals as is their particular importance in fish species without post-hatching parental care, as is the case with salmonids (Gross and Sargent 1985). On this note, semelparous Pacific salmon species die after spawning. Having only one opportunity to mate and to leave descendents makes the mating process a very important part of their history life with strong implications for their evolution, perhaps providing an even more important role for sexual selection than in other species.

In this respect, spawning channels have important advantages compared to hatcheries. Besides needing lower maintenance, they have a much lower human impact

on the fish's life cycle as fish are left to spawn naturally instead of being artificially bred. Therefore, it makes more biological sense to provide the proper spawning grounds than to spawn fish artificially. Since the decline of natural populations is likely a consequence of human activities as discussed above, simply breeding fish artificially and planting eyed eggs, fry and smolts in lakes and rivers may be seen as a short-sighted remedy to the actual problem. These actions may have reached expected results at first but have not demonstrated long-term sustainability. A much more reasonable solution would be to reconstitute and recover the original spawning grounds, and in those places where higher numbers seem tolerable by the local environment, new artificial spawning areas could be created to allow natural processes to rebuild the stocks. Currently, most salmon enhancement programs in BC use artificial breeding through hatchery technologies and rearing practices with mostly unknown consequences on the genetic structure of wild populations. A study of hatchery and wild steelhead on the Kalama River (Chilcote et al., 1986) found that the reproductive performance of hatchery fish was only 28% as successful as the wild fish. In a review of over 300 hatchery programs Steward and Bjorn (1990) found that the majority of captive-bred fish failed to establish natural runs. In a more recent study by Chilcote (2003) it is concluded that the use of hatchery steelhead to rebuild wild populations may be counterproductive by actually lowering overall population productivity. The use of semi-naturally spawning channels instead of the artificial breeding procedures carried out by hatcheries may be beneficial to the offspring survival. To date, no attempts to use spawning channels in the aquaculture industry are known, nor have studies been conducted to evaluate their potential use in commercial production.

The fish nonspecific immune response

As in the rest of jawed vertebrates, the fish immune system can be considered to be constituted by two components. On the one hand, there is an innate or nonspecific immunity whose immune response elements are present before encountering a pathogen. These nonspecific factors recognize invariant molecular patterns found in many microorganisms by means of germline encoded receptors. On the other hand, there is an adaptive or specific immune system which develops after pathogens overwhelm innate immunity. Specific immunity is based on gene-rearranged receptors which recognize specific antigens. It is important to note that this is an artificial classification since generally both systems act intertwined to fight diseases (Lo et al., 1999).

The first defence against infection of the fish innate immune system is constituted by the physical barriers skin and scales, together with gill and gut epitheliums (Ellis 2001). In addition, fish epithelial cells secrete mucus which helps trap and wash away microorganisms (Shephard 1994). The molecular patterns recognized by the nonspecific immune system are collectively designated pathogen-associated molecular patterns (PAMPs) which include lipopolisacharides (LPS) constituent of gram-negative bacteria, peptidoglicans of gram-positive bacteria and double stranded viral RNA among other molecules foreign to cell surfaces of multicellular organisms (Medzhitov and Janeway 2002). These molecules are recognized by germline encoded receptors called pattern recognition receptors (PRRs), which are expressed on surveillance cells such as epithelia and effectors cells of the innate immune system, including antigen presenting cells (APCs; Medzhitov and Janeway 1997).

Teleosts have several humoral innate factors in their secreted mucus such as antimicrobial peptides, lectins, lysozymes and complement factors (Alexander and Ingram 1992). If infectious agents are able to overcome this basic line of defence and enter the organism, they will face more humoral factors in serum such as hepcidin and transferrin which chelate iron essential for bacterial growth (Ellis 2001; Rodrigues et al., 2006). In addition, teleosts possess natural antibodies which are polyreactive immunoglobulins with a low affinity but a broad specificity for both self epitopes and non-self PAMPs (Avrameas and Ternynck 1995). Although immunoglobulins are an important component of the adaptive immune system, natural antibodies produced in the absence of antigen exposure are considered part of the innate immune response (Sinyakov et al., 2002). The innate cellular response of teleosts is carried out by leukocytes that resemble that of mammalian macrophages, neutrophils, monocytes, thrombocytes, eosinophils and natural killer (NK) cells (Hine 1992; Afonso et al., 1997; Miller et al., 1998; Ellis 1999). After macrophages, granulocytes and dendritic cells phagocytose pathogens using scavenger, Fc, and complement receptors (Zapata and Amemiya 2000), these cells secrete cytokines that can lead to inflammation, recruitment of neutrophils and stimulation of APCs to process and present antigens to T cells, the latter initiating an adaptive immune response.

The fish specific immune response

The fish specific immune system is constituted by the main components present in all jawed vertebrates: immunoglobulins (Igs), T cell antigen receptors (TCRs), major histocompatibility (MH) surface molecules, including a thymus and secondary lymphoid

tissues (Flajnik 2002; Cannon et al., 2004). The specificity of TCRs and of B cell receptors (BCRs) is given by the recombination-activating gene (RAG)-mediated V(D)J recombination present in all jawed vertebrates (Flajnik 2002).

BCR diversity generation differs among major vertebrate classes. In chicken they are diversified in the Bursa of Fabricius, in mammals in the bone marrow, and in teleosts in the head kidney or pronephros (Flajnik 2002). On the other hand, the generation of TCR diversity occurs in the thymus as in all jawed vertebrates. T lymphocytes differentiate upon stimulation into two main effector T cells which are functionally characterized by their surface receptors. First, T lymphocytes bearing CD4 co-receptors recognize extracellularly derived antigens presented by MH class II receptors. They are termed "helper" CD4+ T cells due to their main function in humoral immunity as stimulators of B cells to produce antibodies (Dijkstra et al., 2006; Toda et al., 2011). However, subsets of CD4+ T cells can also contribute to cell-mediated immunity by activating macrophages to destroy intracellular infectious agents. Secondly, T lymphocytes expressing CD8 co-receptors recognize intracellularly derived antigens presented by MH class I receptors. They are called cytotoxic CD8+ T cells due to their main role in cell-mediated immune response of lysing virus-infected or altered cells (Fischer et al., 2003; Araki et al., 2008; Toda et al., 2009).

The teleost specific humoral (antibody) response is mediated by B lymphocytes which express IgM and IgD in mature naïve and activated B cells (Wilson et al., 1997). Contrary to the observation in sharks and tetrapods, in which IgM is found in monomeric and pentameric forms, teleosts secrete tetrameric IgM (Kobayashi et al., 1982). Moreover, the tetrameric IgM showed structural diversity due to variation in their cross-

linking of monomeric subunits which may help overcome the lack of class switching seen in mammals (Kaatari et al., 1998). In addition, teleost antibodies are of much lower affinity than those of mammals (Du Pasquier 1982; Warr 1995), and increases in avidity seem to occur late in the immune response of trout (Kaattari et al., 2002). Furthermore, immunological memory has not yet been demonstrated in ectothermic vertebrates (Flajnik 2002).

All the immune responses described above need an accurate and specific cell-to-cell communication allowing the coordination of the effector cells. This function is carried out by cytokines, which are soluble peptides essential for the intercellular communication that initiate and regulate inflammatory and immune responses (Taniguchi 1988). Cytokines are constitutively expressed at low levels, but are quickly upregulated following cell stimulation (Foster 2001). Their effects can be pleiotropic and can influence the expression of other cytokines leading to cascade effects (Secombes 1996; Cavaillon et al., 2003). Cytokines can act exclusively in an innate or adaptive immune response, or they can be secreted by and act upon cells of either division of the immune system. This is another example favouring a more integrative view of the immune system as a coordinated entity instead of separate units. Two pro-inflammatory cytokines produced rapidly after immune stimulation during an acute phase response are tumour necrosis factor α (TNF-α) and interleukin 1-β (IL-1β). Their main physiological function is the recruitment of neutrophils and monocytes to sites of infection in response to gram-negative bacteria and other microbes. TNF-α is produced by macrophages and T cells, whereas IL-1β is produced by a broader array of cells, including macrophages, neutrophils, endothelial and epithelial cells (Secombes et al., 2001). Another important

cytokine that has several important roles in both innate and adaptive immunity is interferon-γ (IFN-γ). IFN-γ is produced in the innate immune phase by NK cells, and in the adaptive immune phase it is secreted by T lymphocytes. Two of its main functions are to activate macrophages to kill phagocytosed microbes and to stimulate MH class II expression on APCs (Zou et al., 2005).

Major histocompatibility genes and mate choice

The human major histocompatibility complex (MHC) is a single 3.6-Mbp DNA region containing 224 loci, with about 60 of them having an immune function (MHC Sequencing Consortium 1999). The immune related genes encode complement and heat shock proteins, cytokines and the highly polymorphic integral membrane glycoproteins MHC class I and II involved in the presentation of peptide antigens to T lymphocytes (Gruen and Weissman 1997). This "complex" organization is found in the rest of tetrapods and in chondrichthyan fishes but not in teleost fishes which have MHC class I and II genes located on different chromosomes (Sato et al., 2000). Thus, it has been suggested that the term "complex" not to be used for teleosts, instead referring to them simply as Major Histocompatibility genes (MH genes; Shand and Dixon 2001).

Major Histocompatibility class I and class II receptor molecules are essential for the specific immune response present in all jawed vertebrates (Flajnik et al., 1999). Class I molecules are expressed on the surface of all nucleated cells and present peptides derived from endogenous viruses and bacteria to CD8+ cytotoxic T lymphocytes (York and Rock 1996). They are comprised of a heavy chain, with three α domains each encoded by different exons, linked non-covalently to a non-polymorphic light chain

called β_2-microglobulin which is encoded outside the MH region (York and Rock 1996). The peptide binding region (PBR) is the highly polymorphic region of the MH class I receptor, which binds antigenic peptides of 8-9 amino acids long and is located at the top of the molecule between the α1 and α2 domains (Engelhard 1994). MH class I has also a role in the non-specific immune response of mammals as it can stimulate cytotoxicity and proliferation of natural killer (NK) cells (Moretta et al., 1995; Warren 1999). NK-like cells have recently been described in several fish species (Fischer et al., 2006), and a study on trout carried out by Utke and colleagues (2007) demonstrated the same regulatory activity of MH class I upon these cytolytic cells.

Class II molecules are expressed on the cellular surface of antigen presenting cells (APCs) such as macrophages, B lymphocytes and dendritic cells, which display extracellularly derived peptides to CD4+ helper T lymphocytes (Watts 1997). The class II receptor is a heterodimer composed of one α and one β chain each encoded in different loci, having two domains each encoded by separate exons (Cresswell 1994). The class II PBR is located in a cleft formed by the α1 and β1 domains with open ends which accommodates peptide fragments up to 19 amino acids long (Engelhard 1994).

MH class I and II are the most polymorphic loci found in the vertebrate genome (Trowsdale 2001). The PBR of each MH molecule shows a degree of specificity allowing it to bind multiple peptides that have common residues at particular anchor positions (Altuvia and Margalit 2004). Individuals having more than one allele are therefore able to recognize and present a broader array of pathogens to T cells (Doherty and Zinkernagel 1975). This has implications on mating preferences as several studies have shown MH-linked mate choice (reviewed in Bernatchez and Landry 2003).

Female mating preferences are expected to occur in resource-based mating systems, in which males provide resources and offspring care. However, in non-resource-based systems such as in salmonids, where males do not contribute to the female and/or offspring survival yet females still express preferences, mate choice could be based on traits other than male phenotype (Andersson 1994). For instance, females may benefit from choosing males with dissimilar MH alleles, which would contribute to their offspring immunocompetence (Apanius et al., 1997).

Studies on the genetic basis of mate preferences have revealed that genes involved in the immune system play a role in sexual selection in humans (Chaix et al., 2008), which was also observed in mice (Yamazaki and Beauchamp 2007), birds (Bonneaud et al., 2006), reptiles (Olsson et al., 2003) and fishes (Milinski et al., 2005). A recent review by Havlicek and Roberts (2009) summarize the current knowledge of human MHC-linked mate choice that supports both odor preference for MHC-dissimilarity and visual preference for MHC-similarity, which together allow reaching an optimum genetic variability. Olfactory-related genes have been found close to the human MHC region on the short-arm of the chromosome 6 (Santos et al., 2010a). A study comparing 16 vertebrate species reperesenting eight taxa further confirmed the evolutionarily conserved MHC linkage of olfactory-related genes (Santos et al., 2010b). Furthermore, a mechanism through which animals can perform kin recognition and discriminate individual's odours (Olsén et al., 1998; Bernatchez and Landry 2003) has been described involving immune-related genes (Singh et al., 1987; Beauchamp and Yamazaki 2003). In general, females may prefer mating with males carrying different alleles than her own (disassortative mating, e.g. Atlantic salmon, Landry et al., 2001), males with high heterozygosity (e.g.

sticklebacks, Reusch et al., 2001), or males with alleles compatible with the female's genotype (reviewed in Tregenza and Wedell 2000). Recent studies conducted by Brian Neff and colleagues on Chinook salmon in spawning channels have demonstrated non-random mating linked to MH haplotypes in this species (Neff et al., 2008).

Assessing the use of spawning channels in the aquaculture of Chinook salmon

Aquaculture is expected to continue its worldwide expansion and Canada may play an important role in it. The increasing demand for seafood poses a challenge to the aquaculture industry in BC, as it needs to keep increasing salmonid production but at the same time it has to protect local populations threatened by escapes of exotic species. Therefore, the aquaculture industry may benefit from the production of local species. In this context, Chinook salmon appears as a valuable alternative species but more knowledge of its performance under cultivation is needed.

Current aquaculture practices employ artificial propagation and rearing methods leading to domestication of farmed stocks (Petersson et al., 1996). This undoubtedly affects fitness-related traits (Fleming and Einum 1997) and may impact negatively in the performance of the cultured species. A different approach used in some enhancement programs takes advantage of the salmonid natural breeding systems. It allows self-spawning and subsequent rearing in semi-natural environments.

The objective of this thesis has been to investigate the effect of mate choice and semi-natural rearing environment on the Chinook salmon immune system. This was performed by comparing the use of spawning channels (Figure 1-1) with standard aquaculture practices which involve artificial mating and artificial rearing environments

(Figure 1-2). In doing so, two particular goals were defined. First, to gain a comprehensive understanding of how these factors may affect the innate and adaptive immune performance of Chinook salmon. Antibody responses to inactivated bacteria were followed in fish held in fresh- and saltwater, and a disease challenge with live bacteria was conducted to assess disease susceptibility, humoral immune response, and gene expression in pre- and post-infection tissues. MH class II heterozygosity was also analyzed among survivors and mortalities. Second, to further study the breeding system of Chinook salmon in spawning channels. This was attempted by performing parentage assignment with microsatellite loci and analyzing the degree of genetic pairwise relatedness among mating individuals.

Figure 1-1. Experimental spawning channels.

Spawning channels used in aquaculture experiments with Chinook salmon. They are approximately 3.5 metres wide, 15 metres long and filled to a depth of approximately 1 metre, with a continuous re-circulated flow to mimic a stream environment. The channels shown in this picture were drained to show the pebbles lining the bottom, which resembles the preferred spawning habitat of wild Chinook salmon in streams.

Figure 1-2. Hatchery throughs used in salmon aquaculture.

Common hatchery tanks are approximately 3x1 m and about 0.6 m in depth. These indoor tanks are under an artificial 16:8 hours light:dark cycle (reflection of light can be seen on the water surface). Automatic feeder is on top. Fish are reared in hatchery tanks from alevin to fry stage before being transferred to sea cages.

Chapter 2. Assessment of humoral immune response and parasite load in Chinook salmon cultivated under different methods: standard hatchery practices versus spawning channel technology.

Introduction

The Canadian salmon aquaculture industry depends extensively on Atlantic salmon (*Salmo salar*). The major provincial producer is British Columbia (BC) where this is a non-native species (BC Aquaculture Statistics 2010). Farmed salmon escapes are a common phenomenon of worldwide salmon farms that are difficult to control. There have been more than a million escapees reported between 1991 and April 1 2002 from salmon farms located in BC and the Washington State (Ginetz 2002), which constitutes a continue threat to natural populations of local species. Atlantic salmon has been shown to be able to spawn in freshwater systems in BC (Volpe et al., 2001), though no self-sustaining population has ever been observed yet. The fact that no established population has been found does not mean it cannot occur; it may be a matter of time or a combination of different parameters that have not come together yet. Therefore, farming native species may be a better sustainable industrial activity.

Chinook salmon (*Oncorhynchus tshawytscha*), a native species that has been cultivated at a small-scale for more than two decades in BC, is a plausible alternative species for the local aquaculture industry. However, initial attempts to cultivate it at large-scales at the beginning of 1970s failed facilitating the introduction of Atlantic salmon for aquaculture production (DFO 2006). Therefore, more studies on its performance under cultivation are needed in order to position Chinook salmon as an alternative to Atlantic salmon.

A different approach to the artificial mating and rearing methods commonly used in aquaculture practices might be the propagation method used in some enhancement programs. This consists in the use of artificial spawning channels that have regulated

water flow and particular gravel size (DFO 2010). Wild brood stock is deviated from the main course of the river to these artificial spawning grounds in which fish breed naturally instead of being artificially bred, allowing natural processes such as mate choice to take place. After spawning, eggs remain undisturbed and early-rearing occurs in the spawning channels for most of the fry stage. This enhancement technique could be adapted to the aquaculture industry provided it produces high quality fish in considerable numbers. This study is an initial step into the investigation of these matters.

The necessity of maintaining pathogen-free health status in cultured fish requires a strong diagnostic effort to reduce exposure to diseases. In this context, assessment of the humoral immune response is an important pursuit. Fish have a broad array of non-specific and specific humoral and cellular immune mechanisms against bacteria pathogens (Ellis 1999). The specific humoral response is mediated by antibody molecules secreted by activated B cells also known as plasma cells. Antibody binds to and coats bacterial cells neutralizing bacterial adherence and toxins and promoting their phagocytosis by immune cells (Ellis 1999). Moreover, antibody coating activates the complement system by the classical pathway which increases opsonisation and lead to the lysis of some bacteria (Ellis 1999). The presence of a specific humoral immune response in fish has been successfully applied in aquaculture by immunization against several diseases such as enteric redmouth disease (Cossarini-Dunier 1986) and vibriosis (Harrell 1979). The strength of the humoral immune response is usually dependent upon the route of administration (Palm et al., 1998).

The work reported here analyzed the immunocompetence of Chinook salmon obtained under common hatchery procedures versus the offspring of fish allowed to self-

spawn in artificial spawning channels, referred to as "hatchery" and "channel" fish respectively. Relative levels of anti-*Vibrio anguillarum* antibodies were measured with the enzyme-linked immunosorbent assay (ELISA) technique, which has proven to be successful in measuring antibodies in salmonid serum (Bøgwald et al., 1991). *V. anguillarum* is a common marine bacterial pathogen which causes severe losses in aquaculture (Toranzo et al., 2005). Vaccine preparations of this pathogen have shown to induce humoral immune responses in salmonids (Harrell et al., 1976) and to be highly efficacious which has spread its use in aquaculture to control the disease. In addition, the relative health status of the two groups of fish was also monitored through histological analyses of gill tissue collected during summertime and early fall. Among the pathogens known to affect Chinook salmon and therefore investigated, were monogeneans and microsporidians (Kent et al., 1998; Scholz 1999). Monogeneans are external and monoxenous parasites, meaning their development occurs in tissues of one host species, and they constitute one of the major groups of parasitic Platyhelminthes that affect fishes (Cribb et al., 2002). Some species of monogeneans cause severe losses to Atlantic salmon farms in Norway, as is the case of *Gyrodactylus salaris* (Olstad et al., 2007). There are two genera known to infect the external body and gills of salmonids in North America. One is *Gyrodactylus,* with five species found in salmonids, three of them considered specific parasites of salmonids: *G. salmonis*, *G. nerkae*, and *G. colemanensis* (Cone et al., 1983). The second genus, *Laminiscus* (Palsson and Beverley-Burton 1983), has one species *L. strelkowi* known to infect two pacific salmon species in BC: *O. gorbuscha* and *O. nerka* (Hoffman 1999). The other group of parasites targeted by the histology analysis were the obligate intracellular parasites microsporidians that have emerged as a disease

problem for the salmon faming industry (Kent 2000). The most infectious agent is *Loma salmonae* which parasites the gill of all species of Pacific salmon, particularly affecting Chinook salmon farms in BC (Scholz 1999). Infection starts in the intestine, meronts are later transported by circulating blood cells to the gill where merogony occurs in cells under the epithelium like the pillar cells, in which xenomas develop hypertrophying the host cells (Kent and Speare 2005).

Finally, direct observations on the body of fish reared in sea cages were conducted in search of other easier to find external parasites known as sea lice. The term sea lice refers to ectoparasitic copepods which include species from two genera: *Caligus* and *Lepeophtheirus* (Boxshall and Defaye 1993). Sea lice have become a critical problem in BC aquaculture industry and for wild salmon populations in recent years (Costello 2009), which merits investigation of the susceptibility to infection of the fish obtained under common hatchery procedures versus fish obtained semi-naturally in spawning channels.

Two experiments were performed to assess the effects of these alternative breeding strategies and rearing environments on the immune system of Chinook salmon. A preliminary study was conducted in 2006 to evaluate the efficacy of two alternative routes of exposure to *V. anguillarum* bacterin while comparing the humoral immune response of hatchery fish with that of channel fish maintained in fresh water. Following the results of this pilot study, an extended second experiment was performed in 2007 with fish reared in sea cages. Serum samples for humoral response and gill samples for histological analysis were collected together with observational data for the presence of external parasites during the saltwater rearing stage.

Materials and methods

Experimental fish

The experiments described in this chapter were conducted in the facilities of Yellow Island Aquaculture Ltd. (YIAL), a family-owned Chinook salmon farm located in Quadra Island, BC. YIAL initiated its operations in 1985 with brood stock from Robertson Creek, a DFO-operated hatchery, and has eradicated the Y chromosome from their stock population maintaining only homogametic (XX) individuals (Hunter et al., 1983). Each spawning season, part of the eggs are treated with testosterone to induce male sexual characters thus creating sex-reversed females which upon maturation are used as brood stock in future spawning seasons (Baker et al., 1988). Homogametic XX males were shown to reach similar sizes to XY males and also present similar plasma concentration of testosterone and 17β-estradiol (Heath et al., 2002). In addition, XX males presented spawning behaviour undistinguishable from normal XY males in a previous study in these channels (Garner et al., 2010).

YIAL became an organic salmon farm in 1989 when it stopped using antibiotics. In the following years, between 1990 and 1994, the farm lost about 65% of their stock in outbreaks due to two common diseases to Pacific salmon: bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum* and vibriosis due to *Vibrio anguillarum*. Thus, the YIAL stock has been naturally selected for BKD and vibriosis resistance.

Two different groups of fish were obtained in the fall of 2005 by applying different breeding methods and different early-rearing environments. The first group called “hatchery” (H) fish, was obtained by using standard hatchery procedures in which

artificial propagation was carried out by mixing eggs and milt in plastic buckets. Fertilized eggs were incubated in vertical stacked trays with a constant freshwater flow. After hatching, alevins were removed to 180 L indoor plastic tanks under artificial light. Hatchery fish were reared in hatchery tanks until the start of the experiments.

The second group of fish, named "channel" (CH) fish, was obtained by allowing the brood stock to spawn semi-naturally in 3.5x15 m and 1 m depth spawning channels supplied with approximately 80 L min^{-1} of fresh water with a water re-circulation rate of approximately 300 L min^{-1}. Inflow of pond water was located at one end of the raceway and outflows were located at each downstream corner. Thirty mature fish were transferred into each of two channels in a 2:1 male to female sex ratio (19:11 and 18:12). All females were 4-year-old and ten males were 3-year-old in each channel and either eight or nine were 4-year-old males. After spawning activity, carcasses were collected, buried eggs left undisturbed and fish were reared in the channels during the alevin and most of the fry stages. Fish were randomly seined from the channels and transferred to hatchery tanks, in which they were acclimatized for a period of two weeks before being used for the experiments.

An additional third group called "egg-transferred fish" (ET) was generated by transferring eyed eggs of channel fish into hatchery tanks. This was done by a technique known as hydrosampling in which a hose connected to a compressor is used to introduce air among the gravel stones thus releasing the buried eggs, which come to the surface and are netted before sinking again. The purpose of this was to have a group produced under a semi-natural spawning method but reared under the artificial hatchery environment, therefore having a control group for environmental effects. Due to their limited numbers

this group was used only in the first experiment carried out in 2006. All three groups of fish were fed with commercial pellets (Ewos Canada Ltd., Surrey, BC).

Freshwater flow to channels and hatchery tanks came from an artificial pond supplied constantly with underground well water. The pond's water input was located on its side at about 35 cm height discharging in a 45 degree angle allowing pumped water to oxygenate and release some nitrogen before entering the pond reservoir.

Experiment 1: Humoral immune response in freshwater

The first experiment was performed with Chinook salmon fry held in freshwater during the months of July and August 2006. It was aimed at evaluating simultaneously two different routes of vaccine administration and the ability to elicit an immune response in channel (CH), hatchery (H) and egg-transferred (ET) fish.

The commercial vaccine MICROViB^{TM}, *Vibrio anguillarum* serotype 01 and 02 bacterin (Microtek Intl. Inc., Saanichton, BC, Canada), was used to immunize fish by employing two different routes of exposure: intraperitoneal (IP) injection and immersion. Briefly, 33 fish were allocated into each of nine 180 L barrels, where sets of three barrels contained hatchery, channel and egg-transferred fish. Mean weight and standard deviation for each group was H = 12.1 ± 2.8 g, CH = 6.2 ± 1.1 g and ET = 9.7 ± 5.4 g. Length measurements were H = 101.5 ± 7.3 cm, CH = 82.5 ± 5.2 cm and ET = 90.5 ± 15.9 cm. To each set of three barrels, one of the following treatments was applied: immersion into a water bath containing 1:10 diluted bacterin, IP injection of 0.1 mL of bacterin and IP injection of 0.1 mL of phosphate-buffered saline (PBS) to sham fish. All fish were fasted for 24 hrs and anaesthetized with 0.5 g L^{-1} MS-222 (Syndel Intl. Inc.,

Vancouver, BC) for immunizations. Fish were fed to satiation three times a day and dead fish were removed regularly and replaced with non-treated fish to maintain uniform density levels throughout the experiment. A second immunization was performed similarly at three weeks. Water temperature ranged between 9.8°C and 11.5°C during the experiment, which was was terminated at five weeks. Fish was euthanized with MS-222 (1 g L^{-1}) and blood samples were collected and left in a refrigerator overnight. Samples were centrifuged 10 min at 3000 g the following morning and serum samples collected into new tubes and placed at -20°C.

Experiment 2: Humoral immune response in saltwater and parasite loads

In June 2006 channel (CH) and hatchery (H) fry having a mean weight of 6.4 ± 1.4 g and 8.3 ± 1.4 g respectively, were immunized by submersion in a water bath containing 1:10 diluted *Vibrio* bacterin and were transferred to sea cages a month later. Each fish received a 21 mm Passive Integrated Transponder (PIT) tag intraperitoneally which allowed identification of the group each fish belonged to (Prentice et al., 1990). Fish was held in a common net in the YIAL's dock beside the cages used for their production fish.

On May 9 2007, two 4.5x4.5x4.5 m sea cages labelled 4A and 19A were set up. Pen 4A received 92 fish (45 channel and 47 hatchery fish) IP injected with 0.1 mL of *Vibrio* bacterin, along with another 15 control fish injected with 0.1 mL of PBS. Pen 19A received 95 fish (54 channel and 41 hatchery fish) injected with 0.1 mL of *Vibrio* bacterin, together with another 15 control fish injected with PBS. Mean weight and

standard deviation was 82.2 ± 16.4 g and 65.3 ± 12.9 g for H and CH respectively. Length measurements for each group were H = 17.9 ± 5.6 cm and CH = 16.3 ± 5.9 cm.

Prior to injection each fish had their PIT tag alphanumerical code recorded, was inspected for sea lice presence and then bled to collect serum samples. This procedure was repeated four times at 30-day intervals. Each time 10 fish from each group per net pen were euthanized with MS-222 (1 g L^{-1}). Serum samples were collected in May, June, July and August. In addition, gill samples for histological analyses were collected in June, July, August and October and stored in 10% buffered formalin for 24hs before being transferred indefinitely to 70% ethanol.

ELISA procedure to measure Chinook salmon anti-*Vibrio* antibodies

Since fish were immunized with *Vibrio* bacterin the humoral immune response was assessed by measuring levels of anti-*Vibrio* antibodies in serum using an indirect enzyme-linked immunosorbent assay (ELISA). Antigen was prepared by homogenizing *Vibrio* bacterin with 0.1 mm zirconia/silicone beads. Polycarbonate 96-well plates (Evergreen Scientific, CA, USA) were coated overnight at RT with 100 µL per well of antigen diluted in one volume of coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6). Plates were rinsed with tris buffered saline containing 0.05 % Tween 80 (T-TBS) and blocked one hour at RT with 300 µL of 5% skim milk in T-TBS per well. Plates were rinsed and probed for two hours at RT using 100 µL of fish serum dilutions in BSA blocking buffer. Each serum sample was assessed by triplicate. After rinsing, a secondary rabbit anti-salmonid antibody diluted 1:1000 was added and incubated for another two hours at RT. Following rinsing, a third antibody goat anti-

rabbit whole molecule alkaline phosphatase conjugate 1:2500 dilution was incubated at 37°C for one hour and rinsed again. Detection was performed using 50 µL per well of p-nitrophenyl phosphate (Fast p-NPP; Sigma, MO, USA) in the dark at RT for 30 minutes. Reactions were stopped by adding 50 µL of 0.03 M NaOH and absorbance was read at 405 nm using a microplate reader (VERSAmax microplate reader, Molecular Devices).

Data analysis of ELISA results

The comparison between the two routes of *Vibrio* bacterin exposure and the assessment of the antibody response afterwards in freshwater tanks were performed with t tests for unequal variances, since in each analysis there were only two groups to be compared at only one sampling point: injection versus submersion and *Vibrio* versus PBS injection.

The analysis of the humoral immune response in saltwater demanded a higher complexity since there were many samples to be analyzed together. Since ELISA plates usually present inter-assay variation due to the many steps involved in each individual plate, this technical variation can be neutralized in part by spreading the samples from each particular sampling group across several plates. In particular, the samples collected in May, June, July and August during the saltwater experiment were arranged in triplicates in six 96-well ELISA plates, ensuring that temporal samples taken from the same fish were in the same plate to avoid inter-assay variation to affect the measuremet of the individuals' immune response development. Another aspect considered in the ELISA analysis was to have in each plate representatives from the two groups under study to homogenize this inter-assay variation. Statistical analysis of the antibody

response was performed using the natural logarithm of spectrophotometer readings at 405 nm. The slope values of the responses for each fish were then calculated and used in a type III ANOVA model in which plate was the fixed factor and fish group was the random factor. ANOVA was carried out in the Statistical analysis system (SAS) v. 9.2 (SAS Institute Inc., Cary, NC).

Histological analyses for detection of monogeneans and microsporidian xenomas

Histological analyses were completed at the Histology Laboratory at the Pacific Biological Station (Nanaimo, BC). The goal of this work was to detect monogenean and microsporidian parasites infecting juvenile Chinook salmon. Gill samples were collected during the summer and fall months of June, July, August and October. Tissue samples were placed into plastic cassettes and were then processed by the automatic tissue processor (Tissue-Tek® VIP) by washing the samples with increasing concentrations of ethanol to dehydrate them. After dehydration it infiltrated the samples with melted paraffin at 62ºC. Samples were finally transferred to the embedding machine, placed into melted paraffin at 62ºC and allowed to solidify. Samples in paraffin were trimmed using a microtome (8 μm slices at a time) until tissue was exposed, followed by sectioning into thinner slices (5 μm) placed in a 42°C water bath. Selected slices were placed over microscope glass slides and kept in dry incubator at 55°C for 30 to 45 minutes.

For monogenean detection, samples were re-hydrated by treatment with xylene and then with decreasing concentration of alcohols. Standard staining and counterstaining were performed with hematoxylin and eosin respectively, followed by de-hydration using a higher alcohol concentration and xylene. Coverslips were mounted with Permount

(Fisher Scientific, cat.# SP15-100) and after dried, slides were inspected at the microscope. Detection of microsporidian xenomas was carried out through immunohistochemical analysis. Sections fixed in microscope slides were de-waxed and re-hydrated with xylene followed by decreasing concentration of alcohols and were probed with 100 to 200 µL of the chicken anti-*Loma salmonae* IgY diluted 1:1000 in PBS-Tween (0.2%) added on top of the sample section making sure was well covered and incubated at RT for 1 hour. Slides were washed 3X for 5 min and a secondary antibody rabbit anti-chicken alkaline phosphatase-conjugated diluted 1:50 in PBS-Tween was added and incubated at RT for 45 min. After rinsing, a chromogen development Blu-Phos (1:1) was incubated at RT for 30 min. Rinsing in distilled water stopped the reaction and a counter stain was then performed with eosin for 5 seconds. Then samples were de-hydrated with higher alcohol concentration and xylene. Finally, coverslips were mounted with Permount (Fisher Scientific, cat.# SP15-100), dried and inspected at the microscope.

Observational data on sea lice

The sea lice genera *Caligus* and *Lepeophtheirus* are known to affect salmon populations in the northeast Pacific Ocean (Boxshall and Defaye 1993). Training for recognition of sea lice species took place in the Pacific Biological Station (Nanaimo, BC). Descriptions were followed from Kabata (1972) and Johnson and Albright (1991) for *Caligus* and *Lepeophtheirus*, respectively. Direct observations on fish held in saltwater were performed at each sampling point in May, June, July and August.

Results

Humoral immune response of fry Chinook salmon in freshwater

A preliminary ELISA analysis compared four different serum dilutions (1:50, 1:100, 1:200 and 1:400) to optimize the dilution factor to be used in group comparisons. A linear trend was obtained with a maximal absorbance for the higher serum concentration as expected (Figure 2-1). The dilution factor 1:100 of primary antiserum was chosen for further analyses to avoid any potential signal saturation that might be produced by a higher concentration.

The analysis of the route of exposure in Chinook salmon fry reared in freshwater showed the IP injection method to elicit an overall stronger antibody response than immersion, although no differences between fish groups were observed (Figure 2-2). A t test for unequal variances confirmed the significant difference between the mean absorbance of *Vibrio* injected fish ($M = 0.4292$, $SD = 0.21992$) and that of the immunized by immersion ($M = 0.1945$, $SD = 0.07959$), $t(32.70) = 5.213$, $p < 0.000$, $\alpha = 0.05$.

Fish injected with bacterin showed a higher antibody response than their PBS injected counterparts (sham fish), although responses within each treatment were similar among groups (Figure 2-3). A t test for unequal variances revealed a significant difference between the mean absorbance of *Vibrio* injected fish ($M = 0.4537$, $SD = 0.17795$) and that of the PBS injected ($M = 0.1632$, $SD = 0.06549$), $t(17.95) = -5.91$, $p < 0.000$, $\alpha = 0.05$. This confirmed *Vibrio* injection ability to elicit an antibody response in Chinook salmon.

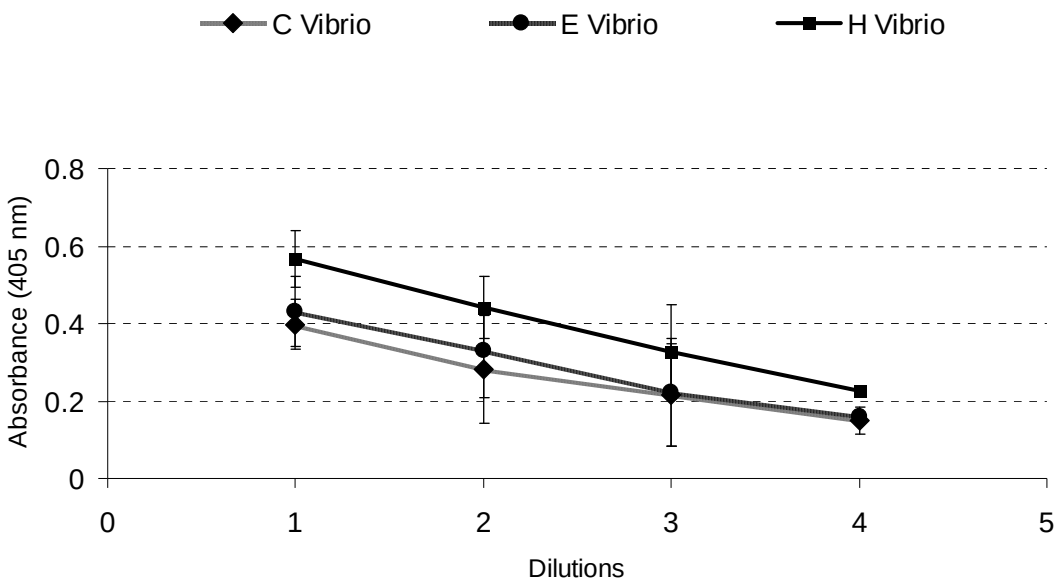


Figure 2-1. Optimization of serum dilution factor.

ELISA assessment of the absorbance range of four serum dilutions: 1 = 1:50, 2 = 1:100, 3 = 1:200, and 4 = 1:400. The dilution factor 1:100 was chosen for further analyses to avoid potential saturation at higher concentrations. Each line represents the average of six fish for a particular group. C: channel fish, E: egg-transferred fish, H: hatchery fish. Error bars: ±1 SD.

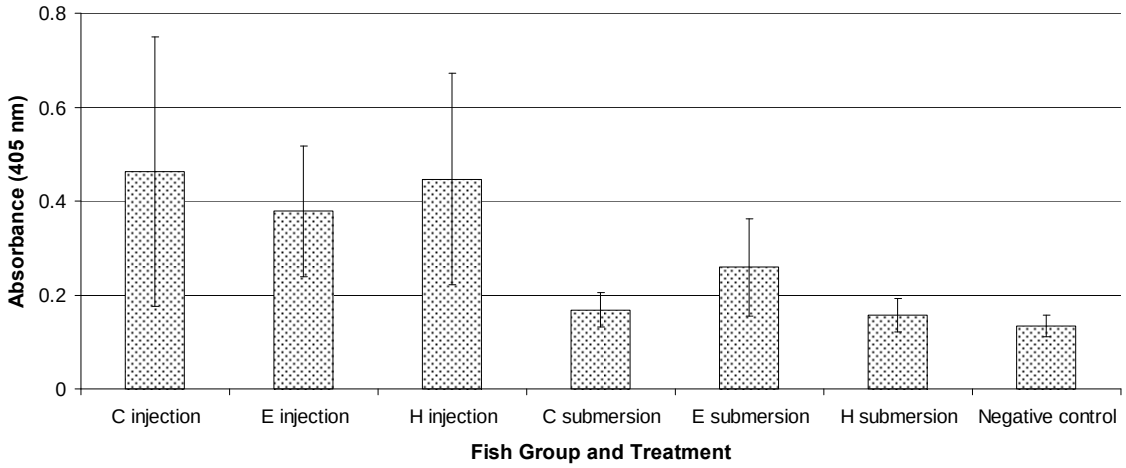


Figure 2-2. Assessement of the route of exposure to *Vibrio* bacterin.

ELISA results in which each bar indicates mean absorbance ±1 SD of nine fish for each combination of group and route of exposure. The last column represents negative control wells coated with coating buffer instead of *Vibrio* antigen. All three injected groups together presented a significantly higher absorbance ($P < 0.001$) than fish immunized by submersion. Note the similarity in absorbance levels of the three *Vibrio* injected groups within each treatment. C: channel fish. E: egg-transferred fish. H: hatchery fish.

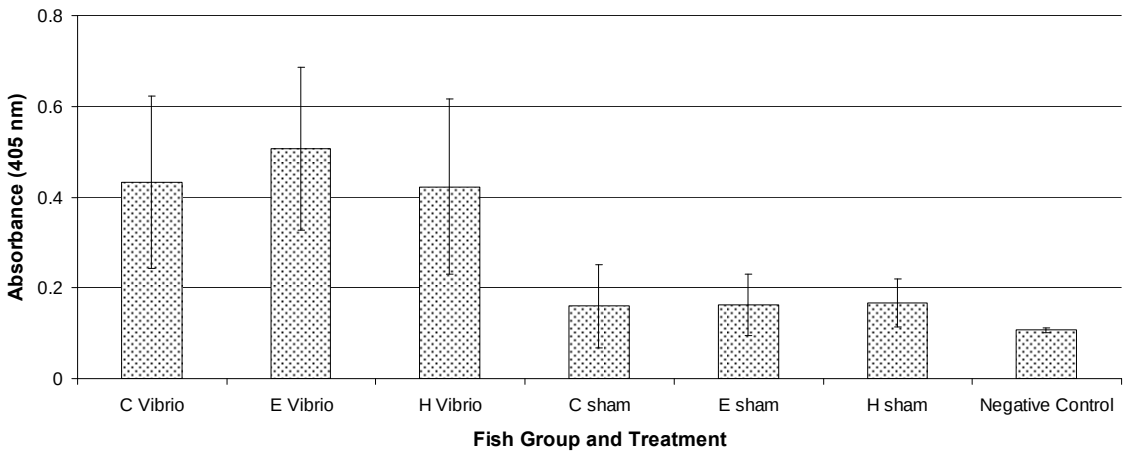


Figure 2-3. Comparison of *Vibrio* injected versus PBS injected Chinook salmon.

ELISA results in which each column indicates mean absorbance ±1 SD of five fish for each combination of group and treatment, except E sham which consisted of four samples. The last column represents negative control wells coated with coating buffer instead of *Vibrio* antigen. All three *Vibrio* immunized groups together presented a significantly higher absorbance ($P < 0.001$) than the sham fish (PBS vaccinated). Note the similarity in absorbance levels of the three *Vibrio* injected groups. C: channel fish. E: egg-transferred fish. H: hatchery fish.

Humoral immune response of juvenile Chinook salmon in saltwater

Antibody response against *V. anguillarum* increased in both groups of fish during the first three months of saltwater rearing, as shown by the absorbance readings obtained with the ELISA assays (Figure 2-4). Fish individual spectrophotometer readings are plotted in Figure 2-5 where it can also be seen a positive trend for all fish under study. Values of the slopes of each individual are represented in Figure 2-6 discriminated for channel and hatchery fish where it can be seen that both groups presented similar values. The ANOVA analysis using the individual slopes as the antibody response data further confirmed that there were no significant differences between channel and hatchery fish ($F_{0.05, 1, 5} = 0.01$, $P > 0.93$).

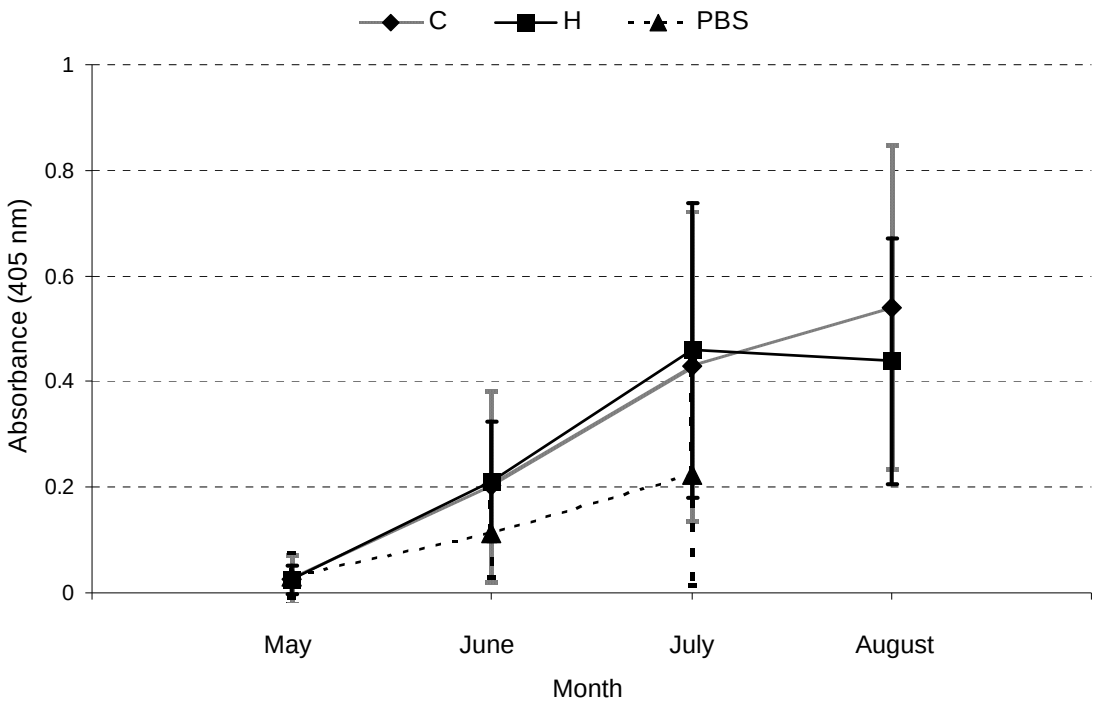


Figure 2-4. Long-term antibody response of Chinook salmon reared in saltwater.

ELISA results of anti-*Vibrio* antibodies in juvenile Chinook salmon. Lines connect measurements representing mean values ±1 SD for each group in the four months analyzed. From May through July the same 46 fish were followed: 21 channel fish and 25 hatchery fish. From those, only 10 channel fish and 14 hatchery fish were measured in August due to mortalities. Channel and hatchery fish presented almost identical development of their antibody response and showed high variability.

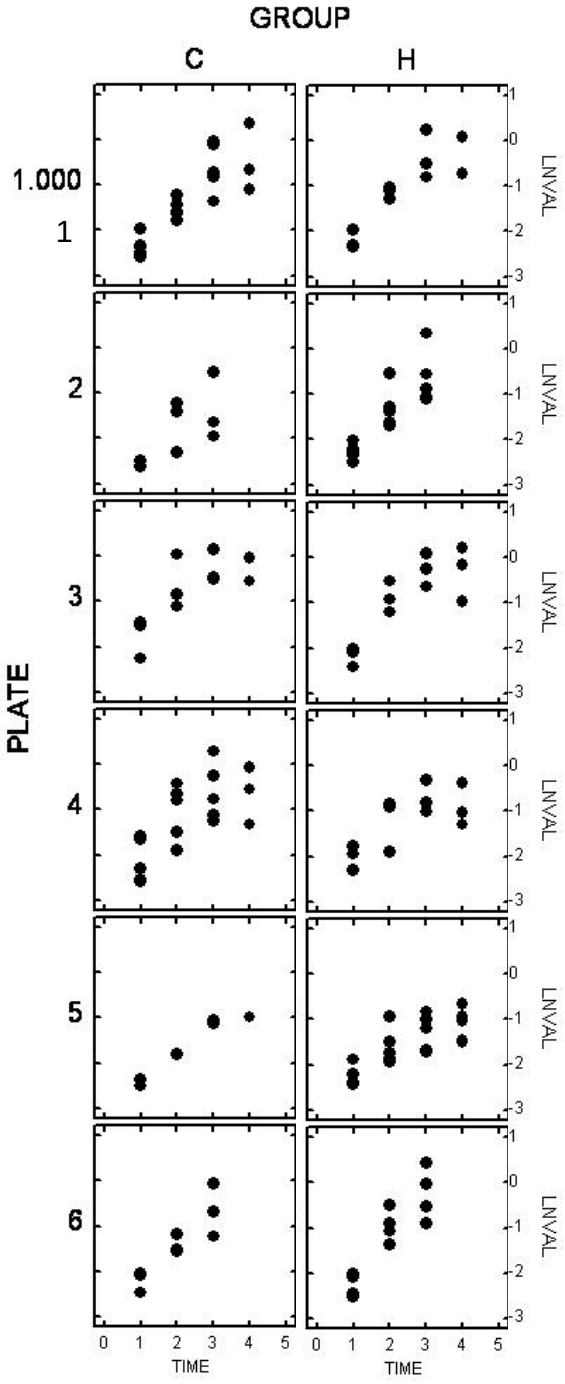


Figure 2-5. Individuals' long-term antibody responses during saltwater rearing.

Scatter plots of the humoral immune response of individual fish as it was analyzed in six ELISA plates (plate numbers on the left). Measurements are the natural logarithm of the absorbance of anti-*Vibrio* antibodies. There is a clear tendency that the immune response increased with the time, indicated by sampling months: 1 = May, 2 = June, 3 = July, 4 = August. From May through July the same 46 fish were followed: 21 channel and 25 hatchery fish. From those, only 10 channel and 14 hatchery fish were measured in August due to mortalities. C: channel fish. H: hatchery fish.

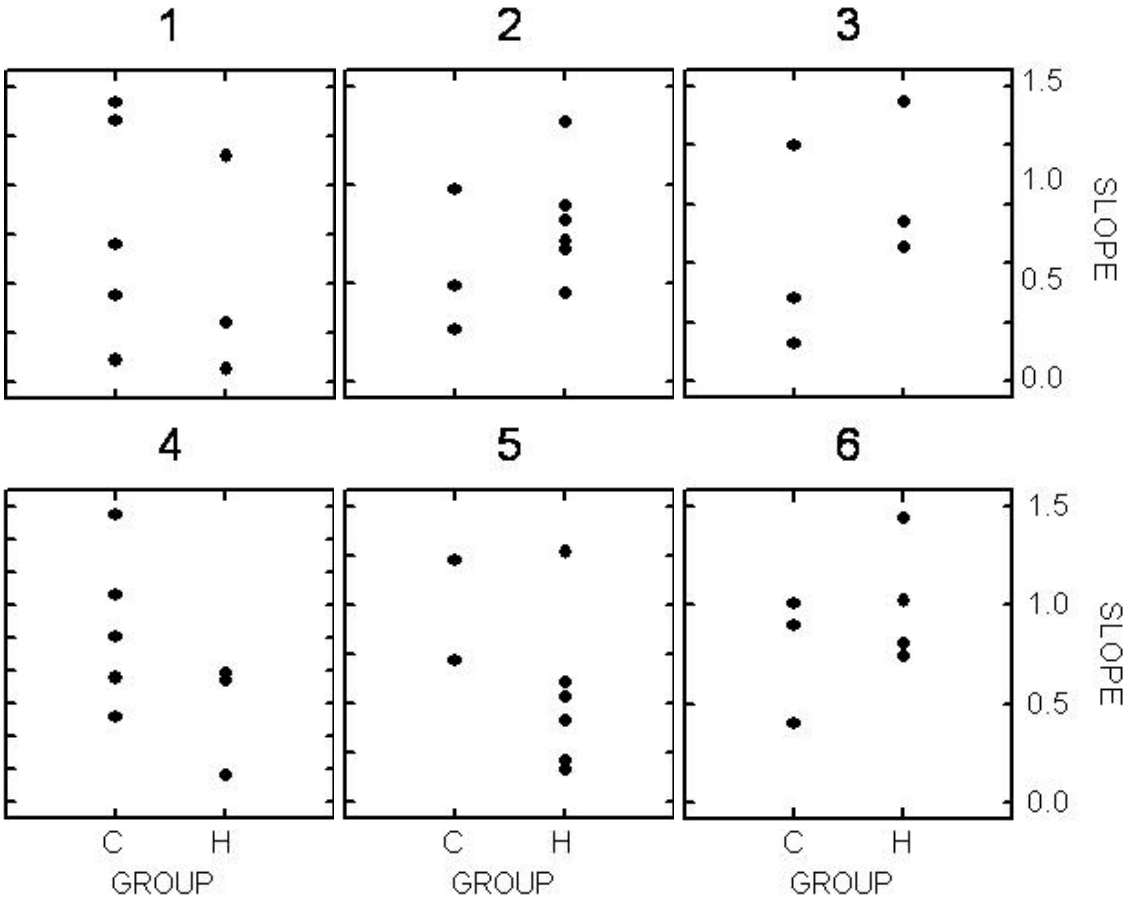


Figure 2-6. Slopes of individuals' developed long-term antibody response.

Scatter plot representations of the slope values obtained for the development of the humoral immune response for each of the 46 fish analyzed during the four-month period of rearing fish in sea cages. Each graph represents one of the six ELISA plates used in the analysis (plate numbers on top of each plot). Individuals are grouped for channel fish (C) and hatchery fish (H) groups.

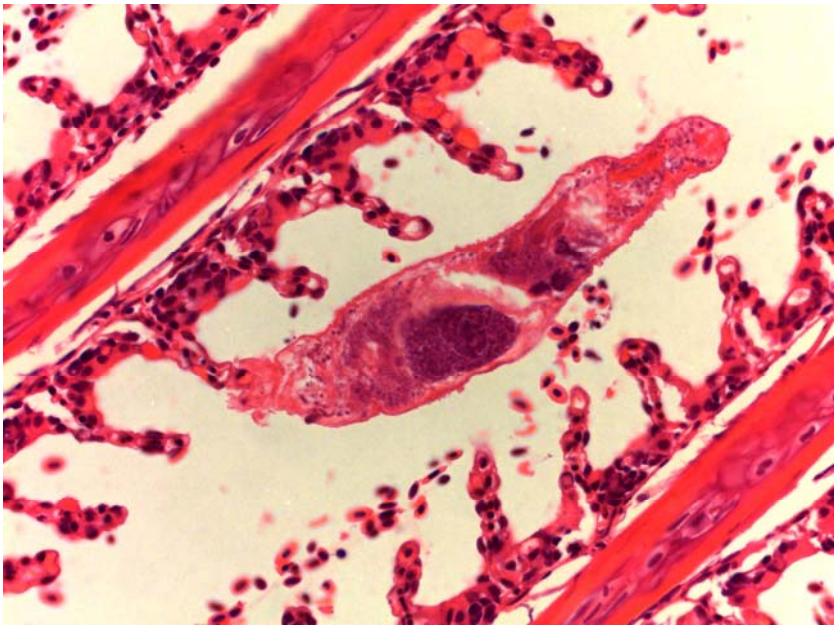
Parasite loads of juvenile Chinook salmon in saltwater

Gill samples were collected for a total of 107 individuals: 24 in June, 26 in July, 27 in August and 30 in October. All 107 samples were treated with hematoxylin and eosin staining (Figure 2-7a) and parasite load was recorded (Appendix A1). The majority (96%) of fish sampled in June (23 out of 24) and July (25 out of 26) were infected with monogeneans (Table 2-1), though differences between CH and H fish were not significant (Tables 2-2 and 2-3 for June and July respectively). Infection in August dropped to 29.6% of fish analyzed (N=27), which included three CH and four H fish (Table 2-1). Four fish (13%) of the 30 inspected in October were infected (Table 2-1), but no group data could be collected at this sampling time due to technical problems with the PIT tag reader.

Immunohistochemistry (Figure 2-7b) was utilized on a total of 76 samples in search for evidence of *Loma salmonae* infection: 19 out of the 26 collected in July, in addition to the 27 and 30 collected in August and October respectively. A total of 13 samples presented xenomas of *L. salmonae* during this study (Appendix A1). Microsporidian infection was very low throughout the summer and early fall (Figure 2-8b). Four samples were infected in July of which two were CH and two were H fish, and only two infected samples were found in August: one H fish and one unknown (Table 2-1b). Finally, seven fish were found infected in October but no group data could be collected.

Only three individuals, one H fish in June, one CH fish in July one H fish in August were detected bearing sea lice during the length of this study.

a)



b)

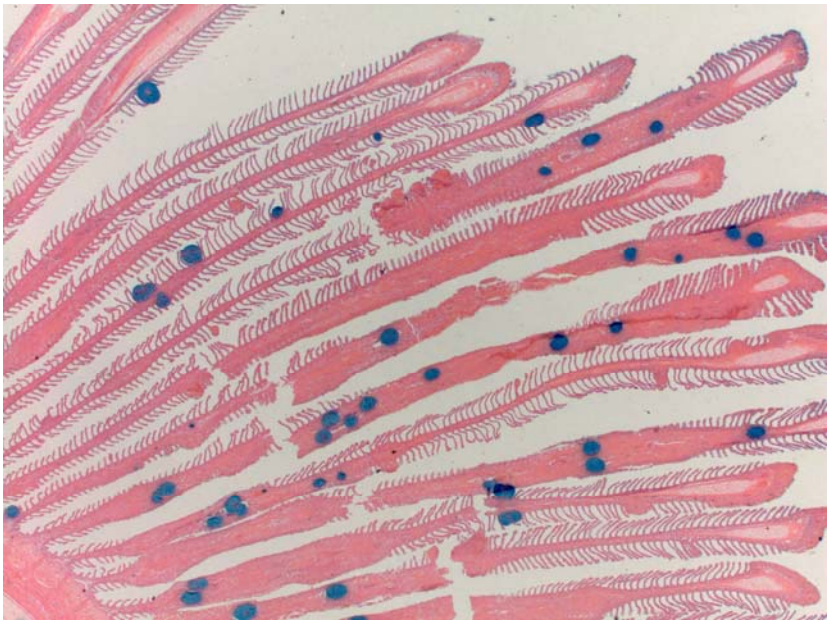
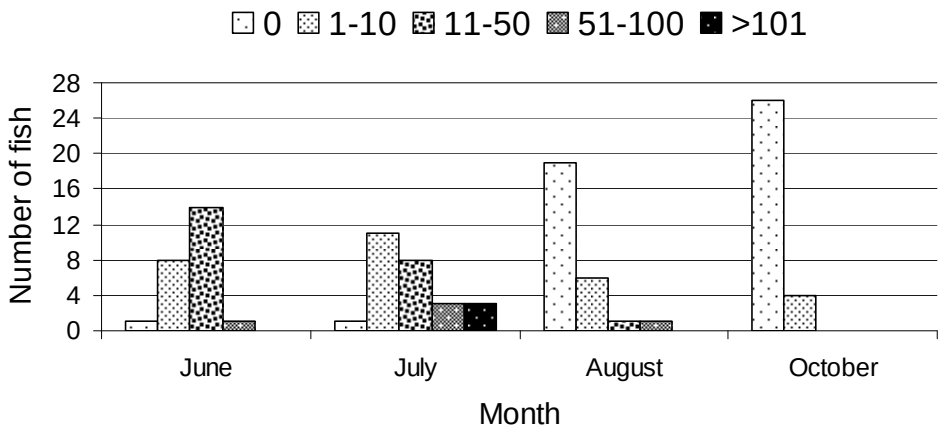


Figure 2-7. Parasites found in gill of Chinook salmon.

Parasites found on gill tissue in histological analyses. Pictures taken with microscope on stained section of gill of Chinook salmon. a) Monogeneans feeding on gill. Stain: hemaetoxylin and eosin. Magnification: 400X. b) Xenomas of *L. salmonae*. Section treated with chicken antibodies specific for xenomas. Counterstain: eosin. Magnification: 25X.

a)



b)

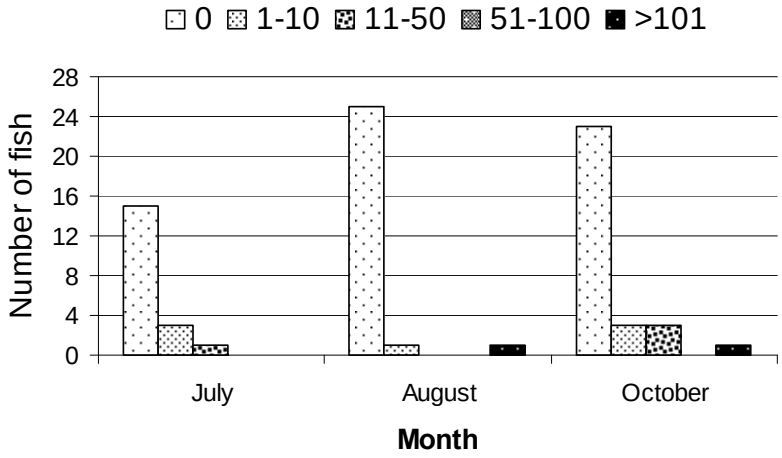


Figure 2-8. Temporal distribution of parasites in Chinook salmon reared in saltwater.

Number of fish with infections of monogeneans (a) and microsporidians (b) on samples collected through the summer and early fall. Monogeneans showed a decreasing level of infection through the summer. Microsporidians tended to be higher in October than in July and August, but number of infected fish remained very low (only 13 fish infected) which impeded to make generalizations. Bars represent number of fish with parasite content in the first left gill arch according to the range indicated. Four ranges were used to indicate number of parasites identified in a particular gill arch: 0, 1-10, 11-50, 51-100, and >100.

Table 2-1. Total number of fish analyzed and found infected for each fish group.

Tables show fish studied for monogenean infection (a) and microsporidian *L. salmonae* infection (b). Group column indicates either channel (CH) or hatchery (H) fish. June and July were months with strong monogenean infections, in which most individuals were infected. A few individuals were infected in August and October. Infections with microsporidian *L. salmonae* were generally very low during the length of the study, affecting both C and H groups. N/A indicates those cases where group discrimination was not possible, either because fish lost the PIT tag (three fish in August) or due to technical problems with the PIT tag reader as happened in October.

a)

		Monogeneans	
	Group	No. fish analyzed	No. fish infected
June	CH	12	11
	H	12	12
July	CH	14	14
	H	12	11
August	CH	10	3
	H	14	4
	N/A	3	1
October	N/A	30	4

b)

		Loma salmonae	
	Group	No. fish analyzed	No. fish infected
July	CH	9	2
	H	10	2
August	CH	10	0
	H	14	1
	N/A	3	1
October	N/A	30	7

Table 2-2. Analysis of monogenean infection in samples collected in June.

Differences between channel (C) and hatchery (H) fish were analyzed with t-tests. (a) Test of equality of variances that resulted in non-significant (p=0.438) therefore a t-test for equal variances was used. (b) T-test of equality of means resulted in no significant differences (p=0.459) between channel and hatchery groups.

a)

GROUP	N	Mean	Variance
C	12	27.583	457.538
H	12	21.667	282.788

95.00% Confidence Interval : 0.466 to 5.620
F-ratio : 1.618
df : 11, 11
p-value : 0.438

b)

GROUP	N	Mean	Standard Deviation
C	12	27.583	21.390
H	12	21.667	16.816

Pooled Variance

Difference in Means : 5.917
95.00% Confidence Interval : -10.373 to 22.206
t : 0.753
df : 22.000
p-value : 0.459

Table 2-3. Analysis of monogenean infection in samples collected in July.

Differences between channel (C) and hatchery (H) fish were analyzed with t-tests. (a) Test of equality of variances that resulted in non-significant (p=0.355) therefore a t-test for equal variances was used. (b) T-test of equality of means resulted in no significant differences (p=0.054) between channel and hatchery fish.

a)

GROUP	N	Mean	Variance
C	14	41.929	1,434.687
H	12	14.833	815.424

95.00% Confidence Interval : 0.519 to 5.626
F-ratio : 1.759
df : 13, 11
p-value : 0.355

b)

GROUP	N	Mean	Standard Deviation
C	14	41.929	37.877
H	12	14.833	28.556

Pooled Variance

Difference in Means : 27.095
95.00% Confidence Interval : -0.449 to 54.640
t : 2.030
df : 24.000
p-value : 0.054

Discussion

Humoral immune response in a short-term study in freshwater

ELISA analyses confirmed an increase in antibody levels specific for *Vibrio anguillarum* in fish immunized with a commercial vaccine administered by IP injection. Serial dilutions showed a linear trend in the range analyzed (Figure 2-1), indicating that 1:100 was a satisfactory dilution factor for comparison studies as has been previously found in other salmonids (Bøgwald et al., 1991). However, fish antibody responses usually have considerable inter-individual variability and the ELISA technique may have not been sensitive enough to detect differences between channel, egg-transferred and hatchery fish in the experiment conducted in freshwater (Figures 2-2 and 2-3). Nevertheless, it was possible to detect an increase in specific antibodies against *V. anguillarum* as reported in previous studies (Harrell et al., 1976), which confirmed the ability of Chinook salmon to develop a humoral immune response. This was especially true for the intraperitoneal injection as the route of exposure since it was very effective at inducing a strong humoral immune response as shown elsewhere (e.g. Palm et al., 1998).

Humoral immune response in a long-term study in saltwater

Fish that were repeatedly IP vaccinated for a period of four months did show increases in antibody levels against *V. anguillarum* as reported previously (Harrell et al., 1976). The increases in antibody levels can be seen between May and June, and June and July (Figure 2-4). Fish sampled in August showed almost similar levels of antibodies as

the previous month. The results found here showed a similar trend found by Kaattari and colleagues studying rainbow trout held in fresh water, in which the peak of the response was at 15 weeks post immunization with decreasing levels afterwards (Ye et al., 2011). However, the standard deviations in this study evidenced the high variability in the humoral response of the fish analyzed, resulting in the ELISA not being able to detect differences at the fish group level.

Parasite load in juvenile Chinook salmon reared in net pens

Three expected types of parasites were detected in the gills of Chinook salmon reared in saltwater through histological analyses, though at not very high levels of infection. Monogeneans constituted the main parasite group and equally affected channel and hatchery fish indicating both were susceptible to monogenean infection. No species identification was performed on the samples collected in this study but PCR-based tests could be employed for this purpose. The genus *Gyrodactylus* is comprised of 409 species and many molecular markers have been applied in their identification (Zietara and Lumme 2002). Gilmore and colleagues (2010) performed a phylogenetic characterization of *G. salmonis* that affect several salmonid species in the East coast of Canada, including Atlantic salmon and rainbow *trout O. mykiss*, using both nuclear and mitochondrial loci. It would be interesting to use those loci to characterize the specimens observed in the gills of Chinook salmon.

Monogeneans presented a decreasing level of infection through the summer that may indicate a seasonal effect (Figure 2-8a), a phenomenon described in other *Gyrodactylus* species such as the highly pathogenic *G. salaris* that affects Norwegian

Atlantic salmon (Appleby and Mo 1997). Studies on *G. salaris* (Jansen and Bakke 1991) and a related species, *G. bullatarudis* infecting guppies *Poecilia reticulate* (Scott and Nokes 1984), showed positive correlations between reproductive rate and water temperature. But interestingly, infection levels of monogeneans in Chinook salmon studied here decreased in late summer when water temperatures are actually higher. This may be a common phenomenon with gyrodactyloids that appears to be related to the ability of the host to develop an immune response (Bakke et al., 2002), which is more efficient at higher temperatures and therefore clearance of the parasite may occur at the end of the summer (Andersen and Buchmann 1998). At lower temperatures gyrodactyloids may persist in low numbers in the host and resist clearance until the next favourable season (Bakke et al., 2002), which agrees with the lower infection level seen in October. The findings by this study may be valuable information for the aquaculture industry as the high infection levels found in early and mid-summer may be indicative of the potential of this parasite to spread through the whole farm stock and produce an epidemic outbreak. Many groups of monogeneans, as is the case of Gyrodactyloids, are higly specific-taxa parasites and may not reproduce in species other than their host (Poulin 1992), but they can use other species as transport hosts (Bakke et al., 2002). Thus, the prevalence of monogeneans in Chinook salmon and other fish species in farm areas should be further studied in the Northeast Pacific Ocean to assess the potential risk salmon farms in BC may be exposed to.

The second group of parasites, the microsporidian *Loma salmonae*, was detected through the immunohistochemical analysis using chicken antibodies (Young et al., 2007). The advantage of this technique is the relatively easy way to identify and quantify pre-

xenomas stages and small xenomas that are difficult to detect with standard hematoxylin and eosin staining. Though the IgY used in this study is an excellent tool to assess infection levels, these polyclonal antibodies have shown to cross-react with *L. branchialis* (recently reassigned *L. morhua*, see Brown et al., 2010) infecting Atlantic cod *Gadus morhua* (Young et al., 2007). However, the possibility of the xenomas analyzed in the present study belonging to *L. branchialis* or any other *Loma sp.* is very remote since *L. salmonae* has been the only microsporidian described for all salmon and trout *Oncorhynchus spp*. (e.g. Brown et al., 2010; Bruno et al., 1995; Kent et al., 1996; Shaw et al., 2000). Moreover, many phylogenetic studies have concluded that there is a strong evolutionary history that relates each microsporidian species to its particular host species (Brown et al., 2010; Smith 2009; Vossbrink et al., 2005), supporting the hypothesis that xenomas found in Chinook salmon are most likely *L. salmonae*. Nevertheless, species level confirmation could be performed by PCR assay that has the advantage of being more sensitive and it is also able to detect earlier stages of infection that may not be possible with histological analyses (Docker et al., 1997).

The low number of fish infected with *L. salmonae* did not allow comparisons between fish groups. However, it is interesting to note that more fish were infected in October than in July and August (Figure 2-8b). This may be due to the pathogenicity of microsporidians that is known to increase with higher water temperatures, therefore they are expected to be more active in early fall (Becker and Speare 2004). Experimental studies have determined that sporogony and xenoma formation of *L. salmonae* occurs in the range of 9 to 20 °C (Beaman et al., 1999). Thus, the low water temperatures at YIAL farm site (8.9 to 9.7 °C at surface level in early fall) may explain the lack of infection

during summertime and the slightly increase in the fall. Another potential reason may be the low rearing density applied by YIAL on its net pens that may help to maintain lower stress levels and healthier fish than the usually intensive commercial salmon farming.

The third group of parasites, the sea lice, were found in only three fish, which did not allow comparisons to be made between CH and H groups. This is not an unexpected result since the majority of sea lice found in cultured fish in BC come from Atlantic salmon farms (Saksida et al., 2007a). In addition, a study on all wild Pacific salmon species found Chinook salmon to have the lowest intensities of sea louse (Beamish et al., 2005). Although Chinook salmon seems to have a naturally low prevalence of sea louse, there are two other potential reasons that may explain the low presence observed by this study in YIAL farm. First, there may be a timing effect as it occurs with the load of sea louse in cultured Atlantic salmon, whose infection level increase by a rate of 1% a month with the time fish is in seawater (Saksida et al., 2007b). The inspection of the fish studied here started right after the first year in seawater. Second, there may be a seasonal effect. Saksida and colleagues (2007b) found that the lowest infection rates occurred between June and September, which is the time when Chinook salmon was checked by this study. Thus, a comparison between CH and H fish at YIAL should be attempted during their second fall season in seawater. This might actually increase the probability of sea louse detection. Finally, the geographical position of a salmon farm may also influence the susceptibility to sea louse in Atlantic salmon (Saksida et al., 2007a). Thus, the YIAL farm site can have a naturally low incidence of sea louse due to the low culture intensities practiced by this salmon farm.

In conclusion, the experiments carried out by this study confirmed the ability of Chinook salmon to develop an immune response when immunized through injection with *Vibrio* bacterin. Antibody responses were detected in Chinook salmon at the fry stage and were also deloped by juveniles reared in saltwater. However, no differences in antibody response were found between hatchery-bred fish and the fish obtained through semi-naturally spawning channel technology. Parasite loads during saltwater rearing were also similar in both groups, either when infection was severe as it was the case with monogeneans or when infection levels were low with microsporidians. In particular, the absence of sea louse in YIAL farm site may have different reasons that should be addressed in further studies by examining different year-age stocks all year round.

Chapter 3. Effects of early-rearing environment and mate choice on the disease resistance of cultured Chinook salmon (*Oncorhynchus tshawytscha*) infected with *Vibrio anguillarum*

Introduction

Current aquaculture practices propagate fish stocks mainly by artificial mating, which may be accompanied by selectively breeding for traits such as growth rate and disease resistance but this practice also results in unintentional selection for negative traits. Hatchery rearing environments also contribute to the fixation of adaptive characters for the artificial environments but maladaptive for natural conditions (Lynch and O'Hely 2001). For instance, Heath and colleagues (2003) found decreasing egg sizes in artificially bred and reared Chinook salmon. Therefore, artificial mating and hatchery rearing environments have many unknown and perhaps undesired consequences for hatchery stock populations. These effects may not be evident at present time but could manifest if changes, as might be the case with oceanic conditions, occur in the future.

An alternative form of salmonid propagation is being used by some enhancement programs for Pacific salmon in a few locations on the west coast of North America. It is based on artificial spawning channels, which have several advantages compared with hatchery breeding practices. First, their maintenance costs, which are basically related to cleaning, are significantly lower than hatchery facilities making them more economical than standard hatchery propagation in tanks. Second, the brood stock is allowed to spawn with only moderate human intervention, which may include the establishment of fish density, allowing the natural process of sexual selection to occur (Neff et al., 2008). It is widely known that salmonids, like other teleosts, have a non-random mating system involving major histocompatibility (MH) gene-linked female mate choice (Bernatchez and Landry 2003). Third, despite being artificial constructs, spawning channels offer a much more natural environment than the stacked trays for holding the eggs and the

fiberglass or aluminum tanks used for fry rearing commonly used in hatcheries. Studies have shown that the early-rearing environment before smoltification can have profound consequences for the development of the brain (Kihslinger and Nevitt 2006). However, given all these advantages, this technology has not yet been developed and optimized for the aquaculture industry, which relies on artificial mating and indoor rearing environments.

The artificial selection process has unknown consequences for the hatchery stock population, particularly with respect to the immune system. To shed light on its consequences, this study conducted a disease challenge infecting cultured Chinook salmon with vibriosis. This disease produces haemorrhagic septicaemia with erythema in fins, vent and mouth (Egidius 1987) and can also cause gills to turn pale reflecting a severe anaemia (Toranzo et al., 2005). Vibriosis affects some 50 marine farmed fish species in which *Vibrio anguillarum*, a gram-negative, short rod-shaped and motile bacterium is the main infectious agent (Austin and Austin 2007). There are a total of 23 O serotypes described for *V. anguillarum*, of which serotypes O1 and O2 are the most pathogenic to farmed fishes such as all salmonids, cod (*Gadus morhua*), seabass (*Dicentrarchus labrax*), seabream (*Sparus aurata*), striped bass (*Morone saxatilis*), and turbot (*Scophthalmus maximus*) (Actis et al., 1999). In particular, 70% of isolates from salmonids belong to serotype O1 and about 20% to serotype O2 (Larsen et al., 1994).

The present study evaluated the potential effects of early-rearing environment and breeding strategy on the immune system of Chinook salmon subjected to vibriosis. To achieve this, two different breeding strategies were applied to common hatchery brood

stock and each of the resulting offspring groups were reared in two different environments, following which both were subjected to a disease challenge.

Materials and methods

Brood stock and breeding treatment

This study was conducted using Chinook salmon from a population maintained for six generations at Yellow Island Aquaculture Ltd. (YIAL), Quadra Island, British Columbia, Canada. YIAL initiated operations in 1985 with brood stock from the Robertson Creek hatchery on Vancouver Island and has eradicated the Y chromosome from their stock population in order to maintain only homogametic (XX) individuals (Hunter et al., 1983). Every spawning season, a fraction of the eggs are masculinised with testosterone to produce sex-reversed females that are used as brood stock in future crosses. Use of all-female stocks is widespread in aquaculture since females usually do not mature before reaching marketable size (Benfey 1996). Homogametic XX males were shown to reach similar sizes to XY males and also present similar plasma concentration of testosterone and 17β-estradiol (Heath et al., 2002). In addition, sexually reversed males have been observed to display similar spawning behaviour as XY wild males in the YIAL spawning channels (Garner et al., 2010).

YIAL became an organic salmon farm in 1989 when it stopped using antibiotics. In the following years, between 1990 and 1994, the farm lost about 65% of their stock in outbreaks due to two common diseases to Pacific salmon: bacterial kidney disease (BKD)

caused by *Renibacterium salmoninarum* and vibriosis due to *V. anguillarum*. Thus, their stock has been naturally selected for BKD and vibriosis resistance.

Two offspring groups were generated in the fall of 2006 using ordinary hatchery fish as brood stock in two different breeding strategies. The first group named "channel" (CH) fish were the offspring of brood stock allowed to spawn semi-naturally in 3.5x15 m spawning channels of about one meter water depth with a partially recirculating flow of approximately 300 L min^{-1}. Spawning channels were populated with 20 females (in an equal ratio of 4- and 5-year-old) and 12 males each. Some channels received an equal mix of 3- and 4-year-old males, whereas others received eight "jacks" (2-year-old mature males) along with two 3- and two 4-year-old males. Carcasses were removed daily from which fin clips and scales were collected. Fertilized eggs remained buried in the gravel until they hatched and alevins swam up in the spring season. Feedways were placed in each channel and fish were fed to satiation with commercial pellets (Ewos Canada Ltd., Surrey, BC). The second group called "hatchery" (H) fish was produced following standard hatchery techniques characterized by artificial mating. Eggs and milt from randomly chosen brood stock were mixed in plastic cups for fertilization in a 2 ♀ x 3 ♂ cross design using 4- and 5-year-old females and 2- ("jacks"), 3- and 4-year-old males. Fertilized eggs were incubated in vertical stacked trays with a constant freshwater flow. After hatching, alevins were removed to indoor fiberglass tanks under artificial light and fed to satiation with commercial pellets (Ewos Canada Ltd., Surrey, BC).

Environmental treatment

Four groups of fish were attained by reciprocally switching the fresh water environments of the two differently bred fish for five months before smoltification. During the first week of May 2007 all channel fry were seined. Two spawning channels were set up to contain a total of 400 channel fry each: 100 from channels that did not receive male jacks plus 300 from channels set up with jacks. Two other spawning channels were stocked with 400 hatchery fish each, 100 fish from families that did not include male jacks in the crosses and 300 fish from families that included jacks. A similar procedure was performed in the hatchery by using four 600 L tanks. Two tanks were set up with 400 channel fish each, 100 fish from channels that did not receive male jacks plus 300 fish from channels set up with jacks. Two other tanks received 400 hatchery fish each, 100 fish from families that did not include male jacks in the crosses and 300 fish from families that included jacks. In short, the four groups obtained were: channel-bred fish reared in the channels (named CH/CH fish), channel-bred fish moved to hatchery tanks (CH/H), hatchery-bred fish reared in hatchery tanks (H/H) and hatchery-bred fish moved to the channels (H/CH). Freshwater supply to channels and hatchery tanks came from an artificial pond filled constantly with underground well water. The pond's water input was located on its side at about 35 cm height discharging in a 45 degree angle allowing pumped water to oxygenate and release some nitrogen before entering the pond reservoir.

Disease challenge set-up

At the end of the five-month environmental switch period, in early October, specimens from each of the four groups of fish were uniquely fin-clipped in one of the pectoral or pelvic fins and were mixed in equal numbers in 600 L tanks with UV-treated pumped seawater. Fish were acclimatized to saltwater tanks for three weeks, during which they went through the smoltification process. Control samples were taken from each group by euthanization with an overdose of MS-222 (Syndel Intl. Inc., Vancouver, BC). Fish numbers at the start of the disease challenge are shown in Table 3-1. Two replicate tanks contained 92 fish from each group, totalling 368 fish per tank while the two other replicate tanks contained only 32 and 34 CH/H fish each due to stock limitations, which was compensated for by adding extra fish from the other three groups to maintain similar density levels (Table 3-1). Contingency table analyses were later performed to test for independence of results from the differences in numbers of individuals per group in each tank.

Table 3-1. Experimental set up of replicate tanks for the disease challenge.

Fish numbers from each group allocated in each of the four replicate tanks (named A1, A2, B1, B2) prior to the disease challenge. (*) CH/H fish group was limited therefore less fish were available for replicate tanks B1 and B2. Fish from the remaining groups were added to maintain similar density levels in all tanks. CH/CH: channel-bred fish reared in the channels. CH/H: channel-bred fish moved to hatchery tanks. H/CH: hatchery-bred fish moved to the channels. H/H: hatchery-bred fish reared in hatchery tanks.

	A1	**A2**	**B1**	**B2**
CH/CH	92	92	114	112
CH/H	92	92	32*	34*
H/CH	92	92	114	112
H/H	92	92	114	112
Total fish	368	368	374	370

Bacteria preparation and disease challenge

V. anguillarum was obtained from a slant maintained at 4°C in the facilities of the Pacific Biological Station (Nanaimo, BC) under case No. 2004-124 that was isolated from an Atlantic salmon individual on June 18 2004. A small amount from the slant was streaked onto trypticase soy agar (TSA) and grown at room temperature (RT) for 48 hours. Identity check by slide agglutination test with rabbit anti-*Vibrio anguillarum* antibodies confirmed that the strain corresponded to O1 serotype. In addition, Gram negative staining and motility on drop glasses were also confirmed.

Bacteria exposure consisted of placing fish from each tank into 50 L water baths containing 20 mL of a bacteria culture for 15 minutes and then returning them to their respective tanks. This methodology was applied to each of the four replicate tanks, preparing a new water bath each time. Mortalities were collected on a daily basis every 8 to 12 hours. Due to the relatively low mortality rates obtained after the first challenge, a one-hour exposure to the bacteria was performed on day 22 of the experiment. This second bacteria suspension was prepared by adding 280 mL of bacteria culture to a 600 L tank containing 160 L of water. The tank was divided into four compartments with a mesh net so that each one received the fish from one replicate tank. All *Vibrio* cultures used in the two disease challenges were prepared by inoculating three colonies grown in TSA plates to 40 mL of TSB placed in 50 mL Falcon® tubes and grown for 30 hours at RT. To estimate the amount of bacteria to which fish were exposed, serial dilutions by the factor of 10 were made for three culture tubes. Twenty-five μL of each dilution factor were plated in replicate TSA plates and grown for 48 hours. Number of colonies/plate

was counted on the plates containing the 10^{-5} dilution factor. Bacteria concentration for the three culture tubes were 1.54 x 10^8 cells/mL, 1.45 x 10^8 cells/mL and 3.44 x 10^8 cells/mL, which resulted in an average of 2.14 x 10^8 cells/mL. Thus, the first challenge had an estimated 8.56 x 10^4 cells/mL and the second exposure was made of an estimated 3.74 x 10^5 cells/mL.

The disease challenge was considered terminated at six weeks when fish stopped dying. Presence of bacteria was confirmed in a total of 64 dead individuals, which were inspected internally and head kidney tissue samples streaked on TSA plates and grown for 48 hours to allow colony formation. Fin clips, gill, spleen, head kidney and blood samples were collected before exposure and post-infection at 24 and 96 hours, and at 3, 4, 4.42, 5, 5.57, and 6 weeks. Remaining survivors were PIT-tagged and moved to a sea cage, after which three additional samplings were performed at 30, 50 and 96 weeks.

MH class II β1 genotyping

Total genomic DNA was extracted from fin clips preserved in 95% ethanol following a standard phenol-chloroform method. Since salmonids have two MH class II beta loci (Harstad et al., 2008), a two-stage PCR + 1 protocol (Borriello and Krauter 1990) was adapted to amplify the hypervariable region (exon 2) and avoid undesired heteroduplexes (L'Abbé et al., 1992). Reactions with 100 ng of DNA were started in a total volume of 25 µL containing 1X reaction buffer, 1mM Cl_2Mg, 0.2 mM dNTPs, 0.02 U μL^{-1} Taq, 0.04 µM of the forward primer B1FA 5'-CTTGGTCTTGACTTG[AC]TCAGTCA and 0.2 µM of the reversed primer B1RAHindIII 5'-CCCGAGAAGCTTCCGATACTCCTCAAAGGACCTGCA. The

amplification conditions were 5 min. at 95°C and 34 cycles of 45 sec. at 95°C, 30 sec. at 57°C, and 45 sec. at 72°C. Following a final extension of 2 min at 72°C, 1 μL of 10 μM B1FAHindIIIb 5'-ATAGAGAAGCTTGGTCTTGACTTG[AC]TCAGTCA was added to each reaction tube and run for one more cycle with a final extension time of 10 min. Amplicons were cloned into p-GEM T-Easy vector (Promega) and plasmid DNA was extracted from putative transformed colonies. Sequences were aligned manually using BioEdit v. 5.0.9 (Hall 1999).

Anti-*Vibrio* antibody detection and statistical analysis

Humoral immune response was assessed by measuring levels of anti-*Vibrio* antibodies in fish serum using an indirect enzyme-linked immunosorbent assay (ELISA). Antigen was prepared by homogenizing *V. anguillarum* serotype O1 and O2 vaccine (Microtek Intl. Inc., Saanichton, BC, Canada) with 0.1 mm zirconia/silicone beads. Polycarbonate 96-well plates (Evergreen Scientific, CA, USA) were coated overnight at RT with 100 μL per well of antigen diluted in one volume of coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6). Plates were rinsed with tris buffered saline containing 0.05 % Tween 80 (T-TBS) and blocked for one hour at RT with 300 μL of 5% skim milk in T-TBS per well. Plates were rinsed and probed for two hours at RT using 100 μL of fish serum dilution in BSA blocking buffer. After rinsing, a secondary rabbit anti-salmonid antibody diluted 1:1000 was added and incubated for another two hours at RT. Following rinsing, a third antibody goat anti-rabbit whole molecule alkaline phosphatase conjugate 1:2500 dilution was incubated at 37°C for one hour and rinsed again. Detection was performed using 50 μL per well of p-nitrophenyl phosphate (Fast p-

NPP; Sigma, MO, USA) in the dark at RT for 30 minutes. Reactions were stopped by adding 50 μL of 0.03 M NaOH and absorbance was read at 405 nm using a microplate reader (VERSAmax microplate reader, Molecular Devices).

The number of serum samples collected during the study made it impossible to compare them in one 96-well plate. As the ELISA is a technique subjected to inter-assay variation due to the many steps involved for each individual plate, an experimental design was essential. The design that made possible to compare all the samples in one whole experiment at once was the unbalanced randomized complete block design (RCBD), in which each 96-well plate is considered a "block". In a RCBD the random allocation of samples in different blocks counteract the "between block variation" therefore the expected ELISA inter-assay variation is cancelled. An unbalanced design was followed to compensate for the fact that the number of samples taken from each fish group not always was the same at each particular sampling time.

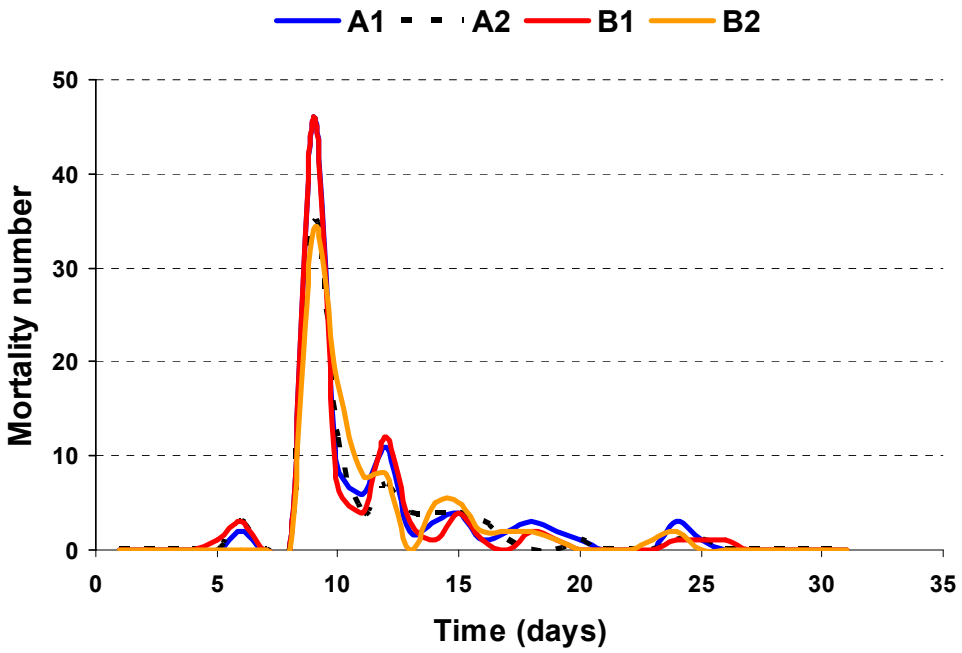
Statistical analysis was performed using the Statistical analysis system (SAS) v. 9.2 (SAS Institute Inc., Cary, NC), using a factorial design Type III ANOVA since there were fixed (plate) and random (environmental and breeding) factors in the model. The plate factor in this case was not relevant for the study as we know plates could be different due to the inter-assay variation. It was the effects of the environmental and breeding factors and their potential interactions that were assessed in this experiment.

Results

Disease challenge mortality

The four replicate tanks presented similar overall daily mortalities (Figures 3-1a) and the cumulative percentages were 24% and 19.9% for tanks A1 and A2, and 22% and 22.1% for replicates B1 and B2 respectively (Figure 3-1b). The contingency tables showed that the mortality numbers were independent of the particular densities for all the fish groups, including the CH/H group which was in lower proportions in two replicates (Table 3-2). A box and whisker plot revealed a strong environmental effect on the percentage mortality of the H fish that was not observed in the CH fish (Figure 3-2). Mean mortality percentage of H/H was 15.38 (S.D. = 2.84) whereas H/CH fish reached 33.64 (S.D. = 5.00). On the other hand, mean mortality percentages for CH fish were very similar in both rearing environments: 20.04 (S.D. = 3.37) and 23.40 (S.D. = 7.72) for CH/CH and CH/H respectively. A two-way ANOVA resulted in a significant genotype-by-environment interaction ($F_{0.05,\ 1,\ 12} = 17.95$, $P < 0.0012$) given by the breeding strategy and the early-rearing environment (Figure 3-3).

a)



b)

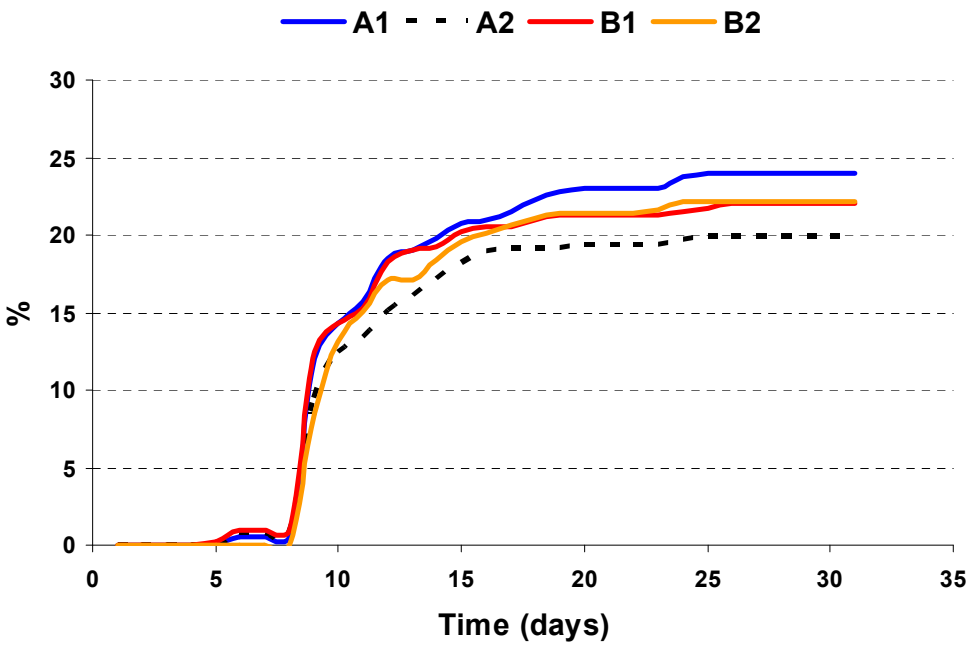


Figure 3-1. Mortality data of Chinook salmon in a disease challenge.

Data collected daily from Chinook salmon susceptible to *Vibrio anguillarum*. (a) Mortality number. (b) Cumulative percentage of daily mortality. Note the similarity among the four replicate tanks in both measures.

Table 3-2. Independence test of mortalities and fish group densities.

Contingency tables and Chi-square results testing independency of mortality of each group fish with respect to particular densities in each tank. a) H/H, b) H/CH, c) CH/H and d) H/H. Despite having two tanks with proportionally lower numbers, CH/H fish withstood independency.

a)

Count

		Fate		
		Dead	Live	Total
Tank	A1	14	78	92
	A2	15	77	92
	B1	21	93	114
	B2	13	99	112
Total		63	347	410

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.099[a]	3	.552
Likelihood Ratio	2.151	3	.542
N of Valid Cases	410		

b)

Count

		Fate		
		Dead	Live	Total
Tank	A1	32	60	92
	A2	25	67	92
	B1	38	76	114
	B2	44	68	112
Total		139	271	410

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.355[a]	3	.340
Likelihood Ratio	3.392	3	.335
N of Valid Cases	410		

Table 3.2. Cont'd.

c)

Count

		Fate		
		Dead	Live	Total
Tank	A1	25	67	92
	A2	17	75	92
	B1	5	27	32
	B2	11	23	34
Total		58	192	250

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.596[a]	3	.204
Likelihood Ratio	4.604	3	.203
N of Valid Cases	250		

d)

Count

		Status		
		Dead	Live	Total
Tank	A1	22	70	92
	A2	20	72	92
	B1	19	95	114
	B2	20	92	112
Total		81	329	410

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.172[a]	3	.537
Likelihood Ratio	2.155	3	.541
N of Valid Cases	410		

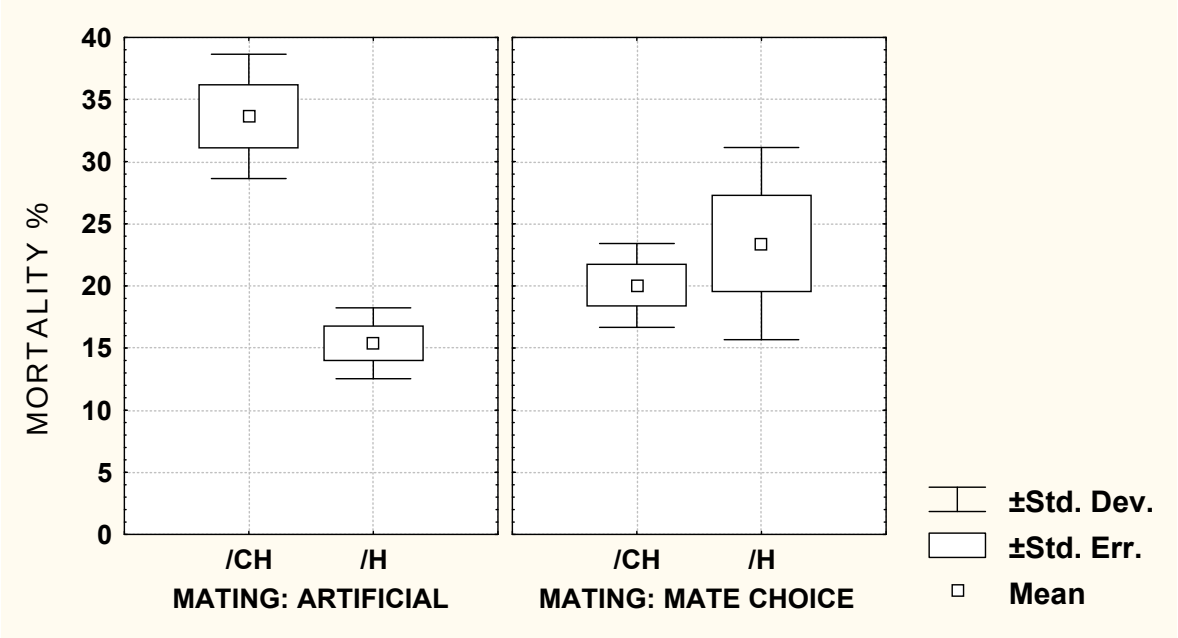


Figure 3-2. Susceptibility of Chinook salmon to *Vibrio anguillarum*.

Box plot showing susceptibility of Chinook salmon to *Vibrio anguillarum* under different breeding and rearing treatments. Graphs present mortality percentages of fish reared in different environments ("/CH": in spawning channels; "/H": in hatchery tanks) grouped by breeding strategy (artificial mating vs. mate choice in spawning channels). Boxes represent ± 1 SE. Whiskers represent ± 1 SD. Clearly artificial mating made offspring more susceptible to early-rearing environment than offspring from spawning channels. A two-way ANOVA indicated interaction of the two factors (breeding strategy and rearing environment) with p=0.0012.

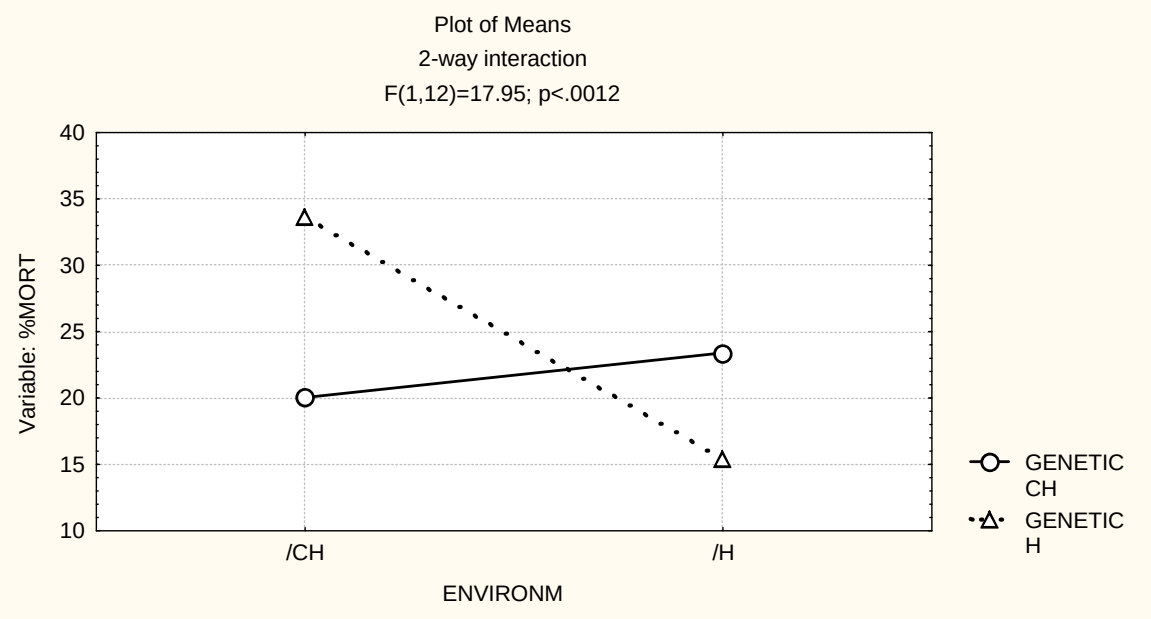


Figure 3-3. Norm of reaction for mortality percentage.

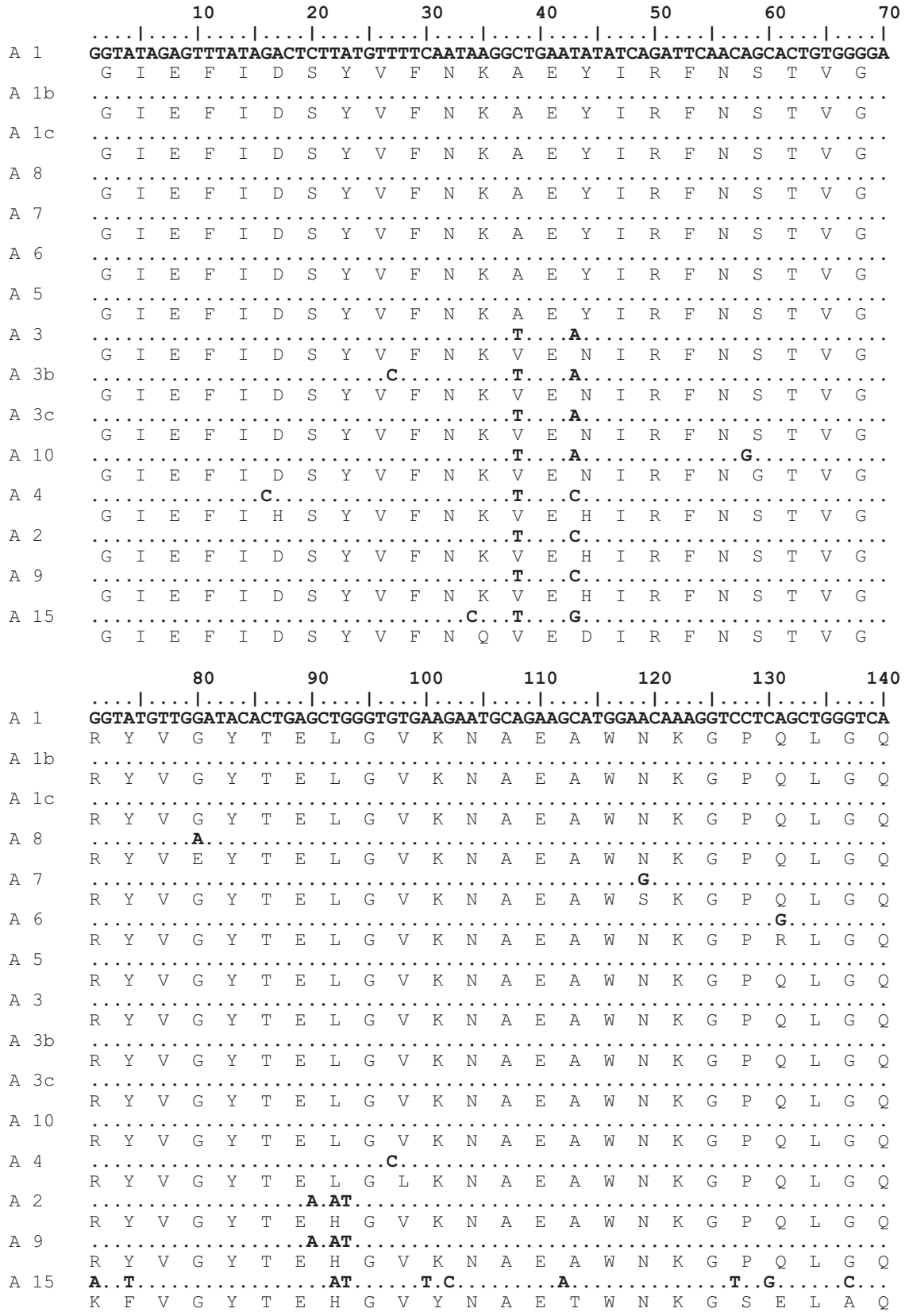
Norm of reaction for mortality percentage in the two breeding strategy groups (CH: mate choice in spawning channels and H: artificial mating) reared in two different rearing environments: outdoor gravelled channels (/CH) and indoor hatchery tanks (/H). Note the higher mortality variation in hatchery-bred fish compared with channel-bred fish.

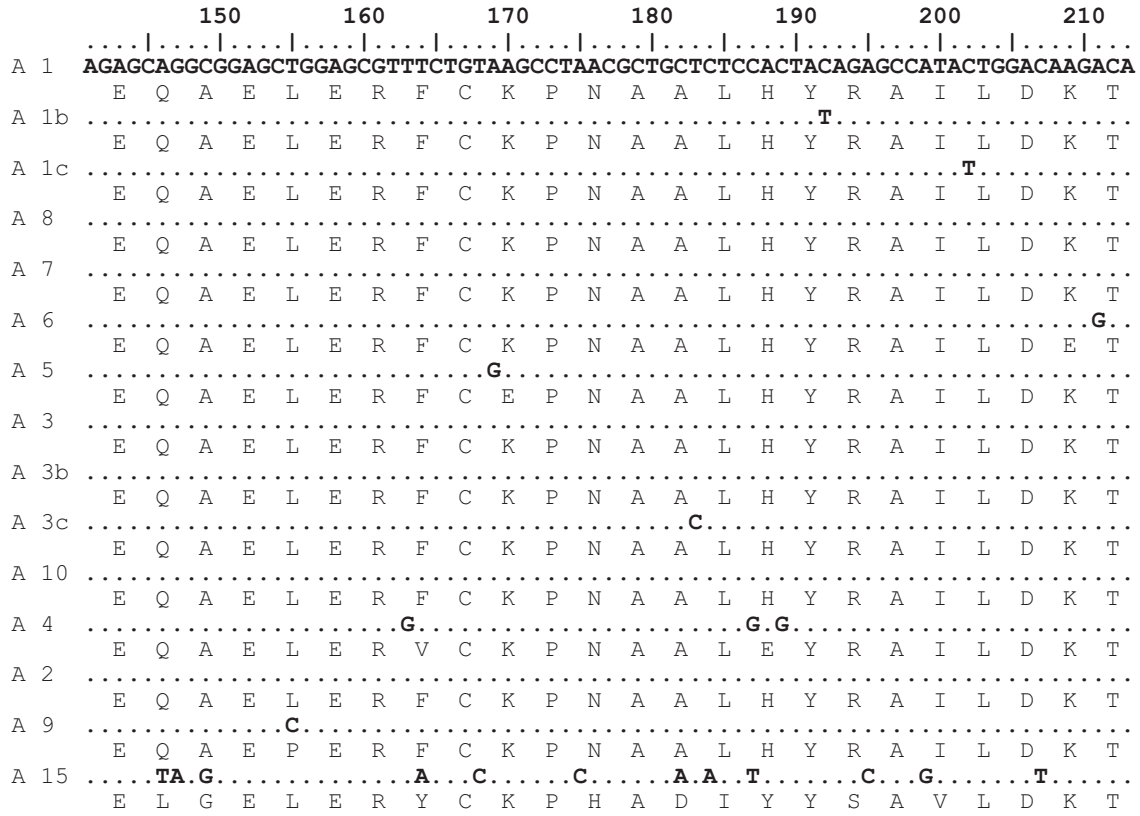
MH class II β1 genotyping

A total of 58 individuals were successfully genotyped for MH class II β1. Fifteen alleles were reported in the population (Figure 3-4). The analyses of MH class II β1 genotypes demonstrated that no particular individual allele was associated with mortalities or survivors (Figure 3-5a). Therefore susceptibility could not be attributed to bearing specific alleles or genotypes for MH class II β1. Out of 30 hatchery fish genotyped, 17 fish shared the same genotypes including eight survivors and nine mortalities (Figure 3-5b). Similarly, out of 28 channel fish genotyped, 15 fish shared genotypes which included five survivors and ten mortalities (Figure 3-5c). There was a tendency of a higher MH class II β1 heterozygosity in survivors compared with mortalities (Figure 3-6a), which was also seen in channel fish compared with hatchery fish (Figures 3-6b).

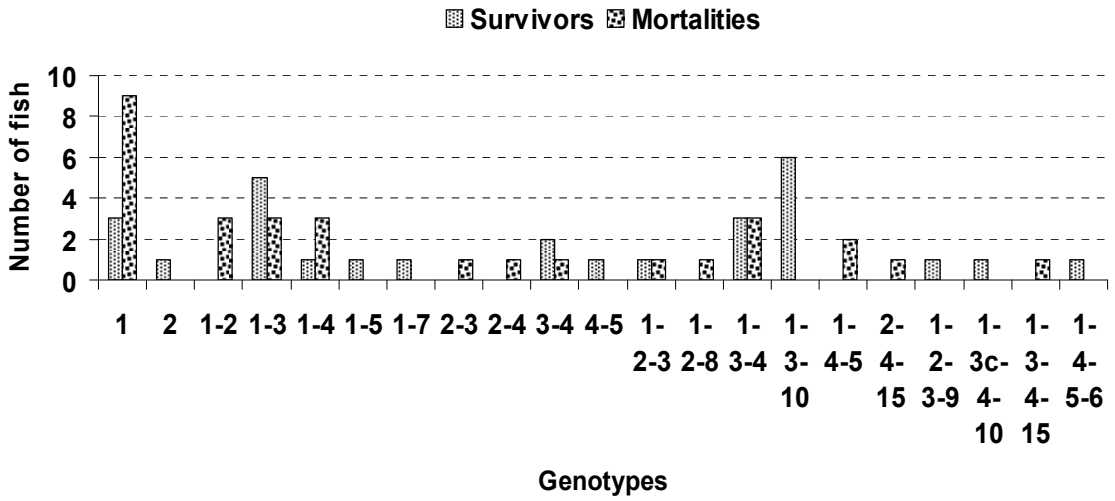
Figure 3-4. Nucleotide sequences of MH class II β1 alleles found in Chinook salmon.

Alignment of the 15 MH class II β1 alleles amplified from 58 samples (28 survivors and 30 mortalities) after a disease challenge. Amino acid translation is indicated below nucleotide sequence. The 15 nuleotide sequences encode a total of 11 different amino acid sequences. Sequences encompass 213 nucleotides from the exon 2 of the MH class II β locus, containing a hypervariable region important in antigen presentation to T cells. Note alleles 1b and 1c each differ in one nucleotide respect to allele 1 but do not alter the amino acid sequence. Similarly, alleles 3b and 3c each differ in one nucleotide with no changes in the amino acid sequence.


10 20 30 40 50 60 70
A 1 GGTATAGAGTTTATAGACTCTTATGTTTTCAATAAGGCTGAATATATCAGATTCAACAGCACTGTGGGGA
G I E F I D S Y V F N K A E Y I R F N S T V G
A 1b
A 1c
A 8
A 7
A 6
A 5
A 3
A 3b
A 3c
A 10
A 4
A 2
A 9
A 15
80 90 100 110 120 130 140
A 1 GGTATGTTGGATACACTGAGCTGGGTGTGAAGAATGCAGAAGCATGGAACAAAGGTCCTCAGCTGGGTCA
R Y V G Y T E L G V K N A E A W N K G P Q L G Q
A 1b
A 1c
A 8
A 7
A 6
A 5
A 3
A 3b
A 3c
A 10
A 4
A 2
A 9
A 15


150 160 170 180 190 200 210
A 1 AGAGCAGGCGGAGCTGGAGCGTTTCTGTAAGCCTAACGCTGCTCTCCACTACAGAGCCATACTGGACAAGACA
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 1b .....T.....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 1c .....T.....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 8 .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 7 .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 6 .....G..
E Q A E L E R F C K P N A A L H Y R A I L D E T
A 5 .....G.....
E Q A E L E R F C E P N A A L H Y R A I L D K T
A 3 .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 3b .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 3c .....C.....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 10 .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 4 .....G.....G.G.....
E Q A E L E R V C K P N A A L E Y R A I L D K T
A 2 .....
E Q A E L E R F C K P N A A L H Y R A I L D K T
A 9 .....C.....
E Q A E P E R F C K P N A A L H Y R A I L D K T
A 15 .....TA.G.....A...C.....C.....A.A..T.....C...G.....T.....
E L G E L E R Y C K P H A D I Y Y S A V L D K T

a)



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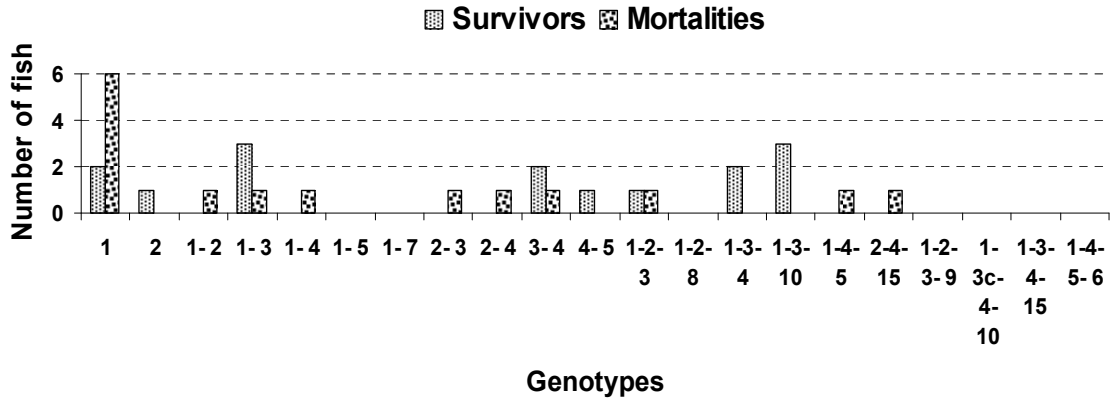

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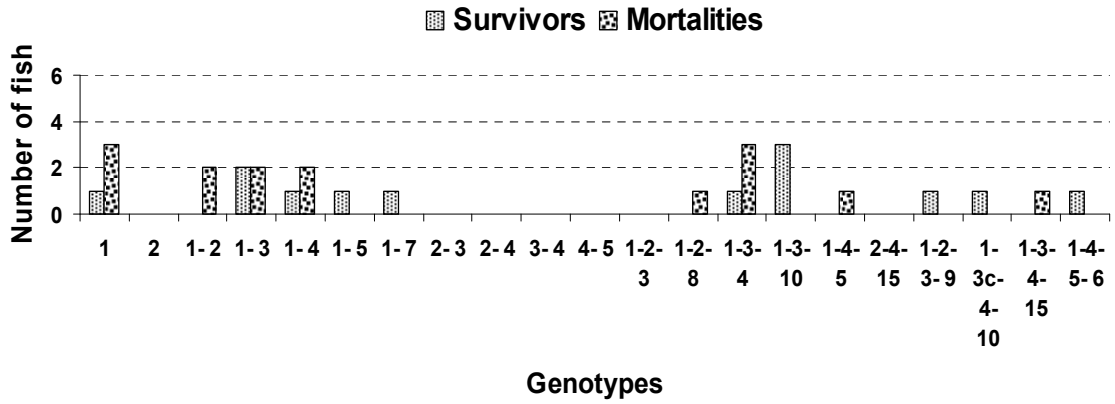
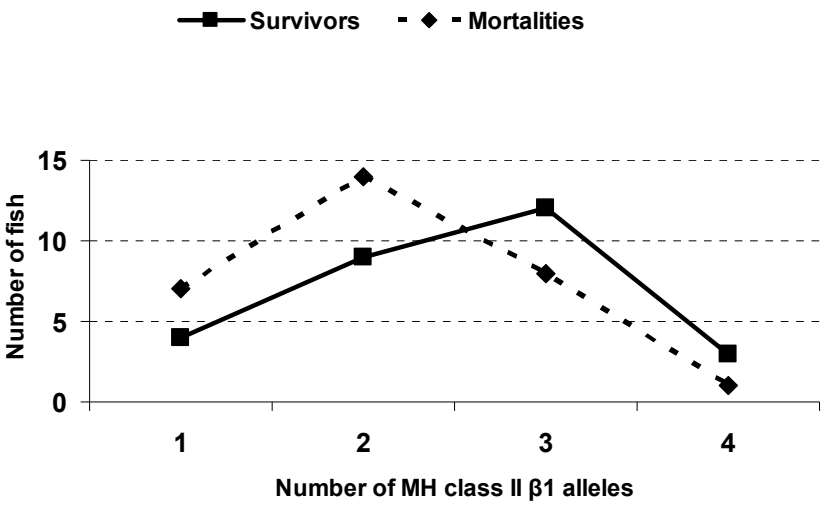


Figure 3-5. MH II β1 genotype distributions in survivors and mortalities.

MH class II β1 genotype distributions obtained from: a) 28 survivors and 30 mortalities. b) 30 hatchery fish, which included 15 survivors and 15 mortalities; and c) 28 channel fish which included 13 survivors and 15 mortalities. Note the contrast between hatchery (b) and channel (c) fish, in which the former did not have tetra-allelic individuals but showed several di-allelic fish.

a)



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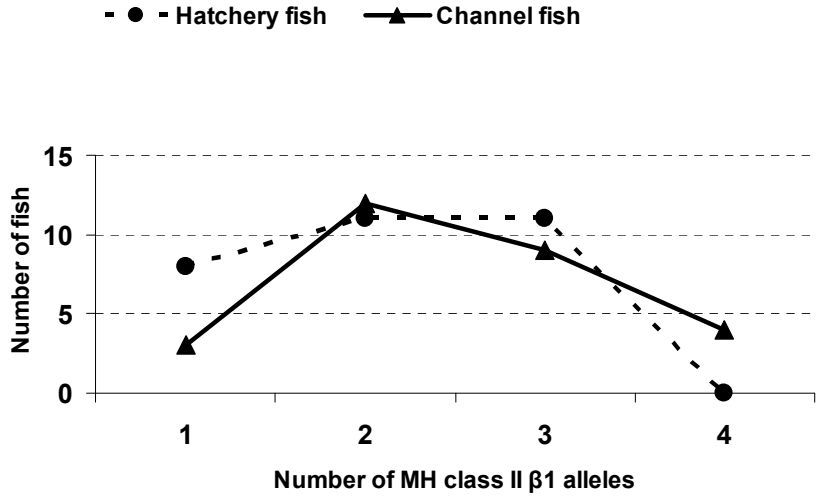


Figure 3-6. Heterozygosity distribution of MH II β1 alleles.

Graphs representing fish having one, two, three or four MH class II β1 alleles. (a) Survivors and mortalities. Survivors tended to have higher number of individuals bearing three and four alleles, whereas mortalities included more individuals with one and two alleles. (b) Hatchery and channel fish. Though both showed a similar overall trend, there were more hatchery fish bearing one allele, whereas more channel fish had four alleles (not one hatchery fish was found having four alleles).

Humoral immune response

A total of 279 serum samples were collected in the 11 sampling times. The outputs of the SAS software analysis showing raw data, ANOVA model and *P* values for each sampling time are presented in Appendix A2. Two sampling times showed significant genotype-by-environment interactions: 4 weeks (P = 0.0014) and 6 weeks (P = 0.0022). Two other time points, 0.57 (P=0.0230) and 5.57 weeks (0.0109), were significant for environmental and breeding effects respectively (Table 3-3). The samples collected at 96 weeks had no representatives of the CH/H fish group since this group had limited individuals. As the analysis was a randomized complete block design, meaning by complete the presence of each treatment group in each block analyzed, this sampling time could not be included in the analysis.

Table 3-3. Humoral immune response of Chinook salmon infected with vibriosis.

Analysis of the humoral immune response of four groups of fish attained after an environmental switch: H/H, H/CH, CH/H and CH/CH (refer to the text for specifications). Samples were collected at 11 sampling points (indicated in weeks), during a disease challenge and afterwards, when fish were transferred to sea cages. Plate column lists the ELISA plates used for each sampling time analyzed, whereas N indicates the total number of fish assessed per sampling time. Source of significance and *P* values were obtained from a type III ANOVA model with unbalanced RCBD analyzed using SAS software. Interactions between the genetic (breeding strategy) and environmental (rearing environment) factors were found at 4 and 6 weeks after exposure to the bacteria. Time 96 weeks was not possible to compare as one group (CH/H) was not represented in the sample due to their limited numbers.

Time (weeks)	Plate numbers	N	*P* value		
			Breed.	Env.	Breed.*Env.
0	1,2	15	0.1140	0.8652	0.1328
0.57	1,2	14	0.4572	**0.0230**	0.4375
3	1,2,3,4	32	0.8543	0.0877	0.1929
4	1,2,3,4,5	32	0.6314	0.0911	**0.0014**
4.42	5,6,7,8	32	0.6958	0.3615	0.1681
5	5,6,7,8	32	0.6318	0.4165	0.9095
5.57	5,6	12	**0.0109**	0.1259	0.3479
6	6,7,8	24	0.6001	0.0117	**0.0022**
30	7,8,9,10	36	0.9735	0.7936	0.1949
50	9,10,11	31	0.6250	0.4994	0.3464
96	9,10	19	N/A	N/A	N/A

Discussion

The rearing environment's effect on the disease resistance of the H fish was a notable result, as the mean mortality percentages doubled in the group reared in the spawning channels. Many studies have addressed how abiotic environmental parameters such as temperature, photoperiod, pH, oxygen level and salinity may affect the fish immune system (reviewed by Bowden 2008). For instance, temperature is known to affect many life history traits of salmonids such as embryo development rate (Hebert et al., 1998) and growth and size (Jonsson and Jonsson 2011). Many of these environmental parameters are to some extent altered in aquaculture conditions such as those YIAL has been using to rear Chinook salmon for six generations now. Therefore, the disease susceptibility observed in the H fish when placed in a different, more natural environment, may be the consequence of a relaxed selection for an efficient immune system in an artificial and protective pathogen-free environment. Thus, important fitness traits such as disease resistance may have alleles that are selected against that would be well adapted in the wild, and on the contrary alleles better suited for the artificial environment may have been favourably selected. This is a common phenomenon of captive breeding and domestication (Petersson et al., 1996).

Interestingly, CH fish showed a similar and moderate disease susceptibility in the two rearing environments compared with that of the H fish that. Thus, the CH fish presented a canalized immune response by cancelling out environmental effects. Environmental canalization refers to genotypes with a small change in the phenotype when subject to environmental variation, as observed in the flat norm of reaction of the

CH fish, as opposed to phenotypic plasticity that means a significant change in phenotype (Van Buskirk and Steiner 2009). The contrasting mortality patterns of H and CH fish groups showed a significant genotype-by-environment interaction effect that was detected both at the whole organism level through the fish susceptibility to vibriosis and at the molecular level through the detection of antibodies generated by the elicited humoral immune response. A recent study found survivorship at the larval and fry stage of Chinook salmon to be affected by genotype-by-environment interaction (Evans et al., 2010). Fitness traits such as disease resistance are inherited and therefore are subject to genotype-by-environment interactions (Garcia de Leaniz et al., 2007), having canalization and phenotypic plasticity as their alternative outcomes.

The canalized disease resistance by the CH fish is likely to have been the result of sexual selection. Mate choice is a very well known process described in many vertebrate taxa, including salmonids (Quinn 2005), and there is ample evidence that MH genes are involved in sexual selection processes (Milinski 2006). A previous study with Chinook salmon brood stock in YIAL spawning channels showed non-random mating with an increase in genetic diversity at the MH class II β1 gene (Neff et al., 2008). The tendency of a slightly higher heterozygosity for that gene in the CH fish in this study may well represent MH-linked mate choice, though a higher number of samples should be analyzed to firmly conclude that. In addition, MH class II β1 heterozygosity tended to be higher in survivors than in mortalities, a result which has been observed previously in Chinook salmon (Arkush et al., 2002). Contrary to the findings in some other studies on salmonids (e.g. Lohm et al., 2002; Grimholt et al., 2003), the 58 individuals genotyped here did not show an association between particular MH class II β1 alleles and susceptibility to the

disease. Thus, considering the lack of association of mortality to MH specific alleles and that disease resistance is a polygenic trait, other immune-related genes that may or may not be linked to MH genes could be implicated in the immune response against *Vibrio*. In this respect, it is important to note that both groups CH and H fish, though obtained through different breeding strategies, came from the same genetic pool which is the YIAL stock that has been naturally selected for *Vibrio* resistance. Therefore, it can be suggested that it is not just a random combination of alleles, but the actual combination of alleles into optimum genotypes that may have implications in the fish immune performance. Artifical propagation is carried out through a random mixture of eggs and milt, whereas mate choice implies non-random mating through sexual selection. There are different models on how females may choose their mates in a non-resourced based mating system as is the case in salmonids. In looking for a genetic quality gain for their offspring, females may look for "good genes" that will increase offspring fitness, or alternatively females may choose "compatible genes" that will maximize fitness when combined with their own. In population genetic models, "good genes" in mate choice contribute to the additive genetic variation whereas "compatible genes" adds to non-additive genetic variation (Neff and Pitcher 2004). Non-additive genetic variation includes interactions between homologs at a gene locus and the interactions between genes at different loci (Neff and Pitcher 2008). Recent publications have shown relationships between mate choice and non-additive genetic benefits in different vertebrate taxa. For instance, in the freshwater fish Chinese rose bitterling, *Rhodeus ocellatu,* female mating preferences for non-aditive benefits was found to correlate with MH dissimilarity (Agbali et al., 2010). Dynamic models of mate choice for non-additive

genetic benefits through directional selection for certain fitness traits in song sparrows showed it to contribute to the maintenance of genetic diversity especially when populations are of small size which eventually may improve their viability (Neff and Pitcher 2009). Particularly in Chinook salmon, not only MH genes showed contributions to survivorship but also many other genes whose epistatic effects seem to be important (Pitcher and Neff 2007). Thus, mate choice for non-additive genetic effects may explain the canalized disease resistance observed in CH fish.

In addition to sexual selection, natural selection may also have had a role in the canalized disease resistance of the CH fish group. The CH fish experienced a more natural environment than their counterpart H fish at the larval as well as the fry stage prior to the start of this experiment. They were exposed to a natural photoperiod and pathogens were not restrained as in the indoor hatchery tanks. Thus, natural selection could act upon the variation of reaction norms of the CH fish group by eliminating maladapted genotypes during early rearing in the channels. Genotype-by-environment interaction effects have been shown to affect the survival at the larval and fry stages of Chinook salmon (Evans et al., 2010), giving support to this argument. Moreover, a recent study on Atlantic salmon (*Salmon salar*) found evidence of pathogen-driven selection in natural environments (Dionne et al., 2009), which cannot be ruled out to happen in the spawning channels.

It is important to note that the Chinook salmon population at YIAL has a long history of artificial breeding. Their founder brood stock was taken from Robertson Creek, a DFO-operated hatchery which has been artificially propagating Chinook salmon since 1972 (DFO 2010). This means the YIAL stock has been under artificial selection for at

least nine generations. Therefore, it is remarkable to have found such significant differences in disease resistance after only one round of sexual selection and early rearing in a semi-natural environment. This may have important consequences for the aquaculture industry as there may be other fitness-related traits that may be favoured by the introduction of more natural propagation methods. Further studies are necessary to comprehend the magnitude of the effects that natural processes such as sexual selection may have in fitness-related traits for the aquaculture industry. Performing similar experiments to the one described here with channel-bred instead of hatchery-bred brood stock will definetely expand our understanding of the long-term effects these natural propagation processes have on important traits.

In conclusion, this study demonstrated that altering important aspects of the salmonid life cycle may have important consequences in a fitness-related trait. Genotype-by-environment interactions were found to affect the disease resistance of Chinok salmon against *V. anguillarum*. Although hatchery-bred fish reared in hatchery tanks performed the best, they also were the more vulnerable to environmental change. The high degree of domestication farmed stocks are exposed to in hatchery environments (Petersson et al., 1996) has the potential to affect their ability to fight bacterial infections in a changing environment clearly reducing their fitness (Fleming and Einum 1997). Considering that salmon aquaculture transfer juveniles to sea cages within a natural environment, canalization of the immune response in face of environmental perturbations may be an important advantage of channel-bred over hatchery-bred fish. Many oceanic conditions such as temperature and salinity may change in the near future which have the capacity for altering the fish immune system (Bowden 2008). Therefore, it is suggested that

introducing sexual selection through mate choice in spawning channels could produce fish with more robust immune systems capable of managing changes in the environment.

Chapter 4. Early-rearing environmental effects on gene expression in farmed Chinook salmon (*Oncorhynchus tshawytscha*) following infection with *Vibrio anguillarum*

Introduction

Vibriosis is the term used to define infection with bacteria belonging to the genus *Vibrio*. This disease affects some 50 marine fish species around the world and is the cause of numerous epizootics in salmon aquaculture (Austin and Austin 2007). It has also been described affecting bivalve molluscs (Bolinches et al., 1986; Freites et al., 1993; Beaz-Hidalgo et al., 2010) and crustaceans (Bowser et al., 1981; De la Peña et al., 1993; Jayasree et al., 2006).

Vibriosis in fish generally manifests as a haemorrhagic septicaemia with erythema in fins, vent and mouth (Egidius 1987), and gills may turn pale reflecting a severe anaemia (Toranzo et al., 2005). The bacterium can be transmitted through the water column (Kanno et al., 1989) through chemotactic motility mediated by its polar flagellum (O'Toole et al., 1996). The species most pathogenic to fish is *Vibrio anguillarum*, a gram-negative, short rod-shaped and motile bacterium (Austin and Austin 2007). It grows in a wide range of salinities and can survive more than one year in the environment (Hoff 1989) thus being a threat not only in saltwater but also in brackish and fresh waters (Eguchi et al., 2000). According to infection trials performed with several species such as rainbow trout *Oncorhynchus mykiss* (Baudin Laurencin and Germon 1987; Spanggaard et al., 2000; Weber et al., 2010), ayu *Plecoglossus altivelis* (Muroga and De La Cruz 1987), and zebrafish *Danio rerio* (O'Toole et al., 2004), *V. anguillarum* uses the host skin, intestinal tract and gills as main routes of penetration. Temperature also plays an important role in the infection process since fish held in lower temperatures (9-12ºC) presented lower bacteria loads than fish held at higher temperatures (16-18ºC) (Baudin Laurencin and Germon 1987).

There are a total of 23 O serotypes described for *V. anguillarum*, of which serotypes O1 and O2 are the most pathogenic to farmed fishes such as all salmonids, cod (*Gadus morhua*), seabass (*Dicentrarchus labrax*), seabream (*Sparus aurata*), striped bass (*Morone saxatilis*), and turbot (*Scophthalmus maximus*) (Actis et al., 1999). In particular, 70% of isolates from salmonids belong to O1 serotype and about 20% to serotype O2 (Larsen et al., 1994). Some O1 serotype strains harbour a 65 kb plasmid called pJM1 that contains the genes essential for the production of the siderophore anguibactin and its associated membrane transport proteins (Koster et al., 1991; Stork et al., 2002). This siderophore can efficiently sequester iron bound to host iron-binding proteins such as haemoglobin, transferrin, lactoferrin and ferritin that are found in the tissues and the interstitial fluids of fish (Crosa 1989; Griffiths 1991). One O1 serotype strain and all O2 serotype isolates lack this virulent plasmid indicating they have other unknown infecting mechanisms (Naka et al., 2011).

In aquaculture, physiological performance and susceptibility to disease may also be determined by the rearing environments fish experience early in life. Evidence of effects of rearing environment on fish development has been known for nearly three decades, discovered during the study of lower survival rates of hatchery fish in salmon enhancement programs (e.g. Hosmer et al., 1979; Fagerlund et al., 1981; Salonius and Iwama 1993; Fleming et al., 1997). Recent studies found differences in brain size between wild and hatchery rearing fish (Marchetti and Nevitt 2003) confirming that early rearing environment affects brain development (Kihslinger and Nevitt 2006). Furthermore, many researchers have analyzed the effects of early rearing environment on

behaviour (Brown et al., 2003; Metcalfe et al., 2003; Sundstrom et al., 2003, 2005; Braithwaite and Salvanes 2005).

Studies on the gene expression in salmonids infected with *V. anguillarum* have been focused mostly on immune-related genes using real-time RT-PCR. Quantitative RT-PCR has shown several cytokines to be up-regulated in Chinook salmon *Oncorhynchus tshawytscha* after 18 hours post- infection with *V. anguillarum* (Ching et al., 2010). In particular, the IL-1, TNF and IL-8 genes were activated, indicating the generation of an immune response.

Thanks to microarray technology it is now possible to conduct studies on immunological and physiological responses at a broad transcriptome level. This innovative method allows thousands of genes to be screened in parallel through the use of spotted pre-synthesized probes on hard (glass) surfaces (Schena et al., 1995). DNA microarrays have revolutionized human disease diagnostics by increasing its speed and accuracy (Yoo et al., 2009), and are being developed for the detection of pathogenic microorganisms in a broad range of hosts, including fish species important for aquaculture (González et al., 2004).

Microarray technology has been applied in studies of physiological and nutritional challenges such as smoltification in Atlantic salmon *Salmo salar* (Seear et al., 2010) and nutritional challenges such as feeding rainbow trout vegetable oils (Leaver et al., 2008) and phosphorus-deficient diets (Kirchner et al., 2007). They have also been employed to study pathogen-host interactions, particularly physiological and immunological reactions of Atlantic salmon to infections (Rise et al., 2004; Martin et al., 2006; Wynne et al., 2008; Young et al., 2008). In addition, microarrays for other fish species have been

developed and are being used to study diseases in Japanese flounder *Paralichthys olivaceus* (Matsuyama et al., 2007) and catfish *Ictalurus* spp. (Peatman et al., 2008). Furthermore, DNA hybridizations with microarrays developed for closely related species can also be performed. For instance, Ching and colleagues (2010) used GRASP (Genomic Research on Atlantic Salmon Project) microarray chips to hybridize cDNA from Chinook salmon infected with vibriosis in studies of gene dosage.

Microarray studies to characterize transcriptional responses to the injection of inactivated *V. anguillarum* have been performed in rainbow trout (Gerwick et al., 2007) and shrimp (Wang et al., 2008). A live disease challenge was performed on Chinook salmon (Ching et al., 2010) but comparisons at pre- and post-infection time were made using quantitative RT-PCR as described above. Therefore, no study has been conducted on a broad transcriptome level using a cDNA microarray on fish infected with vibriosis to study potential physiological changes.

The goal of this work was to assess the potential early-rearing environmental effects on the physiological and immunological responses of hatchery-bred Chinook salmon to a disease challenge with live *V. anguillarum*. The research consisted in the assessment of the physiological and immune response of cultured Chinook salmon in gill, spleen and head kidney tissues sampled at 24 and 96 hours after infection. Microarray hibridizations were performed on gill samples taken at 24 hours post-infection as *Vibrio* forms a biofilm around the fish body quickly after infection. On the other hand, hybridizations on head kidney and spleen tissues were carried out using samples collected at 96 hours post-infection as bacteria takes longer to reach internal tissues. In addition, comparisons were carried out using hatchery fish reared in two different environments

prior to the bacterial exposure. Fish reared only under standard hatchery conditions were compared with fish reared in a semi-natural environment for five months prior to the disease challenge. Therefore, this study was the first to use a cDNA microarray to assess transcriptional differences in pre- and post-*Vibrio* infected Chinook salmon as well as to analyze the potential effects of different early-rearing environments on the immune response to vibriosis.

Materials and methods

Experimental fish

This study was conducted in the facilities of Yellow Island Aquaculture Ltd (YIAL), a Chinook salmon farm located in Quadra Island, BC. YIAL initiated its operations in 1985 with brood stock from the Robertson Creek hatchery on Vancouver Island and has eradicated the Y chromosome from their stock population in order to maintain only homogametic (XX) individuals. Every spawning season sex-reversed females are generated by treating a fraction of the eggs with testosterone. Use of all-female stocks is widespread in aquaculture since females usually do not mature before reaching marketable size (Benfey 1996). Homogametic XX males were shown to reach similar sizes to XY males and also present similar plasma concentration of testosterone and 17β-estradiol (Heath et al., 2002). In addition, XX males presented spawning behaviour undistinguishable from normal XY males in a previous study in these channels (Garner et al., 2010).

YIAL became an organic salmon farm in 1989 when it stopped using antibiotics. In the following years, between 1990 and 1994, the farm lost about 65% of their stock in outbreaks due to two common diseases to Pacific salmon: bacterial kidney disease (BKD) and vibriosis due to *Vibrio anguillarum*. In this way, their stock has been naturally selected for BKD and vibriosis resistance.

The fish used in the present study called "hatchery" (H) fish was produced by artificial mating in the fall of 2006. Eggs and milt from randomly chosen brood stock were mixed in plastic cups for fertilization in a 2x3 cross design using 4- and 5-year-old females and 2-, 3- and 4-year-old males. Fertilized eggs were incubated in vertical stacked trays with a constant freshwater flow. After hatching, alevins were transferred to indoor plastic tanks under artificial light and fed with commercial pellets (Ewos Canada Ltd., Surrey, BC).

In early May 2007, 400 fish randomly taken from hatchery tanks were placed into each of two 3.5x15 m artificial spawning channels and continued being fed with commercial pellets. These outdoor gravelled-based channels had 1 m water depth with a recirculation flow of approximately 300 L min^{-1}. After five months of rearing in the channels, fish were seined and moved back to indoor hatchery tanks to set up the experiments. This group constituted the hatchery fish reared in the channels, named "H/CH" fish. The hatchery fish that was always kept under hatchery rearing conditions constituted the "H/H" group. For clarity, the terms H/CH and H/H will be used throughout the manuscript. Fish were fin clipped for identification purposes and were acclimatized in UV-treated pumped-sea water for three weeks before starting the experiment.

Disease challenge and collection of samples

Fish were subjected to a disease challenge with live *V. anguillarum* as described in Chapter 3. The source of the bacteria was a slant maintained at 4°C in the facilities of the Pacific Biological Station (Nanaimo, BC) under case No. 2004-124 that was isolated from an Atlantic salmon individual on June 18 2004. A small amount from the slant was streaked onto trypticase soy agar (TSA) and grown at room temperature (RT) for 48 hours. Identity check by slide agglutination test with rabbit anti-*Vibrio anguillarum* antibodies confirmed that the strain corresponded to O1 serotype. In addition, Gram negative staining and motility on drop glasses were also confirmed.

Exposure consisted of placing fish into 50 L water baths containing 20 mL of a bacteria culture for 15 minutes. Bacteria density in the water bath was estimated by preparing serial dilutions by the factor of 10 from three culture tubes. Twenty-five μL of each dilution factor were plated in replicate TSA plates and grown for 48 hours. Number of colonies/plate was counted on the plates containing the 10^{-5} dilution factor. Bacteria concentration for the three culture tubes were 1.54×10^8 cells/mL, 1.45×10^8 cells/mL and 3.44×10^8 cells/mL, which resulted in an average of 2.14×10^8 cells/mL. Thus, the water bath in which fish were exposed to *Vibrio* had an estimated 8.56×10^4 cells/mL.

Small tissue samples of approximately 2 mm^3 were collected from gill 24 hs after bacterial exposure, and after 96 hs from head kidney and spleen. Samples were placed immediately in 1.5 mL eppendorf tubes filled with RNA later solution and put transiently at -20 ºC and at -80 ºC for final storage.

Microarray experimental design

Transcriptional profile was analyzed using a 695-gene Chinook salmon cDNA microarray developed in the laboratory of Dr. Daniel Heath in the Great Lakes Institute for Environmental Research (GLIER). The cDNA library containing these Chinook specific transcripts comprised about one-third (234 transcripts) that did not match any known sequences on GenBank and were thus labelled as unknowns. The 461 transcripts that did have a match are listed in Appendix 3.

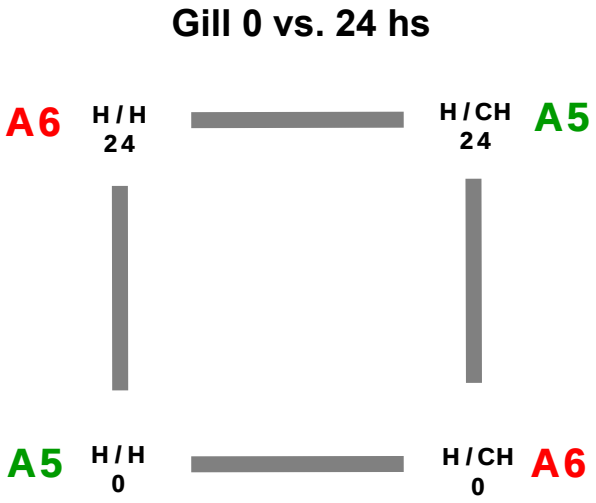
Tissue samples from gill, head kidney and spleen were compared at pre- and post-infection time. Gill tissue was analyzed at 0 (zero) and at 24 hs after exposure since bacteria produces a biofilm on the body during the first day of exposure and may contact the gills early in the infection. On the other hand, the bacteria may reach the fish internal organs after a longer period of time; therefore anterior head kidney and spleen were analyzed at 0 and 96 hours. In addition, comparisons were made for gill and spleen between samples collected from fish reared in different environments. Gill and spleen samples were assessed for gene expression using loop experimental designs as shown in Figures 4-1a and b. This design has the advantage of allowing comparisons in two axes: the horizontal axes compare expression between samples belonging to different rearing environments but from the same sampling time, whereas the vertical axes compare fish reared in the same environment before and after infection. Six loops utilizing four samples each were assessed for gill and spleen tissue, totalling 24 individuals for each tissue type. In the case of head kidney, four direct hybridizations (see Figure 4-1c) before and after infection were performed with fish reared in the spawning channels, totalling eight samples. Table 4-1 shows sample size per tissue and sampling time combinations.

Table 4-1. Sample sizes used in microarray assays.

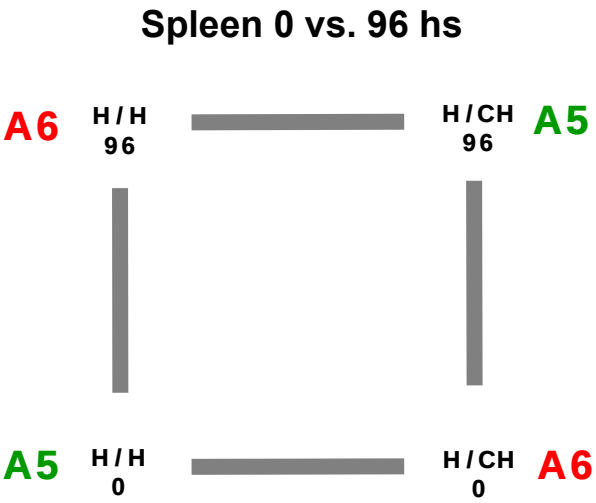
Discrimination of number of samples per tissue per group of fish and per sampling time. A total of 24 samples were analyzed for gill and spleen, whereas 8 samples were analyzed for head kidney. H/H and H/CH means hatchery fish reared in hatchery tanks and in spawning channels, respectively. In each tissue type, comparisons involved pre- and post- infected samples from the same group of fish. Gill and spleen tissue also compared hatchery fish from different rearing environments.

	Gill		Spleen		Head kidney
	N = 24		N = 24		N = 8
	H/H	H/CH	H/H	H/CH	H/CH
0 hs	6	6	6	6	4
24 hs	6	6	--	--	--
96 hs	--	--	6	6	4

a)



b)



c)

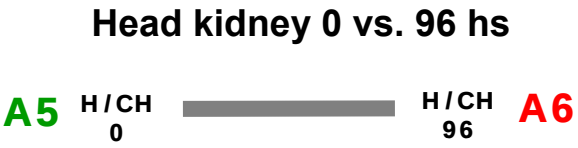


Figure 4-1. Experimental designs used in the cDNA microarray assays.

A loop design was utilized for gill (a) and spleen (b) in which comparisons involved two groups of fish and two time points: pre- and post-infection. In a loop design each sample is used in two assays. A simple pair comparison was used with head kidney tissue (c) in which only one group of fish was compared at pre- and post-infection. Slides are represented by grey bars. H/H and H/CH means hatchery fish reared in hatchery tanks and in spawning channels, respectively. Pre-infection is indicated as zero (0) and post-infection is indicated either at 24 or 96 hours. A5 and A6 indicate samples labelled with green and red fluorescent dye, respectively.

RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol (Invitrogen) following the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). RNA was resuspended in 40 uL of diethylpyrocarbonate (DEPC)-treated water and concentration was determined using a NanoVue Plus spectrophotometer (General Electric, UK) followed by DNase I treatment to avoid genomic contamination. Samples with a minimum $A_{260/280}$ ratio of 1.8 were used in the experiment.

The SuperScript Indirect cDNA Labelling System (Invitrogen) was used to produce the labelled cDNA for microarray hybridizations, called the target. Briefly, synthesis of first-strand cDNA was performed using SuperScript III RNase reverse transcriptase (Invitrogen) and RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). After cDNA synthesis 0.1M EDTA was added and the remaining RNA was hydrolyzed by adding 1N NaOH followed by pH neutralization with 1N HCl. Following purification with ethanol precipitation, cDNA was fluorescently labelled with either Alexa Flour 555 or Alexa Fluor 647 (Invitrogen) and subsequently purified to remove unincorporated dye. Experiments included dye swaps to correct for possible dye fluorescence bias.

Probe amplification and spotting of glass slides

The Chinook salmon cDNA microarray used in the present study consisted of a total of 704 transcripts obtained from cDNA libraries made from brain, liver and muscle, and some clones which were obtained from the GRASP project. The DNA material to be arrayed in the glass slides, called the probe, was obtained by PCR amplification from cDNA libraries and suspended in 30% (vol/vol) dimethyl sulfoxide. Spotting was performed on poly-L-lysine coated glass slides using the SpotArray 24 Micro Array Spotting System (Perkin Elmer). The post-printing process consisted of covalent bonding of DNA to poly-L-lysine with UV irradiation followed by DNA denaturation and blocking of unbound sites. Each array consisted of a total of 6336 spots distributed in three blocks (top, medium, and bottom) each containing 16 subarrays (Figure 4-2). Each subarray contained 132 spots arranged in 11 rows by 12 columns accommodating 44 genes in triplicates. Thus each block contained 704 transcripts in triplicates, totalizing 9 replicates per gene per array.

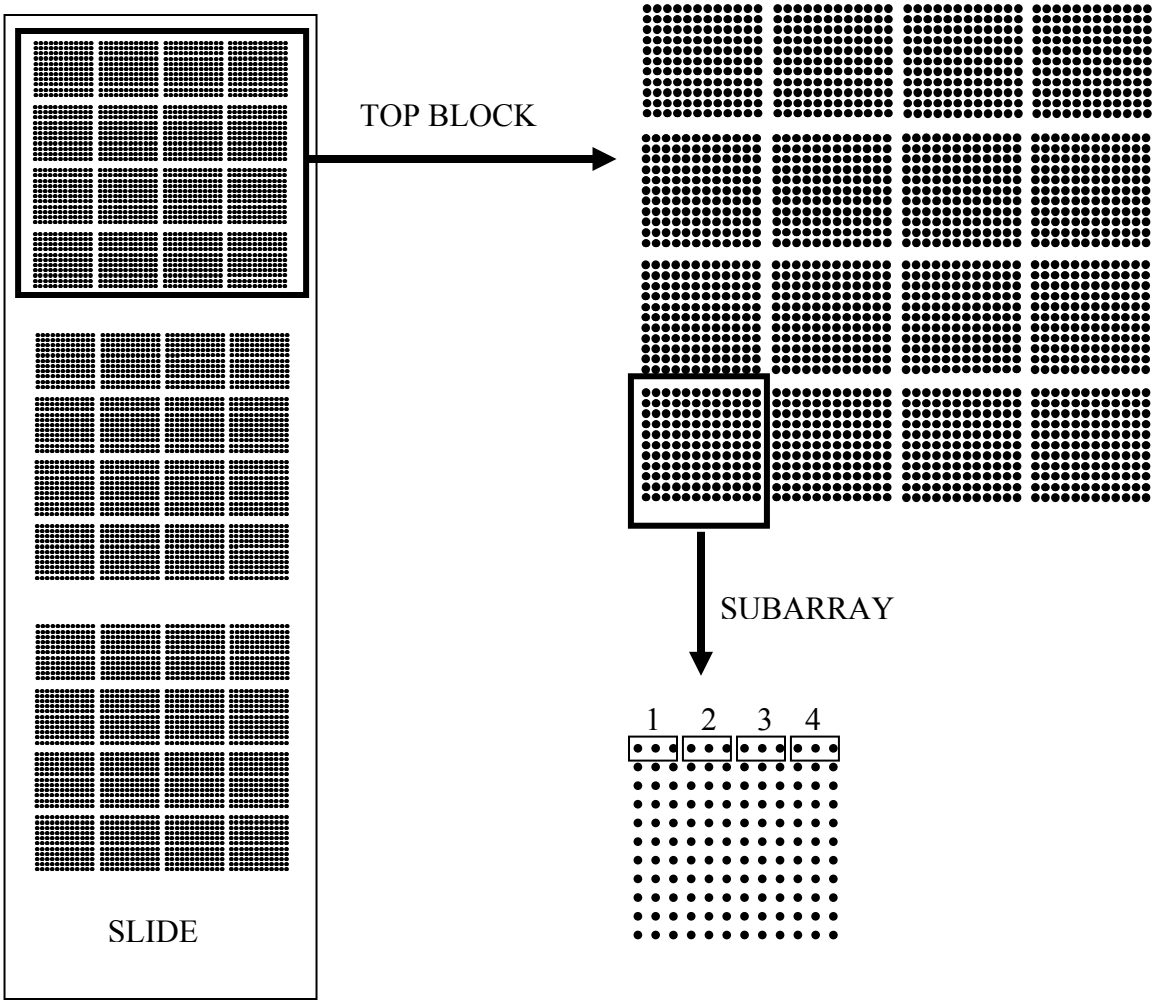


Figure 4-2. Diagram of microarray layouts.

Each microarray slide (left) consisted of a total of 6336 spots distributed in three blocks (top block is magnified on the right). Each block was constituted by 16 subarrays arranged in a 4x4 design. Each subarray consisted of 132 spots arranged in 12 columns and 11 rows. Each row contained triplicate dots of four different transcripts (as indicated by numbers above the first row in the subarray), thus containing 44 cDNA's in triplicate per subarray. Therefore, each block contained triplicates of the 704 transcripts used in the experiment. Thus, each microarray slide contained 704 cDNAs in nonuple totalling 6336 spots.

Hybridization

A hybridization solution was prepared containing 25% Hi-Di formamide (Applied Biosystems, Foster City, USA), 0.1% sodium dodecyl sulfate (SDS), 5X SSPE buffer (3.0 M sodium chloride, 0.2 M sodium hydrogen phosphate, 0.02 M EDTA, pH 7.4), 10% dextran sulfate, 1.5% polyadenylic acid potassium salt (polyA; Sigma-Aldrich, St. Louis, USA) and 6% Human Cot-1 DNA (Invitrogen). To set up the hybridization chambers, 7 uL of each labelled cDNA sample was added to 43.2 uL of hybridization solution and gently placed on the glass slide followed by positioning the coverslip. Wells in the hybridization chambers were filled with 13 uL of 1X TE to keep an appropriate level of humidity followed by incubation in a 42°C water bath for 16 hours. Hybridized arrays were consecutively washed for two minutes in each of the washing buffers: 1X SSC containing 0.2% SDS, 0.2X SSC containing 0.2% SDS, 0.1X SSC and 0.05X SSC. Arrays were dried by placing them in 50 mL Falcon tubes and centrifuged at 1700 rpm for one minute.

Image and data analysis

Slides were read in a ScanArray Express microarray scanner (PerkinElmer) using the accompanying ScanArray Express Microarray Analysis System v. 4.0 (PerkinElmer). Quantitation was obtained using an adaptive circle segmentation method which compensates for morphological changes in the spots. Within-array normalization was performed with a locally weighted scatterplot smoothing algorithm (Lowess; Yang et al., 2001). All the genes on the array were used in the Lowess normalization since only a

small proportion of them were expected to be differentially expressed. The reason for that is that the expected pathways to be dysregulated are mostly related to immune function and the number of immune-related genes in the microarray is relatively low. The Lowess algorithm allows the removal of systematic variation such as the intensity dependent dye biases common in non-commercial arrays such as the one used here (Yang et al., 2001). Spot quality was assessed based on the relative signal to background intensity followed by a manual removal of flagged spots.

The normalized ratio of means after background subtraction was imported into an Excel (Microsoft) spreadsheet to proceed with further analysis. An interquartile range (IQR) was calculated using the replicate spots for each gene and outliers were defined if their value was outside 1.5 times the IQR from the first or third quartile. After removal of outliers, a unique Z-score value for the remaining replicate spots was obtained. A first coefficient of variation (CV%) was calculated with the replicates for each transcript. If the CV% was higher than 20%, the spot with the highest Z-score was deleted. Then a second CV% was calculated and if higher than 20%, the following highest Z-score spot was deleted. Finally a third CV% was calculated and if still was higher than 20% a careful inspection of the remaining replicates was performed, eliminating subsequent highest Z-scores. The transcript was eliminated from the analysis if the number of acceptable replicates was lower than 50% of the total replicates, thus only transcripts with at least 5 out of the 9 replicates were included in the gene expression analysis.

Replicate spots were then averaged obtaining a mean expression value for each dye for each transcript per array. The mean average of each dye was then exported to a text file and array missing values for certain transcripts were imputed using the *impute*

package in R software (R Development Core Team 2010). Between-array normalization was performed separately on each dye using quantile normalization (Bolstad 2001; Bolstad et al., 2003) in R software as described in Deshmukh and Purohit (2007). Quantile normalization makes the distribution of dye intensities for each array the same over the entire set of arrays assuming an underlying common distribution of intensities across the arrays (McLachlan et al., 2004).

Differentially expressed genes were identified using the software Significance Analysis of Microarrays v. 3.06 add-in for Excel (SAM; Tusher et al., 2001). SAM calculates a statistic *d* for each gene, which is a standardized change in expression based on the ratio of change in gene expression to standard deviation in the data for that gene (Tusher et al., 2001). By using repeated permutations it calculates expected *d* scores for each gene and then both observed and expected *d* scores are ranked from higher to lower in magnitude and are represented in a scatter plot (Tusher et al., 2001). The cutoff for significance is given by a threshold (Δ) that sets the difference between observed and expected *d* scores. In addition, SAM algorithm calculates the *q-value* for each gene when setting a particular threshold Δ, which gives the percentage of the false positive genes (false discovery rate, FDR) that are expected in a gene list containing the gene for that q-value and all the genes that are more significant. The method used by SAM for setting thresholds provides asymmetric cutoffs for up- and down-regulated genes allowing the possibility that the *d* scores for those genes show different patterns, therefore producing asymmetric plots (Tusher et al., 2001).

Results

Results showing differentially expressed genes obtained with SAM are presented in several scatter plots (Figures 4-2 through 4-6). Those genes found as up-regulated are in red colour in the first Cartesian quadrant, whereas the down-regulated genes are in green colour in the third quadrant. When comparing tissues at pre- and post-infection time, the control sample was always that of time 0 (zero) or pre-infection. In the case of comparing tissues from hatchery fish reared in different environments, H/H fish was set as the control sample as it represents the standard method in aquaculture. Therefore, in the latter case transcriptional differences correspond to a higher or lower level of gene expression in H/CH fish compared with the H/H fish.

Pre- versus post-infection gene expression in gill

Fourteen and ten genes were up-regulated in gill tissue of H/H and H/CH fish respectively after 24 hours of exposure to *Vibrio*, but neither group showed down-regulated genes (Figures 4-2a and b). Four of those up-regulated genes were common in the two groups of fish (Tables 4-2 and 4-3). Two genes were alpha and beta haemoglobin subunits which are widely known as iron-binding proteins. The third gene transcript up-regulated encodes the putative transthyretin, which is a thyroid hormone-binding protein present in the extracellular fluids of all vertebrates that distributes thyroid hormones such as L-thyroxine in fish (Folli et al., 2003; Richardson et al., 2005). The fourth up-regulated transcript encodes a hypothetical protein for which not much information could be retrieved from genetic databases. It seems to be similar to the gene for a food vacuole

protein of Apicomplexa, particularly *Plasmodium falciparum*, used for the digestion of erythrocyte hemoglobin (Lamarque et al., 2008).

Five transcripts were only up-regulated in gill tissue of H/H fish at 24 hours after infection (Table 4-2), which were not up-regulated in H/CH fish (see Table 4-3). One of them is nucleoside diphosphate kinase (NDK), which has a critical role in the energetic metabolism. NDK are enzymes which catalyze exchanges of phosphate groups between nucleoside phosphates, such as between GTP and ADP to generate ATP. A second phosphotransferase gene, identified as a dolichol kinase (dolk) was also up-regulated. Dolk phosphorylates dolichol which is then used as a glycosyl carrier lipid. Therefore dolk is necessary for the glycosylation of proteins. The third transcript found to be up-regulated codes for a cysteine-rich protein which may be involved in cytoskeleton activity (Tran et al., 2005). The fourth up-regulated transcript encodes a member of the formin-binding-protein family, which participates in deforming the plasma membrane for endocytosis by forming vesicotubular structures (Kamioka et al., 2004). The last up-regulated transcript found in gills of H/H fish was a protein containing a Pleckstrin homology (PH) domain. This domain of 120 amino acids is present in different protein constituents of the cytoskeleton, and it is also found in proteins involved in intracellular signalling.

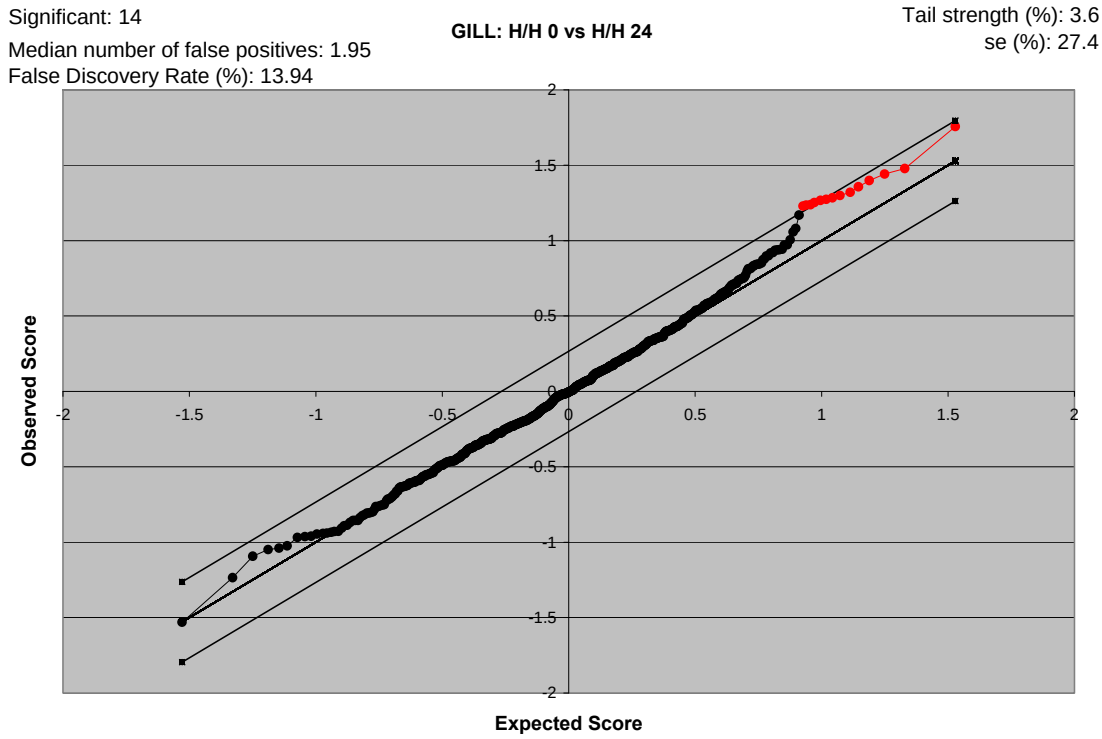
Three were the genes up-regulated only in the H/CH fish group and not in the H/H group (Table 4-3). One was ferritin heavy subunit, which constitutes an important iron-storage molecule. Next, was glyceraldehyde 3-phosphate dehydrogenase or GAPDH, which has metabolic functions in breaking down glucose molecules to obtain energy. Finally, a transcript for a similar protein to Zinc finger CSL domain containing 2 was up-

regulated. This protein has analogs in mouse and humans known as Dph3 (Wu et al., 2008). Dph3 is necessary for posttranslational modification of a histidine residue in the eEF-2 and showed to be essential in mouse development (Liu et al., 2006).

Figure 4-3. Analysis of gene expression in post-infected gill tissue.

Scatter plot for gill tissue analyzed at 24 hours after infection versus pre-infection time (0 hs). a) H/H fish. A total of 14 genes were up-regulated which are indicated as red dots in the first quadrant. No down-regulated genes were detected. The false discovery rate (FDR) was set at 13.94%. b) H/CH fish. A total of 10 genes were up-regulated which are indicated as red dots in the first quadrant. No down-regulated genes were detected. The false discovery rate (FDR) was set at 7.55%.

a)


Significant: 14
Median number of false positives: 1.95
False Discovery Rate (%): 13.94
GILL: H/H 0 vs H/H 24
Tail strength (%): 3.6
se (%): 27.4
Observed Score
Expected Score
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2

b)

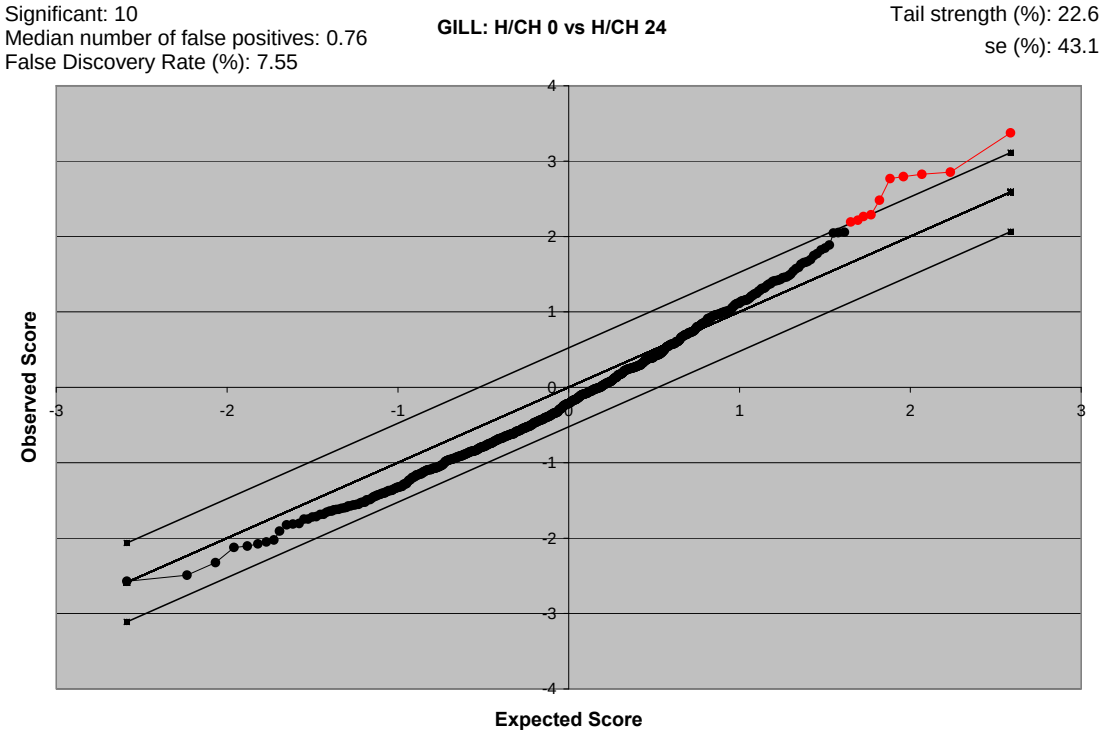

Significant: 10
Median number of false positives: 0.76
False Discovery Rate (%): 7.55
GILL: H/CH 0 vs H/CH 24
Tail strength (%): 22.6
se (%): 43.1
Observed Score
Expected Score
4
3
2
1
0
-1
-2
-3
-4
-3
-2
-1
0
1
2
3

Table 4-2. Up-regulated genes in post-infected gill tissue of H/H fish.

Up-regulated genes found in gill tissue of H/H fish analyzed at 24 hours after infection versus pre-infection time (0 hs). Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without a match in genetic databases.

Gene Name	Score(d)	q-value(%)
Danio rerio pleckstrin homology domain containing, family F	1.76	13.94
Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain).	1.48	13.94
dolichol kinase (dolk) - MGC83595 protein [*Xenopus laevis*]	1.44	13.94
unknown	1.40	13.94
unknown	1.36	13.94
cysteine-rich protein mRNA	1.32	13.94
nucleoside-diphosphate kinase NBR-B [*Bos taurus*].	1.30	13.94
unknown	1.28	13.94
hypothetical protein PF11_0168 [*Plasmodium falciparum* 3D7]	1.27	13.94
unknown	1.27	13.94
putative thyroid hormone carrier; transthyretin [*Cyprinus carpio*]	1.25	13.94
alpha-globin IV [*Oncorhynchus mykiss*]	1.24	13.94
alpha-globin [*Salmo salar*]	1.24	13.94
PRED: sim. to formin binding protein 21 [*Monodelphis domestica*].	1.23	13.94

Table 4-3. Up-regulated genes in post-infected gill tissue of H/CH fish.

Up-regulated genes found in gill tissue of H/CH fish analyzed at 24 hours after infection versus pre-infection time (0 hs). Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. The "unknown" gene was a transcript without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
alpha-globin IV [*Oncorhynchus mykiss*]	3.373415	0
hypothetical protein PF11_0168 [*Plasmodium falciparum* 3D7]	2.854367	0
Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain).	2.825612	0
putative thyroid hormone carrier; transthyretin [*Cyprinus carpio*]	2.792337	0
beta-globin [Oncorhynchus mykiss] (Hemoglobin beta-IV chain)	2.766617	0
similar to Zinc finger, CSL domain containing 2 [*Danio rerio*].	2.479887	7.553648069
unknown	2.288317	7.553648069
glyceraldehyde 3-phosphate dehydrogenase [*Gadus morhua*]	2.264592	7.553648069
Ferritin, heavy subunit (Ferritin H).	2.215587	7.553648069
alpha-globin [*Salmo salar*]	2.192131	7.553648069

Gill expression in H/CH versus H/H

The comparison of gill tissue from H/CH fish with those of H/H fish at basal (pre-infection) transcriptional levels (Figure 4-3a) showed that the H/CH group had a few genes at higher transcriptional level than its counterpart H/H fish group (Table 4-4), while it showed many genes at lower levels than in the H/H fish (Table 4-5). Ten transcripts were found to have higher transcriptional basal levels in gill tissue of H/CH fish compared with H/H fish, though most of them did not have matches in genetic databases (Table 4-4). One of the identified genes was phosphoglucose isomerase-1, which catalyzes the interconversion of glucose and fructose. The other was the transcript for TATA-binding protein-associated factor 1 (TAF1), an essential component of the general transcription factor IID (TFIID) which nucleates the assembly of the pre-initiation complex for transcription by RNA polymerase II (Metcalf and Wassarman 2006).

On the other hand, 28 genes were found at lower basal transcriptional levels in H/CH fish compared with H/H fish (Table 4-5). Four of them, as the alpha and beta haemoglobin subunits, a putative thyroid hormone carrier and a hypothetical protein from *P. falciparum*, are among the ones that were up-regulated at 24 hours post-infection in gill tissue in both groups of fish (see Table 4-2). Three others were also among the genes found to be up-regulated in gill tissue of H/CH after 24 hours of infection (see Table 4-3). These were the iron-storage ferritin heavy subunit, a transcript for a similar protein to Zinc finger CSL domain containing 2, and GAPDH. Other genes found at lower basal levels in gill tissue of H/CH than the H/H fish included pan-epithelial glycoprotein,

which is expressed in normal murine tissues containing epithelial cells and in plasma cells induced by LPS stimulation of spleen B lymphocytes (Bergsagel et al., 1992). Also found was ubiquitin, which participates in numerous cellular functions and is the ATP-dependent proteolytic factor essential to the proteasome. Ubiquitin binds to proteins and directs them to the proteasome (Kimura and Tanaka 2010). Another gene found at lower levels in H/CH fish was integral membrane protein 2B (Itm2b) which is the precursor of the protease inhibitor ABri amyloid protein (Vidal et al., 1999; Martin et al., 2008). Also lower levels in H/CH fish were those of prothymosin alpha-like protein and beta-thymosin, the former involved in chromatin organization and cell proliferation (Gómez-Márquez and Rodriguez 1998) whereas the latter one has many peptide variations with several roles described in vertebrates. One of those, TB_4 has been reported to have an effect on the regulation and differentiation of T lymphocytes (e.g., inducing the expression of terminal deoxynucleotidyltransferase), and also inhibiting migration of macrophages (Hannappel 2007). Finally, mucin 2 precursor which forms mucus glycoproteins (mucins) and an hyperosmotic glycine-rich protein involved in osmorregulation were also detected at lower levels in H/CH fish.

The comparison of gill tissue from H/CH fish with that of H/H fish at 24 hours of infection (Figure 4-3b) found only two genes with higher transcriptional levels in the former fish group and no genes at lower levels (Table 4-6). The two up-regulated genes were cytochrome c oxidase subunit 1 (COX1), a key enzyme in the respiratory electron transport chain of mitochondria; and NaK ATPase with several roles in the cell membrane involving transport of ions and generation of a sodium gradient allowing the cell to import sugars, amino acids and other nutritional requirements.

Figure 4-4. Gene expression in gill tissue of H/CH vs H/H fish.

SAM plot for gill tissue of H/CH fish versus H/H fish analyzed before infection (0 hours). a) Comparison at pre-infection time. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. A total of 38 genes were differentially expressed with a false discovery rate (FDR) set at 6.55%. Ten genes had higher transcriptional levels in the H/CH group, which are indicated as red dots in the first quadrant, whereas 28 were found with lower transcriptional levels indicated as green dots in the third quadrant. b) Compariosn at post-infected time. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Only two genes, indicated as red dots in the first quadrant, had higher transcriptional levels in H/CH fish. False discovery rate (FDR) equalled 0 (zero) %. No genes with lower levels were found.

a)

Significant: 38

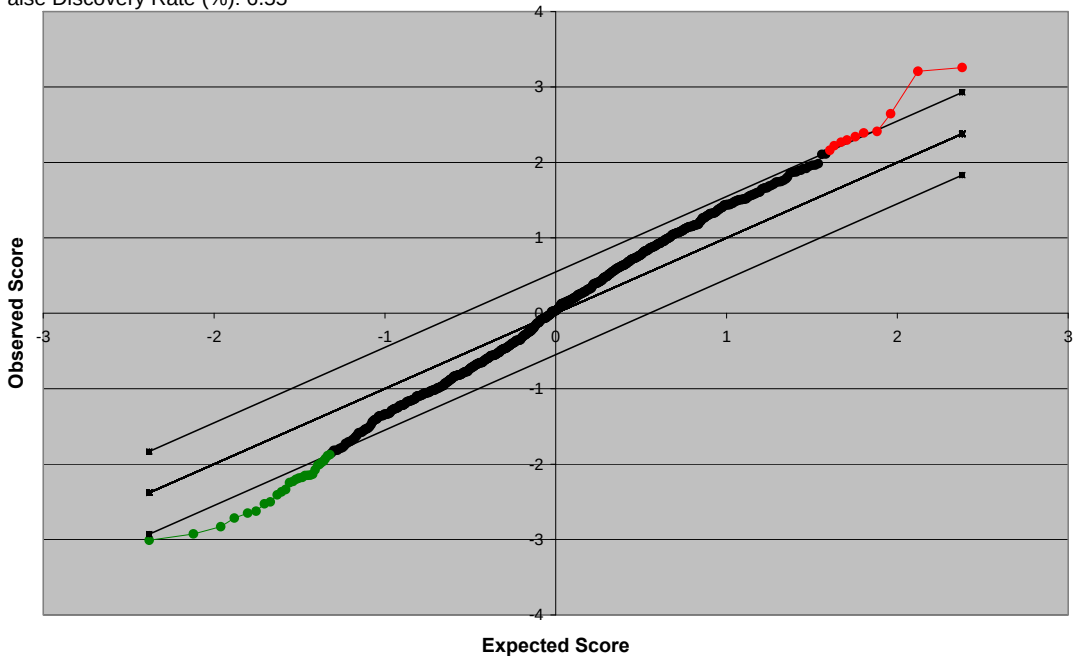
Median number of false positives: 2.49

False Discovery Rate (%): 6.55

GILL: H/CH 0 vs H/H 0

Tail strength (%): 41.3

se (%): 29



b)

Significant: 2

Median number of false positives: 0

False Discovery Rate (%): 0

GILL: H/CH 24 vs H/H 24

Tail strength (%): -54.5

se (%): 35.2

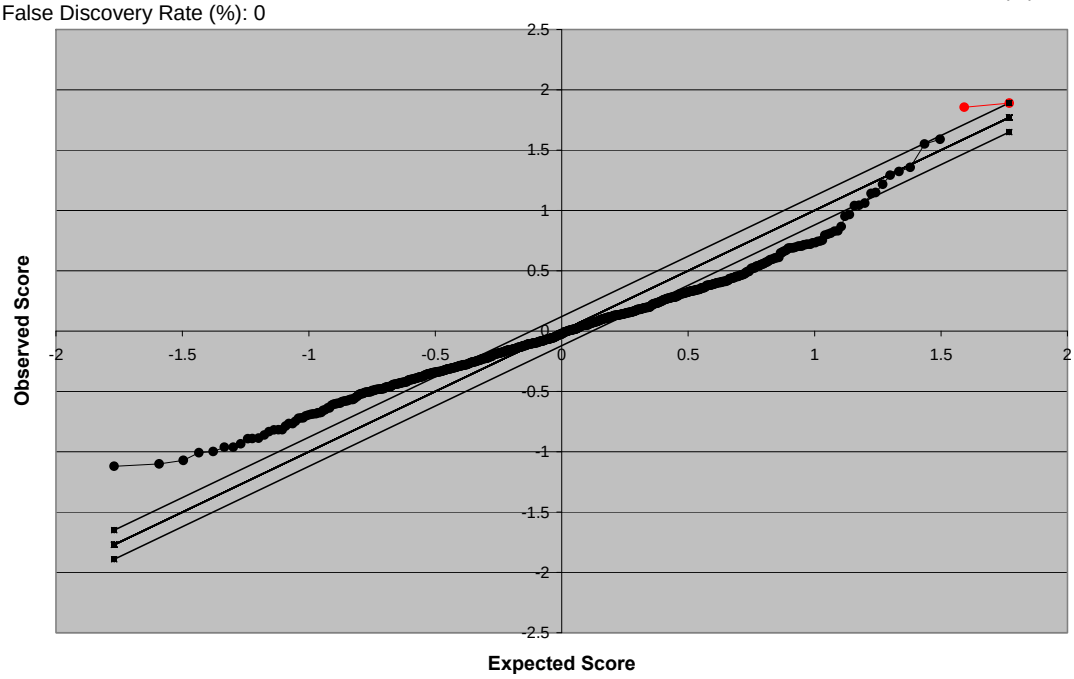


Table 4-4. Genes at higher levels in pre-infected gill tissue of H/CH vs H/H fish.

Genes found at higher basal transcriptional levels (before infection) in gill tissue of H/CH fish compared with H/H fish. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
unknown	3.26	0.00
phosphoglucose isomerase-1 (pgi-1 gene)	3.21	0.00
unknown	2.65	2.70
unknown	2.41	5.33
unknown	2.39	5.33
clone BHMS108 microsatellite	2.34	5.33
unknown	2.30	5.33
unknown	2.27	5.33
DNA sequence from clone DKEY-53P21	2.22	5.33
TAF-Ibeta2 mRNA	2.16	6.55

Table 4-5. Genes at lower levels in pre-infected gill tissue of H/CH vs H/H fish.

Genes found at lower basal transcriptional levels (before infection) in gill tissue of H/CH fish compared with H/H fish. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
alpha-globin IV [*Oncorhynchus mykiss*]	-3.01	0.00
pan-epithelial glycoprotein [*Danio rerio*]	-2.93	0.00
PRED: similar to Mucin 2 precurs (Intestinal mucin 2) [*Gallus gallus*]	-2.83	0.00
alpha-globin [*Salmo salar*]	-2.71	0.00
Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain)	-2.65	0.00
PREDICTED: hypothetical protein XP_511394 [*Pan troglodytes*]	-2.62	0.00
unnamed protein product [*Tetraodon nigroviridis*]	-2.53	0.00
PREDICTED: similar to Zinc finger protein 341 [*Danio rerio*]	-2.50	0.00
unknown	-2.41	0.00
hypothetical protein PF11_0168 [*Plasmodium falciparum* 3D7]	-2.37	4.79
unknown	-2.34	4.79
ubiquitin [*Oncorhynchus mykiss*]	-2.25	4.79
integral membrane protein 2B, like [*Danio rerio*]	-2.23	4.79
Major facilitator superfamily domain-containing protein 4	-2.20	4.79
ferritin-H subunit mRNA	-2.19	4.79
Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain)	-2.18	4.79
glyceraldehyde 3-phosphate dehydrogenase [*Gadus morhua*]	-2.15	4.79
similar to Zinc finger, CSL domain containing 2 [*Danio rerio*]	-2.15	4.79
unknown	-2.15	4.79
Itm2b protein [*Danio rerio*]	-2.14	4.79
hyperosmotic glycine rich protein [*Salmo salar*]	-2.08	5.18
putative thyroid hormone carrier; transthyretin [*Cyprinus carpio*]	-2.02	5.18
prothymosin alpha like-1 protein [*Danio rerio*]	-2.00	5.18
PREDICTED: similar to Ependymin precursor (EPD) [*Danio rerio*]	-1.98	5.18
unknown	-1.96	5.66
beta thymosin mRNA	-1.93	5.66
unknown	-1.89	6.55
unknown	-1.88	6.55

Table 4-6. Genes at higher levels in post-infected gill tissue of H/CH vs H/H fish.

Genes found at higher transcriptional levels at 24 hours after infection in gill tissue of H/CH fish compared with H/H fish. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript.

Gene Name	Score(d)	q-value(%)
NAK ATPASE ALPHA1C	1.89	0.00
cytochrome c oxidase subunit I	1.86	0.00

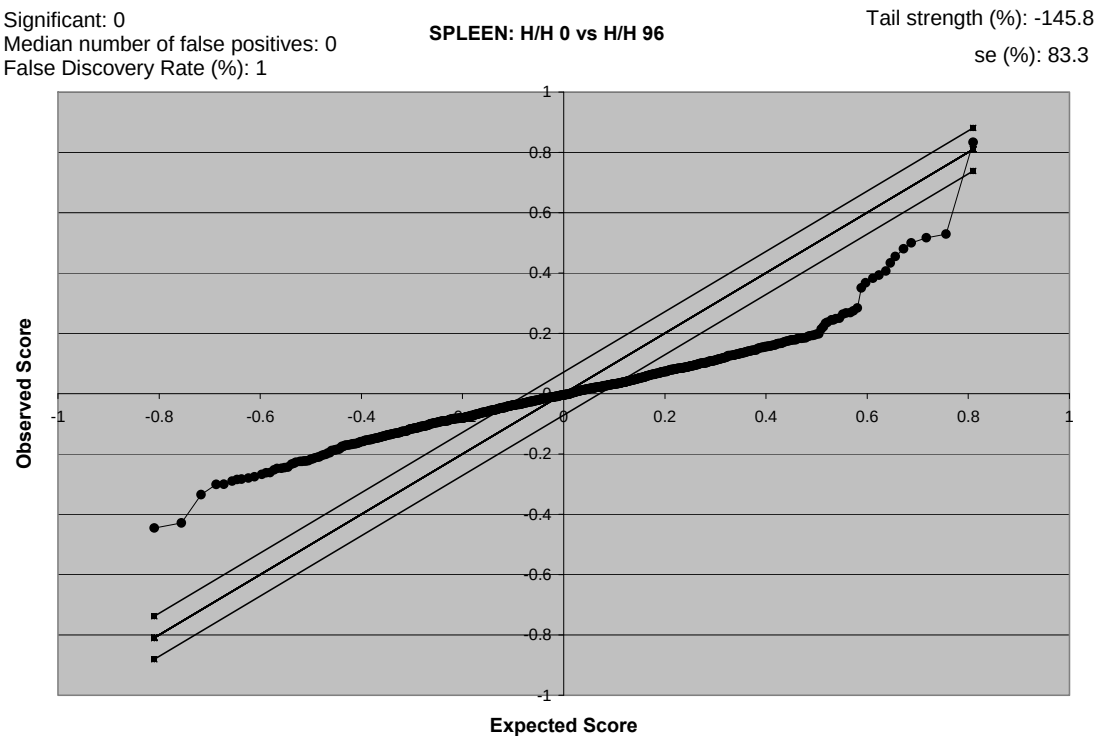
Pre- versus post-infection gene expression in spleen

The analysis of spleen tissue at pre- and post-infection time at 96 hours revealed no transcriptional differences in the H/H fish group for any of the genes included in the microarray (Figure 4-4a). On the other hand, the same analysis did find some differentially expressed genes on the H/CH fish group (Figure 4-4b). Three were the up-regulated genes detected (Table 4-7a). First, apolipoprotein CII that once secreted into plasma it activates the enzyme lipoprotein lipase which hydrolyzes triglycerides and thus provides free fatty acids for cells. Secondly, leukocyte cell-derived chemotaxin-2 (LECT2), a neutrophil chemotactic factor. Finally, C-type lectin 2-1 which encodes a member of the natural killer cell receptor C-type lectin family (Weis et al., 1998). Two were the down-regulated genes identified (Table 4-7b). One was IgM-A heavy chain, which is the main fish antibody molecule. The other transcript down-regulated was prothymosin alpha-like protein which was found at lower basal transcriptional levels in gill tissue of H/CH fish compared with H/H fish (see Table 4-5).

Figure 4-5. Analysis of gene expression in post-infected spleen tissue.

SAM plot for spleen tissue of H/H fish analyzed at 96 hours after infection versus pre-infection time (0 hs). a) H/H fish. No differentially expressed genes were detected. b) H/CH fish. Six genes were found to be differentially expressed: four up-regulated genes indicated as red dots in the first quadrant and two down-regulated genes indicated as green dots in the third quadrant. The false discovery rate (FDR) was set at 17.58%.

a)


Significant: 0
Median number of false positives: 0
False Discovery Rate (%): 1
SPLEEN: H/H 0 vs H/H 96
Tail strength (%): -145.8
se (%): 83.3
Observed Score
Expected Score
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1
-0.8
-0.6
-0.4
0.2
0.4
0.6
0.8
1

b)

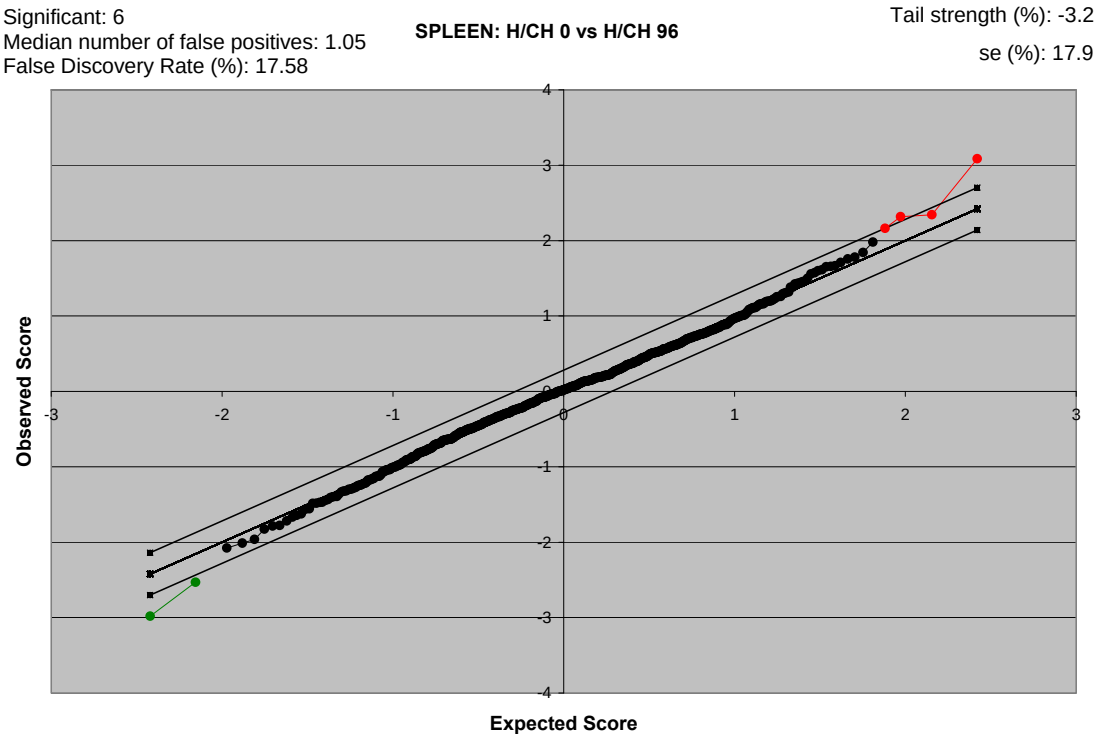

Significant: 6
Median number of false positives: 1.05
False Discovery Rate (%): 17.58
SPLEEN: H/CH 0 vs H/CH 96
Tail strength (%): -3.2
se (%): 17.9
Observed Score
Expected Score
4
3
2
1
0
-1
-2
-3
-4
-3
-2
-1
1
2
3

Table 4-7. Up- and down-regulated genes in post-infected spleen tissue of H/CH fish.

a) Up-regulated genes found in spleen tissue of H/CH fish analyzed at 96 hours after infection versus pre-infection time (0 hs). b) Down-regulated genes found in spleen tissue of H/CH fish analyzed at 96 hours after infection versus pre-infection time (0 hs). Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. The "unknown" gene was a transcript without match found in genetic databases.

a)

Gene Name	Score(d)	q-value(%)
apolipoprotein CII mRNA	3.09	0.00
LECT2 neutrophil chemotactic factor mRNA	2.34	17.58
C-type lectin 2-1 mRNA	2.32	17.58
unknown	2.16	17.58

b)

Gene Name	Score(d)	q-value(%)
IgM-A heavy chain constant region [*Salmo trutta*].	-2.98	0.00
prothymosin alpha like-1 protein [*Danio rerio*]	-2.53	0.00

Spleen expression in H/CH versus H/H

The comparison of spleen tissue from H/CH fish with that of H/H fish at pre-infection time (Figure 4-5a) detected some genes at lower transcriptional levels (Table 4-8) but it did not find any gene at a higher basal transcriptional level. Among the genes with lower basal levels were phosphoglucomutase-3, which facilitates the interconversion of glucose 1-phosphate and glucose 6-phosphate, and a BTB/POZ domain-containing protein 9. BTB/POZ domain is an evolutionarily conserved protein–protein interaction domain present in a variety of eukaryotic proteins, many of which have DNA-related functions (Albagli et al 1995). In *Drosophila melanogaster*, the BTB/POZ domain protein group is made up of transcription factors which play key roles in a variety of developmental programmes (De la Luna et al., 1999). The rest of the genes found at lower transcriptional levels in H/CH fish were two serine peptidases, trypsin 1A and trypsin-3 precursor, usually produced in the pancreas for protein digestion (Male et al., 1995); and a transcript similar to Syncollin, which is involved in granule formation and pancreatic exocytosis of secretory proteins (Kalus et al., 2002).

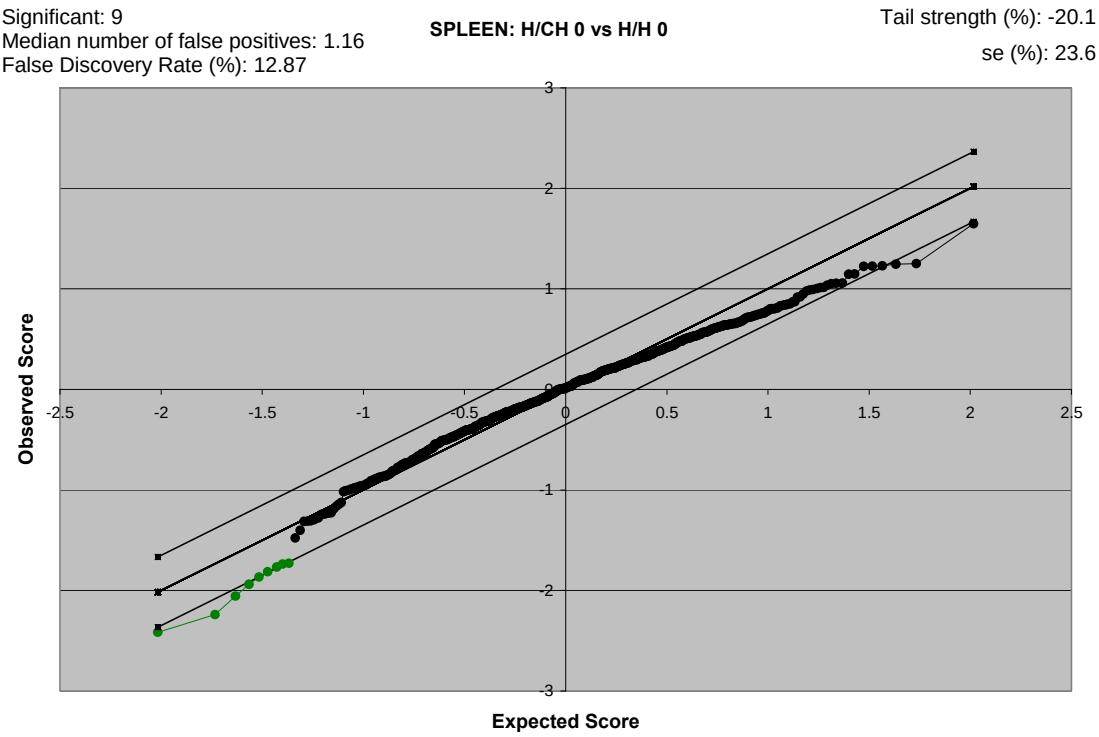
The comparison of spleen tissue from H/CH fish with those of H/H fish at 96 hours of infection (Figure 4-5b) found no genes up-regulated in the former group, whereas it did show 13 down-regulated genes (Table 4-9). Some of these transcripts were already found at lower basal levels in gill tissue of H/CH fish, and were also found equally transcribed in infected gills after 24 hours of infection: alpha and beta haemoglobin subunits, putative thyroid hormone carrier, and a hypothetical protein from *P. falciparum*. Other transcripts with lower levels in spleen of H/CH fish with respect to

H/H fish at 96 hours were the TNF receptor, a viral hemorrhagic septicaemia virus (VHSV)-induced protein-10 found in the nucleus regulating transcription (O'Farrell et al., 2002; Workenhe et al., 2009), and the eukaryotic initiation factor 2 (eIF2)-beta which mediates the binding of $tRNA^{met}$ to the ribosome in a GTP-dependent manner. Another transcript at lower levels in H/CH fish was a coiled-coil domain-containing protein 72, which belongs to the superfamily of coiled-coil proteins, many involved in important biological functions such as the regulation of gene expression (e.g. transcription factors, Landschulz et al., 1988), cell-cell communication, membrane fusion, and proteins which act as molecular spacers and motors (Lupas and Gruber, 2005). The last transcript with lower levels in H/CH fish was one similar to alpha adducin erythrocyte. Adducin plays an essential role in the assembly of actin filaments and it locates at the spectrin-actin junction regulating cytoskeleton-membrane skeleton interactions (Gardner and Bennett 1987), being an effector of many signalling pathways changing the cell shape or the cell movement (Matsuoka et al., 2000). Adducin is expressed in early erythropoiesis when the spectrin-actin network is forming (Matsuoka et al., 2000).

Figure 4-6. Gene expression in spleen tissue of H/CH vs H/H fish.

a) SAM plot for spleen tissue of H/CH fish versus H/H fish analyzed before infection (0 hours). The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Nine genes, indicated as green dots in the third quadrant, were found to have lower transcriptional levels in the H/CH fish, whereas no genes with higher levels were detected. False discovery rate (FDR) equalled 12.87%.
b) SAM plot for spleen tissue of H/CH fish versus H/H fish analyzed after 96 hours of infection. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Thirteen genes, indicated as green dots in the third quadrant, were found to have lower transcriptional levels in the H/CH fish, whereas no genes with higher levels were detected. False discovery rate (FDR) equalled 13.89%.

a)


Significant: 9
Median number of false positives: 1.16
False Discovery Rate (%): 12.87
SPLEEN: H/CH 0 vs H/H 0
Tail strength (%): -20.1
se (%): 23.6
Observed Score
Expected Score
3
2
1
0
-1
-2
-3
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5

b)

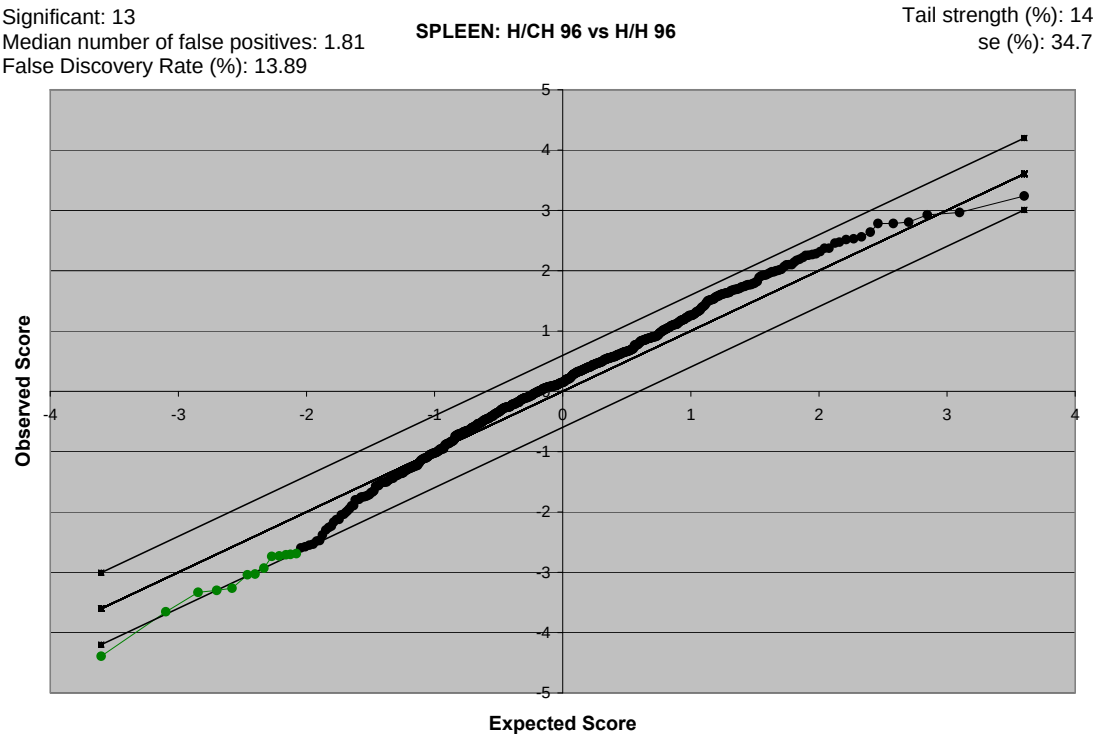

Significant: 13
Median number of false positives: 1.81
False Discovery Rate (%): 13.89
SPLEEN: H/CH 96 vs H/H 96
Tail strength (%): 14
se (%): 34.7
Observed Score
Expected Score
5
4
3
2
1
0
-1
-2
-3
-4
-5
-4
-3
-2
-1
0
1
2
3
4

Table 4-8. Genes at lower levels in pre-infected spleen tissue of H/CH vs H/H fish.

Genes found at lower basal transcriptional levels (before infection) in spleen tissue of H/CH fish compared with H/H fish. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
phosphoglucomutase 3 [*Danio rerio*]	-2.42	0.00
PREDICTED: similar to Syncollin [*Danio rerio*]	-2.24	0.00
trypsin IA [*Salmo salar*]	-2.06	0.00
PRED: similar to BTB/POZ domain-cont protein 9 [*Apis mellifera*]	-1.94	0.00
unnamed protein product [*Tetraodon nigroviridis*]	-1.87	0.00
Trypsin-3 precursor (Trypsin III) [*Salmo salar*]	-1.81	12.87
PREDICTED: hypothetical protein [*Homo sapiens*]	-1.77	12.87
unknown	-1.74	12.87
DNA sequence from clone DKEY-53P21	-1.73	12.87

Table 4-9. Genes at lower levels in post-infected spleen tissue of H/CH vs H/H fish.

Genes found at lower transcriptional levels at 96 hours after infection in spleen tissue of H/CH fish compared with H/H fish. The latter group of fish was set as the "control" group, since it represented the standard aquaculture practices. Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
alpha-globin IV [*Oncorhynchus mykiss*]	-4.39	0.00
unnamed protein product [*Tetraodon nigroviridis*]	-3.65	18.05
Coiled-coil domain-containing protein 72 [*Tetraodon nigroviridis*]	-3.33	18.05
putative thyroid hormone carrier; transthyretin [*Cyprinus carpio*]	-3.30	18.05
beta-globin [*Oncorhynchus mykiss*] (Hemoglobin beta-IV chain)	-3.26	18.05
unknown	-3.04	18.05
VHSV-induced protein-10 [*Oncorhynchus mykiss*]	-3.03	18.05
hypothetical protein PF11_0168 [*Plasmodium falciparum* 3D7]	-2.93	18.05
tumour necrosis factor receptor [*Oncorhynchus mykiss*]	-2.74	18.05
237aa long hypothetical protein APE1937 [*Aeropyrum pernix* K1]	-2.73	18.05
eukaryotic translation initiation factor 2, subunit 2 beta [*Danio rerio*]	-2.71	18.05
unnamed protein product [*Tetraodon nigroviridis*]	-2.70	18.05
Sim. to Alpha adducin (Erythrocyte adducin alpha subunit) [*Danio rerio*]	-2.69	18.05

Pre- versus post-infection gene expression in head kidney

The analysis of head kidney tissue of H/CH fish at pre- and post-infection time at 96 hours revealed a total of 46 genes to be differentially transcribed (Figure 4-6). Among the up-regulated genes (Table 4-10) there were again alpha and beta haemoglobin subunits, a putative thyroid hormone carrier, and the hypothetical protein from *P. falciparum*, that along with nucleoside-diphosphate kinase (NDK) were all found to be up-regulated after 24 hours of infection in gill tissues of both H/H and H/CH fish (see Table 4-2). Other genes up-regulated which, as described above, showed differential transcription in spleen or gill were the eukaryotic initiation factor 2 (eIF2)-beta which was at lower levels in spleen of infected H/CH fish (see Table 4-9), prothymosin already found at lower basal levels in gill tissue of H/CH fish (Table 4-5) and down-regulated in spleen of H/H fish (Table 4-7b), phosphoglucomutase-3 and the transcript similar to Syncollin found at lower basal levels in spleen of H/CH fish (Table 4-8), and phosphoglucose isomerase-1 found at lower basal levels in gill of H/CH fish (Table 4-4). Several other genes, not significant in gill or spleen, were up-regulated in head kidney of infected H/CH fish (Table 4-10). Some of these were well known transcripts such as H3 histone, involved in the structure of the nucleosome and in the regulation of gene expression, leucyl-tRNA synthetase which catalizes the ligation of L-leucine to $tRNA^{(Leu)}$, lactate dehydrogenase-A that converts L-lactate into pyruvate producing NADH during glucolysis, the proteases cathepsin Y and elastase-1, a proteasome subunit for cleaving peptides, three ribosomal proteins and EF1-alpha. Other up-regulated transcripts included interferon-inducible protein Gig-1 with immunoregulatory activities,

connectin/titin which participates in fibre flexion, a chaperonin containing TCP1 required for the production of native actin and tubulin and other proteins involved in cell cycle progression (Brackley and Grantham 2009), and a sop protein which may have functions in the regulation of Hox genes (Zhang et al., 2004),

Among the down-regulated genes (Table 4-11) were C-type lectin already found up-regulated in spleen of H/CH fish and Itm2b which was found at lower basal levels in gill of H/CH. Also among the down-regulated genes were a type-1 growth hormone carrier, a transcript for protein CutA, and the ABC transporter which carries out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair.

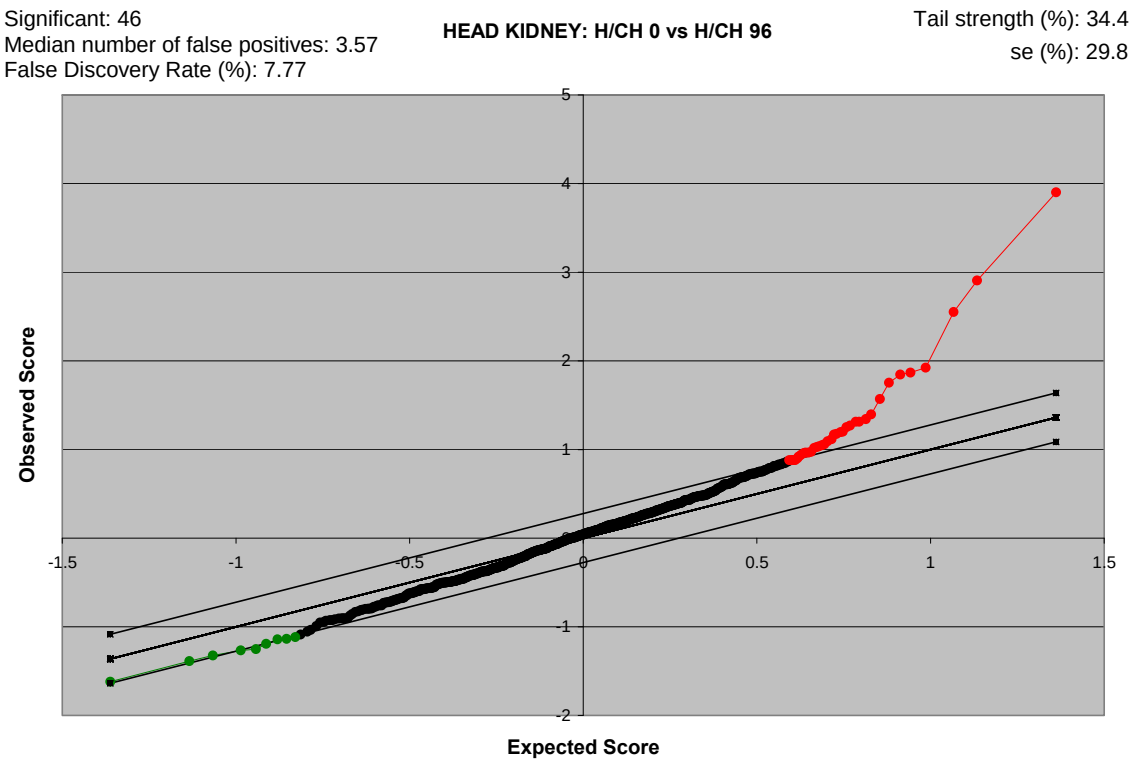


Figure 4-7. Analysis of gene expression in post-infected head kidney of H/CH fish.

SAM plot for head kidney tissue of H/CH fish analyzed at 96 hours after infection versus pre-infection time (0 hs). Forty-six genes were found to be differentially expressed: thirty-seven up-regulated genes indicated as red dots in the first quadrant and nine down-regulated genes indicated as green dots in the third quadrant. The false discovery rate (FDR) was set at 7.77%.

Table 4-10. Up-regulated genes in post-infected head kidney of H/CH fish.

Up-regulated genes found in head kidney tissue of H/CH fish analyzed at 96 hours after infection versus pre-infection time (0 hs). Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain).	3.90	0.00
beta-globin [*Oncorhynchus mykiss*] (Hemoglobin beta-IV chain)	2.91	0.00
alpha-globin [*Salmo salar*]	2.55	0.00
nucleoside-diphosphate kinase NBR-B [*Bos taurus*]	1.92	0.00
unnamed protein product [*Tetraodon nigroviridis*]	1.87	0.00
hypothetical protein PF11_0168 [*Plasmodium falciparum* 3D7]	1.84	0.00
alpha-globin IV [*Oncorhynchus mykiss*]	1.75	0.00
Sop protein [*Xenopus laevis*]	1.57	0.00
prothymosin alpha like-1 protein [*Danio rerio*]	1.39	0.00
hypothetical protein AcidDRAFT_1829 [*Solibacter usitatus* Ellin6076]	1.34	0.00
ribosomal protein L9 mRNA	1.31	0.00
PREDICTED: similar to Ependymin precursor (EPD) [*Danio rerio*]	1.31	0.00
eukaryotic translation initiation factor 2, subunit 2 beta [*Danio rerio*]	1.27	0.00
Elastase-1 >gi|1310934|pdb|1ELT| Mol_id: 1	1.25	0.00
proteasome subunit C10-11 mRNA	1.20	0.00
unknown	1.20	0.00
putative thyroid hormone carrier; transthyretin [*Cyprinus carpio*]	1.18	0.00
phosphoglucomutase 3 [*Danio rerio*]	1.17	0.00
unknown	1.12	2.86
unknown	1.09	2.86
unknown	1.06	2.86
lactate dehydrogenase-A (ldh-a) mRNA	1.05	2.86
PREDICTED: similar to Syncollin [*Danio rerio*]	1.04	2.86
cathepsin Y [*Oncorhynchus mykiss*]	1.03	2.86
40S ribosomal protein mRNA	1.02	2.86
similar to interferon-inducible protein Gig1, partial [*Danio rerio*]	0.97	6.95
unknown	0.97	6.95
elongation factor EF1 alpha mRNA	0.96	6.95
connectin/titin mRNA	0.96	6.95
hypothetical protein CaO19_2537 [*Candida albicans* SC5314]	0.94	6.95
phosphoglucose isomerase-1 (pgi-1 gene)	0.92	6.95
chaperonin containing TCP1, subunit 4 (delta) [*Pan troglodytes*]	0.90	6.95
unknown	0.88	7.77
ribosomal RNA gene	0.88	7.77
H3 histone, family 3A, mRNA	0.88	7.77
unknown	0.88	7.77
leucyl-tRNA synthetase (Kiaa0028 gene) mRNA	0.88	7.77

Table 4-11. Down-regulated genes in post-infected head kidney of H/CH fish.

Down-regulated genes found in head kidney tissue of H/CH fish analyzed at 96 hours after infection versus pre-infection time (0 hs). Gene name lists the transcripts identified along with their score (*d*) calculated by the SAM algorithm and the *q-value* associated to each transcript. "Unknown" genes were transcripts without match found in genetic databases.

Gene Name	Score(d)	q-value(%)
ABC transporter, inner membrane subunit [*Reinekea sp.* MED297].	-1.62	6.95
Itm2b protein [*Danio rerio*]	-1.39	6.95
Unknown	-1.32	6.95
PREDICTED: similar to c-type lectin [*Danio rerio*].	-1.27	6.95
type-1 growth hormone gene	-1.25	6.95
unknown	-1.19	6.95
Protein CutA homolog precursor	-1.14	7.77
unknown	-1.14	7.77
unknown	-1.12	7.77

Discussion

Comparison of basal gene expression of H/CH with H/H fish

The H/CH fish group showed lower transcriptional levels in spleen before infection and not one transcript at higher level than H/H fish. Among those transcripts there were some related to intracellular activities such as phosphoglucomutase 3 related to glucose metabolism, and the BTB/POZ domain-containing protein 9 related to transcription. Other transcripts expressed at lower levels in H/CH were related to exocytosis and protein digestion: trypsin 1A, trypsin-3 precursor and Syncollin.

Several genes were also maintained at lower transcriptional levels in gill tissue of H/CH compared to those of H/H fish. Among those were the alpha and beta haemoglobin subunits and ferritin that, due to their role in iron metabolism, are discussed below when comparing the immune response between the two groups of fish. The lower expression levels of Zinc finger CSL domain-containing protein 2 necessary for the activation of eEF-2 might indicate less transcriptional activity in H/CH fish compared with H/H fish, also evidenced by the lower levels of alpha-like prothymosin, which is involved in chromatin organization and cell proliferation (Gómez-Márquez and Rodriguez 1998). Other transcripts found at lower levels might indicate that H/CH fish maintain lower innate immune defenses, such as beta-thymosin that affects migration of macrophages, ubiquitin that direct proteins to the proteasome and some iron-binding proteins. This, together with the lower levels of GAPDH might indicate a lower basal metabolism in H/CH fish compared to H/H fish. The higher transcriptional level of TATA-binding

protein-associated factor 1 (TAF1), and the phosphoglucose isomerase-1, which can be involved in the generation of free sugars or of fructose for energy storage, makes difficult a final conclusion on the metabolic activity in the gill of H/CH fish. However, given the lack of transcripts expressed at higher levels in spleen and the few observed in gill tissue, and the several transcripts found at lower basal levels in gill and spleen, a hypothesis of a lower general metabolism and energy saving status in the gill and spleen of H/CH fish sounds reasonable.

Finally, a hyperosmotic glycine rich protein which is generally induced under osmotic stress (Pan et al., 2004) was expressed at higher basal levels in gill tissue of H/H fish. This might indicate that this fish group was under more stress than H/CH fish.

Assessing the immune response of H/H fish

The gill tissue of H/H fish did not show any down-regulated transcript after infection at 24 hours, but several up-regulated genes were found (Table 4-2). As expected, the infected fish seemed to be counteracting the iron depletion caused by the *Vibrio* siderophores by up-regulating haemoglobin genes which can reconstitute those proteins broken down by the virulent factors (Fouz et al., 1996). Other transcripts included dolk that participates in glycosylation processes, which is relevant since many immune-related proteins are also glycoproteins. In addition, the up-regulation of a member of the formin-binding-protein family which helps in endocytosis (Kamioka et al., 2004), cysteine-rich proteins, which among other functions regulates actin filament bundling (Tran et al., 2005) and the PH domain containing protein, also involved in cytoskeleton activity, are likely indicators of the activation of the immune response

through phagocytosis by the host cells. All these immune-related transcripts, along with the up-regulation of the NDK enzymes involved in the catalysis of ATP makes it a reasonable hypothesis that the generation of an inflammatory response mediated by macrophages in gill tissue has started 24 hours after infection.

Contrary to the observations in gill, the spleen tissue of H/H fish did not show any differential transcription at 96 hours after infection.

Assessing the immune response of H/CH fish

In a similar pattern as described above for the H/H fish, the gill tissue of H/CH fish did not show any down-regulated transcripts after 24 hours of infection, but several up-regulated genes were found (Table 4-3). Again as expected, the infected H/CH fish up-regulated haemoglobin genes to reconstitute those proteins broken down by the siderophores. Also, a transcript for ferritin heavy subunit was found to be up-regulated, along with GAPDH that releases sugars and a Zinc finger CSL domain containing 2 that may be necessary for the activation of eEF-2 (Liu et al., 2006).

The spleen tissue presented a few differentially transcribed genes at 96 hours. Apolipoprotein was up-regulated which, in a similar way as GAPDH provides free sugars in the gill, it helps to provide free fatty acids for the cells. Two other immune-related transcripts were up-regulated, the neutrophil chemotactic factor LECT2 and a C-type lectin, indicative of an inflammatory response. Interestingly, the antibody constituent IgM-A heavy chain and the prothymosin alpha-like involved in cell proliferation were found to be down-regulated after 96 hours of infection. It is necessary to study their

expression levels at other time points to better understand this expression pattern, as they were expected to be up-regulated during an immune response.

Regarding the head kidney tissue, which was only analyzed for H/CH fish, there were a few down-regulated genes after 96 hours of infection such as C-type lectin protein and Itm2b. On the other hand, there were many up-regulated genes indicating a strong response to bacterial infection. Hemoglobin genes were up-regulated as it was shown in the other tissues, suggesting a response to control iron concentration. The up-regulation of NDK enzyme, lactate dehydrogenase-A, phosphoglucose isomerase-1 and phosphoglucomutase-3 likely ensures energy for the demanding inflammatory processes. Other genes up-regulated suggested a strong immune response in head kidney. Increased expression of proteases such as cathepsin Y and elastase-1, and one proteasome subunit may be implicated in the generation of petides for antigen presentation. Elastase also is found in neutrophils involved in the digestion of engulfed bacteria (Chua and Laurent 2006). An interferon-inducible protein Gig-1 was also up-regulated. IFN-inducible proteins initiate a cascade of activation of a second set of genes, whose expression requires continued protein synthesis (Sen and Lengyel 1992; Koromilas et al., 1995). This makes sense since several transcripts related to gene expression were found to be up-regulated such as eEF2-beta, H3 histone, prothymosin, some ribosomal proteins, leucyl-tRNA synthetase, and EF1-alpha. Moreover, proteins involved in cytoskeleton activity such as a chaperonin containing TCP1 and connectin/titin indicate a strong cellular activity. Taken together, all these up-regulated transcripts evidence major changes in gene expression in head kidney upon infection.

Comparing the immune response of H/CH with H/H fish

Four transcripts presented the same transcriptional behaviour when comparing the immune response of the two groups of fish in several of the experiments performed here. These transcripts were the alpha and beta haemoglobin subunits, the putative transthyretin and the hypothetical protein from *P. falciparum*. The two groups of fish under study, H/H and H/CH, showed these genes to be up-regulated in gill tissue 24 hours after exposure to *Vibrio anguillarum* (Tables 4-2 and 4-3). Although lower levels of these transcripts were found at pre-infection time in gill tissue of H/CH compared to H/H fish (Table 4-5), no differential transcription was observed between the two groups of fish after infection. These results suggest that H/CH fish generated a stronger up-regulation to reach the same transcriptional levels as the H/H fish. Therefore both groups of fish, through different regulatory mechanisms, ended up having the same response in gill tissue at 24 hours post infection. However, this was not the case with the spleen tissue in which the two groups of fish did not show differences in the transcription of these four genes before infection, but then at 96 hours after infection H/H fish presented higher transcriptional levels than H/CH fish (Table 4-9). Therefore, different rearing environments may have different effects on particular tissues. Early-rearing environment seems to influence not only the basal transcriptional levels of the four genes considered here, but also the magnitude of the final response to a bacterial infection.

The four transcripts were again up-regulated in head kidney (Table 4-10), where only H/CH fish was analyzed at pre- and post-infection time. Taken together, these results suggest a common vital role for these four molecules in the development of the

innate immune response of the fish upon bacterial infection. In particular, the up-regulation of the genes coding for iron-binding haemoglobin subunits alpha and beta after infection was expected as these molecules scavenge the iron away from the bacteria thus slowing down the infection. Moreover, the destruction of haemoglobin by the bacteria to obtain iron may trigger the fish to restore normal levels of haemoglobin in blood to recover oxygen transportation capacity.

For bacteria to survive and establish an infection they need to overcome the iron limitation imposed by the host as part of the innate response (Weinberg 1990). Thus, due to the virulence of the bacteria, changes in the iron concentration in the fish plasma are generated. This likely makes more iron enter the macrophage where it combines with apoferritin, which is the natural repressor of ferritin transcription. Macrophages, which are the natural sequesters of iron from dying erythrocytes, must then synthesize the iron-storage protein ferritin in response to the infection. An increase in the transcription of the ferritin gene was observed in the gill cells of H/CH fish 24 hours after exposure (Table 4-3), where an accumulation of macrophages on site after infection is expected. However, H/H fish did not increase the transcription of ferritin in the gill at 24 hours (Table 4-2). This may be a consequence of H/H having higher basal levels than H/CH fish as shown in Table 4-4. Therefore, it follows that both groups of fish had similar levels of ferritin transcription in the gill after 24 hours of infection, which was corroborated by the fact that ferritin levels were not found to be different (Table 4-6). This situation may be indicative of an environmental factor affecting the regulation of the iron storage mechanism in gill tissue of Chinook salmon.

Two molecules with important roles in cellular metabolism, cytochrome c oxidase subunit 1 (COX1) and Na/K ATPase, that were observed at higher levels in gill tissue of infected H/CH fish indicating a higher metabolic activity for this fish during infection.

When analyzing spleen tissue at 96 hours between the two groups of fish, the H/CH fish group presented lower levels of some transcription-related molecules such as VHSV-induced protein, eIF2-beta, the protein similar to Zinc finger CSL domain containing 2, the eEF-2, all of them indicating a decrease in translational activity (i.e. lower protein synthesis). Also interesting was the finding of lower transcriptional levels of the TNF receptor in infected H/CH fish. Mammals activate an iron-withholding system after an acute response is elicited by IL-1 and TNF (Torti et al., 1988; Rogers et al., 1990). Therefore, the lower transcription of TNF receptor in H/CH fish may indicate that its response could be more moderated than in the H/H fish.

Comparison with other studies on fish immune response to infection

Although the cDNA microarray utilized here was developed from liver, muscle and brain tissue and thus it did not contain many specific immune-related genes, a strong response against bacterial infection was evidenced through the many genes found differentially expressed in gill, head kidney and spleen tissue of infected fish. Another recent study on the immune response of Chinook salmon after a disease challenge with *V. anguillarum* found significant changes in gene expression of several cytokines in head kidney using real-time RT-PCR (Ching et al., 2010). The authors found IL-1, IL-8 and TNF to be up-regulated in both triploid and diploid fish and IgM, MHC-II and β-actin to be up-regulated in diploids alone. Even though these genes were not analized in the

present cDNA microarray, the concordance between both studies was based on a detectable immune response developed following a disease challenge with live bacteria.

Several differentially expressed transcripts found on Chinook salmon were also observed in other challenged fish species. For instance, the up-regulation of C-type lectin found in the spleen of Chinook salmon was observed in the spleen and also in head kidney and liver of Atlantic salmon after 13 days of cohabitation with fish infected with *Aeromonas salmonicida* (Ewart et al., 2005). A second study on Atlantic salmon injected with *A. salmonicida* also found C-type lectin to be up-regulated in liver and head kidney, in addition to gill (Martin et al., 2006). Important proteins for withholding iron from bacteria were up-regulated in both species. Chinook salmon showed ferritin up-regulated in gill tissue, what was also found in Atlantic salmon by Martin et al. (2006). Transferrin was not found to be differentially regulated in Chinook but it was up-regulated in Atlantic salmon in spleen and liver (Ewart et al., 2005; Martin et al., 2006). Other important differentially expressed transcripts in Chinook salmon were hemoglobin subunits which were up-regulated in gill and head kidney. These transcripts were also found to be up-regulated in gill of Atlantic salmon salmon (Martin et al., 2006). They were also up-regulated in spleen of an LPS-challenged seabass *Lates calcarifer* (Xia and Yue 2010). Apolipoprotein, which was up-regulated in spleen of Chinook was also up-regulated in spleen of a challenged (Xia and Yue 2010). Interestingly, the same pattern of far more up-regulated genes than down-regulated ones found in gill and head kidney of Chinook salmon was also observed in Atlantic salmon infected with furunculosis (Martin et al., 2006) and in LPS-injected seabass (Xia and Yue 2010).

In conclusion, the present study was the first to use a novel cDNA microarray developed for Chinook salmon to compare pre- and post-infected tissue of this species following a disease challenge with live *V. anguillarum*. The disease challenge allowed to detect an immune response against the bacterial infection through the many genes that were found differentially transcribed in gill and head kidney. The comparison of hatchery-bred fish reared in two different environments showed basal transcriptional differences in gill tissue in which many immune-related genes were at lower basal levels in fish reared in channels compared with fish reared in hatchery tanks. These differences were not present 24 hours after exposure, thus indicating environmental effects on the regulation of the immune response. Environmental effects were also seen at mortality rates and antibody responses in hatchery-bred fish infected with *V. anguillarum* (Chapter 3), thus indicating that early-rearing environments should be carefully examined as they may have profound consequences on the development and regulation of the immune system which eventually may affect the fate of the fish during disease outbreaks.

Chapter 5. A study of the breeding system of Chinook salmon in semi-natural spawning channels using microsatellites

Introduction

Two results obtained in the disease challenge previously described (see Chapter 3) suggested a qualitative difference in the resistance to pathogens between the offspring of artificially-bred fish (hatchery fish) and the offspring of semi-naturally spawned fish (channel fish). First, the disease susceptibility of the hatchery fish was influenced by the early-rearing environment whereas the channel fish showed evidence of canalization of the immune response. The existence of genotype-by-environment interations suggests differences in the genetic structure of the immune system of the two groups of fish. Second, channel fish showed a general tendency towards higher MH-gene heterozygosity than the hatchery fish, further indicating genetic differences between the two groups. Both phenomena are likely to be a consequence of the difference in the breeding strategies: the artificial -random- mating in hatchery fish and the semi-naturally spawning of channel fish which allows natural processes such as sexual selection to occur.

Sexual selection based on MH genes has been recently described in Chinook salmon raised in spawning channels at Yellow Island Aquaculture Ltd (YIAL), Quadra Island, British Columbia (Neff et al., 2008). This is in agreement with studies performed in other species of the Salmonidae such as Atlantic salmon (Landry et al., 2001), Arctic charr (Skarstein et al., 2005) and brown trout (Forsberg et al., 2007). The MH genes constitute a multigene family which produce proteins that process and present pathogen-derived proteins to the effector T cells of the immune system in order to mount a pathogen-specific adaptive immune response against infective agents. An important advantage of heterozygosity at MH genes is the broader array of foreign pathogens that can be detected by the immune system therefore enhancing the immune performance

(Doherty and Zinkernagel 1975). Some evidence for the hypothesis of mate choice for good allele combinations at the MH genes has been found in different species (Mays and Hill 2004). Moreover, particular alleles may confer specific resistance to particular diseases as shown in Atlantic salmon (Grimholt et al., 2003).

Providing the offspring with an appropriate number of compatible alleles makes sense in light of evolutionary biology as this may increase the immune performance of the offspring along with a maximization of female parent fitness. Since salmonids do not have parental care after fertilization, it is the female which invests most of the time and energy to secure the success of the next generation by finding a suitable location in which to prepare the redd (Foote 1990). In this mating system without parental care, the male's contribution is reduced to the haploid genome complement it provides to the zygote and the male's fitness increases with the number of spawning events he participates in. Therefore, there is an advantage for the female to choose a bearer of good genes or genetically compatible partner to fertilize her eggs (Tregenza and Wedell 2000). The paradigm of MH-linked mate choice has gained much support in recent years, supporting a clear evolutionary purpose in leaving fitter offspring to fend off diseases (Bernatchez and Landry 2003; Piertney and Oliver 2006).

The general aim of this study was to contribute to the studies on the breeding system of Chinook salmon in spawning channels. Originally, the work was planned to screen both neutral markers and MH coding loci, but the latter was not possible due to DNA degradation issues in many parental samples. Only short tandem repeats were successfully amplified in all parental samples. The particular goals were 1) to assess individual reproductive success based on the number of matings and on number of

offspring that survived to fry stage; and 2) to analyze the degree of genetic pairwise relatedness among mating individuals. The analysis of the genetic pair relatedness can be used to see if there is evidence of positive or negative assortative mating with respect to genome-wide relatedness.

Materials and methods

Brood stock

The sexually mature fish used in this experiment belonged to a sixth-generation hatchery population at YIAL founded in 1985 with individuals from the Robertson Creek Hatchery, which have been operating by DFO since 1972 (DFO 2010). YIAL has eradicated the Y chromosome from their stock population maintaining only homogametic XX individuals (Hunter et al., 1983). Each spawning season, part of the eggs are treated with testosterone to induce male sexual characters thus creating sex-reversed females which upon maturation are used as brood stock in future spawning seasons (Benfey 1996). Homogametic XX males were shown to reach similar sizes to XY males and also present similar plasma concentration of testosterone and 17β-estradiol (Heath et al., 2002). In addition, XX males presented spawning behaviour undistinguishable from normal XY males in a previous study in these channels (Garner et al., 2010).

In the fall of 2007, sexually mature fish taken from sea cages were transferred into two 3.5x15 m spawning channels, 1 m water depth, supplied with approximately 80 L min^{-1} of fresh water with a water re-circulation rate of 300 L min^{-1}. Fourteen males were placed into each channel labelled CH1 and CH3, along with 16 and 17 females in these

channels respectively. Fin and operculum tissue were collected from the carcasses after the spawning events and preserved in 95% ethanol. Gender, length, weight and date of collection were recorded. All offspring found alive at fry stage in the channels were collected on May 22 2008, totalling 223 and 257 individuals in CH1 and CH3, respectively. Fish were euthanized by overdose with MS-222 (Syndel Intl. Inc., Vancouver, BC) and whole bodies were conserved in 95% ethanol in 50 mL Falcon® tubes.

DNA extraction

Samples were DNA extracted following a protocol given in Elphinstone et al. (2003) with minor modifications. Briefly, fin clips were placed in 96-well plates in 100mM NaCl, 50 mM Tris HCl, pH 8, 10 mM EDTA, 0.5 % SDS and 800 μg mL^{-1} of Proteinase K, and incubated overnight at 37 °C with 300 rpm agitation. Fifty μL of tissue digest were mixed with 150 μL of binding buffer (6 M sodium iodide saturated with 0.2 M sodium sulphite) and 50 μL of 50% Glassmilk, and placed in wells of 96-well filter plate. Binding of DNA was allowed for 1 min and then centrifuged over a waste collection plate followed by the addition of 200 μL of washing buffer (50% ethanol, 50 mM NaCl, 10 mM Tris.HCl, pH 7.4, and 0.5 mM EDTA) and a second centrifugation. Finally, the DNA was eluted with 100 μL of 1mM EDTA, pre-warmed to 65°C, by centrifugation of the filter plate over a collection plate.

Microsatellite genotyping

A total of 12 loci, consisting of either di- (four loci) or tetra-nucleotide (eight loci) repeat sequences were targeted for amplification. Table 5-1 lists the loci name, primers, allele size range (bp), and repeat size. Forward primers were labelled with high sensitivity infrared dyes IRD-700 or IRD-800, which have very low background as infrared wavelengths greatly reduce autofluorescence and enhance detection (Middendorf et al. 1992; Williams and Soper 1995). PCR reactions were performed in 10 μL volume containing 30-50 ng of genomic DNA, 0.02 μg of each primer, 0.2 μM of dNTPs, 1.5 or 2.5 mM $MgCl_2$ depending amplification results and 0.2 units of Taq DNA polymerase. Parental samples were amplified twice in order to assure genotype identification. Offspring samples were amplified and genotyped only once.

The fluorescent amplicons were detected by running the PCR products in acrylamide gels in LI-COR DNA Analysis System (LI-COR, Lincoln, NE, USA). Two PCR products were run together by choosing amplicons which had primers each with a different fluorescent dye. Gel images were analyzed using Gene ImageIR v. 4.05 (Scanalytics, Fairfax, VA, USA) to call amplified bands and to determine individual genotypes.

Table 5-1. Microsatellite primers for the 12 loci used in parentage assignment.

First two columns indicate loci and primer names employed in the microsatellite genotyping of Chinook salmon. Next are the sequences of forward and reverse primers. (*) Forward primers were labelled with infrared dyes either IRD-700 or IRD-800. Reverse primer names end with an "R". Allele size range is indicated in base pairs (bp). Last two columns list nucleotide repeat number (Rt No.) and annealing temperature °C (A.T.).

Locus	Primer name*	Primer sequence	Reference	Size range (bp)	Rt No.	A. T. (°C)
OtsG253	2577 (700)	5'-GAG CAG GCC GAG CAG GTG TCT	Williamson et al., 2002	156-304	4	64-48 °C td
	2548R	5'-GGA GCA TTC CAA TCA AGC CAC TG				
OtsG249	1349 (700)	5'-TTC TCA GAG GGT AAA ATC TCA GTA AG	Williamson et al., 2002	163-271	4	64-48 °C td
	434R	5'-GTA CAA CCC CTC TCA CCT ACC C				
Ots3	1387 (800)	5'-CAC ACT CTT TCA GGA G	Banks et al., 1999	82-100	2	51°C
	16R	5'-AGA ATC ACA ATG GAA G				
Ots4	1382 (700)	5'-GAC CCA GAG CAC AGC ACA A	Olsen et al., 1996	142-156	2	58-52 °C td
	17R	5'-GGA GGA CAC ATT TCA GCA G				
Omy325	1379 (700)	5'-TGT GAG ACT GTC AGA TTT TGC	Olsen et al., 1996	84-104	2	58-52 °C td
	7R	5'-CGG AGT CCG TAT CCT TCC C				
OTS 104	1346 (800)	5'-GCA CTG TAT CCA CCA GTA	Nelson and Beacham 1999	185-277	4	64-48 °C td
	194R	5'-GTA GGA GTT TCA TTT GAA TC				
OTS 107	1353 (800)	5'-ACA GAC CAG ACC TCA ACA	Nelson and Beacham 1999	204-296	4	58°C
	200R	5'-ATA GAG ACC TGA ATC GGT A				
OtsG311	1347 (700)	5'-TGC GGT GCT CAA AGT GAT CTC AGT CA	Williamson et al., 2002	282-374	4	50°C
	436R	5'-TCC ATC CCT CCC CCA TCC ATT GT				
OtsG68	1384 (800)	5'-TAT GAA CTG CAG CTT GTT ATG TTA GT	Williamson et al., 2002	175-291	4	56°C
	430R	5'-CAT GTC GGC TGC TCA ATG TA				
OtsG83b	1380 (700)	5'-TAG CCC TGC ACT AAA ATA CAG TTC	Williamson et al., 2002	165-229	4	60°C
	415R	5'-CAT TAA TCT AGG CTT GTC AGC AGT				
OtsG432	1348 (800)	5'-TGA AAA GTA GGG GAA ACA CAT ACG	Williamson et al., 2002	107-163	4	64-48 °C td
	441R	5'-TAA AGC CCA TTG AAT TGA ATA GAA				
Ots 13	1422 (800)	5'-CAC ACT CTT TCA GGA G	Heath, D.D. pers. comm.	80-100	2	57°C
	16R	5'-AGA ATC ACA ATG GAA G				

Parentage assignment

Parentage assignment analyses were performed following two distinct approaches: an exclusion-based method and a likelihood-based method. The spawning channels constituted a closed system since all parents were sampled and were available for analysis; therefore an exclusion analysis is expected to unambiguously assign female and male parents to each offspring. As exclusion analysis requires having all loci genotyped for every parent and since a few parents did not amplify for two of the loci, a likelihood-based method which allows for missing parental data was also applied in order to use the 12 loci genotyped. Thus this second approach that discriminates the most likely female and male parent for each offspring was used for comparison purposes and as a validation of the general results.

The exclusion analysis was performed using the Family Assignment Program (FAP) v. 3.61 (Taggart 2007). This software has two options for analysis. First, there is a predictive function that uses the parental genotypic data to calculate the proportion of hypothetical individuals that could be assigned to a true family to which they could belong. This expected proportion of assignments is calculated based on the number of alleles each locus presents and also how many alleles in the different loci share the candidate parents. The predictive files are used to evaluate the performance of the loci under study in discriminating among different family genotypes. The second function calculates the actual assignments by excluding parents which have alleles not matching those of the offspring.

The likelihood method for parentage assignment was performed using the program Cervus v. 3.0 (Marshall et al., 1998; Kalinowski et al., 2007), which involves several steps in order to calculate the likelihood of parentage assignment. First, it runs a simulation analysis with the parental genotype data to estimate the resolving power given the allele frequencies in the different loci. This is carried out by generating pairs of parental genotypes along with randomly generated unrelated candidate parents. Then the simulation generates artificial offspring genotypes by Mendelian sampling of the true parents' alleles and calculates the likelihood of parentage of the true parent and the unrelated candidate parents measured by its LOD score. Each offspring will be assigned the most likely candidate parent –which may or may not be the true parent. Each parentage assignment has a LOD score or a Delta score, the latter a derivative obtained as the difference in LOD scores between the most likely candidate parent and the second most likely parent. The distribution of LOD or Delta scores for offspring where the most likely candidate parent was the true parent is compared with the LOD or Delta distribution for offspring where the most likely candidate parent was an unrelated individual in order to obtain a critical LOD or Delta score which will be used to distinguished true parents from unrelated candidate parents with a 95% level of confidence. When analyzing real data, any most likely candidate parent with a LOD or Delta score exceeding the critical values for 95% confidence is assigned parentage with a 95% confidence. For the parental allocation analysis performed with the likelihood method used in the Cervus software, all the genotyping information collected on the 12 screened loci were used. Samples included in the parentage analysis were those having a minimum of 6 loci genotyped.

Genetic analysis of allele frequencies and genotype proportions

Allele frequencies, genotypic matrices and tests for Hardy-Weinberg equilibrium were calculated using GENEPOP (Raymond and Rousset 1995; Rousset 2008). Tests for H-W proportions were carried out estimating exact P-values using the Markov chain method with a dememorization parameter of 10000, 20 batches, and 5000 iterations per batch. Heterozygosity in parental and offspring generations was compared to assess potential changes between generations. In addition, chi-square tests were performed to assess observed versus expected genotype proportions within each family as an indirect assessment for natural selection at the microsatellite loci. Significance was evaluated using sequential Bonferroni correction (Holm 1979; Rice 1989). Briefly, for those families showing one or more tests significant at $\alpha = 0.05$, all tests were ranked according to their *P* values in ascending order. Then the first *P* value was compared against the corrected significance level obtained as α/k. If significant, then the second *P* value was compared with a significance level of $\alpha/(k-1)$, and so on. If a result was not significant, then all the remaining higher *P* values were also rendered not significant.

Genetic pairwise relatedness study

A randomization resampling technique was performed using R software to test the null hypothesis of random mating regarding genetic relatedness. The Queller and Goodnight (1989) mean (QGM) genetic pairwise relatedness was calculated using the equations implemented in the Cervus program. The QGM values were obtained for every potential female-male pair that could have been formed in each channel using the parental

genetic information obtained from the 12 loci genotyped. The bootstrap method was set to sample with replacement from all the potential pairwise relatedness that could have been generated given the parental information. The sampling size was set to an equivalent number of families observed in the channel with 50000 repetitions. The pairwise relatedness values observed in the families identified in each channel were tested for deviation from the mean of the randomized distribution using t-tests and applying the sequential Bonferroni correction.

Reproductive success assessment

Potential relationships of the number of mates and the number of offspring survived to fry stage with weight or Fulton's condition factor (Ricker 1975) were analyzed for females and males separately. In addition, a randomization resampling technique was performed in R software (R Development Core Team 2010) to test the null hypothesis of random mating regarding those biological variables. The bootstrap method was set to sample with replacement from all potential female-male pairs that could be formed. The sampling size was set to an equivalent number of families found in the channel with 50000 repetitions. The pairwise values observed for the families identified in each channel were tested for deviation from the mean of the randomized distribution using t-tests and applying the sequential Bonferroni correction (Holm 1979; Rice 1989).

Chi-squared analyses in an attempt to detect natural selection

Finally, as an attempt to investigate if there has been natural selection on CH fish during larval and early fry stage in the channels, chi-squared analyses were performed within families for all 12 loci. The chi-squared analysis is an useful test to detect any deviation of observed from expected proportions, which in this case was an equal representation of all genotypes within each microsatellite loci among the offspring of each family. Although microsatellites are neutral markers and in so are theoretically exempted from the action of natural selection they can be linked to coding loci in the chromosomes. Therefore, if there is any selection on coding loci then this may be detected in the neutral marker loci. No information regarding linkage to coding loci was available for the 12 loci studied here, but as the data was already collected for the parentage analysis it was a reasonable and simple hypothesis to test.

Results

Microsatellite genotyping

Table 5-2 lists the samples of spawning salmon collected in the fall 2007 from the spawning channels. Ten of the 12 microsatellite loci were successfully genotyped in every parental sample and offspring amplification success ranged from 82.96% to 98.65% (Table 5-3). Table 5-4 presents the number of offspring individuals that were genotyped at a minimum number of loci, where 97.31 % and 94.55% of the individuals for CH1 and CH3 respectively were genotyped at a minimum of 6 loci. The proportions do not decrease much when looking at the individuals which were typed at a minimum of 7, 8 or 9 loci, where the percentages remain over 92%. Lower percentages were obtained for a minimum of 10 and 11 genotyped loci among the offspring, and this drops to approximately 66% of individuals genotyped at all the 12 loci.

The number of alleles for each locus found in parental and offspring samples along with observed and expected heterozygosity values are presented in Table 5-5a and b for CH1 and CH3 respectively. Polymorphism was observed in all loci investigated, with someof them reaching as high as 16 or 17 alleles. Seven loci in CH1 brood stock presented a minimum of 10 alleles, whereas the remaining loci showed a minimum of six alleles. In the CH3 brood stock samples, eight loci had 10 or more alleles, three had seven alleles and one locus had four alleles. Table 5-5a and b also present the P-values for H-W equilibrium tests for each loci in the brood stock and offspring samples respectively. All H-W tests in the brood stock were not significant except for Ots107 and OtsG311 in

CH1, the latter indicated as heterozygosity deficiency (Appendix A12). However, Ots4 ($p=0.0482$) and OtsG83b ($p=0.0232$) were significant for heterozygosity excess even though they were non-significant in the test at a two-tail of H-W equilibrium. All loci in both CH1 and CH3 offspring samples deviated significantly from H-W expected proportions (Appendices A10 and A11). A heterozygosity deficit was found in CH1 for the Ots4 and Ots13 loci, whereas Ots253b, OtsG249, Omy325, Ots104 and Ots107 presented heterozygosity excess. No locus showed deficit of heterozygotes in CH3 and five loci presented excess of heterozygotes: OtsG249, Ots4, Omy325, Ots107, and OtsG83b. In addition, CH1 offspring had 3.44% and 4.25% lower heterozygosity than the brood stock at observed and expected average heterozygosity respectively (Table 5-5a). Offspring at CH3 presented 12.38% and 6.81% lower heterozygosity at observed and expected average heterozygosity respectively (Table 5-5b). Genotypic matrices and allele frequencies at each locus computed with GENEPOP for the offspring populations of CH1 and CH3 are presented in Appendices A4 and A5 respectively.

Table 5-2. Parental carcasses recovered from spawning channels.

Brood stock placed in YIAL's spawning channels in the fall 2007. (a) Channel 1 (CH1) held 17 females and 14 males. (b) Channel 3 (CH3) held 16 females and 14 males.

a)

Date sampled	Sex	Weight (Kg)	Length (cm)
Oct 26th 07	male	3.57	69.0
Oct 26th 07	male	0.15	25.0
Oct 26th 07	male	0.18	26.0
Oct 29th 07	male	0.40	28.5
Oct 29th 07	female	3.03	65.0
Oct 29th 07	female	3.18	63.0
Oct 29th 07	female	4.01	75.0
Oct 31st 07	female	4.09	75.0
Oct 31st 07	female	3.15	66.0
Nov 01st 07	male	2.31	59.0
Nov 01st 07	male	3.25	68.0
Nov 02nd 07	female	4.43	75.0
Nov 02nd 07	female	4.01	71.0
Nov 04th 07	female	3.41	74.0
Nov 04th 07	female	3.48	71.0
Nov 04th 07	female	3.95	72.0
Nov 04th 07	male	2.02	56.0
Nov 04th 07	male	5.00	76.0
Nov 04th 07	female	3.14	70.0
Nov 06th 07	male	5.17	77.0
Nov 07th 07	male	0.58	37.0
Nov 07th 07	female	3.35	70.0
Nov 07th 07	female	3.38	69.0
Nov 08th 07	male	4.35	74.0
Nov 08th 07	female	2.47	62.0
Nov 10th 07	female	2.32	61.0
Nov 10th 07	male	2.66	57.5
Nov 14th 07	male	2.54	56.5
Nov 14th 07	male	0.69	38.0
Nov 19th 07	female	1.69	56.0
Nov 26th 07	female	2.16	59.0

Table 5-2. Cont'd.

b)

Date sampled	Sex	Weight (Kg)	Length (cm)
Oct 26th 07	male	0.51	38.5
Oct 26th 07	female	2.34	62.0
Oct 26th 07	male	0.55	39.0
Oct 29th 07	female	3.15	69.0
Oct 29th 07	female	3.08	65.5
Oct 29th 07	female	4.59	76,0
Oct 29th 07	female	3.55	66.5
Oct 29th 07	female	2.79	65.0
Oct 30th 07	female	2.81	65.5
Oct 30th 07	female	2.73	64.5
Oct 30th 07	female	2.43	63.0
Oct 31st 07	female	4.69	76.0
Oct 31st 07	female	4.65	74.5
Oct 31st 07	male	0.65	39.0
Nov 01st 07	male	2.47	62.0
Nov 01st 07	male	3.86	69.0
Nov 01st 07	male	4.09	73.0
Nov 02nd 07	male	4.51	72.0
Nov 02nd 07	male	5.03	75.0
Nov 02nd 07	male	3.33	67.0
Nov 04th 07	female	3.95	73.5
Nov 04th 07	female	4.93	79.5
Nov 04th 07	female	3.24	73.0
Nov 04th 07	female	4.12	73.0
Nov 05th 07	male	2.93	64.0
Nov 05th 07	male	2.55	61.0
Nov 06th 07	male	2.78	62.0
Nov 06th 07	male	0.24	29.0
Nov 08th 07	female	3.34	70.0
Nov 14th 07	male	2.54	58.0

Table 5-3. Number of parental and offsprig samples genotyped at microsatellite loci.

Number of female and male brood stock and offspring samples genotyped for each of the 12 microsatellite loci screened in the present study. All parental samples from both channels were successfully amplified for the first 10 loci in the list. OtsG432 and Ots13 missed amplification in one or two parental samples. Offspring amplification success percentages are also presented.

	CH1				CH3			
	Females	Males	Offspring N=223		Females	Males	Offspring N=257	
Locus	N=17	N=14	Number	%	N=16	N=14	Number	%
OtsG253b	17	14	204	91.48	16	14	228	88.72
OtsG249	17	14	201	90.13	16	14	227	88.33
Ots3	17	14	220	98.65	16	14	250	97.28
Ots4	17	14	214	95.96	16	14	242	94.16
Omy325	17	14	218	97.76	16	14	243	94.55
Ots104	17	14	217	97.31	16	14	243	94.55
Ots107	17	14	217	97.31	16	14	239	93.00
OtsG311	17	14	205	91.93	16	14	238	92.61
OtsG68	17	14	210	94.17	16	14	224	87.16
OtsG83b	17	14	202	90.58	16	14	222	86.38
OtsG432	16	12	207	92.83	16	12	222	86.38
Ots13	16	13	185	82.96	16	13	242	94.16

Table 5-4. Number of offspring samples genotyped at a minimum number of loci.

Number and respective percentages of offspring in each channel genotyped at a determined minimum number of loci, from six to twelve loci. It can be seen that 97% and almost 95% of the offspring in CH1 and CH3 respectively, were genotyped at least in six of the 12 loci screened.

	CH1 N=223		CH3 N=257	
Minimum typed loci	No.	%	No.	%
6	217	97.31	243	94.55
7	213	95.52	241	93.77
8	211	94.62	240	93.39
9	208	93.27	237	92.22
10	201	90.13	227	88.33
11	195	87.44	215	83.66
12	148	66.37	168	65.37

Table 5-5. Observed and expected heterozygosity in Chinook salmon samples.

Summary presenting number of alleles and observed and expected heterozygosity in parental and offspring samples in CH1 (a) and CH3 (b). k: number of alleles at the locus. N: number of individuals typed at the locus. Hobs: observed heterozygosity. Hexp: expected heterozygosity. H=: average heterozygosity over all loci. *P*: P-values obtained from H-W test. □: indicates significance when testing null hypothesis H1 = heterozygosity excess. ◆: indicates significance when testing null hypothesis H1 = heterozygosity deficit. *: indicates loci that were significant for H-W equilibrium test but were not significant when testing separately excess or deficit as the null hypothesis in Genepop (see Appendix A10 and A11).

a)

CH1	Brood stock					Offspring				
Locus	k	N	Hobs	Hexp	*P*	k	N	Hobs	Hexp	*P*
OtsG253b	13	31	0.871	0.804	0.3247	10	204	0.77	0.741	0.0182□
OtsG249	11	31	0.935	0.892	0.5750	13	201	0.920	0.847	0.0024□
Ots3	7	31	0.581	0.567	0.3691	7	220	0.564	0.567	*
Ots4	6	31	0.742	0.597	0.4761	5	214	0.528	0.536	0.0366◆
Omy325	6	31	0.871	0.830	0.1584	6	218	0.803	0.734	0.0000□
Ots104	15	31	0.806	0.851	0.2589	12	217	0.908	0.829	0.0034□
Ots107	10	31	0.806	0.851	0.0228	9	217	0.862	0.837	0.0252□
OtsG311	12	31	0.742	0.891	0.0010	11	205	0.800	0.833	*
OtsG68	16	31	0.871	0.855	0.2513	14	210	0.829	0.794	*
OtsG83b	12	31	1.000	0.891	0.1839	13	202	0.842	0.858	*
OtsG432	9	28	0.857	0.858	0.8352	9	207	0.821	0.829	*
Ots13	7	29	0.483	0.512	0.1575	8	185	0.589	0.595	0.0038◆
	H =		0.797	0.783		H =		0.770	0.750	

b)

CH3	Broodstock					Offspring				
Locus	k	N	Hobs	Hexp	*P*	k	N	Hobs	Hexp	*P*
OtsG253b	13	30	0.833	0.814	0.4741	10	228	0.68	0.757	*
OtsG249	13	30	1.000	0.908	0.4515	11	227	0.969	0.857	0.0021□
Ots3	7	30	0.767	0.646	0.6617	6	250	0.560	0.568	*
Ots4	4	30	0.733	0.571	0.0890	5	242	0.591	0.544	0.0000□
Omy325	7	30	0.933	0.828	0.3142	7	243	0.872	0.822	0.0490□
Ots104	14	30	0.900	0.858	0.8621	12	243	0.753	0.749	0.0414□
Ots107	10	30	0.967	0.866	0.3270	9	239	0.854	0.773	*
OtsG311	12	30	0.967	0.883	0.7160	17	238	0.912	0.869	*
OtsG68	16	30	0.900	0.844	0.9679	15	224	0.835	0.795	*
OtsG83b	14	30	0.967	0.906	0.1268	12	222	0.982	0.887	0.0000□
OtsG432	10	28	0.964	0.877	0.9745	9	222	0.797	0.805	*
Ots13	7	29	0.759	0.646	0.7171	6	242	0.562	0.564	*
	H =		0.891	0.803		H =		0.781	0.749	

Parentage assignment

A *sine qua non* condition to run an exclusion method is to have all parental samples genotyped for every locus used in the assignment. Therefore only the 10 loci successfully amplified in every parental sample (Table 5-3) were included, leaving out loci OtsG432 and Ots13. The probability of assignment for the 238 and 224 potential parental genotypes in CH1 and CH3 in an exclusion analysis are presented in Appendices A6 and A7, respectively. In general, most families had a high probability of assignment using 10 loci, with a few parental combinations with probabilities as low as 0.75.

Exclusion analysis was run four times for each channel population allowing 0, 1, 2 or 3 mismatches (Tables 5-6a and b). Knowing that genotyping errors cannot be completely excluded from laboratory practices, not considering samples with mismatches would most likely leave many offspring unassigned as can be seen in the relatively low percentages of families assigned with single matches. Allowing for one or two mismatches resulted in a sensible increase in the total number of assignments by about 15%. Therefore, allowing two allele mismatches successfully assigned some three quarters of each population from CH1 and CH3 (Table 5-6a and b).

Parentage results: family assignments

Family assignments obtained with the likelihood and exclusion methods for CH1 and CH3 are presented in Appendices A8 and A9, respectively. The tables present the offspring genotyped at a minimum of 6 loci assigned with the likelihood method at a 95%

confidence considering individuals whose parents were all genotyped at 10 loci and individuals whose parents were genotyped at all 12 loci. The results obtained with the exclusion method included individuals with one family match. The tables identify those individuals for which the exclusion method found more than one family match, but that were still assigned with a 95% confidence by the likelihood method. Individuals having more than two mismatches were discarded in both methods.

A total of 183 of the 223 offspring individuals of CH1 were assigned by the likelihood method with a 95% confidence. From those, 171 coincided with the exclusion analysis having one single family match and 8 had more than a single match by exclusion analysis. A total of 217 of the 257 individuals of CH3 were assigned by the likelihood method with a 95% confidence. From those, 181 coincided with one single match by the exclusion analysis and 22 had more than a single match.

A total of 17 and 23 families were identified in CH1 and CH3, respectively. Six families out of the 17 in CH1 were major families with at least 9 individuals assigned each. Five major families were identified in CH3 with at least 19 individuals assigned to each. The remaining families were considered minor families as they had one to four individuals assigned. From the total of 171 offspring assigned to a single parent pair for CH1 by the exclusion method, 153 individuals were assigned to six major families with a minimum of nine offspring each, and 18 individuals assigned to other 11 minor families (Table 5-7). From the total of 181 offspring assigned to a single parent pair for CH3, 148 individuals were assigned to five major families with a minimum of 19 offspring assigned each and 34 individuals assigned to other 18 minor families (Table 5-7). The parentage analysis revealed that 90% of the offspring successfully assigned were spawned by just

three females in each channel, and were sired by six and five males in CH1 and CH3 respectively.

Table 5-6. Family assignments with mismatch tolerance.

Number and percentage of individuals from CH1 (a) and CH3 (b) for which a single parent pair was found, more than one parent pair was found, or no assignment was performed with different levels of allele mismatch tolerance in the exclusion analysis by FAP.

a)

CH1 (N=223)	Allele mismatch tolerance							
	0		**1**		**2**		**3**	
Single matches found	130	(58.3 %)	163	(73.1 %)	169	(75.8 %)	171	(76.7 %)
Multiple matches found	7	(3.1 %)	20	(9.0 %)	22	(9.9 %)	24	(10.8 %)
No matches found	86	(38.6 %)	40	(17.9 %)	32	(14.3 %)	28	(12.6 %)

b)

CH3 (N=257)	Allele mismatch tolerance							
	0		**1**		**2**		**3**	
Single matches found	129	(50.2 %)	170	(66.1 %)	185	(72 %)	194	(75.5 %)
Multiple matches found	26	(10.1 %)	34	(13.2 %)	38	(14.8 %)	44	(17.1 %)
No matches found	102	(39.7 %)	53	(20.6 %)	34	(13.2 %)	19	(7.4 %)

Table 5-7. Offspring assignment to families identified through exclusion analysis.

Offspring number assigned to families by the exclusion method. CH1 presented six major and eleven minor families. CH3 presented five major and eighteen minor families. QGM: Queller and Goodnight (1989) mean pairwise relatedness coefficient. Major families are in bold.

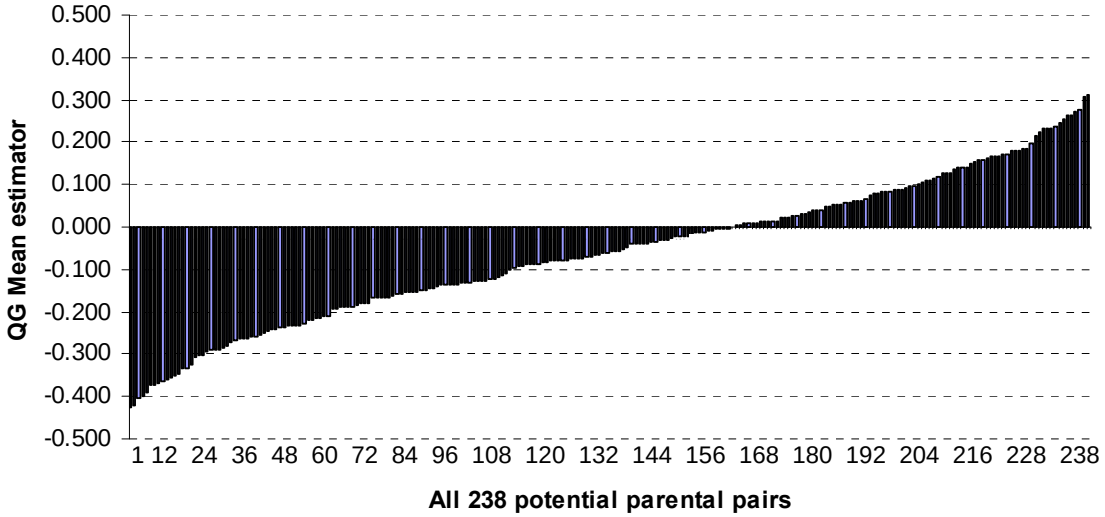
Families CH1	N	QGM
F228xM234	21	**-0.058**
F228xM240	12	**0.053**
F228xM252	9	**0.156**
F230xM243	12	**-0.015**
F231xM233	55	**-0.153**
F231xM247	44	**-0.039**
F230xM234	1	-0.079
F230xM244	3	-0.399
F230xM252	1	-0.090
F236xM241	2	-0.150
F236xM243	3	0.031
F236xM247	1	-0.014
F237xM251	1	-0.121
F242xM240	1	-0.162
F242xM250	1	-0.240
F246xM252	3	-0.348
F248xM247	1	-0.127
Total families = 17	N = 171	

Families CH3	N	QGM
F267xM258	26	**-0.145**
F267xM276	28	**-0.092**
F270xM275	55	**0.089**
F270xM276	20	**0.006**
F281xM271	19	**-0.189**
F263xM272	1	-0.137
F263xM273	3	0.074
F265xM275	1	-0.104
F266xM283	3	0.166
F266xM285	1	-0.124
F267xM273	1	-0.069
F267xM285	4	-0.211
F267xM287	1	0.216
F269xM272	1	-0.004
F269xM273	1	-0.180
F270xM271	4	-0.306
F270xM282	3	0.018
F270xM283	1	-0.056
F270xM285	1	-0.166
F279xM272	4	0.239
F280xM274	1	0.159
F280xM287	1	0.261
F281xM283	1	-0.062
Total families = 23	N = 181	

Relatedness study in parental samples

The Queller and Goodnight mean (QGM) genetic pairwise relatedness values for the families identified in each channel are indicated in table 5-7. Distributions of QGM relatedness coefficient for every potential female-male pair are represented in figures 5-1a and b for CH1 and CH3 respectively. The histograms of the randomized distributions are presented in figures 5-2a and b. Three families in CH1, one of them a major family, and six families in CH3 with no major families involved, significantly deviated from the random distribution (Tables 5-8 and 5-9).

a)



b)

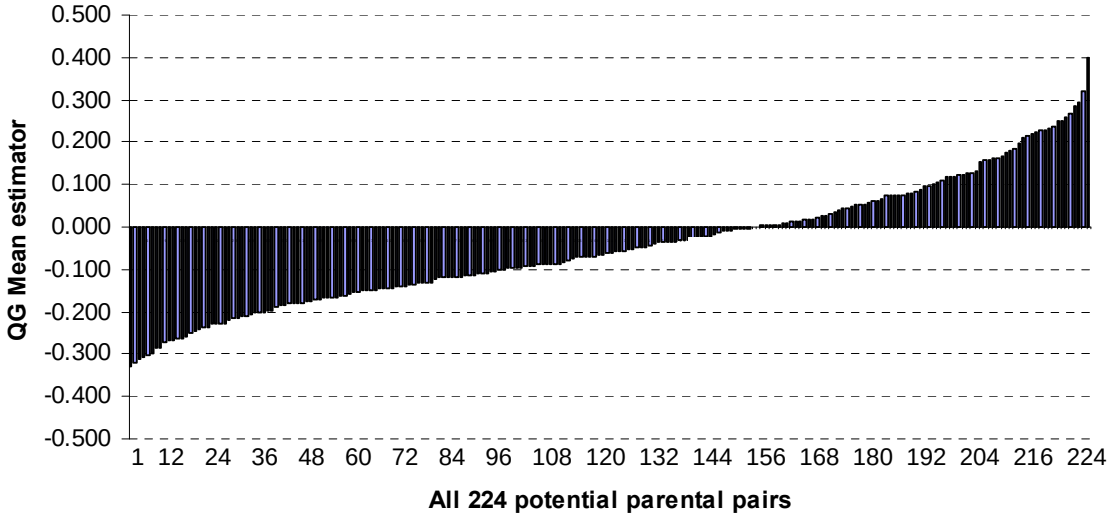
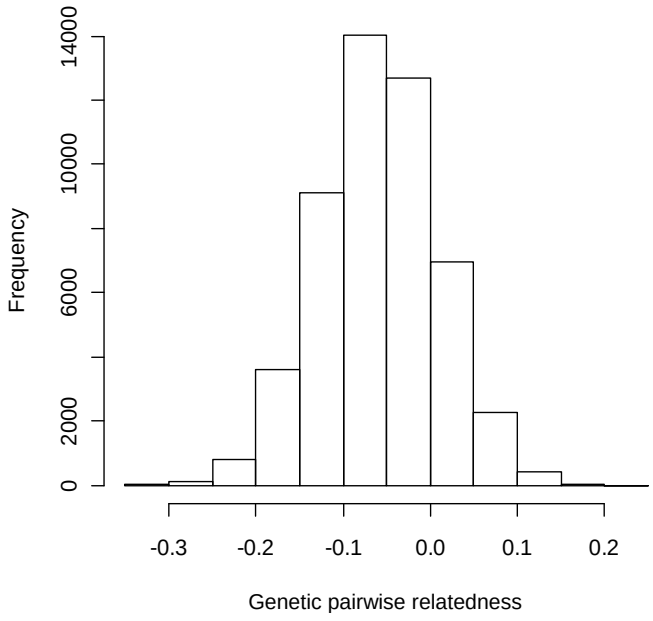


Figure 5-1. Mean pairwise relatedness distributions.

Arrangement from lowest to highest mean pairwise relatedness values (QGM) for every potential family that could have originated given the parental information in CH1 (a) and CH3 (b). Given that there were 17 females and 14 males in CH1, there were 238 potential pairs to be formed. Similarly, CH3 had 16 females and 14 males that could have formed 224 pairs. The coefficients were calculated using all 12 loci.

a)



b)

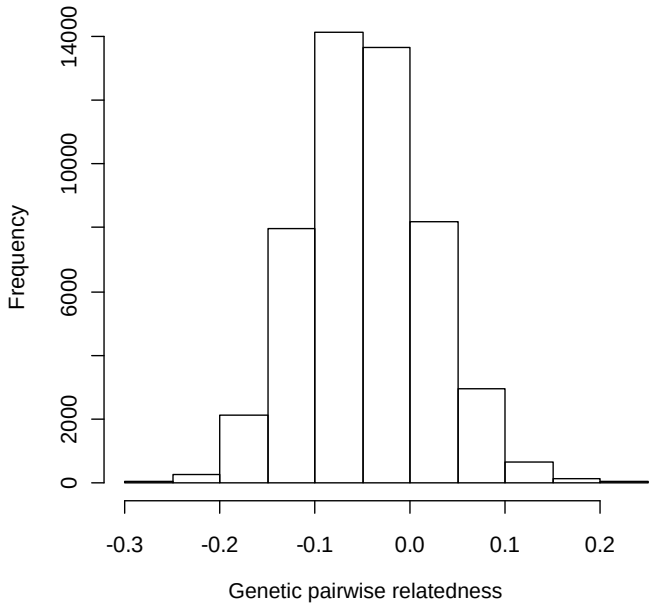


Figure 5-2. Randomized distributions of mean genetic pairwise relatedness.

Histograms obtained from a randomization test for genetic pairwise relatedness (QGM) values for CH1 (a) and CH3 (b) female-male potential pairs.

Table 5-8. Sequential Bonferroni correction for pairwise relatedness in CH1.

Sequential Bonferroni correction for CH1 families showing significant t-tests results. Only 3 families remained significant after correction of the critical α value, of which one was a major family. Pairwise relatedness QGM values of major successful families are in bold.

Families CH1	QGM	*P-values*	Seq. Bonf.	α value	Result
F230xM244	-0.399	0.00000	α / k	0.00294	SIGNIF
F246xM252	-0.348	0.00002	α / (k-1)	0.00313	SIGNIF
F228xM252	**0.156**	0.00144	α / (k-2)	0.00333	SIGNIF
F242xM250	-0.240	0.00739	α / (k-3)	0.00357	NS
F228xM240	**0.053**	0.09657			
F242xM240	-0.162	0.12872			
F231xM233	**-0.153**	0.16518			
F236xM241	-0.150	0.18005			
F236xM243	0.031	0.18117			
F248xM247	-0.127	0.31783			
F237xM251	-0.121	0.36048			
F236xM247	-0.014	0.50048			
F230xM243	**-0.015**	0.51242			
F230xM252	-0.090	0.65071			
F231xM247	**-0.039**	0.76434			
F230xM234	-0.079	0.77049			
F228xM234	**-0.058**	0.98524			
	k =	17			

Table 5-9. Sequential Bonferroni correction for pairwise relatedness in CH3.

Sequential Bonferroni correction for CH3 families showing significant t-tests results. Only 6 families remained significant after correction of the critical α value, of which none was a major family. Pairwise relatedness QGM values of major successful families are in bold.

Families CH3	QGM	*P-values*	Seq. Bonf.	α value	Result
F280xM287	0.261	0.00001	α / k	0.00217	SIGNIF
F279xM272	0.239	0.00003	α / (k-1)	0.00227	SIGNIF
F267xM287	0.216	0.00011	α / (k-2)	0.00238	SIGNIF
F270xM271	-0.306	0.00011	α / (k-3)	0.00250	SIGNIF
F266xM283	0.166	0.00173	α / (k-4)	0.00263	SIGNIF
F280xM274	0.159	0.00250	α / (k-5)	0.00278	SIGNIF
F267xM285	-0.211	0.01414	α / (k-6)	0.00294	NS
F281xM271	**-0.189**	0.03429			
F269xM273	-0.180	0.04662			
F270xM275	**0.089**	0.04667			
F270xM285	-0.166	0.07568			
F263xM273	0.074	0.07623			
F267xM258	**-0.145**	0.14197			
F263xM272	-0.137	0.17864			
F266xM285	-0.124	0.24455			
F270xM282	0.018	0.34913			
F265xM275	-0.104	0.38479			
F270xM276	**0.006**	0.44495			
F267xM276	**-0.092**	0.49232			
F269xM272	-0.004	0.53689			
F267xM273	-0.069	0.73504			
F281xM283	-0.062	0.81385			
F270xM283	-0.056	0.87426			
	k =	23			

Chi-squared analyses

Chi-square tests for major families in CH1 and CH3 are presented in Appendices A14 and A15 respectively. Some families with a low number of assigned offspring had expected frequency cells lower than 5 and therefore results should be taken with caution in these cases, though the problem is more critical in cases where there is significance. There were eight significant chi-squared tests in CH1, of which three remained significant after applying sequential Bonferroni correction (Table 5-10). No significant tests were obtained for CH3.

Table 5-10. Sequential Bonferroni correction for chi-squared tests in CH1.

Sequential Bonferroni correction for CH1 families showing significant chi-squared results (Appendix A11). Only three tests out of the eight originally significant were confirmed as truly significant.

F228xM240	*P-values*	Seq. Bonf.	α value corr.	Result
Ots3	0.00053	α / k	0.00455	SIGNIF
Ots107	0.00389	α / (k-1)	0.00500	SIGNIF
Ots4	0.01111	α / (k-2)	0.00556	NS
OtsG83b	0.03407			
OtsG68	0.24821			
Omy325	0.34303			
OtsG432	0.34303			
OtsG253b	0.48356			
Ots104	0.56370			
OtsG311	0.56370			
OtsG249	0.72123			
Ots13	N/A			
k =	11			
F230xM243	*P-values*	Seq. Bonf.	α value	Result
Ots4	0.00083	α / k	0.00417	SIGNIF
OtsG83b	0.11161	α / (k-1)	0.00455	NS
Ots107	0.11161			
Ots13	0.13167			
Ots3	0.24821			
Omy325	0.24821			
OtsG432	0.34303			
Ots104	0.44592			
OtsG249	0.52709			
OtsG311	0.56370			
OtsG68	0.57241			
OtsG253b	1			
k =	12			
F231xM247	*P-values*	Seq. Bonf.	α value	Result
Ots3	0.00679	α / k	0.00417	NS
Ots13	0.02517			
Omy325	0.03187			
OtsG253b	0.11048			
OtsG432	0.11980			
Ots107	0.13167			
OtsG249	0.15772			
OtsG68	0.16139			
Ots4	0.35454			
Ots104	0.43561			
OtsG83b	0.65508			
OtsG311	0.87879			
k =	12			

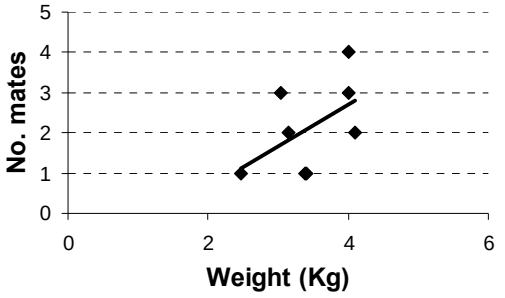
Reproductive success

Regressions of the number of mates versus weight and Fulton's condition factor did not find any significant relationship (Figure 5-3). Similarly, regressions of the number of offspring versus weight and Fulton's condition factor failed to show significant relationships of these variables (Figure 5-4). The histograms of the randomized distributions of pairwise differences in weight and in the Fulton's condition factor are presented in figures 5-5 and 5-6 respectively. Ten families in CH1 significantly deviated from the random distribution of pairwise differences in weight (Table 5-11). Three of them showed higher differential and seven, including three major families, presented lower pairwise differences than the random distribution (Table 5-11). Fifteen were the significant families for pairwise differences in weight in CH3 (Table 5-12). Seven of them had higher differential and eight, including four of the five major families, presented lower differences than the random distribution. Pairwise differences in the Fulton's index of seven families were significant and were lower then the random distribution, including one major familiy (Table 5-13). The same was observed for the Fulton's pairwise differences in CH3, in which 15 families, two of them major ones, were found significant and all lower than the randomly expected pairwise differences (Table 5-14).

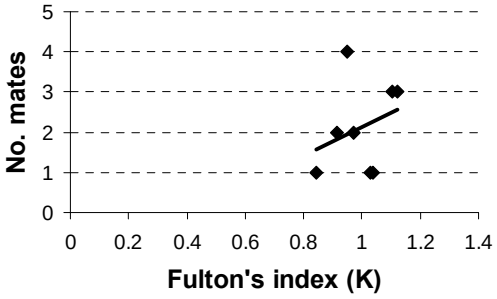
Figure 5-3. Regression of No. of mates and weight and Fulton's condition factor.

Regressions of females (a) and males (b) from CH1 in which the dependent variable is number of mates and the independent variable is weight (Kg). Linear equations are in the upper right corner of each figure. Variability in the number of mates is hardly explained by the variance in weight. Number of mates does not seem to be a function of body weight in either females or males.

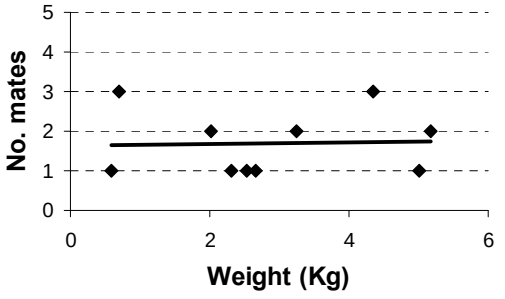
a) Females CH1 $y = 1.038x - 1.4486$, $R^2 = 0.276$



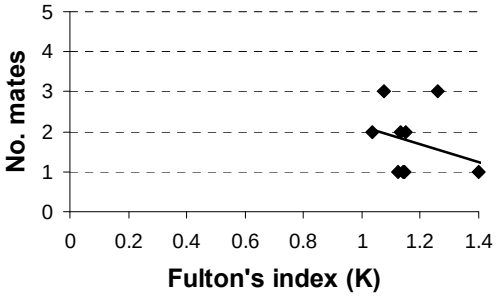
b) Females CH1 $y = 3.5385x - 1.3989$, $R^2 = 0.0889$



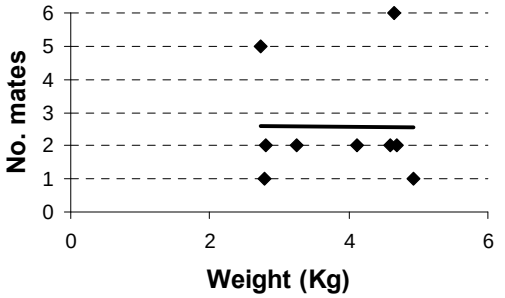
c) Males CH1 $y = 0.0223x + 1.6362$, $R^2 = 0.0019$



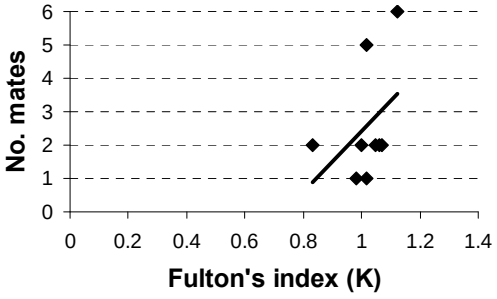
d) Males CH1 $y = -2.21x + 4.3219$, $R^2 = 0.1182$



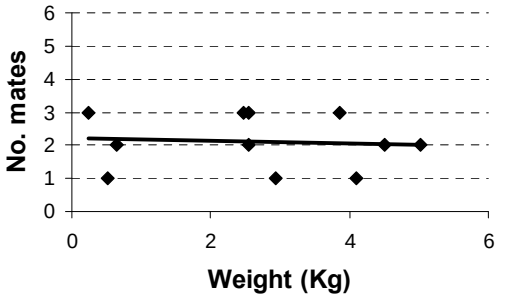
e) Females CH3 $y = -0.0179x + 2.6242$, $R^2 = 9E\text{-}05$



f) Females CH3 $y = 9.0439x - 6.634$, $R^2 = 0.1765$



g) Males CH3 $y = -0.0398x + 2.1971$, $R^2 = 0.0062$



h) Males CH3 $y = 1.151x + 0.8164$, $R^2 = 0.0248$

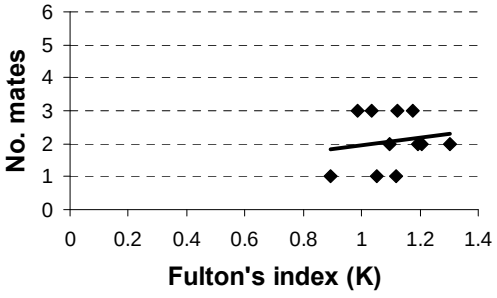
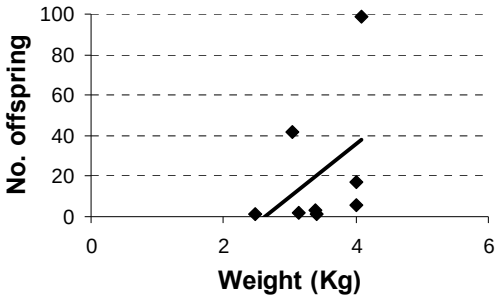


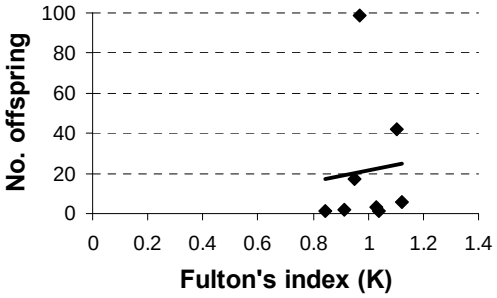
Figure 5-4. Regression of No. of offspring and weight and Fulton's condition factor.

Regressions of females (a) and males (b) from CH1 in which the dependent variable is number of offspring and the independent variable is weight (Kg). Linear equations are in the upper right corner of each figure. Variability in the number of offspring is hardly explained by the variance in weight. Number of offspring does not seem to be a function of body weight in either females or males.

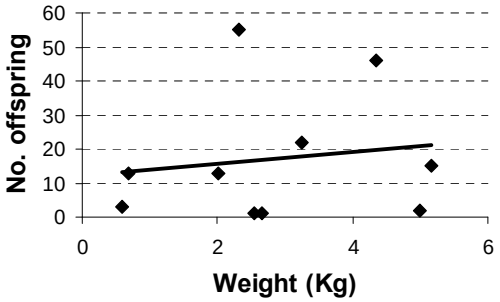
a) Females CH1 y = 25.55x - 66.59, R^2 = 0.1799



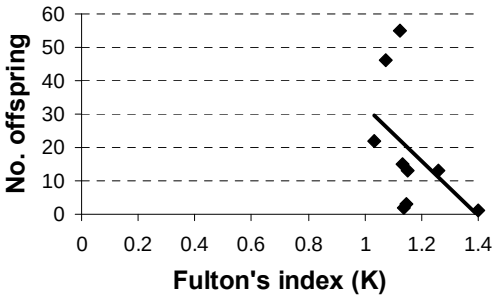
b) Females CH1 y = 27.648x - 6.1585, R^2 = 0.0058



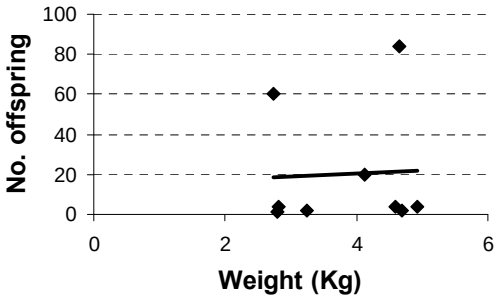
c) Males CH1 y = 1.7058x + 12.227, R^2 = 0.0207



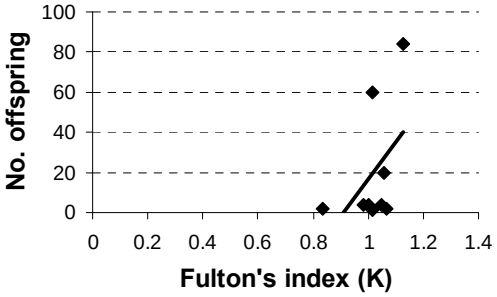
d) Males CH1 y = -81.811x + 114.16, R^2 = 0.3014



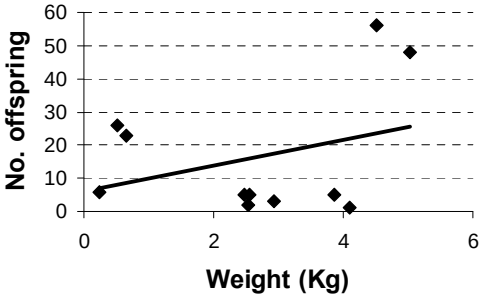
e) Females CH3 y = 1.4263x + 14.636, R^2 = 0.0019



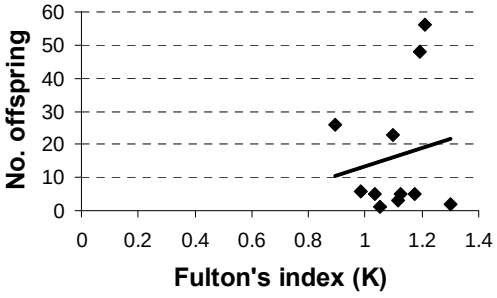
f) Females CH3 y = 185.52x - 168.4, R^2 = 0.2408



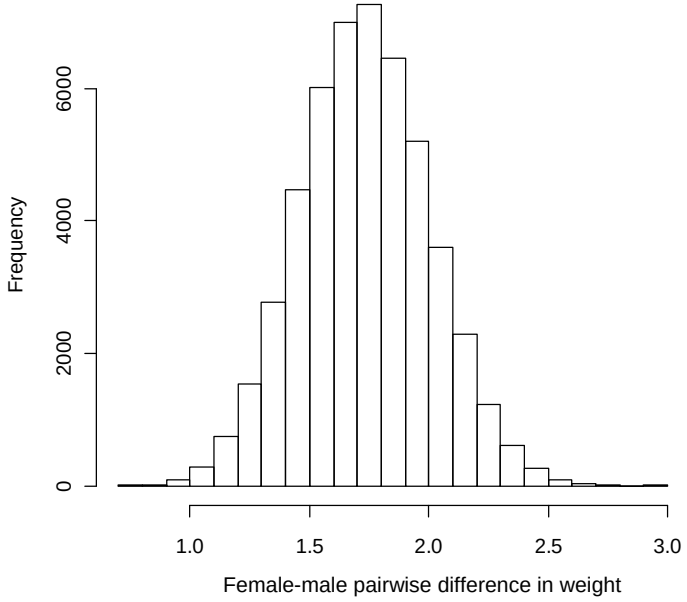
g) Males CH3 y = 3.8726x + 6.0204, R^2 = 0.1066



h) Males CH3 y = 27.948x - 14.582, R^2 = 0.0264



a)



b)

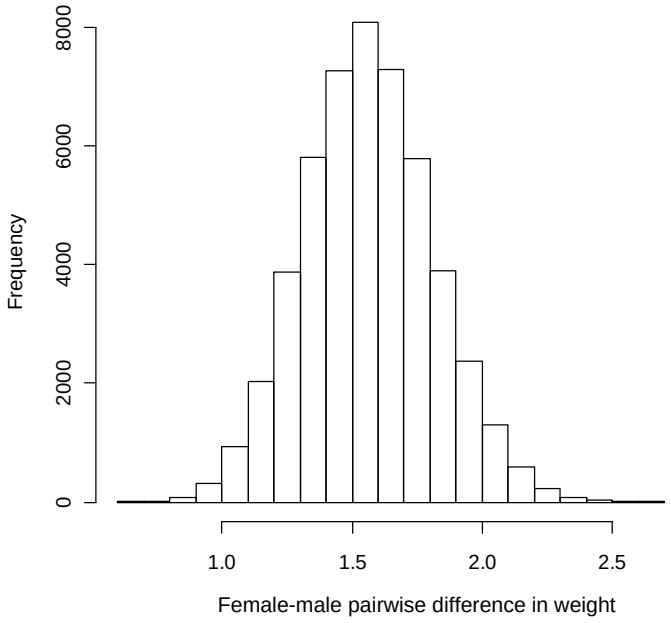
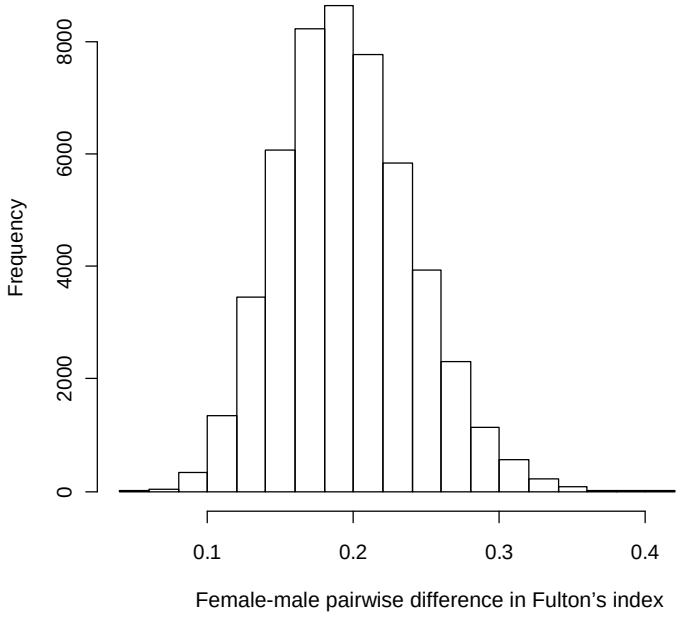


Figure 5-5. Randomized distributions of pairwise differences in weight.

Histograms obtained from a randomization test for the pairwise differences in weight for female-male potential pairs of CH1 (a) and CH3 (b).

a)



b)

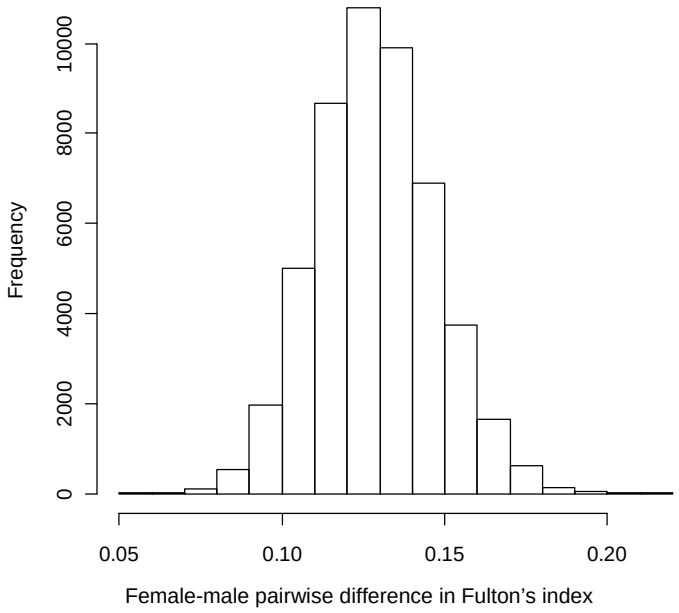


Figure 5-6. Randomized distributions of pairwise differences in Fulton's index.

Histograms obtained from a randomization test for the pairwise differences in the Fulton's index for female-male potential pairs of CH1 (a) and CH3 (b).

Table 5-11. Sequential Bonferroni corr. for pairwise differences in weight in CH1.

Sequential Bonferroni correction for CH1 families showing significant t-tests results for pairwise differences in weight. Ten families remained significant after correction of the critical α value, three of them being major families. Major successful families are in bold.

Families CH1	Diff.Weight	*P-values*	Seq. Bonf.	α value corr.	Result
F236xM241	3.43	0.00000	α / k	0.00294	SIGNIF
F236xM243	3.32	0.00000	α / (k-1)	0.00313	SIGNIF
F230xM244	0.22	0.00000	α / (k-2)	0.00333	SIGNIF
F242xM240	0.26	0.00000	α / (k-3)	0.00357	SIGNIF
F236xM247	0.34	0.00000	α / (k-4)	0.00385	SIGNIF
F231xM247	0.48	0.00000	α / (k-5)	0.00417	SIGNIF
F231xM233	0.76	0.00034	α / (k-6)	0.00455	SIGNIF
F230xM234	2.69	0.00041	α / (k-7)	0.00500	SIGNIF
F230xM243	0.87	0.00148	α / (k-8)	0.00556	SIGNIF
F248xM247	0.99	0.00623	α / (k-9)	0.00625	SIGNIF
F246xM252	1.01	0.00801	α / (k-10)	0.00714	NS
F230xM252	1.12	0.02406			
F228xM252	2.34	0.02427			
F242xM250	1.16	0.03498			
F237xM251	1.16	0.03498			
F228xM234	1.88	0.58470			
F228xM240	1.78	0.85892			
	k =	17			

Table 5-12. Sequential Bonferroni corr. for pairwise differences in weight in CH3.

Sequential Bonferroni correction for CH3 families showing significant t-tests results for pairwise differences in weight. Fifteen families remained significant after correction of the critical α value, four of them being major families. Major successful families are in bold.

Families CH3	Diff.Weight	*P-values*	Seq. Bonf.	α value corr.	Result
F270xM285	4.41	0.00000	α / k	0.00217	SIGNIF
F270xM271	4.00	0.00000	α / (k-1)	0.00227	SIGNIF
F281xM271	3.47	0.00000	α / (k-2)	0.00238	SIGNIF
F270xM275	0.14	0.00000	α / (k-3)	0.00250	SIGNIF
F267xM287	0.19	0.00000	α / (k-4)	0.00263	SIGNIF
F266xM283	0.26	0.00000	α / (k-5)	0.00278	SIGNIF
F270xM276	0.38	0.00000	α / (k-6)	0.00294	SIGNIF
F266xM285	2.57	0.00005	α / (k-7)	0.00313	SIGNIF
F267xM285	2.49	0.00019	α / (k-8)	0.00333	SIGNIF
F279xM272	2.46	0.00031	α / (k-9)	0.00357	SIGNIF
F280xM287	0.70	0.00052	α / (k-10)	0.00385	SIGNIF
F263xM273	0.73	0.00081	α / (k-11)	0.00417	SIGNIF
F267xM276	2.30	0.00306	α / (k-12)	0.00455	SIGNIF
F269xM273	0.83	0.00319	α / (k-13)	0.00500	SIGNIF
F280xM274	0.85	0.00412	α / (k-14)	0.00556	SIGNIF
F267xM258	2.22	0.00829	α / (k-15)	0.00625	NS
F269xM272	2.22	0.00829			
F263xM272	2.12	0.02521			
F270xM283	2.10	0.03095			
F267xM273	1.13	0.08138			
F265xM275	1.72	0.52888			
F270xM282	1.72	0.52888			
F281xM283	1.57	0.97883			
	k =	23			

Table 5-13. Sequential Bonferroni corr. for pairwise diffs. in Fulton's index in CH1.

Sequential Bonferroni correction for CH1 families showing significant t-tests results for pairwise differences in Fulton's index. Seven families remained significant after correction of the critical α value, one of them being major families. Major successful families are in bold.

Families CH1	Diff.Fulton	*P-values*	Seq. Bonf.	α value corr.	Result
F237xM251	0.57	0.00000	α / k	0.00294	SIGNIF
F242xM250	0.48	0.00000	α / (k-1)	0.00313	SIGNIF
F236xM243	0.01	0.00005	α / (k-2)	0.00333	SIGNIF
F236xM241	0.02	0.00010	α / (k-3)	0.00357	SIGNIF
F248xM247	0.04	0.00048	α / (k-4)	0.00385	SIGNIF
F228xM240	0.05	0.00098	α / (k-5)	0.00417	SIGNIF
F236xM247	0.05	0.00106	α / (k-6)	0.00455	SIGNIF
F228xM234	0.07	0.00594	α / (k-7)	0.00500	NS
F230xM234	0.08	0.01320			
F230xM252	0.31	0.01422			
F231xM247	0.10	0.04360			
F228xM252	0.15	0.34962			
F231xM233	0.16	0.37407			
F242xM240	0.23	0.38853			
F246xM252	0.23	0.46805			
F230xM243	0.18	0.76287			
F230xM244	0.19	0.98062			
	k =	17			

Table 5-14. Sequential Bonferroni corr. for pairwise diffs. in Fulton's index in CH3.

Sequential Bonferroni correction for CH3 families showing significant t-tests results for pairwise differences in Fulton's index. Fifteen families remained significant after correction of the critical α value, two of them being major families. Major successful families are in bold.

Families CH3	Diff.Fulton	*P-values*	Seq. Bonf.	α value corr.	Result
F280xM287	0.47	0.00000	α / k	0.00217	SIGNIF
F267xM287	0.28	0.00000	α / (k-1)	0.00227	SIGNIF
F270xM283	0.00	0.00000	α / (k-2)	0.00238	SIGNIF
F270xM282	0.01	0.00000	α / (k-3)	0.00250	SIGNIF
F263xM272	0.01	0.00000	α / (k-4)	0.00263	SIGNIF
F266xM285	0.02	0.00000	α / (k-5)	0.00278	SIGNIF
F270xM271	0.03	0.00000	α / (k-6)	0.00294	SIGNIF
F269xM272	0.03	0.00000	α / (k-7)	0.00313	SIGNIF
F267xM285	0.03	0.00000	α / (k-8)	0.00333	SIGNIF
F281xM271	0.04	0.00000	α / (k-9)	0.00357	SIGNIF
F280xM274	0.22	0.00000	α / (k-10)	0.00385	SIGNIF
F279xM272	0.06	0.00006	α / (k-11)	0.00417	SIGNIF
F281xM283	0.06	0.00042	α / (k-12)	0.00455	SIGNIF
F265xM275	0.19	0.00048	α / (k-13)	0.00500	SIGNIF
F270xM276	0.07	0.00083	α / (k-14)	0.00556	SIGNIF
F267xM276	0.17	0.01126	α / (k-15)	0.00625	NS
F270xM275	0.08	0.01370			
F267xM273	0.16	0.11268			
F269xM273	0.11	0.22573			
F270xM285	0.14	0.51706			
F266xM283	0.12	0.77478			
F267xM258	0.12	0.78344			
F263xM273	0.13	0.96979			
	k =	23			

Discussion

The number of alleles observed in the microsatellite loci used in the present study provided an appropriate level of polymorphism to be used for parentage assignment (Koskinen et al., 2004). Most loci presented high allelic diversity, many exceeding 10 alleles and some reaching 16 or 17 alleles (Tables 5-5a and b). This, in addition to the high percentage of offspring samples that were successfully genotyped at a minimum of six loci (Table 5-4), provided the confidence to include in the parentage analysis all those samples with a minimum of six loci genotyped (Bernatchez and Duchesne 2000). Parentage analysis performed with exclusion and likelihood methods provided similar results, with a few cases in which exclusion resulted in multiple matching whereas likelihood assigned parentage with a 95% confidence level. No major improvements were observed when comparing the likelihood results using 12 versus 10 loci (Appendices A8 and A9). Thus, 10 loci seemed to be enough loci for proper identification and resolution of parentage assignments regardless the method employed. This is in agreement to the conclusions reached in the study performed by Bernatchez and Duchesne (2000).

H-W probability tests performed on the brood stock were not significant except for one locus in CH1, OtsG311, which was indicated as heterozygosity deficient by GENEPOP (Appendices A12 and A13). When looking at the H-W expected proportions in the offspring samples, the opposite situation was observed in which five loci in each channel deviated significantly having heterozygosity excess. Several causes may have produced an heterozygote excess in these loci in the offspring sampled at the fry stage. First, there could have been negative assortative mating happening in the channels by which female and male mated with partners sharing low genetic relatedness, thus yielding

higher than expected heterozygosity in the microsatellite loci analyzed. But this hypothesis is likely incorrect as the genetic pairwise relatedness randomized distributions (Figures 5-2a and b) showed that the relatedness values of most families identified did not significantly deviate from the random distribution (Tables 5-8 and 5-9). Second, there could have been a selection advantage favouring heterozygotes for these particular loci, but this hypothesis lacks evidence as most loci were not significant in the chi-square tests which tested genotype proportions within each family (Appendices A14 and A15). The three significant tests after Bonferroni correction may be due to the lower numbers of samples in those families, though natural selection cannot be ruled out since it could be that the neutral markers were loosely linked to loci under selection. A broader study should be performed including many more markers, perhaps known to be under selection, to help increase the capacity to detect selection. A third alternative explanation may be that males and females in each spawning channel differed at their allele frequencies therefore originating a new generation bearing a mixture of their original alleles. This is partially supported by the absolute differences between females and males (Table 5-15a and b), in which several loci had a difference in allele frequency that was higher than 20%, reaching more than 30% in one case (Table 5-15a). The differences in the frequencies of shared alleles adds to another observation in which about one third of the alleles carried by females and males were not present in the opposite sex. Moreover, some loci had less than half of the alleles shared between females and males (Table 5-15a and b). Therefore, having females and males carrying alleles at different frequencies will undoubtedly generate a higher than expected amount of heterozygotes at those loci. This

then may be a plausible explanation to the heterozygosity excess observed in the offspring versus the brood stock.

The existence of only two loci, Ots4 and Ots13, with excess of homozygotes in the CH1 offspring group suggested that inbreeding is not an issue in this captive-bred population, because it this was not observed in the other 10 loci analyzed. A possible explanation for these two loci with homozygosity excess could be the presence of null alleles that are the result of undetectable PCR products due to mutations in the primer binding region that do not allow amplification (Brookfield 1996). In the presence of null alleles, heterozygotes are actually interpreted as homozygotes as only one allele amplifies, and homozygotes for the null allele will result in no amplification. However, if null alleles would be present in the YIAL stock population, the homozygosity excess for these two loci should be observed in the two channels and that was not found here.

This study did not find any significant relationship in the analyses performed on the reproductive success. In addition, the randomization resampling technique employed to test random mating regarding pairwise differences of weight did not show any clear tendency as pairs presented both higher and lower differences with respect the random distribution. On the other hand, the seven and fifteen significant female-male pairs in CH1 and CH3 respectively for the Fulton's condition index presented all low pairwise differentials. Morevoer, the pairwise differences were all lower than the randomized distribution and were all close to zero, indicating that pairs were formed among fish with similar condition factors. This suggested that fish mated non-randomly regarding the Fulton's condition index. Furthermore, the fact that the vast majority of the new generations in both channels were represented by a selected and a small number of

families may also indicate that mating was not random. This is in agreement with a previous study conducted by Neff and colleagues (2008) with Chinook salmon in these spawning channels, who demonstrated that female mate choice increased genetic diversity at the MH genes. The general tendency of higher MH heterozygosity observed in channel-bred fish with respect to randomly-mated hatchery fish (see Chapter 3) supports this argument. MH-linked mate choice has been already described in many different fish species such as sticklebacks (Reusch et al 2001), Atlantic salmon (Landry et al. 2001), brown trout (Forsberg et al., 2007), and Arctic charr (Skarstein et al 2005).

Finally, the pairwise relatedness randomization test indicated that most females and males paired randomly regarding genetic relatedness. This is what would be expected if they mated according to a trait under selection. Genetic markers associated to fitness-related traits are needed to continue studying the breeding system of Chinook salmon in spawning channels. The MH genes are excellent candidates as they already have been linked to mate choice in this species (Neff et al., 2008). Moreover, MH genes play a critical role in the immune system of vertebrates which makes them an ideal marker for continuing studies on semi-natural propagation systems in aquaculture.

In conclusion, a successful parentage assignment was obtained following two different methods, likelihood and exclusion, that produced similar results. In general, parental samples revealed observed heterozygosity as expected whereas about half of the loci in the offspring presented higher observed than expected heterozygosity. The excess in heterozygosity in those particular loci may be the result of females and males carrying alleles at different frequencies. In addition, no evidence for inbreeding effects or presence

of null alleles was obtained from the two channel populations. Interestingly, evidence of non-random mating was found using Fulton's condition factor in 22 families.

Table 5-15. Allelic differences between males and females.

Allelic differences in females and males of CH1 (a) and CH3 (b). The last three columns present the minimum, maximum and mean values of the absolute differences in the frequencies between the sexes.

a)

CH1	No. alleles in females	No. alleles in males	Differential No. Alleles	Proportion of shared alleles	Absolute diff. in freq. in female and male		
					MIN	MAX	MEAN
OtsG253b	10	9	1	0.4615	0.0126	0.1786	0.0598
OtsG249	9	10	1	0.7273	0.0042	0.2353	0.0630
Ots3	6	6	0	0.7143	0.0042	0.1071	0.0378
Ots4	4	5	1	0.5000	0.0294	0.1912	0.0735
Omy325	6	6	0	1.0000	0.0147	0.2038	0.0910
Ots104	7	13	6	0.3333	0.0063	0.2752	0.0661
Ots107	8	9	1	0.7000	0.0063	0.1450	0.0626
OtsG311	9	10	1	0.5833	0.0168	0.2647	0.0704
OtsG68	10	14	4	0.5000	0.0063	0.3571	0.0701
OtsG83b	9	11	2	0.6667	0.0147	0.1702	0.0732
OtsG432	9	7	2	0.7778	0.0104	0.1146	0.0602
Ots13	6	5	1	0.5714	0.0216	0.0769	0.0495
Average proportion of shared alleles =				0.6280			

b)

CH3	No. alleles in females	No. alleles in males	Differential No. Alleles	Proportion of shared alleles	Absolute diff. in freq. in female and male		
					MIN	MAX	MEAN
OtsG253b	8	12	4	0.5385	0.0089	0.1786	0.0522
OtsG249	9	12	3	0.6154	0.0045	0.1875	0.0776
Ots3	6	7	1	0.8571	0.0045	0.0893	0.0485
Ots4	3	4	1	0.7500	0.0357	0.1741	0.0871
Omy325	7	5	2	0.7143	0.0045	0.0982	0.0408
Ots104	11	11	0	0.5714	0.0045	0.2277	0.0491
Ots107	9	8	1	0.7000	0.0000	0.2589	0.0813
OtsG311	9	11	2	0.6667	0.0268	0.1071	0.0580
OtsG68	11	12	1	0.4375	0.0045	0.2232	0.0552
OtsG83b	11	12	1	0.6429	0.0045	0.1473	0.0631
OtsG432	8	9	1	0.7778	0.0000	0.1458	0.0604
Ots13	6	7	1	0.8571	0.0072	0.0865	0.0453
Average proportion of shared alleles =				0.6774			

Chapter 6. Discussion and conclusions

General discussion

The work presented here described genotype-by-environment interactions affecting the immune performance of Chinook salmon. These interactions were induced by altering the breeding system and early rearing environment. These genotype-by-environment interactions should be taken into account when propagating cultured stocks to help expand the aquaculture industry of Chinook salmon in BC.

Humoral immune response, parasite load and disease resistance

A specific humoral immune response was detectable in Chinook salmon injected with *Vibrio anguillarum* bacterin by measuring the antibody response by ELISA, as shown previously in other salmonids (Harrell et al., 1975; Bøgwald et al., 1991). Intraperitoneal injection of *Vibrio* bacterin was confirmed as the route of vaccine administration that produces higher detectable levels of antibody than bath vaccination (Figure 2-2) as has been described in salmonids held in freshwater (Palm et al., 1998). The present study also assessed the antibody response of juvenile Chinook salmon reared in saltwater (Figure 2-4). The humoral immune response of hatchery-bred and channel-bred fish were found to be similar in freshwater as well as in saltwater following injection of *Vibrio* bacterin. Therefore, no differences in mortality would have been predicted based on the antibody response. However, exposure to live *V. anguillarum* revealed that the antibody response was influenced by genotype-by-environment interaction effects (Table 3-3). These interaction effects were also detected at the whole organism level through the disease resistance to vibriosis (Figures 3-2 and 3-3). Moreover, survivors of

the disease challenge showed a general tendency to have higher MH class II β1 heterozygosity than mortalities (Figure 3-6b). This result is in agreement with the hypothesis of Doherty and Zinkernagel (1975) that a higher heterozygosity at these immunological genes that encode pathogen recognition receptors will increase the efficiency of the immune response, as it was shown elsewhere (e.g. Penn et al., 2002; Grimholt et al., 2003; Consuegra and Garcia de Leaniz 2008). Interestingly, no differences in parasite loads were detected during saltwater rearing of juvenile Chinook salmon. Monogeneans infected 96% of both channel-bred and hatchery-bred fish during June and July, whereas microsporidians did not present a high infection rate. The absence of sea lice infection could have been due to the low incidence of this parasite in Chinook salmon farms (Beamish et al., 2005), though a seasonal effect may have contributed to these results as it has been described that sea lice infection is low during summer (Saksida et al., 2007).

Transcriptomic differences following infection with *Vibrio anguillarum*

A strong immune response to infection with *V. anguillarum* was detected using a cDNA microarray developed for Chinook salmon containing 695 genes from a cDNA library developed from liver, brain and muscle tissues. Although not many immune-related genes were present on the microarray, an increase in expression of genes important for limiting bacterial growth was observed. Among those, transcripts for iron-binding proteins such as ferritin and hemoglobin subunits were detected (Rogers et al., 1990). In general, metabolic-related genes in hatchery-bred fish were expressed at lower basal (pre-infection) levels in gill and spleen when reared in the channels, a semi-natural

environment, than when reared in artificial hatchery tanks (Tables 4-5 and 4-8). This suggested that fish maintained a lower metabolism in the semi-natural environment. However after infection, hatchery fish reared in a semi-natural environment presented higher transcriptional levels for COX1 and NAK ATPase than hatchery fish reared in hatchery tanks. This may indicate metabolic differences between fish reared in different environments. Moreover, it is quite possible that hatchery fish reared in artificial tanks suffered higher stress as shown by the higher basal expression of hyperosmotic glycine rich protein (Table 4-5; Pan et al., 2004). Hatchery fish reared in both environments up-regulated hemoglobin genes for iron metabolism in gill tissue (Tables 4-2 and 4-3), which are important for controlling bacterial infection (Weinberg 1990). However, fish reared in the channels again were found with lower basal transcription of these and other immune-related genes in gill (Table 4-5), such as ferritin, ubiquitin and pan-epithelial glycoproteins. However, once exposed to the disease they were able to reach the same expression level as fish reared in hatchery tanks. Therefore, the differences observed in healthy, pre-infected, fish may be related to influences of early-rearing environmental factors upon the development of the immune system. A different scenario was found for gene expression of hemoglobin subunits in spleen tissue, in which no basal transcriptional differences were observed, but differences were found after infection where fish reared in artificial environments had higher transcriptional levels than those reared in the channels.

Mate choice and rearing environmental effects on the immune system of Chinook salmon

The tendency observed in the channel-bred fish of a higher MH class II β1 heterozygosity than hatchery-bred fish may have been the result of semi-natural spawning involving non-random mating (Landry et al., 2001) as opposed to the artificial random mating performed with hatchery fish. Alternatively, it could have been the result of natural selection favouring heterozygotes in the channel fish prior to the experiments. Although both hypotheses seem possible, evidence of MH-based mate choice in Chinook salmon has been found in a previous study in the YIAL spawning channels (Neff et al., 2008). These authors found that female mate choice produced offspring with greater genetic diversity at the MH genes in Chinook salmon as found in this study. Moreover, a comparison of genotype proportions on 12 microsatellite loci in families from two spawning channels did not show evidence of selection, though this was not conclusive as loci subjected directly to selection would be most appropriate to test that hypothesis. However, many other studies have corroborated the effects of mate choice in MH gene diversity (Penn 2002). Reusch and colleagues (2001) suggested that in vertebrate species with multiple MH loci, as is the case in salmonids that went through tetraploidization, females will increase MH heterozygosity among offspring by choosing the males with many alleles.

As mentioned above, channel-bred fish seemed to use mate choice to produce offspring with a canalized disease resistance, i.e. independent of the rearing environment. On the other hand, hatchery-bred fish was severely influenced by the rearing environment as was evidenced by the significant differences in mortalities during the disease

challenge. This demonstrated the existence of genotype-by-environment interaction effects on the immune response of Chinook salmon. Differences in brain development in salmonids have already been demonstrated in relation to different rearing environments (Marchetti and Nevitt 2003; Kihslinger and Nevitt 2006). Therefore, a hypothesis of the existence of environmental effects on the development of the immune system sounds reasonable and may well occur in the early stages of rearing. The finding that disease susceptibility and gene expression are influenced by early-rearing environment is in accordance with previous studies. Hosmer and colleagues (1979) found evidence of water flow and rearing density affecting adult returns of Atlantic salmon. Early-rearing density was shown to affect many physiological variables in Coho salmon (Fagerlund et al., 1981). Stress levels were also affected by rearing environment in studies performed with wild Coho salmon (Salonius and Iwama 1993). Reproductive capacity was also reduced in hatchery-reared Atlantic salmon compared with a natural-like environment (Fleming et al., 1997).

Finally, it is important to note that the hatchery fish at YIAL have been under artificial mating for six generations, but because the founder brood stock were brought from the Robertson Creek Hatchery, a DFO-operated hatchery artificially propagating salmon since 1972 (DFO 2010), the YIAL hatchery fish can be considered to have been artificially mated for at least nine generations. Thus, the effects of mate choice on the disease resistance seen in this thesis after just one round of semi-naturally spawning constitutes an exciting result that deserves further investigation.

Conclusion

Canada has a tremendous potential to continue growing salmonids on the west coast, though currently the industry depends mainly on an exotic species. The native species Coho and Chinook salmon have been cultured at a small-scale in BC for the last 25 years, but lack of knowledge on species-specific aquaculture methods has limited large-scale development. The work presented here made use of the spawning channel technology, which allows the natural process of mate choice, to assess the immune performance of Chinook salmon as an attempt to improve aquaculture conditions of native species. Channel-bred and artificially propagated hatchery-bred fish presented similar antibody responses and parasite load, however differences were found following a disease challenge with live *Vibrio anguillarum*. Hatchery-bred fish were affected by the rearing environment whereas channel-bred fish presented a more stable –canalized– disease resistance which was independent of rearing environments. Thus, genotype-by-environment interaction effects were seen to affect the ability to mount an immune response against a bacterial infection. Moreover, since these effects were seen after just one round of semi-naturally spawning using hatchery fish, it is suggested that studies involving channel-bred fish as broodstock in spawning channels would further highlight the benefits of semi-natural propagation methods. More studies involving spawning channel technology may contribute to the necessary knowledge for a change in paradigm of aquaculture production systems, allowing the expansion of aquaculture of native salmonids in BC.

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Chapter 1

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Appendix

Appendix A1. Results of histological analysis of the first left gill arch collected from juvenile Chinook salmon reared in saltwater between June and October 2007. Group indicates C: channel and H: hatchery origin. Mon.: number of monogeneans counted on hematoxylin and eosin stained samples. Xen.: number of xenomas detected with immunohistochemistry using chicken anti-*Loma salmonae* antibodies.

	Sample#	Group	Mon.	Xen.
June	1	C	38	N/A
	2	C	7	N/A
	3	C	29	N/A
	4	C	5	N/A
	5	C	47	N/A
	6	C	33	N/A
	7	C	44	N/A
	8	C	10	N/A
	9	C	6	N/A
	10	C	47	N/A
	11	C	0	N/A
	12	C	65	N/A
	13	H	5	N/A
	14	H	5	N/A
	15	H	44	N/A
	16	H	15	N/A
	17	H	3	N/A
	18	H	40	N/A
	19	H	15	N/A
	20	H	4	N/A
	21	H	13	N/A
	22	H	37	N/A
	23	H	36	N/A
	24	H	43	N/A

	Sample#	Group	Mon.	Xen.
July	1	C	17	N/A
	2	C	101	N/A
	3	C	57	N/A
	4	C	26	N/A
	5	C	135	12
	6	C	3	N/A
	7	C	9	0
	8	C	54	0
	9	C	47	0
	10	C	1	0
	11	C	30	0
	12	C	15	0
	13	C	51	3
	14	C	41	0
	15	H	6	4
	16	H	2	N/A
	17	H	16	1
	18	H	23	N/A
	19	H	5	0
	20	H	103	0
	21	H	7	0
	22	H	1	0
	23	H	6	0
	24	H	8	0
	25	H	0	0
	26	H	1	0

Appendix A1 cont'd.

	Sample#	Group	Mon.	Xen.
August	1	C	0	0
	2	C	2	0
	3	C	0	0
	4	C	0	0
	5	C	0	0
	6	C	1	0
	7	C	79	0
	8	C	0	0
	9	C	0	0
	10	C	0	0
	11	H	0	0
	12	H	0	0
	13	H	5	0
	14	H	0	0
	15	H	6	0
	16	H	0	0
	17	H	0	0
	18	H	0	0
	19	H	0	0
	20	H	0	0
	21	H	1	0
	22	H	0	0
	23	H	0	94
	24	H	25	0
	25	N/A	1	5
	26	N/A	0	0
	27	N/A	0	0

	Sample#	Group	Mon.	Xen.
October	1	N/A	0	104
	2	N/A	0	10
	3	N/A	0	0
	4	N/A	0	0
	5	N/A	0	0
	6	N/A	7	7
	7	N/A	1	0
	8	N/A	0	0
	9	N/A	0	0
	10	N/A	0	0
	11	N/A	0	0
	12	N/A	0	0
	13	N/A	0	0
	14	N/A	0	0
	15	N/A	0	0
	16	N/A	0	7
	17	N/A	0	0
	18	N/A	10	11
	19	N/A	0	0
	20	N/A	0	15
	21	N/A	0	0
	22	N/A	1	21
	23	N/A	0	0
	24	N/A	0	0
	25	N/A	0	0
	26	N/A	0	0
	27	N/A	0	0
	28	N/A	0	0
	29	N/A	0	0
	30	N/A	0	0

Appendix A2. Output of the SAS software Statistical analyses of ELISA obtained with the software SAS.

The SAS System – Time (weeks) = 0

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	1	66	H/H	H	H	0	0.31827	-1.14487	1
2	1	65	H/CH	H	CH	0	0.48267	-0.72843	2
3	1	76	CH/H	CH	H	0	0.20707	-1.57471	3
4	1	67	CH/CH	CH	CH	0	0.22537	-1.49003	4
5	1	68	H/CH	H	CH	0	0.21753	-1.52540	5
6	1	78	CH/H	CH	H	0	0.28450	-1.25702	6
7	1	71	CH/CH	CH	CH	0	0.30990	-1.17151	7
8	2	75	H/H	H	H	0	0.51403	-0.66547	38
9	2	70	H/CH	H	CH	0	0.20980	-1.56160	39
10	2	79	CH/H	CH	H	0	0.27637	-1.28603	40
11	2	72	CH/CH	CH	CH	0	0.29227	-1.23009	41
12	2	77	H/H	H	H	0	0.31810	-1.14539	42
13	2	73	H/CH	H	CH	0	0.36393	-1.01078	43
14	2	80	CH/H	CH	H	0	0.19347	-1.64265	44
15	2	74	CH/CH	CH	CH	0	0.37007	-0.99407	45

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	2	1 2
Breed	2	CH H
Env	2	CH H

Number of Observations Read	15
Number of Observations Used	15

Appendix A2 cont'd.

The SAS System – Time (weeks) = 0

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 1.7778 Var(PLATE*Breed*Env) + 7.1111 Var(PLATE)
Breed	Var(Error) + 1.7778 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 1.7778 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 1.7778 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 1.8182 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	1	0.013931	0.013931	0.38	0.5775
* Breed	1	0.164222	0.164222	4.44	0.1140
* Env	1	0.001235	0.001235	0.03	0.8652
Breed*Env	1	0.142284	0.142284	3.85	0.1328
Error	3.4369	0.127026	0.036959		

Error: 0.9778*MS(PLATE*Breed*Env) + 0.0222*MS(Error)
* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	3	0.105828	0.035276	0.32	0.8125
Error: MS(Error)	7	0.777080	0.111011		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 0.57

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	1	99	H/H	H	H	0.57	0.24800	-1.39433	8
2	1	104	H/CH	H	CH	0.57	0.56923	-0.56346	9
3	1	93	CH/H	CH	H	0.57	0.30237	-1.19611	10
4	1	94	CH/CH	CH	CH	0.57	0.66250	-0.41173	11
5	1	103	H/H	H	H	0.57	0.35047	-1.04849	12
6	1	105	H/CH	H	CH	0.57	0.47277	-0.74915	13
7	1	95	CH/H	CH	H	0.57	0.43843	-0.82455	14
8	1	96	CH/CH	CH	CH	0.57	0.57237	-0.55798	15
9	2	106	H/H	H	H	0.57	0.27907	-1.27630	32
10	2	108	H/CH	H	CH	0.57	0.42783	-0.84902	33
11	2	97	CH/H	CH	H	0.57	0.29603	-1.21728	34
12	2	98	CH/CH	CH	CH	0.57	0.35743	-1.02881	35
13	2	107	H/H	H	H	0.57	0.26850	-1.31490	36
14	2	101	CH/H	CH	H	0.57	0.33223	-1.10192	37

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	2	1 2
Breed	2	CH H
Env	2	CH H

Number of Observations Read	14
Number of Observations Used	14

Appendix A2 cont'd.

The SAS System – Time (weeks) = 0.57

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 1.6 Var(PLATE*Breed*Env) + 6.4 Var(PLATE)
Breed	Var(Error) + 1.6 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 1.6 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 1.6 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 1.6444 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	1	0.184370	0.184370	5.75	0.0922
* Breed	1	0.022950	0.022950	0.72	0.4572
* Env	1	0.556413	0.556413	17.35	0.0230
Breed*Env	1	0.025264	0.025264	0.79	0.4375
Error	3.1424	0.100790	0.032074		

Error: 0.973*MS(PLATE*Breed*Env) + 0.027*MS(Error)
* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	3	0.096615	0.032205	1.18	0.3940
Error: MS(Error)	6	0.164166	0.027361		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 3

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	1	243	H/H	H	H	3	0.30810	-1.17733	16
2	1	242	H/CH	H	CH	3	0.69593	-0.36250	17
3	1	254	CH/H	CH	H	3	0.60880	-0.49627	18
4	1	245	CH/CH	CH	CH	3	0.49170	-0.70989	19
5	1	244	H/H	H	H	3	0.44930	-0.80006	20
6	1	246	H/CH	H	CH	3	0.37517	-0.98038	21
7	1	255	CH/H	CH	H	3	0.69620	-0.36212	22
8	1	248	CH/CH	CH	CH	3	0.29077	-1.23523	23
9	2	247	H/H	H	H	3	0.67197	-0.39755	52
10	2	251	H/CH	H	CH	3	0.35010	-1.04954	53
11	2	256	CH/H	CH	H	3	0.60890	-0.49610	54
12	2	250	CH/CH	CH	CH	3	0.33747	-1.08629	55
13	2	249	H/H	H	H	3	0.49913	-0.69488	56
14	2	253	H/CH	H	CH	3	0.46760	-0.76014	57
15	2	257	CH/H	CH	H	3	0.66633	-0.40597	58
16	2	252	CH/CH	CH	CH	3	0.29407	-1.22395	59
17	3	259	H/H	H	H	3	0.20840	-1.56830	62
18	3	262	H/CH	H	CH	3	0.18113	-1.70852	63
19	3	258	CH/H	CH	H	3	0.19417	-1.63904	64
20	3	261	CH/CH	CH	CH	3	0.18330	-1.69663	65
21	3	260	H/H	H	H	3	0.17833	-1.72410	66
22	3	266	H/CH	H	CH	3	0.39777	-0.92189	67
23	3	265	CH/H	CH	H	3	0.44487	-0.80998	68
24	3	268	CH/CH	CH	CH	3	0.17773	-1.72747	69
25	4	263	H/H	H	H	3	0.51107	-0.67126	93
26	4	267	H/CH	H	CH	3	0.17803	-1.72578	94
27	4	272	CH/H	CH	H	3	0.18880	-1.66707	95
28	4	270	CH/CH	CH	CH	3	0.20473	-1.58605	96
29	4	264	H/H	H	H	3	0.20533	-1.58312	97
30	4	269	H/CH	H	CH	3	0.20073	-1.60578	98
31	4	273	CH/H	CH	H	3	0.21313	-1.54584	99
32	4	271	CH/CH	CH	CH	3	0.24320	-1.41387	100

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	4	1 2 3 4
Breed	2	CH H
Env	2	CH H

Number of Observations Read	32
Number of Observations Used	32

Appendix A2 cont'd.

The SAS System – Time (weeks) = 3

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 2 Var(PLATE*Breed*Env) + 8 Var(PLATE)
Breed	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	3	4.030317	1.343439	11.19	0.0022
* Breed	1	0.004292	0.004292	0.04	0.8543
* Env	1	0.440613	0.440613	3.67	0.0877
Breed*Env	1	0.237888	0.237888	1.98	0.1929
Error	9	1.080972	0.120108		

Error: MS(PLATE*Breed*Env)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	9	1.080972	0.120108	1.19	0.3661
Error: MS(Error)	16	1.619461	0.101216		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 4

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	1	391	H/H	H	H	4	0.21817	-1.52250	24
2	1	397	H/CH	H	CH	4	0.97923	-0.02099	25
3	1	396	CH/H	CH	H	4	0.45140	-0.79540	26
4	1	390	CH/CH	CH	CH	4	0.39243	-0.93539	27
5	1	392	H/H	H	H	4	0.27207	-1.30171	28
6	1	398	H/CH	H	CH	4	0.23463	-1.44973	29
7	2	393	H/H	H	H	4	0.28657	-1.24978	46
8	2	399	H/CH	H	CH	4	0.61180	-0.49135	47
9	2	401	CH/H	CH	H	4	0.55830	-0.58286	48
10	2	395	CH/CH	CH	CH	4	0.54953	-0.59869	49
11	2	402	CH/H	CH	H	4	0.37777	-0.97348	50
12	2	400	CH/CH	CH	CH	4	0.41743	-0.87363	51
13	3	394	H/H	H	H	4	0.24533	-1.40514	80
14	3	405	H/CH	H	CH	4	0.39217	-0.93607	81
15	3	403	CH/H	CH	H	4	0.33963	-1.07989	82
16	3	404	CH/CH	CH	CH	4	0.25853	-1.35273	83
17	3	406	H/H	H	H	4	0.19530	--1.63322	84
18	3	411	H/CH	H	CH	4	0.44813	-0.80266	85
19	4	407	H/H	H	H	4	0.20407	-1.58931	101
20	4	413	H/CH	H	CH	4	0.29240	-1.22963	102
21	4	409	CH/H	CH	H	4	0.21833	-1.52173	103
22	4	408	CH/CH	CH	CH	4	0.19133	-1.65374	104
23	4	418	CH/H	CH	H	4	0.17500	-1.74297	105
24	4	412	CH/CH	CH	CH	4	0.18863	-1.66795	106
25	5	410	H/H	H	H	4	0.31633	-1.15096	115
26	5	415	H/CH	H	CH	4	0.21697	-1.52801	116
27	5	420	CH/H	CH	H	4	0.42610	-0.85308	117
28	5	416	CH/CH	CH	CH	4	0.22570	-1.48855	118
29	5	414	H/H	H	H	4	0.18680	-1.67772	119
30	5	417	H/CH	H	CH	4	0.33117	-1.10513	120
31	5	421	CH/H	CH	H	4	0.35510	-1.03536	121
32	5	419	CH/CH	CH	CH	4	0.23280	-1.45758	122

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	5	1 2 3 4 5
Breed	2	CH H
Env	2	CH H

Number of Observations Read	32
Number of Observations Used	32

Appendix A2 cont'd.

The SAS System – Time (weeks) = 4

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 1.4545 Var(PLATE*Breed*Env) + 5.8182 Var(PLATE)
Breed	Var(Error) + 1.4286 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 1.4286 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 1.4286 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 1.5265 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	4	1.690473	0.422618	7.51	0.0017
Error	14.698	0.826821	0.056253		

Error: 0.9529*MS(PLATE*Breed*Env) + 0.0471*MS(Error)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
* Breed	1	0.013752	0.013752	0.24	0.6314
* Env	1	0.186135	0.186135	3.24	0.0911
Breed*Env	1	0.860355	0.860355	14.98	0.0014
Error	15.695	0.901329	0.057427		

Error: 0.9358*MS(PLATE*Breed*Env) + 0.0642*MS(Error)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	12	0.636011	0.053001	0.43	0.9185
Error: MS(Error)	12	1.463845	0.121987		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 4.42

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	5	444	H/H	H	H	4.42	0.16623	-1.79436	123
2	5	451	H/CH	H	CH	4.42	0.39680	-0.92432	124
3	5	442	CH/H	CH	H	4.42	0.19903	-1.61428	125
4	5	443	CH/CH	CH	CH	4.42	0.18973	-1.66214	126
5	5	446	H/H	H	H	4.42	0.39540	-0.92786	127
6	5	452	H/CH	H	CH	4.42	0.30600	-1.18417	128
7	5	445	CH/H	CH	H	4.42	0.64513	-0.43830	129
8	5	455	CH/CH	CH	CH	4.42	0.37137	-0.99057	130
9	6	449	H/H	H	H	4.42	0.23530	-1.44689	147
10	6	453	H/CH	H	CH	4.42	0.40357	-0.90741	148
11	6	447	CH/H	CH	H	4.42	0.22833	-1.47695	149
12	6	456	CH/CH	CH	CH	4.42	0.36977	-0.99488	150
13	6	450	H/H	H	H	4.42	0.18693	-1.67700	151
14	6	454	H/CH	H	CH	4.42	0.24440	-1.40895	152
15	6	448	CH/H	CH	H	4.42	0.21180	-1.55211	153
16	6	457	CH/CH	CH	CH	4.42	0.24620	-1.40161	154
17	7	458	H/H	H	H	4.42	0.23537	-1.44661	195
18	7	459	H/CH	H	CH	4.42	0.20473	-1.58605	196
19	7	465	CH/H	CH	H	4.42	0.19293	-1.64541	197
20	7	463	CH/CH	CH	CH	4.42	0.21660	-1.52970	198
21	7	461	H/H	H	H	4.42	0.19010	-1.66021	199
22	7	460	H/CH	H	CH	4.42	0.36257	-1.01455	200
23	7	467	CH/H	CH	H	4.42	0.36890	-0.99723	201
24	7	464	CH/CH	CH	CH	4.42	0.23313	-1.45614	202
25	8	469	H/H	H	H	4.42	0.48007	-0.73383	227
26	8	462	H/CH	H	CH	4.42	0.40260	-0.90981	228
27	8	471	CH/H	CH	H	4.42	0.22563	-1.48884	229
28	8	468	CH/CH	CH	CH	4.42	0.28670	-1.24932	230
29	8	470	H/H	H	H	4.42	0.22023	-1.51307	231
30	8	466	H/CH	H	CH	4.42	0.18680	-1.67772	232
31	8	472	CH/H	CH	H	4.42	0.27797	-1.28025	233
32	8	473	CH/CH	CH	CH	4.42	0.21100	-1.55590	234

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	4	5 6 7 8
Breed	2	CH H
Env	2	CH H

Number of Observations Read	32
Number of Observations Used	32

Appendix A2 cont'd.

The SAS System – Time (weeks) = 4.42

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 2 Var(PLATE*Breed*Env) + 8 Var(PLATE)
Breed	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	3	0.220602	0.073534	1.41	0.3013
* Breed	1	0.008477	0.008477	0.16	0.6958
* Env	1	0.048048	0.048048	0.92	0.3615
Breed*Env	1	0.116854	0.116854	2.25	0.1681
Error	9	0.467934	0.051993		

Error: MS(PLATE*Breed*Env)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	9	0.467934	0.051993	0.32	0.9577
Error: MS(Error)	16	2.630019	0.164376		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 5

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	5	497	H/H	H	H	5	0.34093	-1.07607	131
2	5	494	H/CH	H	CH	5	0.40663	-0.89984	132
3	5	501	CH/H	CH	H	5	0.18633	-1.68022	133
4	5	500	CH/CH	CH	CH	5	0.74290	-0.29719	134
5	5	499	H/H	H	H	5	0.24797	-1.39446	135
6	5	495	H/CH	H	CH	5	0.26270	-1.33674	136
7	5	502	CH/H	CH	H	5	0.53377	-0.62780	137
8	5	505	CH/CH	CH	CH	5	0.25020	-1.38549	138
9	6	507	H/H	H	H	5	0.45760	-0.78176	155
10	6	496	H/CH	H	CH	5	0.17757	-1.72841	156
11	6	503	CH/H	CH	H	5	0.35977	-1.02230	157
12	6	506	CH/CH	CH	CH	5	0.48813	-0.71717	158
13	6	509	H/H	H	H	5	0.19407	-1.63955	159
14	6	498	H/CH	H	CH	5	0.60647	-0.50011	160
15	6	504	CH/H	CH	H	5	0.23760	-1.43717	161
16	6	508	CH/CH	CH	CH	5	0.17260	-1.75678	162
17	7	514	H/H	H	H	5	0.23167	-1.46246	179
18	7	516	H/CH	H	CH	5	0.22917	-1.47331	180
19	7	519	CH/H	CH	H	5	0.19120	-1.65444	181
20	7	510	CH/CH	CH	CH	5	0.22517	-1.49091	182
21	7	515	H/H	H	H	5	0.46720	-0.76100	183
22	7	517	H/CH	H	CH	5	0.22247	-1.50298	184
23	7	524	CH/H	CH	H	5	0.26053	-1.34502	185
24	7	511	CH/CH	CH	CH	5	0.30377	-1.19150	186
25	8	518	H/H	H	H	5	0.20490	-1.58523	219
26	8	521	H/CH	H	CH	5	0.20997	-1.56081	220
27	8	525	CH/H	CH	H	5	0.18957	-1.66301	221
28	8	512	CH/CH	CH	CH	5	0.18850	-1.66866	222
29	8	520	H/H	H	H	5	0.18050	-1.71202	223
30	8	522	H/CH	H	CH	5	0.53440	-0.62661	224
31	8	526	CH/H	CH	H	5	0.61473	-0.48657	225
32	8	513	CH/CH	CH	CH	5	0.44293	-0.81434	226

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	4	5 6 7 8
Breed	2	CH H
Env	2	CH H

Number of Observations Read	32
Number of Observations Used	32

Appendix A2 cont'd.

The SAS System – Time (weeks) = 5

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 2 Var(PLATE*Breed*Env) + 8 Var(PLATE)
Breed	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	3	0.316339	0.105446	1.29	0.3366
* Breed	1	0.020140	0.020140	0.25	0.6318
* Env	1	0.059361	0.059361	0.73	0.4165
Breed*Env	1	0.001119	0.001119	0.01	0.9095
Error	9	0.736736	0.081860		

Error: MS(PLATE*Breed*Env)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	9	0.736736	0.081860	0.27	0.9746
Error: MS(Error)	16	4.881347	0.305084		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 5.57

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	5	543	H/H	H	H	5.57	0.43453	-0.83348	139
2	5	544	H/CH	H	CH	5.57	0.35143	-1.04574	140
3	5	545	CH/H	CH	H	5.57	0.33397	-1.09671	141
4	5	551	CH/CH	CH	CH	5.57	0.29170	-1.23203	142
5	5	546	H/H	H	H	5.57	0.37660	-0.97657	143
6	5	548	H/CH	H	CH	5.57	0.48667	-0.72018	144
7	6	547	CH/H	CH	H	5.57	0.33870	-1.08264	171
8	6	553	CH/CH	CH	CH	5.57	0.18803	-1.67114	172
9	6	549	H/H	H	H	5.57	0.52510	-0.64417	173
10	6	552	H/CH	H	CH	5.57	0.45473	-0.78804	174
11	6	550	CH/H	CH	H	5.57	0.39087	-0.93939	175
12	6	554	CH/CH	CH	CH	5.57	0.41997	-0.86758	176

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	2	5 6
Breed	2	CH H
Env	2	CH H

Number of Observations Read	12
Number of Observations Used	12

Appendix A2 cont'd.

The SAS System – Time (weeks) = 5.57

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 1.3333 Var(PLATE*Breed*Env) + 5.3333 Var(PLATE)
Breed	Var(Error) + 1.3333 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 1.3333 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 1.3333 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 1.3333 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	1	0.027222	0.027222	2.72	0.1974
* Breed	1	0.321518	0.321518	32.18	0.0109
* Env	1	0.044284	0.044284	4.43	0.1259
Breed*Env	1	0.012317	0.012317	1.23	0.3479
Error	3	0.029974	0.009991		

Error: MS(PLATE*Breed*Env)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	3	0.029974	0.009991	0.10	0.9554
Error: MS(Error)	4	0.396343	0.099086		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 6

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	6	582	H/H	H	H	6	0.32570	-1.12178	163
2	6	579	H/CH	H	CH	6	0.24090	-1.42337	164
3	6	578	CH/H	CH	H	6	0.25157	-1.38005	165
4	6	581	CH/CH	CH	CH	6	0.69143	-0.36899	166
5	6	586	H/H	H	H	6	0.47227	-0.75021	167
6	6	580	H/CH	H	CH	6	0.28750	-1.24653	168
7	6	583	CH/H	CH	H	6	0.18347	-1.69572	169
8	6	585	CH/CH	CH	CH	6	0.44697	-0.80527	170
9	7	588	H/H	H	H	6	0.45187	-0.79437	187
10	7	589	H/CH	H	CH	6	0.50633	-0.68056	188
11	7	584	CH/H	CH	H	6	0.27673	-1.28470	189
12	7	594	CH/CH	CH	CH	6	0.59293	-0.52267	190
13	7	592	H/H	H	H	6	0.50530	-0.68260	191
14	7	591	H/CH	H	CH	6	0.49743	-0.69829	192
15	7	587	CH/H	CH	H	6	0.21467	-1.53867	193
16	7	595	CH/CH	CH	CH	6	0.58563	-0.53506	194
17	8	599	H/H	H	H	6	0.38393	-0.95729	211
18	8	597	H/CH	H	CH	6	0.44323	-0.81366	212
19	8	590	CH/H	CH	H	6	0.19160	-1.65235	213
20	8	596	CH/CH	CH	CH	6	0.50503	-0.68313	214
21	8	600	H/H	H	H	6	0.26233	-1.33814	215
22	8	601	H/CH	H	CH	6	0.19433	-1.63818	216
23	8	593	CH/H	CH	H	6	0.28697	-1.24839	217
24	8	598	CH/CH	CH	CH	6	0.35137	-1.04592	218

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	3	6 7 8
Breed	2	CH H
Env	2	CH H

Number of Observations Read	24
Number of Observations Used	24

Appendix A2 cont'd.

The SAS System – Time (weeks) = 6

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 2 Var(PLATE*Breed*Env) + 8 Var(PLATE)
Breed	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 2 Var(PLATE*Breed*Env)

The SAS System

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	2	0.480652	0.240326	4.65	0.0602
* Breed	1	0.015808	0.015808	0.31	0.6001
* Env	1	0.660883	0.660883	12.80	0.0117
Breed*Env	1	1.351393	1.351393	26.17	0.0022
Error	6	0.309889	0.051648		

Error: MS(PLATE*Breed*Env)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	6	0.309889	0.051648	0.75	0.6224
Error: MS(Error)	12	0.828236	0.069020		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 30

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	7	1	H/H	H	H	30	0.46177	-0.77270	203
2	7	10	H/CH	H	CH	30	0.72407	-0.32287	204
3	7	3	CH/H	CH	H	30	0.40627	-0.90075	205
4	7	2	CH/CH	CH	CH	30	0.42730	-0.85027	206
5	7	11	H/H	H	H	30	0.63390	-0.45586	207
6	7	13	H/CH	H	CH	30	0.46950	-0.75609	208
7	8	12	H/H	H	H	30	0.44757	-0.80393	235
8	8	15	H/CH	H	CH	30	0.27460	-1.29244	236
9	8	4	CH/H	CH	H	30	0.34707	-1.05824	237
10	8	7	CH/CH	CH	CH	30	0.86243	-0.14800	238
11	8	5	CH/H	CH	H	30	0.46593	-0.76371	239
12	8	8	CH/CH	CH	CH	30	0.48063	-0.73265	240
13	9	18	H/H	H	H	30	0.21873	-1.51990	243
14	9	17	H/CH	H	CH	30	0.37757	-0.97401	244
15	9	6	CH/H	CH	H	30	0.29047	-1.23627	245
16	9	9	CH/CH	CH	CH	30	0.39657	-0.92491	246
17	9	19	H/H	H	H	30	0.42220	-0.86228	247
18	9	26	H/CH	H	CH	30	0.36043	-1.02045	248
19	9	16	CH/H	CH	H	30	0.42967	-0.84475	249
20	9	14	CH/CH	CH	CH	30	0.32443	-1.12568	250
21	9	22	H/H	H	H	30	0.24690	-1.39877	266
22	9	31	H/CH	H	CH	30	0.30200	-1.19733	267
23	9	20	CH/H	CH	H	30	0.30633	-1.18308	268
24	9	21	CH/CH	CH	CH	30	0.37157	-0.99003	269
25	9	34	H/H	H	H	30	0.23743	-1.43787	270
26	9	33	H/CH	H	CH	30	0.27473	-1.29195	271
27	10	36	H/H	H	H	30	0.44340	-0.81328	290
28	10	46	H/CH	H	CH	30	0.33243	-1.10132	291
29	10	23	CH/H	CH	H	30	0.26170	-1.34056	292
30	10	27	CH/CH	CH	CH	30	0.21213	-1.55054	293
31	10	41	H/H	H	H	30	0.33873	-1.08254	294
32	10	48	H/CH	H	CH	30	0.26047	-1.34528	295
33	10	24	CH/H	CH	H	30	0.28097	-1.26952	296
34	10	28	CH/CH	CH	CH	30	0.34410	-1.06682	297
35	10	25	CH/H	CH	H	30	0.21497	-1.53727	301
36	10	29	CH/CH	CH	CH	30	0.34227	-1.07217	302

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	4	7 8 9 10
Breed	2	CH H
Env	2	CH H

Number of Observations Read 36
Number of Observations Used 36

Appendix A2 cont'd.

```
The SAS System – Time (weeks) = 30

                                The GLM Procedure

Source           Type III Expected Mean Square

PLATE            Var(Error) + 2.005 Var(PLATE*Breed*Env) + 8.0199 Var(PLATE)

Breed            Var(Error) + 1.8113 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)

Env              Var(Error) + 1.8113 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)

Breed*Env        Var(Error) + 1.8113 Var(PLATE*Breed*Env) + Q(Breed*Env)

PLATE*Breed*Env  Var(Error) + 2.0757 Var(PLATE*Breed*Env)



                                The GLM Procedure

            Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE   LNVALUE

Source           DF     Type III SS     Mean Square    F Value    Pr > F

PLATE             3        1.042656        0.347552       3.59    0.0576

Error        9.3448        0.905889        0.096941
Error: 0.9659*MS(PLATE*Breed*Env) + 0.0341*MS(Error)


Source           DF     Type III SS     Mean Square    F Value    Pr > F

*    Breed        1        0.000107        0.000107       0.00    0.9735
*    Env          1        0.006679        0.006679       0.07    0.7936
Breed*Env         1        0.177894        0.177894       1.92    0.1949

Error       10.446        0.968464        0.092714
Error: 0.8726*MS(PLATE*Breed*Env) + 0.1274*MS(Error)
* This test assumes one or more other fixed effects are zero.


Source           DF     Type III SS     Mean Square    F Value    Pr > F

PLATE*Breed*Env   9        0.886366        0.098485       1.85    0.1204

Error: MS(Error) 20        1.063665        0.053183
```

Appendix A2 cont'd.

The SAS System – Time (weeks) = 50

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	9	3	H/H	H	H	50	0.43487	-0.83272	251
2	9	8	H/CH	H	CH	50	0.34020	-1.07822	252
3	9	7	CH/H	CH	H	50	0.41500	-0.87948	253
4	9	1	CH/CH	CH	CH	50	0.71640	-0.33352	254
5	9	9	H/CH	H	CH	50	0.31273	-1.16240	255
6	9	14	CH/H	CH	H	50	0.32860	-1.11291	256
7	9	2	CH/CH	CH	CH	50	0.25300	-1.37437	257
8	10	16	H/H	H	H	50	0.37773	-0.97357	274
9	10	10	H/CH	H	CH	50	0.26173	-1.34043	275
10	10	17	CH/H	CH	H	50	0.45883	-0.77907	276
11	10	4	CH/CH	CH	CH	50	0.24643	-1.40066	277
12	10	18	H/H	H	H	50	0.30773	-1.17852	278
13	10	13	H/CH	H	CH	50	0.23587	-1.44449	279
14	10	28	CH/H	CH	H	50	0.26150	-1.34132	280
15	10	5	CH/CH	CH	CH	50	0.27723	-1.28290	281
16	11	22	H/H	H	H	50	0.33243	-1.10132	305
17	11	15	H/CH	H	CH	50	0.27730	-1.28266	306
18	11	32	CH/H	CH	H	50	0.29360	-1.22554	307
19	11	6	CH/CH	CH	CH	50	0.63623	-0.45219	308
20	11	26	H/H	H	H	50	0.25730	-1.35751	309
21	11	20	H/CH	H	CH	50	0.21700	-1.52786	310
22	11	33	CH/H	CH	H	50	0.32447	-1.12557	311
23	11	12	CH/CH	CH	CH	50	0.48230	-0.72919	312
24	11	27	H/H	H	H	50	0.47683	-0.74059	320
25	11	21	H/CH	H	CH	50	0.53327	-0.62873	321
26	11	25	H/CH	H	CH	50	0.41347	-0.88318	322
27	11	19	CH/CH	CH	CH	50	0.25137	-1.38084	323
28	11	29	H/H	H	H	50	0.19367	-1.64162	324
29	11	23	H/CH	H	CH	50	0.35123	-1.04630	325
30	11	30	H/H	H	H	50	0.34717	-1.05795	326
31	11	24	CH/CH	CH	CH	50	0.28900	-1.24133	327

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	3	9 10 11
Breed	2	CH H
Env	2	CH H

Number of Observations Read	31
Number of Observations Used	31

Appendix A2 cont'd.

The SAS System – Time (weeks) = 50

The GLM Procedure

Source	Type III Expected Mean Square
PLATE	Var(Error) + 2.2211 Var(PLATE*Breed*Env) + 8.8845 Var(PLATE)
Breed	Var(Error) + 2.1239 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)
Env	Var(Error) + 2.1239 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)
Breed*Env	Var(Error) + 2.1239 Var(PLATE*Breed*Env) + Q(Breed*Env)
PLATE*Breed*Env	Var(Error) + 2.2944 Var(PLATE*Breed*Env)

The GLM Procedure

Tests of Hypotheses for Mixed Model Analysis of Variance

Dependent Variable: LNVALUE LNVALUE

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE	2	0.253261	0.126630	2.20	0.1828
Error	6.8744	0.396073	0.057615		

Error: 0.9681*MS(PLATE*Breed*Env) + 0.0319*MS(Error)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
* Breed	1	0.015562	0.015562	0.26	0.6250
* Env	1	0.030134	0.030134	0.50	0.4994
Breed*Env	1	0.060219	0.060219	1.00	0.3464
Error	8.1833	0.493826	0.060346		

Error: 0.9257*MS(PLATE*Breed*Env) + 0.0743*MS(Error)

* This test assumes one or more other fixed effects are zero.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLATE*Breed*Env	6	0.333346	0.055558	0.46	0.8269
Error: MS(Error)	19	2.279689	0.119984		

Appendix A2 cont'd.

The SAS System – Time (weeks) = 96

Obs	PLATE	FISH	GROUP	Breed	Env	TIMEWEEKS	VALUE	LNVALUE	case
1	9	1	CH/CH	CH	CH	96	0.22817	-1.47768	258
2	9	2	CH/CH	CH	CH	96	0.69910	-0.35796	259
3	9	3	H/CH	H	CH	96	0.29567	-1.21852	260
4	9	4	H/H	H	H	96	0.32697	-1.11790	261
5	9	6	CH/CH	CH	CH	96	0.31000	-1.17118	262
6	9	7	CH/CH	CH	CH	96	0.24103	-1.42282	263
7	9	5	H/CH	H	CH	96	0.67213	-0.39730	264
8	9	12	H/H	H	H	96	0.84140	-0.17269	265
9	10	11	CH/CH	CH	CH	96	0.31203	-1.16465	282
10	10	13	CH/CH	CH	CH	96	0.97747	-0.02279	283
11	10	15	H/CH	H	CH	96	0.45297	-0.79194	284
12	10	14	H/H	H	H	96	0.29640	-1.21605	285
13	10	8	CH/CH	CH	CH	96	0.38263	-0.96068	286
14	10	9	CH/CH	CH	CH	96	0.55967	-0.58041	287
15	10	10	CH/CH	CH	CH	96	0.63470	-0.45460	288
16	10	17	H/CH	H	CH	96	0.45500	-0.78746	289
17	10	16	CH/CH	CH	CH	96	0.35913	-1.02406	298
18	10	18	H/CH	H	CH	96	0.54910	-0.59947	299
19	10	19	CH/CH	CH	CH	96	0.63373	-0.45613	300

The GLM Procedure

Class Level Information

Class	Levels	Values
PLATE	2	9 10
Breed	2	CH H
Env	2	CH H

Number of Observations Read	19
Number of Observations Used	19

Appendix A2 cont'd.

```
The SAS System – Time (weeks) = 96

                                The GLM Procedure

Source           Type III Expected Mean Square

PLATE            Var(Error) + 2.2009 Var(PLATE*Breed*Env) + 6.6026 Var(PLATE)

Breed            Var(Error) + 3.2621 Var(PLATE*Breed*Env) + Q(Breed,Breed*Env)

Env              Var(Error) + 1.7143 Var(PLATE*Breed*Env) + Q(Env,Breed*Env)

Breed*Env        0

PLATE*Breed*Env  Var(Error) + 2.5165 Var(PLATE*Breed*Env)
```

Appendix A3. List of the transcripts included in the microarray analysis. There were a total of 695 transcripts spotted in the microarray slides, of which 465 have been found similar to sequences in gene repositories. A total of 230 transcripts did not match to known sequences and are listed as unknowns. ID: indicates transcript cDNA library code from Dr. Heath's lab.

ID	Transcript name
MT_EE1a7	antiquitin [Acanthopagrus schlegelii]
MT_EE1b8	PREDICTED: hypothetical protein XP_677980 [Danio rerio]
MT_EE1b10	hypothetical protein PFF0720w [Plasmodium falciparum 3D7]
MT_EE1c4	Zn-dependent alcohol dehydrogenases [Brevibacterium linens BL2]
MT_EE1c7	microsomal glutathione S-transferase [Oreochromis mossambicus]
MT_EE1d5	U88 [Human herpesvirus 6]
MT_EE1e8	Calrl protein [Danio rerio]
MT_EE1e12	alpha-1-microglobulin/bikunin precursor [Oncorhynchus mykiss]
MT_EE1f4	LamG-like jellyroll fold [Burkholderia cenocepacia AU 1054]
MT_EE1f8	clone 2B3 [Homo sapiens]
MT_EE2a3	Transducin-like enhancer of split 1 [Mus musculus]
MT_EE2a5	Hypothetical protein CBG05643 [Caenorhabditis briggsae]
MT_EE2a10	sim to complement factor H isoform a precursor 1 [Macaca mulatta]
MT_EE2b3	unnamed protein product [Tetraodon nigroviridis]
MT_EE2b9	cyclic nucl-bind dom containing protein [Tetrahymena thermophila]
MT_EE2b10	hypothetical protein [Dictyostelium discoideum AX4]
MT_EE2d3	PREDICTED: hypothetical protein [Danio rerio].
MT_EE2e1	unnamed protein product [Tetraodon nigroviridis]
MT_EE2e7	unnamed protein product [Tetraodon nigroviridis]
MT_EE2f5	orotidine 5'-phosphate decarboxylase [Psychroflexus torquis]
MT_EE2f7	cytochrome c oxidase subunit I [Pelecus cultratus].
MT_EE2g8	putative ribosomal protein S8 [Oncorhynchus mykiss].
MT_EE2g9	LDL receptor 2 precursor - African clawed frog
MT_EE2g11	Zgc:64114 [Danio rerio] hypothetical protein LOC378866
MT_EE2h2	Zgc:114044 [Danio rerio] | hypothetical protein LOC564370
MT_EE2h9	unnamed protein product [Tetraodon nigroviridis]
MT_EE2h11	ribosomal protein L36 [Ictalurus punctatus]
MT_EE3a2	hypothetical protein [Tetrahymena thermophila SB210]
MT_EE3a3	unnamed protein product [Tetraodon nigroviridis]
MT_EE3a5	novel ankyrin repeat containing protein [Mus musculus]
MT_EE3a6	(protein for MGC:151926) [Bos taurus]
MT_EE3a8	unnamed protein product [Tetraodon nigroviridis]
MT_EE3a9	sim to Ig kappa chain V-IV region S107B precursor [Mus musculus]
MT_EE3a10	RNase 1 [Danio rerio].
MT_EE3a11	Proteasome 26S subunit, ATPase, 1a [Danio rerio]
MT_EE3a12	PREDICTED: similar to Syncollin [Danio rerio]
MT_EE3b2	phosphoglucomutase 3 [Danio rerio]
MT_EE3b3	phosphate ABC transporter [Geobacillus kaustophilus]
MT_EE3b4	nephrosin [Plecoglossus altivelis altivelis]

Appendix A3 cont'd.

ID	Transcript name
MT_EE3c5	phosphoglucose isomerase [Mugil cephalus]
MT_EE3c9	NS5 protein [Spondweni virus]
MT_EE3c10	PREDICTED: similar to Hornerin [Homo sapiens]
MT_EE3d5	hypothetical protein MapoCp023 [Marchantia polymorpha]
MT_EE3e2	putative polyprotein [Oryza sativa (japonica cultivar-group)]
MT_EE3e3	sim to BTB/POZ domain-containing protein 9 [Apis mellifera]
MT_EE3e5	ubiquitous gelsolin; U-gelsolin [Danio rerio]
MT_EE3e8	Zgc:103738 protein [Danio rerio]
MT_EE3g5	6-pyruvoyl-tetrahydropterin synth of hepat nuclear factor 1 [Danio rerio]
MT_EE3g7	transferrin [Oncorhynchus mykiss]
MT_EE3g8	hemagglutinin [Influenza A virus (A/equine/Santiago/77(H7N7))]
MT_EE3g9	hypothetical protein VAS14_03503 [Vibrio angustum S14]
MT_EE3g12	putative NADH dehydrogenase I chain A [Thermofilum pendens Hrk 5].
MT_EE3h4	immunoglobulin light chain L2 [Oncorhynchus mykiss]
MT_EE3h6	unnamed protein product [Tetraodon nigroviridis]
MT_EE3h8	PREDICTED: similar to Protein Wnt-8a precursor [Rattus norvegicus]
MT_EE3h9	phosphatidylethanolamine binding protein [Danio rerio].
MT_EE4a1	Elastase-1.
MT_EE4a6	Tm4sf3-prov protein [Xenopus laevis]
MT_EE4b4	Zgc:153186 [Danio rerio] hypothetical protein LOC751758
MT_EE4b6	unnamed protein product [Aspergillus oryzae]
MT_EE4b7	serine protease-like protein precursor [Salvelinus fontinalis]
MT_EE4b10	integral membrane protein 2B, like [Danio rerio].
MT_EE4b11	hypothetical protein LOC553723 [Danio rerio]
MT_EE4c6	Integral membrane protein 2B, like [Danio rerio]
MT_EE4c7	hypothetical protein VAS14_12969 [Vibrio angustum S14]
MT_EE4d1	ferritin heavy subunit; ferritin H [Salmo salar]
MT_EE4d2	chemokine (C-C motif) ligand 13 [Canis familiaris]
MT_EE4d5	complement factor H1 protein [Oncorhynchus mykiss]
MT_EE4d9	PREDICTED: similar to Zinc finger protein 341 [Danio rerio]
MT_EE4d11	PREDICTED: hypothetical protein XP_511394 [Pan troglodytes]
MT_EE4e6	epididymal secretory protein E1 [Danio rerio]| Npc2 protein
MT_EE4e7	hypothetical protein [Croceibacter atlanticus HTCC2559]
MT_EE4e8	cAMP-dependent protein kinase catalytic subunit beta [Xenopus laevis]
MT_EE4e11	trypsin III [Salmo salar] TRY3_SALSA Trypsin-3 precursor (Trypsin III)
MT_EE4f5	hypothetical protein [Cryptococcus neoformans var. neoformans]
MT_EE4f7	unnamed protein product [Tetraodon nigroviridis]
MT_EE4f10	putative aminopeptidase [Mytilus galloprovincialis].
MT_EE4f11	anterior gradient-2-like protein 2 [Salmo salar]
MT_EE4g3	glycosyl transferase, family 28 [Nitrobacter winogradskyi Nb-255]
MT_EE4g5	Peptidylprolyl isomerase B (cyclophilin B) [Danio rerio]
MT_EE4g9	PREDICTED: hypothetical protein isoform 1 [Tribolium castaneum]
MT_EE4g11	PREDICTED: hypothetical protein XP_677792 isoform 1 [Danio rerio]

Appendix A3 cont'd.

ID	Transcript name
MT_EE4h2	VHSV-induced protein-10 [Oncorhynchus mykiss]
MT_EE4h7	elastase A [Scophthalmus maximus]
MT_EE4h11	Lysozyme Complex With 4-Methyl-Umbelliferyl Chitobiose
MT_EE5a4	similar to formin binding protein 21 [Monodelphis domestica].
MT_EE5a5	similar to Ependymin precursor (EPD) [Danio rerio]
MT_EE5b5	beta thymosin [Oncorhynchus mykiss]
MT_EE5b8	cytochrome c oxidase subunit I [Oncorhynchus clarkii henshawi]
MT_EE5c1	prothrombin [Oncorhynchus mykiss]
MT_EE5c4	immunoglobulin light chain precursor [Salmo salar]
MT_EE5c7	(protein for IMAGE:8109385) [Danio rerio]
MT_EE5c9	similar to vertebrate alanyl aminopeptidase [Danio rerio]
MT_EE5c10	PREDICTED: hypothetical protein XP_698441 [Danio rerio]
MT_EE5c11	unnamed protein product [Mus musculus]
MT_EE5d4	hypothetical protein AcidDRAFT_1829 [Solibacter usitatus Ellin6076]
MT_EE5e1	unnamed protein product [Tetraodon nigroviridis]
MT_EE5e4	unnamed protein product [Tetraodon nigroviridis]
MT_EE5e5	unnamed protein product [Tetraodon nigroviridis]
MT_EE5e6	PREDICTED: hypothetical protein [Homo sapiens]
MT_EE5e7	Chain A, Trypsin Specificity
MT_EE5e11	elastase A precursor [Gadus morhua]
MT_EE5f2	similar to Heat shock 71 kDa protein (HS 70 kDa) [Canis familiaris]
MT_EE5f9	unnamed protein product [Tetraodon nigroviridis]
MT_EE5g7	complement factor H precursor [Oncorhynchus mykiss]
MT_EE5g8	hypothetical protein PF11_0168 [Plasmodium falciparum 3D7]
MT_EE5h12	immunoglobulin light chain [Oncorhynchus mykiss]
MT_EE6a3	prothymosin alpha like-1 protein [Danio rerio]
MT_EE6a9	elastase 3 precursor [Paralichthys olivaceus]
MT_EE6b5	CC chemokine SCYA113 [Ictalurus punctatus]
MT_EE6b7	glyceraldehyde 3-phosphate dehydrogenase [Gadus morhua]
MT_EE6c2	unnamed protein product [Salmo salar] Serum albumin 1 precursor
MT_EE6d1	unnamed protein product [Tetraodon nigroviridis]
MT_EE6d5	unnamed protein product [Tetraodon nigroviridis]
MT_EE6d6	glucosyltransferase-11 [Vigna angularis]
MT_EE6d8	unnamed protein product [Tetraodon nigroviridis]
MT_EE6e3	eukaryotic translation initiation factor 2, subunit 2 beta [Danio rerio].
MT_EE6e5	unnamed protein product [Tetraodon nigroviridis]
MT_EE6e11	H3 histone, family 3A [Danio rerio] isoform A [Drosophila melanogaster]
MT_EE6f1	beta-globin [Oncorhynchus mykiss] Hemoglobin subunit beta-4
MT_EE6f3	Sb:cb742 protein [Danio rerio]
MT_EE6f9	similar to (Large fibroblast proteoglycan) (GHAP) [Danio rerio]
MT_EE6g2	metabotropic GluR1 [Taeniopygia guttata]
MT_EE6g10	hypothetical protein CC1G_12922 [Coprinopsis cinerea okayama]
MT_EE7a3	Fatty acid binding protein 10, liver basic [Danio rerio]

Appendix A3 cont'd.

ID	Transcript name
MT_EE7a4	unnamed protein product [Tetraodon nigroviridis]
MT_EE7a9	Elastase-1 Mol_id: 1; Molecule: Elastase; Chain: Null; Ec: 3.4.21.36
MT_EE7a10	putative thyroid hormone carrier; transthyretin [Cyprinus carpio]
MT_EE7b4	pentraxin [Oncorhynchus mykiss]
MT_EE7b6	PREDICTED: hypothetical protein XP_689361 [Danio rerio]
MT_EE7b7	cathepsin L.1 [Danio rerio].
MT_EE7b12	complement component C9 [Oncorhynchus mykiss]
MT_EE7c1	complement factor H1 protein [Oncorhynchus mykiss].
MT_EE7c2	Lipopolysaccharide heptosyltransferase I [Pseudomonas fluorescens]
MT_EE7c5	simple repeat sequence-containing transcript [Mus musculus]
MT_EE7c10	alcohol dehydrogenase Class VI [Oryzias latipes]
MT_EE7d3	hypothetical protein CaO19_2537 [Candida albicans SC5314]
MT_EE7d5	procathepsin B [Oncorhynchus mykiss]
MT_EE7d7	Protein CutA homolog precursor
MT_EE7d8	alpha-globin [Salmo salar]
MT_EE7e1	S6 ribosomal protein [Oncorhynchus mykiss] 40S
MT_EE7e9	Coiled-coil domain-containing protein 72 [Tetraodon nigroviridis]
MT_EE7f5	237aa long hypothetical protein [Aeropyrum pernix K1]
MT_EE7f10	similar to Mucin 2 precursor (Intestinal mucin 2) [Gallus gallus]
MT_EE7g1	unnamed protein product [Tetraodon nigroviridis]
MT_EE7g5	DNA primase [Pelodictyon phaeoclathratiforme BU-1]
MT_EE7g7	PREDICTED: similar to F-box protein 38 isoform b [Danio rerio]
MT_EE7g8	sim to Protein Wnt-8a precursor (Stimulated by retinoic acid protein 11)
MT_EE7g9	alpha-globin IV [Oncorhynchus mykiss]
MT_EE7g10	NADH dehydrogenase (ubiquinone) [Mus musculus]
MT_EE7h1	hypothetical protein TTHERM_00661480 [Tetrahymena thermophila]
MT-EE2a7	cathepsin Y [Oncorhynchus mykiss]
MT-EE2h3	pan-epithelial glycoprotein [Danio rerio]
MT-EE2h4	elastase 3 precursor [Paralichthys olivaceus]
MT-EE3b8	C-type MBL-2 protein [Oncorhynchus mykiss]
MT-EE3c11	PREDICTED: similar to c-type lectin [Danio rerio].
MT-EE3c11	PREDICTED: similar to c-type lectin [Danio rerio].
MT-EE3h7	sim to dipeptidyl peptidase 8 isoform 1 isoform 5 [Canis familiaris].
MT-EE4a9	syntaxin 7 [Gallus gallus]
MT-EE4b5	hyperosmotic glycine rich protein [Salmo salar]
MT-EE4b12	Itm2b protein [Danio rerio]
MT-EE4c4	ABC transporter, inner membrane subunit [Reinekea sp. MED297].
MT-EE4g4	PREDICTED: hypothetical protein [Danio rerio]
MT-EE4g8	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa].
MT-EE4h4	PREDICTED: similar to c-type lectin [Danio rerio].
MT-EE4h10	Trypsin-3 precursor (Trypsin III).
MT-EE5a1	Carboxylesterase, type B [Bacillus coagulans 36D1].
MT-EE5a9	protein kinase regulator [Cryptococcus neoformans var. neoformans

Appendix A3 cont'd.

ID	Transcript name
MT-EE5a12	Ig light chain - rainbow trout (fragment)
GRASP-4897	Src kinase-assoc phosphoprotein 2 (Src family-assoc phosphoprotein 2).
GRASP-4943	similar to ubiquitin and ribosomal protein S27a precursor
GRASP-5008	chaperonin containing TCP1, subunit 4 (delta) [Pan troglodytes].
GRASP-5057	pituitary tumor-transforming 1 interacting protein [Xenopus tropicalis].
GRASP-5135	TPA_inf: RTN4-M [Oncorhynchus mykiss]
GRASP-5225	Eef1g protein [Danio rerio]
GRASP-5310	tissue inhibitor of metalloproteinase 2 [Oncorhynchus mykiss].
GRASP-5367	ribulose-5-phosphate-3-epimerase [Danio rerio].
GRASP-5640	novel protein sim to vertebrate integrin beta 4 bind prot [Danio rerio].
GRASP-5701	IgM-A heavy chain constant region [Salmo trutta].
GRASP-5762	similar to calpain regulatory subunit isoform 1 [Danio rerio].
GRASP-4912	Tc1-like transporase [Oncorhynchus mykiss]
GRASP-4953	oocyte protease inhibitor-2 [Oncorhynchus mykiss].
GRASP-5018	similar to Sec24A protein [Gallus gallus].
GRASP-5065	IgM-B heavy chain constant region [Salmo trutta].
GRASP-5142	Cytochrome c oxidase polypeptide VIa, mitochondrial precursor.
GRASP-5227	cathepsin [Paralabidochromis chilotes]
GRASP-5317	similar to mitogen-activated protein kinase organizer 1 [Danio rerio].
GRASP-5368	enolase 1 isoform 8 [Macaca mulatta].
GRASP-5514	lysozyme g [Salmo salar]
GRASP-5652	S-phase kinase-assoc protein 1A, isoform CRA_d [Homo sapiens].
GRASP-5705	Zgc:153855 protein [Danio rerio]
GRASP-5769	glia maturation factor beta [Cyprinus carpio]
GRASP-4916	Phospholipase D family, member 4 [Mus musculus]
GRASP-4954	ubiquinol-cytochrome c reductase, complex III subunit VII [Danio rerio].
GRASP-5020	proliferating cell nuclear antigen [Pagrus major].
GRASP-5073	similar to NADH-ubiquinone oxidoreductase 39 kDa subunit [Danio rerio].
GRASP-5149	CCAAT/enhancer-binding protein beta [Oncorhynchus mykiss].
GRASP-5233	pigment epithelium-derived factor [Paralichthys olivaceus].
GRASP-5318	NADH dehydrogenase subunit 2 [Coregonus lavaretus].
GRASP-5392	hyaluronoglucosaminidase 2 isoform 1 [Pan troglodytes].
GRASP-5522	similar to UDP-glucuronosyltransferase 2A1 precursor[Danio rerio].
GRASP-5663	Down syndrome critical region gene 5 [Danio rerio].
GRASP-5710	glutathione S-transferase theta-class [Kryptolebias marmoratus].
GRASP-5774	S100 calcium binding protein, beta (neural) [Danio rerio].
GRASP-4919	prepro-vasotocin-I [Oncorhynchus keta].
GRASP-4963	similar to Alpha adducin (Erythrocyte adducin alpha subunit) [Danio rerio]
GRASP-5024	Hemoglobin subunit beta-4 (Hemoglobin beta-IV chain).
GRASP-5078	fatty acid binding protein 2, intestinal [Danio rerio].
GRASP-5161	cox-i1b protein [Candida stellata]
GRASP-5239	NEDD4 family-interacting protein 1.
GRASP-5327	solute carrier family 27 (fatty acid transporter), member 2 [Danio rerio].

Appendix A3 cont'd.

ID	Transcript name
GRASP-5416	L-plastin [Danio rerio]
GRASP-5533	RNA-binding protein PNO1.
GRASP-5666	solute carrier family 25 alpha, member 5 [Danio rerio].
GRASP-5717	SM22 alpha-b [Danio rerio]
GRASP-5777	20S proteasome beta 6 subunit [Pagrus major]
GRASP-4923	similar to Sm protein G isoform 1 [Gallus gallus].
GRASP-4975	Thioredoxin (Trx).
GRASP-5027	eukaryotic translation initiation factor 2, subunit 2 beta [Danio rerio].
GRASP-5107	hip2 [Danio rerio]
GRASP-5183	nucleoside-diphosphate kinase NBR-B [Bos taurus].
GRASP-5247	serine protease-like protein precursor [Salvelinus fontinalis].
GRASP-5343	agmatine ureohydrolase [Danio rerio]
GRASP-5419	transposase [Oncorhynchus mykiss]
GRASP-5534	similar to interferon-inducible protein Gig1, partial [Danio rerio]
GRASP-5670	rpL14 protein [Takifugu rubripes]
GRASP-5719	similar to eukaryotic translation initiation factor 3, 47kDa [Danio rerio].
GRASP-5806	calmyrin [Ictalurus punctatus]
GRASP-4932	tumour necrosis factor receptor [Oncorhynchus mykiss].
GRASP-4988	tubulin, alpha 1 (testis specific), isoform CRA_a [Homo sapiens].
GRASP-5032	cathepsin Y [Oncorhynchus mykiss]
GRASP-5115	similar to dicarbonyl L-xylulose reductase isoform 1 [Danio rerio].
GRASP-5198	similar to cellular retinoic acid-binding protein; CRABPI [Pan troglodytes].
GRASP-5255	eukaryotic translation elongation factor 1 delta [Xenopus tropicalis].
GRASP-5350	novel protein (zgc:112282) [Danio rerio]
GRASP-5439	reverse transcriptase-like protein [Paralichthys olivaceus].
GRASP-5535	Tcp1 protein [Danio rerio]
GRASP-5676	similar to Cathepsin H [Gallus gallus].
GRASP-5723	metallothionein-like protein [Jasus edwardsii]
GRASP-5814	ubiquitin [Oncorhynchus mykiss]
GRASP-4940	nephrosin [Plecoglossus altivelis altivelis]
GRASP-4998	ATP synthase F0 subunit 6 [Dorosoma cepedianum].
GRASP-5038	Sop protein [Xenopus laevis]
GRASP-5119	PLAC8-like 1 [Mus musculus]
GRASP-5207	putative interferon-alpha/beta receptor alpha chain [Oncorhynchus mykiss]
GRASP-5266	C-type natriuretic peptide 1 precursor (C-type natriuretic peptideI)
GRASP-5353	Mrps34-prov protein [Xenopus laevis].
GRASP-5445	plastin 3 (T isoform) [Danio rerio]
GRASP-5573	Atp5a1 protein [Xenopus laevis]
GRASP-5686	alpha tubulin subunit [Oncorhynchus nerka]
GRASP-5738	similar to H2A histone family, member V isoform 1 [Rattus norvegicus].
GRASP-4857	similar to Zinc finger, CSL domain containing 2 [Danio rerio].
GRASP-4941	heat shock 90kDa protein 1 beta isoform b [Oncorhynchus mykiss]
GRASP-5007	interleukin-15 precursor [Oncorhynchus mykiss].

Appendix A3 cont'd.

ID	Transcript name
GRASP-5047	cyclin B2 [Oncorhynchus mykiss]
GRASP-5131	integrin, beta-like 1 [Danio rerio]
GRASP-5224	formiminotransferase cyclodeaminase [Danio rerio].
GRASP-5279	Major facilitator superfamily domain-containing protein 4.
GRASP-5363	cytochrome oxidase subunit 1 [Oncorhynchus masou masou].
GRASP-5450	glyceraldehyde-3-phosph dehydrogenase [Oncorhynchus tshawytscha].
GRASP-5610	retinoblastoma binding protein 4 [Danio rerio].
GRASP-5698	eukaryotic translation initiation factor 4E binding protein 3 [Danio rerio].
GRASP-5748	oncomodulin B [Danio rerio]
(MT_EE5b3)	sensor histidine regulator [Bacteroides thetaiotaomicron]
(MT_EE5c3)	Ferritin, heavy subunit (Ferritin H).
(MT_EE5d6)	PREDICTED: similar to c-type lectin [Danio rerio]
(MT_EE5f1)	Protein disulfide isomerase associated 4 [Danio rerio]
(MT_EE5g6)	trypsin IA [Salmo salar]
(MT_EE6a8)	pregnancy-zone protein [Rattus norvegicus]
(MT_EE6d2)	integral membrane protein 2B, like [Danio rerio].
(MT_EE6d11)	cathepsin L.1 [Danio rerio]
(MT_EE6f12)	MGC83595 protein [Xenopus laevis]
(MT_EE6e8)	unnamed protein product [Macaca fascicularis]
(MT_EE6g11)	PREDICTED: similar to olfactory receptor Olr78 [Canis familiaris]
(MT_EE7c7)	ribosomal protein S28 [Branchiostoma belcheri tsingtaunese].
GRASP_4868	Heat shock cognate 70 kDa protein (HSP70). Oncorhynchus mykiss
GRASP_4871	prenyl (decaprenyl) diphosphate synthase, subunit 2 [Danio rerio].
GRASP_4867	novel protein similar to vertebrate sulfotransferase family [Danio rerio].
GRASP_4824	cyclin B3 [Gallus gallus].
GRASP_4861	cytochrome P450 monooxygenase CYP2K1v2 [Oncorhynchus mykiss
N/A	IL8-A
N/A	IL8-B
N/A	NAK ATPASE ALPHA1B
N/A	NAK ATPASE ALPHA1C
N/A	CFTRII
N/A	HTPASE B1
N/A	TNFA
N/A	IGM H
N/A	IL8
N/A	IL8-R
N/A	MHC B1 REGION
N/A	MHC TM REGION
BR-TS1-16	CA protein mRNA
BR-TS1-48	transmembrane 9 superfamily member 1 mRNA
BR-TS1-50	clone CM310 mRNA
BR-TS1-78	OSU immune-type receptor 2 (NITR2) gene
BR-TS1-84	hypothetical protein MGC63622 mRNA

Appendix A3 cont'd.

ID	Transcript name
BR-TS1-114	ribosomal protein S9 mRNA
BR-TS1-146	apolipoprotein E mRNA
BR-TS1-188	ATP synthase beta-subunit mRNA
BR-TS1-194	14-3-3G1 protein mRNA
BR-TS1-199	high mobility group protein HMG-T mRNA
BR-TS1-215	TATA-binding protein-associated 55 kd factor (taf7) mRNA
BR-TS1-253	Cu/Zn-superoxide dismutase (SOD1) mRNA
BR-TS1-260	wu:fk58f09 mRNA
BR-TS1-358	guanine nucleotide binding protein beta polypeptide 2-like 1
BR-TS1-434	KIAA1160 protein mRNA
BR-TS1-455	ATPase, H+ transporting, V1 subunit G isoform 1, mRNA
BR-TS1-463	calmodulin mRNA
BR-TS1-482	neuroplasticin-2 (NPC2) mRNA
BR-TS1-517	20S proteasome beta 6 subunit mRNA
BR-TS1-527	prostaglandine D synthase mRNA
BR-TS1-546	catenin (cadherin-associated protein), alpha, mRNA
BR-TS1-572	nuclease sensitive element binding protein 1 mRNA
BR-TS1-611	clone RZ146A4G07 chromosome 20 open reading frame 20
BR-TS1-626	activated protein kinase C (RACK1) mRNA
BR-TS1-650	aldehyde dehydrogenase 2 precursor (ALDH2) mRNA
BR-TS1-698	clone BUSM1-150F13 in linkage group 8
BR-TS1-709	RTN7 mRNA
BR-TS1-754	leucyl-tRNA synthetase (Kiaa0028 gene) mRNA
BR-TS1-761	clone CH211-222L21 in linkage group 11
BR-TS1-799	ependymin (Om-I) mRNA
BR-TS1-800	origin recognition complex subunit 1 mRNA
BR-TS1-807	inhibitor of growth family, member 3 (ING3) mRNA
BR-TS1-813	zgc:55817 mRNA (cDNA clone MGC:55817 IMAGE:3817803)
BR-TS1-814	TAF-Ibeta2 mRNA
BR-TS1-818	phosphoglucose isomerase-1 (pgi-1 gene)
BR-TS1-842	lysosomal-associated protein transmembrane 4 alpha mRNA
BR-TS1-871	hypothetical protein LOC227619 (LOC227619) mRNA
BR-TS1-900	ran protein mRNA
BR-TS1-1005	tenascin-X mRNA
BR-TS1-1015	14-3-3C1 protein mRNA
BR-TS1-1051	cysteine string protein mRNA
BR-TS1-1079	RASD family, member 2, mRNA
BR-TS1-1081	hypothetical protein LOC284723 mRNA
BR-TS1-1120	beta thymosin mRNA
BR-TS1-1124	polyubiquitin mRNA
BR-TS1-1128	coatomer protein complex, subunit beta 2 (beta prime) (COPB2), mRNA
BR-TS1-1136	ATPase H+ transporting lysosomal vacuolar proton pump mRNA
BR-TS1-1162	cytosolic malate dehydrogenase

Appendix A3 cont'd.

ID	Transcript name
BR-TS1-1164	eps8 binding protein (e3B1) mRNA
BR-TS1-1169	clone 247M18
BR-TS1-1173	clone BHMS108 microsatellite
BR-TS1-1179	SL gene for somatolactin
BR-TS1-1183	myosin light chain 3 mRNA
BR-TS1-1185	outer mitochondrial membrane
BR-TS1-1203	ATP-synthase subunit D mRNA
BR-TS1-1250	stathmin-like mRNA
BR-TS1-1255	gamma-aminobutyric acid (GABA) A receptor, beta 1(GABRB1), mRNA
BR-TS1-1266	brain-subtype creatine kinase mRNA
BR-TS1-1270	clone Hae453 microsatellite sequence
BR-TS1-1308	NBRP cDNA clone:XL448a11ex
BR-TS1-1317	type-1 growth hormone gene
BR-TS1-1348	muscle-specific beta 1 integrin binding protein 2 mRNA
BR-TS1-1350	cyclin G1 (CCNG1) mRNA
BR-TS1-1351	transcription factor BTF3a mRNA
BR-TS1-1354	prolactin II
BR-TS1-1366	DNA sequence from clone DKEY-29H23 in linkage group 15
BR-TS1-1372	actin-related protein 2/3 complex mRNA
BR-TS1-1373	DNA sequence from clone DKEY-11F14 in linkage group 19
BR-TS1-1379	cysteine-rich protein mRNA
BR-TS1-1395	insulin-like growth factor I (IGF-I.1) gene
BR-TS1-1425	zgc:73197 mRNA
BR-TS1-1434	proteasome subunit alpha Type 6-A
BR-TS1-1437	similar to Triple functional domain pr
BR-TS1-1442	N-myc downstream regulated family
BR-TS1-1450	alpha tubulin mR
BR-TS1-1455	transposon SSTN11 tn gene for putat
BR-TS1-1476	pituitary-specific transcription factor P
BR-TS1-1503	enolase 2, (gamma, neuronal) mRN
BR-TS1-1505	proteasome activator subunit 2 (psme2) mRNA
LV-TS1-25	V-Fos transformation effector (fte-1) mRNA
LV-TS1-42	pyruvate dehydrogenase (lipoamide) alpha 1 mRNA
LV-TS1-82	hyperosmotic glycine rich protein mRNA
LV-TS1-83	ferritin middle subunit
LV-TS1-104	carbonyl reductase/20beta-hydroxysteroid dehydrogenase B gene
LV-TS1-153	H3 histone, family 3A, mRNA
LV-TS1-163	uridine phosphorylase 1 mRNA
LV-TS1-178	thrombin mRNA
LV-TS1-186	interferon inducible protein (IIP gene) mRNA
LV-TS1-218	DNA sequence from clone DKEY-259B21 in li
LV-TS1-238	DNA sequence from clone DKEYP-75B4 in
LV-TS1-239	hepcidin (Hep1) precursor RNA

Appendix A3 cont'd.

ID	Transcript name
LV-TS1-240	C1q-like adipose specific protein mRNA
LV-TS1-243	40S ribosomal protein S2 mRNA
LV-TS1-315	ribosomal protein L10 mRNA
LV-TS1-318	40S ribosomal protein Sa mRNA
LV-TS1-335	C-type lectin 2-1 mRNA
LV-TS1-337	keratinocytes associated protein 2 (KCP2) mRNA
LV-TS1-339	glycoprotein, synaptic 2 (gpsn2), mRNA
LV-TS1-346	pentraxin mRNA
LV-TS1-349	hemopexin-like protein variant 1 mRNA
LV-TS1-360	complement component C9 mRNA
LV-TS1-374	LECT2 neutrophil chemotactic factor mRNA
LV-TS1-380	tyrosine aminotransferase mRNA
LV-TS1-384	cystein inhibitor protein (salarin gene) mRNA
LV-TS1-393	ceruloplasmin mRNA
LV-TS1-398	complement component C7 mRNA
LV-TS1-405	putative fibrinogen gamma A chain mRNA
LV-TS1-408	C1 inhibitor (c1 Inh gene) mRNA
LV-TS1-409	retinol-binding protein (RBP) mRNA
LV-TS1-410	apolipoprotein CII mRNA
LV-TS1-418	alcohol dehydrogenase class III mRNA
LV-TS1-445	S6 ribosomal protein mRNA
LV-TS1-499	complement component C3-3 mRNA
LV-TS1-501	DNA sequence from clone CH211-243G18 in
LV-TS1-520	ribosomal protein L9 mRNA
LV-TS1-522	zinc finger, DHHC domain containing 14; NEW1
LV-TS1-533	clone ChEST377o20
LV-TS1-556	DNA sequence from clone DKEY-53P21
LV-TS1-557	cysteine proteinase gene
LV-TS1-580	clone OSU natural killer cell enhancement factor (
LV-TS1-657	id2 protein mRNA
LV-TS1-677	procathepsin L mRNA
LV-TS1-680	proteasome subunit C10-11 mRNA
LV-TS1-686	dispersed repeat AvaIII, clone Ava(CAr)-1507
LV-TS1-693	haptoglobin fragment 1 mRNA
LV-TS1-698	DNA sequence from clone DKEY-7F3
LV-TS1-712	fuse-binding protein-interacting repressor transcript variant 2
LV-TS1-717	endothelial-derived gene 1
LV-TS1-731	lysosomal associated protein transmembrane 4 beta mRNA
LV-TS1-760	ferritin-H subunit mRNA
LV-TS1-761	haptoglobin mRNA
LV-TS1-762	40S ribosomal protein S3 (RPS3) mRNA
LV-TS1-772	secreted phosphoprotein 24 (spp2 gene) mRNA
MS-RD1-15	connectin/titin mRNA

Appendix A3 cont'd.

ID	Transcript name
MS-RD1-16	ribosomal protein L7a mRNA
MS-RD1-27	NBRP cDNA clone:XL441h11ex, 5' end
MS-RD1-41	creatine kinase mitochondrial isoform mRNA
MS-RD1-44	glycogen phosphorylase mRNA
MS-RD1-86	elongation factor EF1 alpha mRNA
MS-RD1-89	40S ribosomal protein S4 mRNA
MS-RD1-131	60S ribosomal protein L8 mRNA
MS-RD1-155	adenylate kinase 1 (AK1) mRNA.
MS-RD1-157	creatine kinase mRNA
MS-RD1-179	cDNA FLJ43303 fis, clone NOVAR2000136
MS-RD1-193	carbonyl reductase 1 mRNA
MS-RD1-258	ribosomal RNA gene
MS-RD1-262	aldolase mRNA
MS-RD1-279	enolase 1 mRNA
MS-RD1-296	putative ribosomal protein S8 mRNA
MS-RD1-305	sarcoplasmic/endoplasmic reticulum calcium ATPase 1B mRNA
MS-RD1-327	fast myotomal muscle tropomyosin mRNA
MS-RD1-337	troponin C mRNA
MS-RD1-339	cDNA clone MGC:66039 IMAGE:6794716
MS-RD1-347	myosin heavy chain mRNA
MS-RD1-352	glyceraldehyde 3-phosphate dehydrogenase mRNA
MS-RD1-374	sarcoplasmic/endoplasmic reticulum calcium ATPase 1B mRNA
MS-RD1-413	fast myotomal muscle actin mRNA
MS-RD1-425	ribosomal protein L17 mRNA
MS-RD1-435	lactate dehydrogenase-A (ldh-a) mRNA
MS-RD1-441	RNA binding motif protein 4 mRNA
MS-RD1-445	phosphoglycerate mutase 2 (muscle) mRNA
MS-RD1-454	18S rRNA gene
MS-RD1-459	xMSS1 protein mRNA
MS-RD1-460	mitochondrion related protein
MS-RD1-470	28S ribosomal RNA gene
MS-RD1-472	solute carrier family 25 member 4 (SLC25A4), mRNA.
MS-RD1-612	myosin regulatory light chain 2 (mlc-2 gene) mRNA
MS-RD1-618	ribosomal protein L5b mRNA
MS-RD1-644	cDNA clone MGC:56073 IMAGE:5409859

Appendix A4. Genotypic matrices and allele frequencies output files from Genepop program for the CH1 offspring.

```
Number of populations detected : 1
Number of loci detected        : 12

CH1 Pop: 223    Locus: Ots253b
----------------------------------------

 Genotypic matrix:
    160 164 188 192 204 208 216 236 256 260
    ___________________________________________
160   0
164   0   3
188  17   0   1
192   0   0   0   0
204   0   4   0   0   0
208  17  13  29   2   5  43
216   0   7   0   0   0   3   0
236   0   3  14   0   0  18   0   0
256   0   4   9   0   0  11   0   0   0
260   0   0   0   1   0   0   0   0   0   0


Expected number of homozygotes  : 52.8329
Observed number of homozygotes  : 47
Expected number of heterozygotes: 151.1671
Observed number of heterozygotes: 157



Allele frequencies and Fis:
-----------------------------------------------------
                                      Fis
                                     ----------------
Allele     Sample count     Frequency   W&C
 160           34             0.0833   -0.0885
 164           37             0.0907    0.0810
 188           71             0.1740   -0.1742
 192           3              0.0074   -0.0050
 204           9              0.0221   -0.0201
 208           184            0.4510    0.0323
 216           10             0.0245   -0.0227
 236           35             0.0858   -0.0914
 256           24             0.0588   -0.0601
 260           1              0.0025    0.0000
Tot            408                     -0.0387
-----------------------------------------------------
```

Appendix A4 cont'd.

CH1 Pop: 223 Locus: OtsG249

Genotypic matrix:

	163	199	207	211	215	223	227	231	235	243	247	251	259
163	1												
199	0	0											
207	0	0	0										
211	0	7	0	6									
215	21	4	0	22	8								
223	0	0	0	0	13	0							
227	1	0	0	0	0	0	0						
231	1	0	0	1	0	0	0	0					
235	24	0	1	17	0	5	0	7	0				
243	0	0	0	2	9	0	0	2	3	1			
247	0	0	0	0	0	0	0	0	1	0	0		
251	0	0	0	4	4	0	0	0	1	0	0	0	
259	0	0	0	4	5	0	0	0	23	3	0	0	0

Expected number of homozygotes : 30.7182
Observed number of homozygotes : 16
Expected number of heterozygotes: 170.2818
Observed number of heterozygotes: 185

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
163	49	0.1219	-0.0899
199	11	0.0274	-0.0256
207	1	0.0025	-0.0000
211	69	0.1716	0.0052
215	94	0.2338	-0.0806
223	18	0.0448	-0.0444
227	1	0.0025	-0.0000
231	11	0.0274	-0.0256
235	82	0.2040	-0.2539
243	21	0.0522	0.0479
247	1	0.0025	-0.0000
251	9	0.0224	-0.0204
259	35	0.0871	-0.0929
Tot	402		-0.0867

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Ots3

Genotypic matrix:

	82	84	88	90	92	98	100
82	0						
84	0	0					
88	0	0	0				
90	6	1	2	93			
92	0	0	0	16	3		
98	3	0	0	45	8	0	
100	0	0	0	18	5	20	0

Expected number of homozygotes : 95.1845
Observed number of homozygotes : 96
Expected number of heterozygotes: 124.8155
Observed number of heterozygotes: 124

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
82	9	0.0205	-0.0186
84	1	0.0023	0.0000
88	2	0.0045	-0.0023
90	274	0.6227	0.1509
92	35	0.0795	0.1021
98	76	0.1727	-0.2066
100	43	0.0977	-0.1061
Tot	440		0.0065

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Ots4

Genotypic matrix:

	144	146	150	152	156
144	0				
146	11	79			
150	4	73	21		
152	0	2	2	1	
156	0	20	1	0	0

Expected number of homozygotes : 99.3607
Observed number of homozygotes : 101
Expected number of heterozygotes: 114.6393
Observed number of heterozygotes: 113

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
144	15	0.0350	-0.0340
146	264	0.6168	-0.0455
150	122	0.2850	0.0851
152	6	0.0140	0.3259
156	21	0.0491	-0.0493
Tot	428		0.0143

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Omy325

Genotypic matrix:

	88	90	94	98	100	104
88	0					
90	0	0				
94	0	24	9			
98	0	0	2	0		
100	4	24	36	0	30	
104	3	11	36	0	35	4

Expected number of homozygotes : 58.0276
Observed number of homozygotes : 43
Expected number of heterozygotes: 159.9724
Observed number of heterozygotes: 175

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
88	7	0.0161	-0.0140
90	59	0.1353	-0.1543
94	116	0.2661	-0.1488
98	2	0.0046	-0.0023
100	159	0.3647	0.0223
104	93	0.2133	-0.1596
Tot	436		-0.0942

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Ots104

Genotypic matrix:

	185	205	209	213	217	221	225	229	233	237	241	253
185	0											
205	0	4										
209	0	0	0									
213	0	0	0	0								
217	2	11	5	5	8							
221	0	0	0	0	2	0						
225	0	12	0	4	12	3	1					
229	1	11	1	2	38	0	4	5				
233	0	0	0	0	0	0	5	6	0			
237	0	0	0	0	0	0	5	7	0	0		
241	0	0	0	0	0	0	1	0	0	0	0	
253	0	0	0	0	1	0	33	26	0	0	0	2

Expected number of homozygotes : 37.1178
Observed number of homozygotes : 20
Expected number of heterozygotes: 179.8822
Observed number of heterozygotes: 197

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
185	3	0.0069	-0.0047
205	42	0.0968	0.1060
209	6	0.0138	-0.0117
213	11	0.0253	-0.0237
217	92	0.2120	-0.0460
221	5	0.0115	-0.0093
225	81	0.1866	-0.1969
229	106	0.2442	-0.1961
233	11	0.0253	-0.0237
237	12	0.0276	-0.0261
241	1	0.0023	-0.0000
253	64	0.1475	-0.0974
Tot	434		-0.0954

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Ots107

Genotypic matrix:

	204	208	224	228	236	240	244	248	252
204	0								
208	0	0							
224	0	1	0						
228	0	27	5	4					
236	0	2	0	1	0				
240	0	1	1	19	3	9			
244	0	17	8	7	1	15	1		
248	14	7	0	12	3	17	13	16	
252	0	3	0	1	0	2	1	6	0

Expected number of homozygotes : 35.4596
Observed number of homozygotes : 30
Expected number of heterozygotes: 181.5404
Observed number of heterozygotes: 187

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
204	14	0.0323	-0.0310
208	58	0.1336	-0.1520
224	15	0.0346	-0.0335
228	80	0.1843	-0.1011
236	10	0.0230	-0.0213
240	76	0.1751	0.0771
244	64	0.1475	-0.1341
248	104	0.2396	0.0918
252	13	0.0300	-0.0286
Tot	434		-0.0301

Appendix A4 cont'd.

CH1 Pop: 223 Locus: OtsG311

Genotypic matrix:

	294	298	302	306	310	314	326	330	334	346	374
294	0										
298	7	2									
302	0	5	3								
306	0	6	0	0							
310	10	21	8	1	29						
314	1	1	0	0	1	0					
326	9	0	1	5	6	0	1				
330	13	13	5	5	23	0	0	1			
334	0	2	0	2	0	0	2	0	0		
346	0	0	0	1	0	0	0	0	0	0	
374	2	0	0	9	1	1	0	0	3	0	5

Expected number of homozygotes : 34.3056
Observed number of homozygotes : 41
Expected number of heterozygotes: 170.6944
Observed number of heterozygotes: 164

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
294	42	0.1024	-0.1117
298	59	0.1439	-0.0865
302	25	0.0610	0.1930
306	29	0.0707	-0.0737
310	129	0.3146	0.1993
314	4	0.0098	-0.0074
326	25	0.0610	0.0227
330	61	0.1488	-0.1339
334	9	0.0220	-0.0200
346	1	0.0024	-0.0000
374	26	0.0634	0.3451
Tot	410		0.0393

Appendix A4 cont'd.

CH1 Pop: 223 Locus: OtsG68

Genotypic matrix:

	175	183	191	211	219	223	235	239	243	247	251	259	267	291
175	0													
183	0	0												
191	1	0	0											
211	0	0	1	1										
219	0	0	1	0	0									
223	0	0	0	0	0	0								
235	0	0	0	13	0	0	20							
239	0	0	3	0	0	0	2	1						
243	0	1	5	22	1	3	21	2	14					
247	0	0	0	0	0	0	0	0	0	0				
251	0	0	0	0	0	0	0	0	10	0	0			
259	0	0	2	0	0	0	26	1	33	4	0	0		
267	0	0	0	0	0	0	6	1	4	1	0	0	0	
291	0	0	3	0	0	0	0	1	6	0	0	0	0	0

Expected number of homozygotes : 43.3461
Observed number of homozygotes : 36
Expected number of heterozygotes: 166.6539
Observed number of heterozygotes: 174

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
175	1	0.0024	-0.0000
183	1	0.0024	-0.0000
191	16	0.0381	-0.0372
211	38	0.0905	-0.0392
219	2	0.0048	-0.0024
223	3	0.0071	-0.0048
235	108	0.2571	0.1548
239	12	0.0286	0.1445
243	136	0.3238	-0.1721
247	5	0.0119	-0.0097
251	10	0.0238	-0.0220
259	66	0.1571	-0.1841
267	12	0.0286	-0.0270
291	10	0.0238	-0.0220
Tot	420		-0.0442

Appendix A4 cont'd.

CH1 Pop: 223 Locus: OtsG83b

Genotypic matrix:

	165	169	173	177	185	193	197	201	205	209	221	225	229
165	0												
169	0	0											
173	1	4	0										
177	1	0	0	0									
185	6	0	3	3	12								
193	0	0	0	0	7	0							
197	0	0	0	0	0	0	0						
201	1	0	7	0	11	2	15	10					
205	0	0	3	0	0	0	0	1	0				
209	0	0	1	0	0	0	0	3	0	0			
221	1	0	0	0	2	0	0	15	0	0	1		
225	0	0	0	1	9	1	13	14	3	6	16	9	
229	4	0	0	1	8	6	0	1	0	0	0	0	0

Expected number of homozygotes : 28.6352
Observed number of homozygotes : 32
Expected number of heterozygotes: 173.3648
Observed number of heterozygotes: 170

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
165	14	0.0347	-0.0334
169	4	0.0099	-0.0075
173	19	0.0470	-0.0469
177	6	0.0149	-0.0126
185	73	0.1807	0.1831
193	16	0.0396	-0.0388
197	28	0.0693	-0.0720
201	90	0.2228	0.0018
205	7	0.0173	-0.0152
209	10	0.0248	-0.0229
221	36	0.0891	-0.0344
225	81	0.2005	0.0297
229	20	0.0495	-0.0496
Tot	404		0.0195

Appendix A4 cont'd.

CH1 Pop: 223 Locus: OtsG432

Genotypic matrix:

	107	111	123	127	131	135	155	159	163
107	23								
111	7	6							
123	0	15	7						
127	27	10	1	1					
131	0	0	6	7	0				
135	5	6	6	7	0	0			
155	0	1	1	0	0	0	0		
159	11	16	5	33	0	5	0	0	
163	0	1	0	0	0	0	0	0	0

Expected number of homozygotes : 35.3680
Observed number of homozygotes : 37
Expected number of heterozygotes: 171.6320
Observed number of heterozygotes: 170

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
107	96	0.2319	0.3241
111	68	0.1643	0.0170
123	48	0.1159	0.2011
127	87	0.2101	-0.2347
131	13	0.0314	-0.0300
135	29	0.0700	-0.0729
155	2	0.0048	-0.0024
159	70	0.1691	-0.2012
163	1	0.0024	0.0000
Tot	414		0.0095

Appendix A4 cont'd.

CH1 Pop: 223 Locus: Ots13

Genotypic matrix:

	76	82	84	88	90	92	98	100
76	1							
82	0	0						
84	0	0	0					
88	0	0	0	0				
90	0	6	1	5	71			
92	0	0	0	0	17	3		
98	0	3	0	0	35	6	1	
100	0	0	0	0	16	4	16	0

Expected number of homozygotes : 74.8699
Observed number of homozygotes : 76
Expected number of heterozygotes: 110.1301
Observed number of heterozygotes: 109

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
76	2	0.0054	1.0000
82	9	0.0243	-0.0222
84	1	0.0027	-0.0000
88	5	0.0135	-0.0110
90	222	0.6000	0.1018
92	33	0.0892	0.1044
98	62	0.1676	-0.1599
100	36	0.0973	-0.1051
Tot	370		0.0103

Appendix A5. Genotypic matrices and allele frequencies output files from Genepop program for the CH3 offspring.

```
Number of populations detected : 1
Number of loci detected        : 12

CH3 Pop: 257    Locus: Ots253b
----------------------------------------

 Genotypic matrix:
    160 164 188 192 196 204 208 216 220 236
    ___________________________________________
160   0
164   0  19
188   3  16   0
192   0   0   0   0
196   0   1   0   0   0
204   4  22   8   0   0  13
208   8  35   9   1   0   9  41
216   0   3   0   0   0   2  12   0
220   0   2   0   0   0   2   0   0   0
236   0   2   0   0   0   2  14   0   0   0


Expected number of homozygotes  : 55.3648
Observed number of homozygotes  : 73
Expected number of heterozygotes: 172.6352
Observed number of heterozygotes: 155

Allele frequencies and Fis:
-------------------------------------------------------
                                        Fis
                                       ----------------
Allele     Sample count     Frequency   W&C
 160           15             0.0329   -0.0318
 164           119            0.2610    0.0812
 188           36             0.0789   -0.0835
 192           1              0.0022    0.0000
 196           1              0.0022    0.0000
 204           75             0.1645    0.2202
 208           170            0.3728    0.1768
 216           17             0.0373   -0.0365
 220           4              0.0088   -0.0067
 236           18             0.0395   -0.0389
Tot            456                       0.1024
-------------------------------------------------------
```

Appendix A5 cont'd.

CH3 Pop: 257 Locus: OtsG249

Genotypic matrix:

	163	211	215	219	223	227	231	235	243	251	259
163	0										
211	12	1									
215	0	0	2								
219	5	8	4	0							
223	0	1	0	2	0						
227	0	0	0	0	0	0					
231	3	0	6	0	0	0	2				
235	17	3	4	14	1	1	0	1			
243	0	8	1	18	1	0	1	25	1		
251	0	0	0	9	0	0	6	3	0	0	
259	0	11	0	20	0	0	0	36	0	0	0

Expected number of homozygotes : 32.3532
Observed number of homozygotes : 7
Expected number of heterozygotes: 194.6468
Observed number of heterozygotes: 220

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
163	37	0.0815	-0.0865
211	45	0.0991	-0.0585
215	19	0.0419	0.1782
219	80	0.1762	-0.2118
223	5	0.0110	-0.0089
227	1	0.0022	-0.0000
231	20	0.0441	0.1653
235	106	0.2335	-0.2779
243	56	0.1233	-0.0978
251	18	0.0396	-0.0391
259	67	0.1476	-0.1710
Tot	454		-0.1306

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Ots3

```
----------------------------------------
```

Genotypic matrix:

	84	88	90	92	94	100
84	0					
88	1	2				
90	25	33	107			
92	5	27	29	0		
94	0	0	1	0	0	
100	1	3	11	4	0	1

Expected number of homozygotes : 108.0000
Observed number of homozygotes : 110
Expected number of heterozygotes: 142.0000
Observed number of heterozygotes: 140

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
84	32	0.0640	-0.0664
88	68	0.1360	-0.0873
90	313	0.6260	0.1562
92	65	0.1300	-0.1475
94	1	0.0020	-0.0000
100	21	0.0420	0.0576
Tot	500		0.0141

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Ots4

Genotypic matrix:

	144	146	148	150	156
144	0				
146	1	94			
148	0	5	0		
150	0	87	0	5	
156	0	18	6	26	0

Expected number of homozygotes : 110.4224
Observed number of homozygotes : 99
Expected number of heterozygotes: 131.5776
Observed number of heterozygotes: 143

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
144	1	0.0021	-0.0000
146	299	0.6178	0.0308
148	11	0.0227	-0.0212
150	123	0.2541	-0.2298
156	50	0.1033	-0.1132
Tot	484		-0.0870

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Omy325

Genotypic matrix:

	84	88	90	94	100	104	108
84	1						
88	10	2					
90	4	5	5				
94	14	21	14	7			
100	9	29	1	13	13		
104	23	5	10	18	35	3	
108	0	0	1	0	0	0	0

Expected number of homozygotes : 43.1691
Observed number of homozygotes : 31
Expected number of heterozygotes: 199.8309
Observed number of heterozygotes: 212

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
84	62	0.1276	-0.1072
88	74	0.1523	-0.1138
90	45	0.0926	0.1449
94	94	0.1934	-0.0531
100	113	0.2325	-0.0011
104	97	0.1996	-0.1701
108	1	0.0021	-0.0000
Tot	486		-0.0610

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Ots104

Genotypic matrix:

	201	209	213	217	221	225	229	237	245	253	265	273
201	0											
209	0	5										
213	0	0	0									
217	0	26	7	43								
221	0	0	0	7	3							
225	0	0	0	10	1	1						
229	0	0	7	50	0	0	5					
237	0	0	0	13	0	0	0	0				
245	0	0	0	1	0	0	0	0	0			
253	2	4	0	6	8	2	30	6	0	3		
265	0	0	0	2	0	0	0	0	0	0	0	
273	0	0	0	0	0	1	0	0	0	0	0	0

Expected number of homozygotes : 61.0206
Observed number of homozygotes : 60
Expected number of heterozygotes: 181.9794
Observed number of heterozygotes: 183

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
201	2	0.0041	-0.0021
209	40	0.0823	0.1847
213	14	0.0288	-0.0276
217	208	0.4280	-0.0233
221	22	0.0453	0.2402
225	16	0.0329	0.0973
229	97	0.1996	-0.1185
237	19	0.0391	-0.0386
245	1	0.0021	-0.0000
253	64	0.1317	-0.0416
265	2	0.0041	-0.0021
273	1	0.0021	-0.0000
Tot	486		-0.0056

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Ots107

Genotypic matrix:

	208	224	228	232	236	240	244	248	288
208	1								
224	0	0							
228	1	0	0						
232	0	0	0	0					
236	2	0	1	0	1				
240	0	0	44	1	7	4			
244	1	1	26	0	11	41	29		
248	2	3	27	3	7	2	23	0	
288	0	0	0	0	0	0	1	0	0

Expected number of homozygotes : 54.1530
Observed number of homozygotes : 35
Expected number of heterozygotes: 184.8470
Observed number of heterozygotes: 204

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
208	8	0.0167	0.2392
224	4	0.0084	-0.0063
228	99	0.2071	-0.2593
232	4	0.0084	-0.0063
236	30	0.0628	0.0063
240	103	0.2155	-0.1736
244	162	0.3389	0.0310
248	67	0.1402	-0.1610
288	1	0.0021	0.0000
Tot	478		-0.1039

Appendix A5 cont'd.

CH3 Pop: 257 Locus: OtsG311

Genotypic matrix:

	282	290	294	298	302	306	310	314	318	326	330	334	346	358	362	374	378
282	0																
290	0	0															
294	0	2	3														
298	0	0	6	3													
302	0	0	0	1	0												
306	1	0	0	0	0	0											
310	0	0	15	25	0	2	4										
314	0	3	0	0	0	0	11	0									
318	0	0	0	0	0	0	0	0	0								
326	0	0	1	4	0	1	1	0	0	0							
330	0	0	0	5	1	2	5	0	0	6	4						
334	0	0	0	0	0	0	0	1	0	0	2	1					
346	0	7	0	1	1	0	3	0	0	0	0	0	0				
358	0	0	0	15	0	0	6	0	1	2	6	1	0	0			
362	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0		
374	0	0	19	23	2	2	12	0	0	2	8	0	2	6	0	6	
378	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Expected number of homozygotes : 31.0926
Observed number of homozygotes : 21
Expected number of heterozygotes: 206.9074
Observed number of heterozygotes: 217

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
282	1	0.0021	0.0000
290	12	0.0252	-0.0238
294	49	0.1029	0.0239
298	88	0.1849	-0.1411
302	5	0.0105	-0.0085
306	8	0.0168	-0.0150
310	89	0.1870	-0.1173
314	15	0.0315	-0.0304
318	1	0.0021	0.0000
326	17	0.0357	-0.0349
330	43	0.0903	0.1073
334	6	0.0126	0.3267
346	14	0.0294	-0.0282
358	37	0.0777	-0.0822
362	2	0.0042	-0.0021
374	88	0.1849	-0.0574
378	1	0.0021	0.0000
Tot	476		-0.0489

Appendix A5 cont'd.

CH3 Pop: 257 Locus: OtsG68

Genotypic matrix:

	175	183	191	207	211	219	227	231	235	239	243	255	259	267	275
175	0														
183	0	1													
191	0	1	0												
207	0	0	0	0											
211	0	0	0	0	0										
219	0	0	0	0	0	0									
227	0	0	0	5	0	0	0								
231	0	0	0	0	0	0	2	0							
235	6	0	0	0	4	6	11	4	20						
239	0	0	0	0	0	0	5	0	7	0					
243	3	0	1	7	0	2	1	5	56	20	16				
255	6	0	0	0	1	4	4	0	1	0	2	0			
259	0	0	1	0	0	0	1	0	0	0	2	0	0		
267	0	0	0	0	0	0	11	0	0	0	7	0	0	0	
275	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Expected number of homozygotes : 45.9329
Observed number of homozygotes : 37
Expected number of heterozygotes: 178.0671
Observed number of heterozygotes: 187

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
175	15	0.0335	-0.0324
183	3	0.0067	0.6657
191	3	0.0067	-0.0045
207	12	0.0268	-0.0253
211	5	0.0112	-0.0090
219	12	0.0268	-0.0253
227	40	0.0893	-0.0958
231	11	0.0246	-0.0229
235	135	0.3013	-0.0050
239	32	0.0714	-0.0747
243	139	0.3103	-0.1139
255	18	0.0402	-0.0396
259	4	0.0089	-0.0068
267	18	0.0402	-0.0396
275	1	0.0022	0.0000
Tot	448		-0.0503

Appendix A5 cont'd.

CH3 Pop: 257 Locus: OtsG83b

Genotypic matrix:

	165	173	185	193	197	201	205	209	213	221	225	229
165	0											
173	7	1										
185	7	4	2									
193	2	1	0	0								
197	7	17	12	0	0							
201	4	19	14	0	0	0						
205	0	10	2	0	0	1	0					
209	0	2	2	0	0	0	0	0				
213	0	0	1	0	0	0	0	0	0			
221	9	1	2	0	3	9	9	8	0	1		
225	13	2	2	1	8	11	15	7	0	3	0	
229	0	0	1	0	0	0	0	0	0	2	0	0

Expected number of homozygotes : 25.1242
Observed number of homozygotes : 4
Expected number of heterozygotes: 196.8758
Observed number of heterozygotes: 218

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
165	49	0.1104	-0.1218
173	65	0.1464	-0.1332
185	51	0.1149	-0.0389
193	4	0.0090	-0.0068
197	47	0.1059	-0.1162
201	58	0.1306	-0.1481
205	37	0.0833	-0.0887
209	19	0.0428	-0.0425
213	1	0.0023	0.0000
221	48	0.1081	-0.0722
225	62	0.1396	-0.1601
229	3	0.0068	-0.0045
Tot	444		-0.1076

Appendix A5 cont'd.

CH3 Pop: 257 Locus: OtsG432

Genotypic matrix:

	107	111	123	127	131	135	155	159	163
107	15								
111	24	29							
123	4	4	1						
127	6	5	8	0					
131	0	1	0	0	0				
135	19	32	7	1	0	0			
155	0	1	0	8	0	6	0		
159	16	3	1	0	0	19	0	0	
163	7	0	1	0	0	4	0	0	0

Expected number of homozygotes : 43.2551
Observed number of homozygotes : 45
Expected number of heterozygotes: 178.7449
Observed number of heterozygotes: 177

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
107	106	0.2387	0.0604
111	128	0.2883	0.2337
123	27	0.0608	0.0164
127	28	0.0631	-0.0651
131	1	0.0023	0.0000
135	88	0.1982	-0.2451
155	15	0.0338	-0.0327
159	39	0.0878	-0.0941
163	12	0.0270	-0.0255
Tot	444		0.0098

Appendix A5 cont'd.

CH3 Pop: 257 Locus: Ots13

Genotypic matrix:

	84	88	90	92	94	100
84	0					
88	1	2				
90	26	36	102			
92	4	22	28	1		
94	0	0	1	0	0	
100	1	3	10	4	0	1

Expected number of homozygotes : 105.5093
Observed number of homozygotes : 106
Expected number of heterozygotes: 136.4907
Observed number of heterozygotes: 136

Allele frequencies and Fis:

Allele	Sample count	Frequency	Fis W&C
84	32	0.0661	-0.0687
88	66	0.1364	-0.0857
90	305	0.6302	0.1067
92	60	0.1240	-0.1014
94	1	0.0021	-0.0000
100	20	0.0413	0.0633
Tot	484		0.0036

Appendix A6. Proportion of each family that can be assigned in CH1 by FAP.

Family CH1	Proportion
F228xM224	1.00
F228xM225	1.00
F228xM226	1.00
F228xM227	1.00
F228xM233	0.93
F228xM234	1.00
F228xM240	0.91
F228xM241	1.00
F228xM243	1.00
F228xM244	1.00
F228xM247	1.00
F228xM250	1.00
F228xM251	1.00
F228xM252	1.00
F229xM224	1.00
F229xM225	1.00
F229xM226	1.00
F229xM227	1.00
F229xM233	0.94
F229xM234	1.00
F229xM240	0.91
F229xM241	1.00
F229xM243	1.00
F229xM244	1.00
F229xM247	1.00
F229xM250	1.00
F229xM251	1.00
F229xM252	1.00
F230xM224	0.98
F230xM225	0.99
F230xM226	0.99
F230xM227	0.99
F230xM233	0.93
F230xM234	0.99
F230xM240	0.90
F230xM241	0.99
F230xM243	0.99
F230xM244	0.99
F230xM247	0.99
F230xM250	0.99
F230xM251	0.99
F230xM252	0.99

Family CH1	Proportion
F231xM224	1.00
F231xM225	1.00
F231xM226	1.00
F231xM227	1.00
F231xM233	0.94
F231xM234	1.00
F231xM240	0.94
F231xM241	1.00
F231xM243	1.00
F231xM244	1.00
F231xM247	1.00
F231xM250	1.00
F231xM251	1.00
F231xM252	1.00
F232xM224	1.00
F232xM225	1.00
F232xM226	1.00
F232xM227	1.00
F232xM233	0.94
F232xM234	1.00
F232xM240	0.91
F232xM241	1.00
F232xM243	1.00
F232xM244	1.00
F232xM247	1.00
F232xM250	1.00
F232xM251	1.00
F232xM252	1.00
F235xM224	1.00
F235xM225	1.00
F235xM226	1.00
F235xM227	1.00
F235xM233	0.94
F235xM234	0.94
F235xM240	0.94
F235xM241	0.97
F235xM243	1.00
F235xM244	1.00
F235xM247	1.00
F235xM250	1.00
F235xM251	1.00
F235xM252	1.00

Family CH1	Proportion
F236xM224	0.94
F236xM225	0.94
F236xM226	0.94
F236xM227	0.90
F236xM233	0.88
F236xM234	0.94
F236xM240	0.85
F236xM241	0.94
F236xM243	0.93
F236xM244	0.94
F236xM247	0.93
F236xM250	0.93
F236xM251	0.93
F236xM252	0.94
F237xM224	1.00
F237xM225	1.00
F237xM226	1.00
F237xM227	1.00
F237xM233	0.91
F237xM234	1.00
F237xM240	0.86
F237xM241	1.00
F237xM243	1.00
F237xM244	1.00
F237xM247	1.00
F237xM250	1.00
F237xM251	1.00
F237xM252	1.00
F238xM224	1.00
F238xM225	1.00
F238xM226	1.00
F238xM227	1.00
F238xM233	0.94
F238xM234	1.00
F238xM240	0.94
F238xM241	1.00
F238xM243	1.00
F238xM244	1.00
F238xM247	1.00
F238xM250	1.00
F238xM251	1.00
F238xM252	1.00

Appendix A6 cont'd.

Family CH1	Proportion
F239xM224	0.94
F239xM225	0.94
F239xM226	0.94
F239xM227	0.94
F239xM233	0.88
F239xM234	0.94
F239xM240	0.85
F239xM241	0.94
F239xM243	0.94
F239xM244	0.94
F239xM247	0.94
F239xM250	0.94
F239xM251	0.94
F239xM252	0.94
F242xM224	1.00
F242xM225	1.00
F242xM226	1.00
F242xM227	1.00
F242xM233	0.94
F242xM234	1.00
F242xM240	0.94
F242xM241	1.00
F242xM243	1.00
F242xM244	1.00
F242xM247	1.00
F242xM250	1.00
F242xM251	1.00
F242xM252	1.00
F245xM224	1.00
F245xM225	1.00
F245xM226	1.00
F245xM227	1.00
F245xM233	0.94
F245xM234	1.00
F245xM240	0.89
F245xM241	1.00
F245xM243	1.00
F245xM244	1.00
F245xM247	1.00
F245xM250	1.00
F245xM251	1.00
F245xM252	1.00

Family CH1	Proportion
F246xM224	1.00
F246xM225	1.00
F246xM226	1.00
F246xM227	1.00
F246xM233	0.94
F246xM234	1.00
F246xM240	0.94
F246xM241	1.00
F246xM243	1.00
F246xM244	1.00
F246xM247	1.00
F246xM250	1.00
F246xM251	1.00
F246xM252	1.00
F248xM224	1.00
F248xM225	1.00
F248xM226	1.00
F248xM227	1.00
F248xM233	0.92
F248xM234	0.94
F248xM240	0.89
F248xM241	0.97
F248xM243	0.98
F248xM244	1.00
F248xM247	1.00
F248xM250	0.99
F248xM251	0.97
F248xM252	1.00
F249xM224	1.00
F249xM225	1.00
F249xM226	1.00
F249xM227	1.00
F249xM233	0.94
F249xM234	1.00
F249xM240	0.91
F249xM241	1.00
F249xM243	1.00
F249xM244	1.00
F249xM247	1.00
F249xM250	1.00
F249xM251	1.00
F249xM252	1.00

Family CH1	Proportion
F253xM224	0.99
F253xM225	1.00
F253xM226	0.99
F253xM227	0.99
F253xM233	0.93
F253xM234	0.93
F253xM240	0.93
F253xM241	0.97
F253xM243	1.00
F253xM244	0.99
F253xM247	0.99
F253xM250	1.00
F253xM251	0.99
F253xM252	0.99
F254xM224	1.00
F254xM225	1.00
F254xM226	1.00
F254xM227	1.00
F254xM233	0.93
F254xM234	1.00
F254xM240	0.90
F254xM241	1.00
F254xM243	1.00
F254xM244	1.00
F254xM247	1.00
F254xM250	1.00
F254xM251	0.99
F254xM252	1.00

Appendix A7. Proportion of each family that can be assigned in CH3 by FAP.

Family CH3	Proportion
F259xM258	1
F259xM260	1
F259xM271	1
F259xM272	1
F259xM273	0.875
F259xM274	1
F259xM275	1
F259xM276	0.75
F259xM277	1
F259xM282	1
F259xM283	1
F259xM284	1
F259xM285	1
F259xM287	1
F261xM258	1
F261xM260	1
F261xM271	1
F261xM272	1
F261xM273	0.875
F261xM274	1
F261xM275	1
F261xM276	0.75
F261xM277	1
F261xM282	1
F261xM283	1
F261xM284	0.998
F261xM285	1
F261xM287	1
F262xM258	1
F262xM260	1
F262xM271	1
F262xM272	1
F262xM273	0.875
F262xM274	1
F262xM275	1
F262xM276	0.75
F262xM277	1
F262xM282	1
F262xM283	1
F262xM284	1
F262xM285	1
F262xM287	1

Family CH3	Proportion
F263xM258	1
F263xM260	1
F263xM271	1
F263xM272	1
F263xM273	0.875
F263xM274	0.997
F263xM275	0.999
F263xM276	0.75
F263xM277	1
F263xM282	1
F263xM283	1
F263xM284	1
F263xM285	1
F263xM287	1
F264xM258	1
F264xM260	1
F264xM271	1
F264xM272	1
F264xM273	0.875
F264xM274	1
F264xM275	1
F264xM276	0.75
F264xM277	1
F264xM282	1
F264xM283	1
F264xM284	1
F264xM285	1
F264xM287	0.999
F265xM258	1
F265xM260	1
F265xM271	1
F265xM272	1
F265xM273	0.875
F265xM274	1
F265xM275	1
F265xM276	0.75
F265xM277	1
F265xM282	1
F265xM283	1
F265xM284	1
F265xM285	1
F265xM287	1

Family CH3	Proportion
F266xM258	1
F266xM260	1
F266xM271	1
F266xM272	1
F266xM273	0.875
F266xM274	1
F266xM275	1
F266xM276	0.75
F266xM277	1
F266xM282	1
F266xM283	1
F266xM284	0.997
F266xM285	1
F266xM287	1
F267xM258	1
F267xM260	1
F267xM271	1
F267xM272	1
F267xM273	0.875
F267xM274	1
F267xM275	1
F267xM276	0.75
F267xM277	1
F267xM282	1
F267xM283	1
F267xM284	1
F267xM285	1
F267xM287	0.998
F268xM258	1
F268xM260	1
F268xM271	1
F268xM272	1
F268xM273	0.875
F268xM274	1
F268xM275	1
F268xM276	0.75
F268xM277	1
F268xM282	0.999
F268xM283	1
F268xM284	1
F268xM285	1
F268xM287	0.998

Appendix A7 cont'd.

Family CH3	Proportion
F269xM258	1
F269xM260	1
F269xM271	1
F269xM272	1
F269xM273	0.875
F269xM274	0.999
F269xM275	0.998
F269xM276	0.75
F269xM277	0.988
F269xM282	1
F269xM283	1
F269xM284	1
F269xM285	1
F269xM287	1
F270xM258	1
F270xM260	1
F270xM271	1
F270xM272	1
F270xM273	0.875
F270xM274	1
F270xM275	1
F270xM276	0.75
F270xM277	1
F270xM282	1
F270xM283	1
F270xM284	1
F270xM285	1
F270xM287	1
F278xM258	1
F278xM260	1
F278xM271	1
F278xM272	1
F278xM273	0.875
F278xM274	1
F278xM275	0.997
F278xM276	0.75
F278xM277	1
F278xM282	1
F278xM283	1
F278xM284	1
F278xM285	1
F278xM287	0.996

Family CH3	Proportion
F279xM258	1
F279xM260	1
F279xM271	1
F279xM272	1
F279xM273	0.875
F279xM274	1
F279xM275	1
F279xM276	0.75
F279xM277	1
F279xM282	1
F279xM283	1
F279xM284	1
F279xM285	1
F279xM287	1
F280xM258	1
F280xM260	1
F280xM271	1
F280xM272	1
F280xM273	0.875
F280xM274	1
F280xM275	1
F280xM276	0.75
F280xM277	1
F280xM282	1
F280xM283	1
F280xM284	1
F280xM285	1
F280xM287	0.997
F281xM258	1
F281xM260	1
F281xM271	1
F281xM272	1
F281xM273	0.875
F281xM274	1
F281xM275	1
F281xM276	0.75
F281xM277	1
F281xM282	1
F281xM283	1
F281xM284	1
F281xM285	1
F281xM287	1

Family CH3	Proportion
F286xM258	1
F286xM260	1
F286xM271	1
F286xM272	1
F286xM273	0.874
F286xM274	1
F286xM275	1
F286xM276	0.75
F286xM277	1
F286xM282	1
F286xM283	1
F286xM284	0.998
F286xM285	1
F286xM287	1

Appendix A8. Parentage assignment in CH1 performed with the likelihood method using Cervus software and exclusion with FAP software. L.T.: Loci typed, L.M.: Loci mismatched, C.M.: Candidate Mother, C.F.: Candidate Father. Delta: likelihood difference between the most likely and the second most likely parent. (ø) indicates an offspring with less than six loci typed among the 10 loci. (#) indicates the family assignment that differed between the two methods. (§) indicates an offspring with more than one family match in the exclusion analysis. (‡) indicates that likelihood-based method differed in the assignment when using 10 and 12 loci. N/A: no assignment made.

CH1	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
1	11	0	228	240	11.27	10	0	228	240	6.65	F228xM240
4	12	2	236	243	13.55	10	1	236	243	15.50	F236xM243
6	12	0	231	247	17.84	10	0	231	247	14.69	F231xM247
8(#)	11	0	231	240	0.01	10	0	231	240	0.01	F231xM233
9	12	0	231	233	16.24	10	0	231	233	11.38	F231xM233
11	12	0	231	233	10.90	10	0	231	233	6.04	F231xM233
12	10	0	231	247	11.76	9	0	231	247	10.25	F231xM247
14	11	0	231	247	17.88	10	0	231	247	16.38	F231xM247
15	12	0	246	252	32.15	10	0	246	252	25.85	F246xM252
16	11	0	228	252	17.13	10	0	228	252	11.25	F228xM252
17	12	0	231	233	15.32	10	0	231	233	10.74	F231xM233
18	12	0	228	252	12.17	10	0	228	252	7.51	F228xM252
19	12	1	228	234	11.68	10	1	228	234	6.63	F228xM234
21	11	1	231	233	7.09	10	1	231	233	5.71	F231xM233
22	11	1	230	244	6.43	10	1	230	244	7.11	F230xM244
23	11	0	231	233	5.70	9	0	231	233	0.68	F231xM233
24	12	0	230	244	13.94	10	0	230	244	13.01	F230xM244
25	11	1	228	240	9.49	10	1	228	240	7.55	F228xM240
26	11	0	231	247	14.78	10	0	231	247	13.79	F231xM247
27	12	0	231	233	14.57	10	0	231	233	10.58	F231xM233
28	12	0	231	233	10.43	10	0	231	233	5.42	F231xM233
29	12	0	231	233	10.50	10	0	231	233	5.48	F231xM233
30	12	1	228	234	11.92	10	1	228	234	6.54	F228xM234
31	11	0	231	233	10.17	10	0	231	233	5.30	F231xM233
32	12	0	248	247	19.45	10	0	248	247	11.09	F248xM247
34	12	0	231	247	14.47	10	0	231	247	12.80	F231xM247
35	12	0	231	247	20.23	10	0	231	247	16.56	F231xM247
36	6	0	231	233	5.99	6	0	231	233	5.99	F231xM233
37	11	1	228	240	5.39	10	1	228	240	0.01	(§)
38	11	0	231	247	13.92	10	0	231	247	12.55	F231xM247
39	12	1	228	234	10.95	10	1	228	234	6.44	F228xM234
40(‡)	12	0	231	233	4.55	10	0	231	240	0.01	F231xM233
41	12	0	231	233	10.34	10	0	231	233	5.48	F231xM233
43	11	0	231	247	16.95	10	0	231	247	15.81	F231xM247
44	10	0	231	233	5.71	9	0	231	233	5.71	F231xM233
46	12	1	231	247	10.89	10	1	231	247	8.61	F231xM247

Appendix A8 cont'd.

CH1	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
47	12	0	231	247	18.96	10	0	231	247	15.80	F231xM247
48	12	0	228	252	16.92	10	0	228	252	16.31	F228xM252
49	11	0	231	233	11.31	10	0	231	233	11.31	F231xM233
50	12	0	231	247	15.35	10	0	231	247	13.22	F231xM247
51	11	0	231	233	15.44	10	0	231	233	11.25	F231xM233
53	12	0	231	233	12.63	10	0	231	233	10.58	F231xM233
54	12	0	231	233	16.28	10	0	231	233	11.47	F231xM233
55	11	0	228	234	12.45	9	0	228	234	11.77	F228xM234
56	8	0	231	233	6.04	8	0	231	233	6.04	F231xM233
57	12	0	231	233	14.45	10	0	231	233	11.47	F231xM233
59	12	0	231	233	13.60	10	0	231	233	9.87	F231xM233
60	11	0	231	233	9.05	10	0	231	233	9.05	F231xM233
61	10	0	231	233	10.96	9	0	231	233	5.95	F231xM233
62	12	0	228	234	12.11	10	0	228	234	11.64	F228xM234
65	12	0	228	234	15.63	10	0	228	234	10.54	F228xM234
66	12	0	228	240	15.50	10	0	228	240	10.41	F228xM240
67	12	0	231	247	14.65	10	0	231	247	12.98	F231xM247
68	12	1	228	234	13.59	10	1	228	234	8.50	F228xM234
70	11	1	228	234	4.09	10	1	228	234	4.09	F228xM234
71	12	0	246	252	33.02	10	0	246	252	27.28	F246xM252
72	9	0	231	233	11.67	9	0	231	233	11.67	F231xM233
73	12	1	230	243	17.53	10	1	230	243	14.40	F230xM243
74	11	0	231	233	10.50	10	0	231	233	10.50	F231xM233
76	12	0	231	233	15.75	10	0	231	233	11.31	F231xM233
77	12	0	230	234	23.74	10	0	230	234	18.82	F230xM234
78	11	2	228	234	1.85	9	1	228	234	4.25	F228xM234
79	12	0	231	233	9.37	10	0	231	233	9.36	F231xM233
81	12	0	228	240	6.29	10	0	228	240	6.29	F228xM240
82	12	0	231	247	17.98	10	0	231	247	14.45	F231xM247
83	11	0	231	233	9.60	10	0	231	233	9.60	F231xM233
84	10	0	230	243	18.92	8	0	230	243	15.22	F230xM243
85	11	0	231	247	14.65	10	0	231	247	13.11	F231xM247
86	11	0	231	247	12.76	9	0	231	247	10.63	F231xM247
87	12	0	230	243	29.09	10	0	230	243	25.38	F230xM243
88	10	0	231	247	11.37	9	0	231	247	10.38	F231xM247
89	12	2	230	252	13.48	10	2	230	252	9.95	F230xM252
90	12	0	231	233	10.80	10	0	231	233	10.80	F231xM233
91	12	0	228	240	5.74	10	0	228	240	5.74	F228xM240
92	11	1	231	247	7.79	10	1	231	247	7.79	F231xM247
93	12	0	231	233	12.62	10	0	231	233	9.56	F231xM233
95	11	0	228	240	9.29	9	0	228	240	4.68	F228xM240
96	6	0	231	233	0.68	6	0	231	233	0.68	F231xM233
97	12	0	231	247	16.73	10	0	231	247	14.60	F231xM247
98	11	0	231	233	9.89	9	0	231	233	5.35	F231xM233

Appendix A8 cont'd.

CH1	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
99	12	0	246	252	31.68	10	0	246	252	25.62	F246xM252
100	12	1	231	247	14.83	10	0	231	247	16.62	F231xM247
101	12	0	231	247	18.59	10	0	231	247	15.29	F231xM247
102	12	0	236	243	13.10	10	0	236	243	6.02	F236xM243
103	12	1	231	233	11.92	10	1	231	233	9.68	F231xM233
104	12	0	231	247	16.19	10	0	231	247	13.92	F231xM247
105	12	0	231	233	9.66	10	0	231	233	9.66	F231xM233
106(‡)	12	0	231	233	4.85	10	0	231	240	0.01	F231xM233
107	8	0	246	250	3.67			246	250	3.67	N/A
108	11	1	231	233	9.39	10	1	231	233	7.81	F231xM233
109	12	0	231	247	17.31	10	0	231	247	14.15	F231xM247
110	12	0	228	240	9.17	10	0	228	240	4.68	F228xM240
112	12	2	231	233	4.64	10	1	231	233	7.46	F231xM233
113	12	0	228	240	11.26	10	0	228	240	6.60	F228xM240
114	12	1	231	247	9.04	10	1	231	247	7.37	F231xM247
118(‡)	11	1	231	233	5.00	9	1	231	240	0.01	(§)
120	12	1	228	252	7.51	10	1	228	252	7.51	F228xM252
121	12	1	228	234	11.90	10	1	228	234	6.63	F228xM234
122	9	1	231	233	5.50	8	1	231	233	4.50	F231xM233
123	9	2	228	233	0.24	8	1	228	233	1.57	(§)
124	12	1	236	241	6.08	10	1	236	241	4.43	F236xM241
125	12	0	228	252	14.25	10	0	228	252	13.77	F228xM252
127	12	0	228	234	8.02	10	0	228	234	7.53	F228xM234
128	11	0	231	233	14.03	10	0	231	233	13.04	F231xM233
129	12	0	242	240	17.41	10	0	242	240	11.37	F242xM240
130	12	2	231	247	3.02	10	2	231	247	0.74	F231xM247
131	12	0	231	247	14.40	10	0	231	247	12.49	F231xM247
132	12	1	231	247	13.60	10	1	231	247	10.08	F231xM247
133	12	0	231	247	18.36	10	0	231	247	14.28	F231xM247
134	12	0	231	233	14.16	10	0	231	233	12.51	F231xM233
135	12	0	228	234	15.19	10	0	228	234	12.61	F228xM234
136	12	0	231	233	10.11	10	0	231	233	5.29	F231xM233
138	12	0	231	233	9.05	10	0	231	233	9.05	F231xM233
139	12	0	231	247	13.19	10	0	231	247	11.43	F231xM247
140	9	1	228	234	5.74	7	1	228	234	0.69	(§)
141	12	0	228	240	9.29	10	0	228	240	4.67	F228xM240
142(‡)	11	1	236	243	1.65	9	1	N/A	N/A		(§)
143	12	1	228	234	10.47	10	1	228	234	9.57	F228xM234
144(‡)	6	2	230	243	7.35	5	2	(ø)	(ø)		(ø)
145	12	1	236	247	10.59	10	1	236	247	10.90	F236xM247
147	11	0	231	233	11.45	10	0	231	233	10.20	F231xM233
148	12	0	231	233	14.28	10	0	231	233	11.93	F231xM233
149	12	0	228	234	10.55	10	0	228	234	5.46	F228xM234
150	12	0	228	234	11.10	10	0	228	234	6.59	F228xM234

Appendix A8 cont'd.

CH1	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
152	12	0	231	233	14.36	10	0	231	233	11.34	F231xM233
153	12	1	228	234	11.42	10	1	228	234	8.00	F228xM234
154	11	1	231	233	8.88	9	1	231	233	6.64	F231xM233
155	12	0	237	251	14.95	10	0	237	251	14.95	F237xM251
157	12	1	239	241	0.20	10	1	239	241	0.00	(§)
158	11	0	231	233	11.36	9	0	231	233	10.70	F231xM233
159	7	1	254	233	3.58			254	233	0.68	N/A
160	12	1	228	234	10.50	10	1	228	234	10.50	F228xM234
161	12	0	230	243	14.41	10	0	230	243	15.07	F230xM243
163	12	0	228	252	18.31	10	0	228	252	12.43	F228xM252
164	11	0	231	247	18.28	9	0	231	247	14.75	F231xM247
165	12	0	228	240	5.28	10	0	228	240	0.01	(§)
166	12	0	231	247	11.79	10	0	231	247	11.04	F231xM247
167	9	0	230	244	15.18	7	0	230	244	12.56	F230xM244
169	11	0	231	247	16.62	9	0	231	247	13.46	F231xM247
173	12	0	230	243	24.88	10	0	230	243	21.32	F230xM243
174	12	0	228	234	13.48	10	0	228	234	7.53	F228xM234
175	12	0	228	240	10.33	10	0	228	240	5.67	F228xM240
176	10	1	236	243	10.25	8	1	236	243	4.52	F236xM243
177	12	0	231	247	13.94	10	0	231	247	13.35	F231xM247
178	12	1	230	243	8.73	10	0	230	243	5.75	F230xM243
179	12	0	231	233	10.44	10	0	231	233	5.42	F231xM233
182	12	0	231	247	17.70	10	0	231	247	14.17	F231xM247
183	12	1	228	234	13.62	10	1	228	234	8.19	F228xM234
185	12	0	228	252	13.32	10	0	228	252	11.25	F228xM252
186	12	0	231	233	15.02	10	0	231	233	10.90	F231xM233
187	12	0	231	233	14.09	10	0	231	233	12.81	F231xM233
188	12	0	231	233	10.73	10	0	231	233	5.71	F231xM233
189	12	0	231	247	17.30	10	0	231	247	14.14	F231xM247
190	12	1	231	247	11.27	10	1	231	247	8.99	F231xM247
191	12	0	230	243	17.15	10	0	230	243	16.36	F230xM243
192	12	0	236	241	7.07	10	0	236	241	5.41	F236xM241
193	12	1	231	247	8.07	10	1	231	247	7.10	F231xM247
194	12	0	230	243	26.22	10	0	230	243	22.66	F230xM243
195	12	0	230	243	23.75	10	0	230	243	20.05	F230xM243
196	12	0	228	234	13.60	10	0	228	234	12.58	F228xM234
197	12	0	228	234	11.91	10	0	228	234	11.23	F228xM234
198	12	0	231	247	17.07	10	0	231	247	12.61	F231xM247
199	11	0	230	243	20.96	9	0	230	243	17.40	F230xM243
201	12	0	231	247	18.93	10	0	231	247	15.40	F231xM247
202	12	1	231	233	8.22	10	1	231	233	6.17	F231xM233
203	12	0	242	250	24.55	10	0	242	250	18.83	F242xM250
205	11	0	231	247	14.20	9	0	231	247	10.79	F231xM247

Appendix A8 cont'd.

CH1	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
206(‡)	6	1	228	243	0.52			N/A	N/A		N/A
207	12	0	231	247	12.13	10	0	231	247	8.04	F231xM247
208	12	0	228	252	19.17	10	0	228	252	16.61	F228xM252
209	12	0	230	243	30.54	10	0	230	243	26.98	F230xM243
211	11	2	231	233	5.46	9	2	231	233	3.60	F231xM233
212	11	1	228	252	9.77	9	1	228	252	8.83	F228xM252
213	12	0	230	243	7.07	10	0	230	243	7.07	F230xM243
214	12	1	228	240	9.95	10	0	228	240	4.67	F228xM240
215	12	0	231	247	14.21	10	0	231	247	13.24	F231xM247
217	11	0	231	233	10.73	9	0	231	233	5.71	F231xM233
218	12	0	231	247	11.54	10	0	231	247	10.43	F231xM247
219	12	0	231	247	11.76	10	0	231	247	10.79	F231xM247
220(‡)	11	2	231	233	1.67	9	2	231	240	0.01	(§)
221	12	0	231	247	18.14	10	0	231	247	14.61	F231xM247
222	12	0	228	240	16.02	10	0	228	240	10.64	F228xM240
223	12	2	231	233	6.31	10	2	231	233	4.06	F231xM233

Appendix A9. Parentage assignment in CH3 performed with the likelihood method using Cervus software and exclusion with FAP software. L.T.: Loci typed, L.M.: Loci mismatched, C.M.: Candidate Mother, C.F.: Candidate Father. Delta: likelihood difference between the most likely and the second most likely parent. (*) indicates more than two loci mismatches. (§) indicates an offspring with more than one family match in the exclusion analysis. (‡) indicates that likelihood-based method differed in the assignment when using 10 and 12 loci. N/A: no assignment made.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
1	12	0	281	271	25.54	10	0	281	271	21.62	F281xM271
2	12	0	267	276	1.29	10	0	267	276	0.69	(§)
3(‡)	9	1	270	277	3.22	7	1	269	277	0.99	N/A
4	12	0	270	271	16.27	10	0	270	271	16.27	F270xM271
6	12	0	267	276	1.29	10	0	267	276	0.69	(§)
7	11	1	270	276	6.26	9	1	270	276	4.77	F270xM276
9	12	0	267	276	8.52	10	0	267	276	6.78	F267xM276
10	12	0	270	275	15.34	10	0	270	275	13.71	F270xM275
11	12	0	266	283	22.48	10	0	266	283	17.51	F266xM283
12	12	0	281	271	29.68	10	0	281	271	23.23	F281xM271
13	12	1	270	275	8.38	10	1	270	275	6.93	F270xM275
14	12	0	267	276	8.52	10	0	267	276	6.78	F267xM276
15	12	0	270	276	7.92	10	0	270	276	6.17	F270xM276
16	12	0	263	273	17.92	10	0	263	273	19.23	F263xM273
17	11	2	270	273	0.44	9	2	270	273	0.52	(§)
18	12	3	(*)	(*)		10	2	267	287	5.64	F267xM287
19	12	0	270	275	13.21	10	0	270	275	11.68	F270xM275
20	12	1	267	258	13.24	10	1	267	258	12.28	F267xM258
21	12	1	270	275	12.73	10	1	270	275	9.51	F270xM275
22	12	3	(*)	(*)		10	2	270	283	3.44	F270xM283
23	8	1	270	282	1.53	8	1	270	282	1.53	F270xM282
24	12	0	267	258	14.49	10	0	267	258	9.99	F267xM258
25	12	0	270	276	14.07	10	0	270	276	10.60	F270xM276
26	12	0	270	275	9.86	10	0	270	275	10.54	F270xM275
27	12	0	267	258	16.53	10	0	267	258	16.53	F267xM258
28	11	0	267	276	4.21	9	0	267	276	4.21	F267xM276
29	12	0	281	271	28.90	10	0	281	271	18.68	F281xM271
30	12	1	270	275	11.47	10	1	270	275	4.99	F270xM275
31	12	0	267	258	10.88	10	0	267	258	10.88	F267xM258
32	12	1	267	276	8.52	10	1	267	276	6.78	F267xM276
33	12	0	267	276	8.52	10	1	267	276	6.78	F267xM276
34	12	1	270	275	4.27	10	1	270	275	4.95	F270xM275
35	11	0	270	275	12.50	9	0	270	275	10.82	F270xM275
36	11	0	266	283	21.72	9	0	266	283	16.07	F266xM283
37	11	0	267	276	6.51	10	0	267	276	6.51	F267xM276
38	12	0	267	258	15.52	10	0	267	258	10.18	F267xM258

Appendix A9 cont'd.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
39	12	0	267	276	7.39	10	0	267	276	6.78	F267xM276
40	12	0	270	275	18.41	10	0	270	275	15.53	F270xM275
41	10	1	269	272	6.52	8	1	269	272	5.83	F269xM272
42	11	0	270	275	10.13	9	0	270	275	9.91	F270xM275
43	12	1	270	276	6.59	10	1	270	276	6.51	F270xM276
44	12	1	270	275	7.88	10	1	270	275	4.08	F270xM275
45	11	0	270	276	1.30	9	0	270	276	0.69	(§)
46	12	0	270	275	11.68	10	0	270	275	9.84	F270xM275
47	11	0	270	275	13.78	10	0	270	275	13.36	F270xM275
50	12	0	279	272	31.90	10	0	279	272	29.52	F279xM272
51	12	0	267	258	20.02	10	0	267	258	16.87	F267xM258
52	12	0	281	271	34.08	10	0	281	271	28.69	F281xM271
53	10	1	267	285	4.89	8	2	267	285	4.89	F267xM285
54	12	0	270	276	12.94	10	0	270	276	12.33	F270xM276
55	12	0	269	273	18.85	10	0	269	273	19.46	F269xM273
56	12	0	267	285	20.95	10	0	267	285	16.77	F267xM285
57	12	0	267	276	7.11	10	0	267	276	6.51	F267xM276
58	12	0	270	275	18.99	10	0	270	275	15.77	F270xM275
59	12	0	270	275	12.52	10	0	270	275	12.07	F270xM275
60	12	1	270	275	8.02	10	1	270	275	7.57	F270xM275
61	9	2	281	271	8.64	8	2	281	271	5.78	F281xM271
62	12	0	281	271	30.75	10	0	281	271	18.65	F281xM271
63	9	0	270	275	10.40	7	0	270	275	8.76	F270xM275
66	12	0	270	275	15.39	10	0	270	275	13.76	F270xM275
68	12	0	267	276	8.52	10	0	267	276	6.78	F267xM276
70	12	0	270	276	2.43	10	0	270	276	0.69	(§)
74	11	0	270	275	13.13	10	0	270	275	10.65	F270xM275
75	12	1	270	275	10.58	10	1	270	275	7.70	F270xM275
76	12	0	270	275	16.68	10	0	270	275	13.46	F270xM275
79	12	0	270	275	17.02	10	0	270	275	14.37	F270xM275
80	12	0	263	272	11.75	10	0	263	272	11.75	F263xM272
81	10	1	279	272	13.54	8	1	279	272	12.86	F279xM272
82	12	0	267	276	8.52	10	0	267	276	6.78	F267xM276
83	12	1	270	276	2.43	10	1	270	276	0.69	(§)
84	12	0	270	275	16.59	10	0	270	275	13.71	F270xM275
85	11	0	270	276	7.24	9	0	270	276	6.63	F270xM276
86	11	0	267	276	2.43	9	0	267	276	0.69	(§)
87	12	0	270	276	8.25	10	0	270	276	6.51	F270xM276
89	11	1	266	285	11.38	9	1	266	285	11.38	F266xM285
90	12	0	263	273	18.73	10	0	263	273	19.34	F263xM273
91	12	0	267	276	8.25	10	0	267	276	6.51	F267xM276
92	12	0	267	285	20.90	10	0	267	285	17.04	F267xM285
93	12	1	270	276	12.96	10	1	270	276	10.20	F270xM276

Appendix A9 cont'd.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
94(‡)	9	2	266	275	0.09	7	2	278	283	0.27	N/A
96	12	0	270	275	13.65	10	0	270	275	12.20	F270xM275
97	11	2	281	283	8.58	9	1	281	283	11.31	F281xM283
98	12	1	270	275	3.12	10	1	270	275	3.80	F270xM275
99	12	0	267	258	13.13	10	0	267	258	12.45	F267xM258
100	12	0	267	258	21.52	10	0	267	258	17.04	F267xM258
101	12	1	267	276	1.04	10	0	267	276	0.69	(§)
103	12	0	267	258	10.24	10	0	267	258	10.24	F267xM258
104	12	0	267	258	10.63	10	0	267	258	10.63	F267xM258
105	11	1	270	285	12.92	9	1	270	285	11.24	F270xM285
106	12	1	267	276	14.35	10	1	267	276	11.64	F267xM276
108	12	1	270	276	2.43	10	1	270	276	0.69	(§)
109	12	0	267	258	10.81	10	0	267	258	10.81	F267xM258
110	12	0	281	271	30.82	10	0	281	271	25.43	F281xM271
111	12	0	270	275	14.67	10	0	270	275	11.90	F270xM275
112	11	0	270	275	11.51	9	0	270	275	10.40	F270xM275
113(‡)	6	1	263	274	3.03	10		N/A	N/A		N/A
114	12	0	267	276	14.60	10	0	267	276	12.86	F267xM276
115	12	0	267	258	22.77	10	0	267	258	19.39	F267xM258
116	12	1	263	276	2.43	10	1	263	276	0.69	(§)
117	12	0	267	258	20.92	10	0	267	258	16.43	F267xM258
118	12	0	267	276	8.25	10	0	267	276	6.51	F267xM276
119	12	0	281	271	25.87	10	0	281	271	22.56	F281xM271
120	12	1	267	276	12.75	10	1	267	276	11.18	F267xM276
121	12	0	267	258	21.54	10	0	267	258	19.84	F267xM258
122	12	0	267	258	15.99	10	0	267	258	15.99	F267xM258
123	11	0	270	275	16.52	9	0	270	275	13.87	F270xM275
125	11	1	270	275	6.18	9	1	270	275	5.62	F270xM275
126	12	0	270	275	12.58	10	0	270	275	9.05	F270xM275
127	12	2	270	282	12.30	10	2	270	282	9.84	F270xM282
128	12	0	267	276	7.11	10	0	267	276	6.51	F267xM276
129	11	0	270	276	1.30	10	0	270	276	0.69	(§)
130	12	0	267	276	7.11	10	0	267	276	6.50	F267xM276
131	12	0	267	276	10.01	10	0	267	276	10.01	F267xM276
133	12	0	270	276	12.61	10	0	270	276	12.00	F270xM276
135	12	0	267	258	16.81	10	0	267	258	16.81	F267xM258
136	12	0	279	272	12.60	10	0	279	272	11.92	F279xM272
137	12	0	270	275	8.89	10	0	270	275	9.57	F270xM275
138	12	0	267	258	23.29	10	0	267	258	20.57	F267xM258
139	12	0	270	276	7.12	10	0	270	276	6.51	F270xM276
140	11	0	267	276	1.29	9	0	267	276	0.69	(§)
141	12	0	270	275	7.64	10	0	270	275	8.32	F270xM275
142	12	0	270	275	18.40	10	0	270	275	15.51	F270xM275

Appendix A9 cont'd.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
143	12	0	270	275	15.79	10	0	270	275	13.14	F270xM275
145	12	0	270	276	8.25	10	0	270	276	6.51	F270xM276
146	12	1	270	276	11.74	10	1	270	276	9.48	F270xM276
147	12	0	270	271	19.79	10	0	270	271	18.00	F270xM271
148	12	0	270	276	12.94	10	0	270	276	12.33	F270xM276
150	12	0	270	275	13.66	10	0	270	275	12.25	F270xM275
151	12	0	270	275	12.66	10	0	270	275	10.31	F270xM275
152	11	0	270	275	14.23	10	0	270	275	13.35	F270xM275
153	11	1	270	276	5.90	10	1	270	276	5.82	F270xM276
154	11	0	267	258	6.28	9	0	267	258	5.60	F267xM258
155	12	1	267	285	6.48	10	1	267	285	6.48	F267xM285
156	12	0	270	275	16.33	10	0	270	275	13.67	F270xM275
158	11	1	267	276	7.39	9	1	267	276	6.78	F267xM276
159	12	0	270	275	13.27	10	0	270	275	12.16	F270xM275
160	12	0	267	276	8.53	10	0	267	276	6.78	F267xM276
162	11	0	267	258	10.62	9	0	267	258	10.62	F267xM258
163	11	0	270	275	8.55	9	0	270	275	9.23	F270xM275
164	9	2	270	275	3.88	8	2	270	275	1.87	F270xM275
165	11	0	267	276	2.43	9	0	267	276	0.69	(§)
166	11	2	280	287	9.01	10	2	280	287	8.55	F280xM287
167	12	0	267	276	7.64	10	0	267	276	7.04	F267xM276
168	8	1	270	282	6.61	7	1	270	282	4.60	N/A
173(‡)	12	2	270	271	4.10	10	2	263	271	0.98	N/A
174	11	0	267	258	10.88	10	0	267	258	10.88	F267xM258
175	10	0	267	276	0.69	9	0	267	276	0.69	(§)
176	12	2	267	258	13.03	10	2	267	258	15.40	F267xM258
177	12	0	270	275	15.21	10	0	270	275	13.58	F270xM275
178	11	0	270	276	12.45	10	0	270	276	12.45	F270xM276
179	12	0	263	273	17.72	10	0	263	273	17.17	F263xM273
180	12	2	266	285	9.06	10	2	266	285	8.89	F266xM285
181	12	0	281	271	25.09	10	0	281	271	21.90	F281xM271
183	10	1	270	276	6.51	9	1	270	276	6.51	F270xM276
185	12	2	267	273	0.40	10	2	267	273	1.01	N/A
186	12	0	267	276	8.25	10	0	267	276	6.51	F267xM276
187	9	1	270	275	4.03	8	1	270	275	4.46	F270xM275
188	11	0	270	275	15.67	9	0	270	275	12.67	F270xM275
189	12	0	267	276	8.78	10	0	267	276	7.04	F267xM276
190	12	0	270	276	7.12	10	0	270	276	6.51	F270xM276
191	11	0	270	275	9.50	9	0	270	275	9.94	F270xM275
192	12	1	270	276	2.43	10	1	270	276	0.69	(§)
193	12	0	270	276	7.91	10	0	270	276	6.17	F270xM276
194	12	2	270	282	3.06	10	2	270	282	3.06	F270xM282
195	12	1	270	275	12.93	10	1	270	275	9.71	F270xM275

Appendix A9 cont'd.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
196	10	2	270	271	5.55	9	2	270	271	5.09	F270xM271
198	12	0	267	273	4.92	10	0	267	273	5.53	F267xM273
199	11	0	270	275	16.32	9	0	270	275	13.44	F270xM275
200	12	0	267	276	7.11	10	0	267	276	6.51	F267xM276
201	12	1	270	275	8.28	10	1	270	275	7.49	F270xM275
202	12	0	279	272	12.57	10	0	279	272	11.89	F279xM272
204	12	0	267	276	1.29	10	0	267	276	0.69	(§)
205	12	0	281	271	21.61	10	0	281	271	11.23	F281xM271
206	11	2	281	271	18.43	9	2	281	271	11.90	N/A
207	12	2	270	275	5.32	10	2	270	275	2.32	N/A
208	11	2	281	271	12.27	9	2	281	271	7.33	F281xM271
209	12	2	270	276	5.69	10	2	270	276	2.57	N/A
210	12	1	270	276	5.57	10	1	270	276	5.49	F270xM276
211	11	0	267	276	2.43	9	0	267	276	0.69	(§)
212	12	0	265	275	22.31	10	0	265	275	19.60	F265xM275
213	11	1	280	274	15.82	9	1	280	274	13.18	F280xM274
214	12	0	267	276	8.52	10	0	267	276	6.78	F267xM276
215	12	0	281	271	32.53	10	0	281	271	27.58	F281xM271
216	12	0	281	271	19.67	10	0	281	271	19.89	F281xM271
217	12	0	267	258	15.85	10	0	267	258	15.85	F267xM258
218	12	2	281	271	12.71	10	2	281	271	8.43	N/A
219	12	0	281	271	24.02	10	0	281	271	23.84	F281xM271
223	11	0	266	283	17.48	9	0	266	283	16.80	F266xM283
224	11	0	270	275	9.40	10	0	270	275	10.08	F270xM275
225	11	0	267	258	5.70	9	0	267	258	5.70	F267xM258
226	11	0	270	275	8.94	9	0	270	275	4.84	F270xM275
227	12	1	267	258	11.82	10	1	267	258	11.82	F267xM258
228	12	0	263	276	1.30	10	0	263	276	0.69	(§)
229	12	1	270	276	8.07	10	1	270	276	5.82	F270xM276
230	12	2	281	271	15.33	10	2	281	271	9.42	F281xM271
232	12	2	267	276	2.43	10	2	267	276	0.69	(§)
233	12	0	267	276	8.25	10	0	267	276	6.51	F267xM276
234	10	0	270	275	5.16	8	0	270	275	0.68	(§)
235	10	0	270	275	13.42	9	0	270	275	12.95	F270xM275
236	12	1	270	275	11.81	10	1	270	275	8.93	F270xM275
237	9	1	270	275	5.05	8	1	270	275	4.59	F270xM275
238	12	0	281	271	31.50	10	0	281	271	25.08	F281xM271
239	12	0	281	271	26.87	10	0	281	271	20.83	F281xM271
240	12	0	270	271	22.39	10	0	270	271	19.62	F270xM271
241	11	0	267	276	8.24	9	0	267	276	6.50	F267xM276
242	12	2	281	271	13.03	10	2	281	271	9.11	N/A
243	12	2	267	276	2.43	10	2	267	276	0.69	(§)
244	12	2	267	258	5.49	10	2	267	258	2.11	F267xM258
246	12	0	281	271	32.89	10	0	281	271	25.54	F281xM271

Appendix A9 cont'd.

CH3	12 Loci		Likelihood			10 Loci		Likelihood			Exclusion
ID	L.T.	L.M.	C.M.	C.F.	Delta	L.T.	L.M.	C.M.	C.F.	Delta	Assignment
248(‡)	9	0	270	275	2.85	7	0	270	277	0.69	(§)
250	11	0	267	276	7.12	9	0	267	276	6.51	F267xM276
252	12	0	270	275	11.40	10	0	270	275	10.00	F270xM275
253	12	1	267	258	14.19	10	1	267	258	12.95	F267xM258
254	9	0	281	271	20.83	7	0	281	271	14.06	F281xM271
255	11	1	270	275	7.17	9	1	270	275	5.83	F270xM275
256	12	2	267	276	1.61	10	2	267	276	0.04	N/A

Appendix A10. H-W tests output files from Genepop program for the CH1 offspring.

Offspring CH1 N=223

Hardy Weinberg: Probability test
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.0000	0.0000	-0.0387	18368 switches
OtsG249	0.0000	0.0000	-0.0867	13701 switches
Ots3	0.0000	0.0000	0.0065	14358 switches
Ots4	0.0404	0.0066	0.0143	27806 switches
Omy325	0.0008	0.0005	-0.0942	30211 switches
Ots104	0.0000	0.0000	-0.0954	14530 switches
Ots107	0.0000	0.0000	-0.0301	38510 switches
OtsG311	0.0000	0.0000	0.0393	24302 switches
OtsG68	0.0000	0.0000	-0.0442	7698 switches
OtsG83b	0.0000	0.0000	0.0195	21154 switches
OtsG432	0.0000	0.0000	0.0095	24701 switches
Ots13	0.0019	0.0012	0.0103	12003 switches

Hardy Weinberg test when H1= heterozygote deficit
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.9718	0.0125	-0.0387	18546 switches
OtsG249	0.9992	0.0008	-0.0867	13464 switches
Ots3	0.8489	0.0292	0.0065	14543 switches
Ots4	0.0366	0.0036	0.0143	27858 switches
Omy325	0.9988	0.0008	-0.0942	30369 switches
Ots104	0.9994	0.0006	-0.0954	14553 switches
Ots107	0.9786	0.0050	-0.0301	38251 switches
OtsG311	0.0925	0.0147	0.0393	24271 switches
OtsG68	0.7671	0.0435	-0.0442	8009 switches
OtsG83b	0.7174	0.0307	0.0195	21026 switches
OtsG432	0.4739	0.0262	0.0095	24996 switches
Ots13	0.0038	0.0017	0.0103	12022 switches

Hardy Weinberg test when H1= heterozygote excess
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.0182	0.0056	-0.0387	18547 switches
OtsG249	0.0024	0.0024	-0.0867	13697 switches
Ots3	0.1746	0.0256	0.0065	14379 switches
Ots4	0.9675	0.0039	0.0143	27870 switches
Omy325	0.0000	0.0000	-0.0942	30448 switches
Ots104	0.0034	0.0034	-0.0954	14581 switches
Ots107	0.0252	0.0054	-0.0301	38266 switches
OtsG311	0.8975	0.0122	0.0393	24201 switches
OtsG68	0.2224	0.0362	-0.0442	8084 switches
OtsG83b	0.3201	0.0383	0.0195	21572 switches
OtsG432	0.4899	0.0240	0.0095	24730 switches
Ots13	0.9951	0.0018	0.0103	12412 switches

Appendix A11. H-W tests output files from Genepop program for the CH3 offspring.

Offspring CH3 N=257

Hardy Weinberg: Probability test
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.0000	0.0000	0.1024	13912 switches
OtsG249	0.0000	0.0000	-0.1306	27764 switches
Ots3	0.0000	0.0000	0.0141	26746 switches
Ots4	0.0000	0.0000	-0.0870	22012 switches
Omy325	0.0000	0.0000	-0.0610	40040 switches
Ots104	0.0000	0.0000	-0.0056	10383 switches
Ots107	0.0000	0.0000	-0.1039	17432 switches
OtsG311	0.0000	0.0000	-0.0489	9126 switches
OtsG68	0.0000	0.0000	-0.0503	9063 switches
OtsG83b	0.0000	0.0000	-0.1076	25110 switches
OtsG432	0.0000	0.0000	0.0098	29288 switches
Ots13	0.0001	0.0001	0.0036	26207 switches

Hardy Weinberg test when H1= heterozygote deficit
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.2144	0.0411	0.1024	13842 switches
OtsG249	0.9986	0.0012	-0.1306	27395 switches
Ots3	0.8969	0.0124	0.0141	26500 switches
Ots4	0.9992	0.0004	-0.0870	22029 switches
Omy325	0.9671	0.0071	-0.0610	40033 switches
Ots104	0.0872	0.0112	-0.0056	10454 switches
Ots107	0.9222	0.0219	-0.1039	17405 switches
OtsG311	0.5284	0.0534	-0.0489	9020 switches
OtsG68	0.1348	0.0274	-0.0503	9439 switches
OtsG83b	1.0000	0.0000	-0.1076	25262 switches
OtsG432	0.8362	0.0144	0.0098	29121 switches
Ots13	0.8407	0.0131	0.0036	26056 switches

Hardy Weinberg test when H1= heterozygote excess
****

locus	P-val	S.E.	W&C	Steps
Ots253b	0.8445	0.0135	0.1024	13995 switches
OtsG249	0.0021	0.0014	-0.1306	27584 switches
Ots3	0.1018	0.0091	0.0141	26608 switches
Ots4	0.0000	0.0000	-0.0870	22152 switches
Omy325	0.0490	0.0124	-0.0610	39907 switches
Ots104	0.9252	0.0227	-0.0056	10438 switches
Ots107	0.0414	0.0180	-0.1039	17469 switches
OtsG311	0.4294	0.0579	-0.0489	9338 switches
OtsG68	0.8605	0.0276	-0.0503	9279 switches
OtsG83b	0.0000	0.0000	-0.1076	24768 switches
OtsG432	0.1826	0.0183	0.0098	29265 switches
Ots13	0.1633	0.0138	0.0036	26051 switches

Appendix A12. H-W tests output files from Genepop program for the CH1 brood stock.

Brood stock CH1 N=31

Hardy Weinberg: Probability test
****

locus	P-val	S.E.	W&C	Steps	
Ots253b	0.3247	0.0209	-0.0843	13411	switches
OtsG249	0.5750	0.0101	-0.0501	41380	switches
Ots3	0.3691	0.0111	-0.0237	34507	switches
Ots4	0.4761	0.0078	-0.2489	30879	switches
Omy325	0.1584	0.0033	-0.0499	171252	switches
Ots104	0.2589	0.0211	0.0530	9919	switches
Ots107	0.0228	0.0030	0.0536	39637	switches
OtsG311	0.0010	0.0004	0.1692	28381	switches
OtsG68	0.2513	0.0250	-0.0195	9260	switches
OtsG83b	0.1839	0.0095	-0.1245	32316	switches
OtsG432	0.8352	0.0048	0.0008	54063	switches
Ots13	0.1575	0.0086	0.0588	23043	switches

Hardy Weinberg test when H1= heterozygote deficit
****

locus	P-val	S.E.	W&C	Steps	
Ots253b	0.4661	0.0190	-0.0843	12937	switches
OtsG249	0.5230	0.0110	-0.0501	41422	switches
Ots3	0.7275	0.0088	-0.0237	34088	switches
Ots4	0.9724	0.0032	-0.2489	30551	switches
Omy325	0.7491	0.0040	-0.0499	171119	switches
Ots104	0.5307	0.0263	0.0530	9811	switches
Ots107	0.1615	0.0085	0.0536	39619	switches
OtsG311	0.0008	0.0003	0.1692	28392	switches
OtsG68	0.8239	0.0188	-0.0195	9268	switches
OtsG83b	1.0000	0.0000	-0.1245	32294	switches
OtsG432	0.5249	0.0098	0.0008	54198	switches
Ots13	0.4902	0.0100	0.0588	22790	switches

Hardy Weinberg test when H1= heterozygote excess
****

locus	P-val	S.E.	W&C	Steps	
Ots253b	0.4990	0.0174	-0.0843	12959	switches
OtsG249	0.5037	0.0108	-0.0501	41536	switches
Ots3	0.3964	0.0098	-0.0237	35014	switches
Ots4	0.0482	0.0044	-0.2489	30757	switches
Omy325	0.2400	0.0040	-0.0499	170975	switches
Ots104	0.6584	0.0232	0.0530	9800	switches
Ots107	0.8458	0.0070	0.0536	39437	switches
OtsG311	0.9974	0.0008	0.1692	28090	switches
OtsG68	0.2717	0.0219	-0.0195	9327	switches
OtsG83b	0.0232	0.0027	-0.1245	32416	switches
OtsG432	0.4895	0.0090	0.0008	54531	switches
Ots13	0.5848	0.0088	0.0588	22933	switches

Appendix A13. H-W tests output files from Genepop program for the CH3 brood stock.

Brood stock CH3 N=30

Hardy Weinberg: Probability test
****

locus	P-val	S.E.	W&C	Steps	
Ots253b	0.4741	0.0173	-0.0247	13135	switches
OtsG249	0.4515	0.0117	-0.1027	25859	switches
Ots3	0.6617	0.0079	-0.1900	42038	switches
Ots4	0.0890	-	-0.2915	215	matrices
Omy325	0.3142	0.0055	-0.1301	88261	switches
Ots104	0.8621	0.0122	-0.0503	13143	switches
Ots107	0.3270	0.0097	-0.1191	44020	switches
OtsG311	0.7160	0.0127	-0.0965	27839	switches
OtsG68	0.9679	0.0064	-0.0675	7907	switches
OtsG83b	0.1268	0.0116	-0.0686	18710	switches
OtsG432	0.9745	0.0019	-0.1020	47215	switches
Ots13	0.7171	0.0086	-0.1778	42015	switches

Hardy Weinberg test when H1= heterozygote deficit
****

locus	P-val	S.E.	W&C	Steps	
Ots253b	0.6879	0.0180	-0.0247	13198	switches
OtsG249	1.0000	0.0000	-0.1027	25965	switches
Ots3	0.9914	0.0012	-0.1900	41968	switches
Ots4	0.9708	-	-0.2915	215	matrices
Omy325	0.9715	0.0017	-0.1301	88031	switches
Ots104	0.6390	0.0202	-0.0503	13340	switches
Ots107	0.9882	0.0018	-0.1191	44148	switches
OtsG311	0.9759	0.0033	-0.0965	27936	switches
OtsG68	0.8521	0.0186	-0.0675	7833	switches
OtsG83b	0.8919	0.0096	-0.0686	18461	switches
OtsG432	0.9822	0.0018	-0.1020	47421	switches
Ots13	0.9887	0.0015	-0.1778	41286	switches

Hardy Weinberg test when H1= heterozygote excess
****

locus	P-val	S.E.	W&C	R&H	Steps	
Ots253b	0.3597	0.0187	-0.0247	13316	switches	
OtsG249	0.0571	0.0055	-0.1027	26140	switches	
Ots3	0.0293	0.0025	-0.1900	42549	switches	
Ots4	0.0437	-	-0.2915	215	matrices	
Omy325	0.0384	0.0018	-0.1301	88202	switches	
Ots104	0.3462	0.0213	-0.0503	13337	switches	
Ots107	0.0414	0.0028	-0.1191	43945	switches	
OtsG311	0.0541	0.0054	-0.0965	27916	switches	
OtsG68	0.1893	0.0210	-0.0675	8083	switches	
OtsG83b	0.1474	0.0123	-0.0686	18782	switches	
OtsG432	0.0663	0.0042	-0.1020	47044	switches	
Ots13	0.0478	0.0040	-0.1778	41935	switches	

Appendix A14. Chi-squared tests for the six major families in CH1 for all 12 loci genotyped. O: observed number of individuals with a particular genotype. E: expected number of individuals with a particular genotype. N: number of individuals assigned to that family genotyped for a particular locus. X^2: chi-squared value. d.f.: degrees of freedom. *P*: probability associated to the X^2 value. Significant tests are highlighted. (*) indicate families with a low number of assigned offspring which had expected frequency cells lower than 5. A total of eight tests resulted significant.

Appendix A14 cont'd.

CH1	F228xM234			F228xM240			F228xM252			F230xM243			F231xM233			F231xM247		
OtsG253b	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E	Genotype	O	E
	164 208	4	5.25	164 208	4	2.75	164 204	3	2.25	160 208	6	6	188 208	14	12.75	160 188	17	10.75
	164 216	7	5.25	164 236	2	2.75	164 256	3	2.25	208 208	6	6	188 236	14	12.75	188 208	11	10.75
	208 208	7	5.25	208 208	1	2.75	204 208	3	2.25				208 208	12	12.75	160 208	9	10.75
	208 216	3	5.25	208 236	4	2.75	208 256	0	2.25				208 236	11	12.75	208 208	6	10.75
N =			21			11			9			12			51			43
X^2 =			2.43			2.45			3.00			0.00			0.53			6.02
d.f. =			3			3			3			1			3			3
P =			*0.49*			*0.48*			*0.39*			*1.00*			*0.91*			*0.11*
OtsG249	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E	Genotype	O	E
	199 211	6	5.25	211 211	4	3	211 211	1	2.25	211 259	4	5	163 215	9	12.5	163 215	11	10
	211 215	4	5.25	211 243	2	3	211 251	2	2.25	235 259	6	5	211 215	13	12.5	215 259	5	10
	199 215	4	5.25	211 215	2	3	211 215	2	2.25				163 235	15	12.5	163 235	9	10
	215 215	7	5.25	215 243	4	3	215 251	4	2.25				211 235	13	12.5	235 259	15	10
N =			21			12			9			10			50			40
X^2 =			1.29			1.33			2.11			0.40			1.52			5.20
d.f. =			3			3			3			1			3			3
P =			*0.73*			*0.72*			*0.55*			*0.53*			*0.68*			*0.16*
Ots3	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E	Genotype	O	E
	90 90			82 90	0	6	82 90	3	4.5	90 90	4	6	90 90	23	27.5	90 90	7	11
				90 90	12	6	90 90	6	4.5	90 92	8	6	90 98	32	27.5	90 100	12	11
																90 98	5	11
																98 100	20	11
N =						12			9			12			55			44
X^2 =			N/A			12.00			0.32			1.33			1.47			12.18
d.f. =			N/A			1			1			1			1			3
P =			*N/A*			***0.00***			*0.44*			*0.25*			*0.22*			***0.01***

Appendix A14 cont'd.

CH1	F228xM234			F228xM240			F228xM252			F230xM243			F231xM233			F231xM247		
Ots4	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	144 146	5	4.75	146 146	2	2.75	146 146	6	4.5	146 146	1	3	146 146	34	27	146 146	24	21
	146 150	3	4.75	146 150	2	5.5	146 150	3	4.5	146 152	2	3	146 156	20	27	146 150	18	21
	144 150	4	4.75	150 150	7	2.75				146 150	9	3						
	150 150	7	4.75							150 152	0	3						
N =			19			11			9			12			54			42
X^2 =			1.84			9.00			1.00			16.67			3.63			0.86
d.f. =			3			3			1			3			1			1
P =			*0.61*			***0.01***			*0.32*			***0.00***			*0.06*			*0.35*
Omy325	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	94 100	8	5.25	90 100	1	3	88 100	2	2.25	94 100	4	6	90 94	20	13.25	94 100	6	10.75
	100 100	3	5.25	100 100	2	3	90 100	4	2.25	94 104	8	6	94 100	9	13.25	94 104	17	10.75
	94 104	4	5.25	90 104	5	3	88 104	3	2.25				90 100	11	13.25	100 100	6	10.75
	100 104	6	5.25	100 104	4	3	90 104	0	2.25				100 100	13	13.25	100 104	14	10.75
N =			21			12			9			12			53			43
X^2 =			2.81			3.33			3.89			1.33			5.19			5.19
d.f. =			3			3			3			1			3			3
P =			*0.42*			*0.34*			*0.27*			*0.25*			*0.16*			***0.03***
Ots104	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	217 229			205 217	7	6	213 217	3	4.5	217 221	1	3	205 225	12	13.5	217 225	10	11
				209 217	5	6	217 217	6	4.5	217 229	5	3	225 253	19	13.5	225 253	14	11
										221 225	3	3	205 229	11	13.5	217 229	7	11
										225 229	3	3	229 253	12	13.5	229 253	13	11
N =						12			9			12			54			44
X^2 =			N/A			0.33			1.00			2.67			3.04			2.73
d.f. =			N/A			1			1			3			3			3
P =			*N/A*			*0.56*			*0.32*			*0.45*			*0.39*			*0.44*

Appendix A14 cont'd.

CH1	F228xM234			F226xM240			F228xM252			F230xM243			F231xM233			F231xM247		
Ots107	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	204 248	12	10.5	240 248	1	6	236 248	3	4.5	208 240	0	3	228 240	18	13.5	208 228	27	22
	240 248	9	10.5	248 248	11	6	248 252	6	4.5	208 248	5	3	228 248	12	13.5	208 244	17	22
										240 240	5	3	240 244	14	13.5			
										240 248	2	3	244 248	10	13.5			
N =		21			12			9			12			54			44	
X^2 =		0.43			8.33			1.00			6.00			2.59			2.27	
d.f. =		1			1			1			3			3			1	
P =		*0.51*			***0.00***			*0.32*			*0.11*			*0.46*			*0.13*	
OtsG311	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E	Genotype	O	E
	298 310	4	5	294 298	5	6	298 302	4	1.75	306 374	7	6	294 310	8	12	310 310	21	21.5
	310 326	6	5	294 326	7	6	298 334	0	1.75	374 374	5	6	298 310	16	12	310 330	22	21.5
							302 326	1	1.75				294 330	11	12			
							326 334	2	1.75				298 330	13	12			
N =		10			12			7			12			48			43	
X^2 =		0.40			0.33			5.00			0.33			2.83			0.02	
d.f. =		1			1			3			1			3			1	
P =		*0.53*			*0.56*			*0.17*			*0.56*			*0.42*			*0.88*	
OtsG68	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	235 243	12	10.5	211 243	8	6	243 243			191 239	2	2.5	211 235	11	12.5	235 235	13	10.25
	243 251	9	10.5	243 259	4	6				191 291	3	2.5	235 259	14	12.5	235 259	12	10.25
										239 243	1	2.5	211 243	11	12.5	235 243	4	10.25
										243 291	4	2.5	243 259	14	12.5	243 259	12	10.25
N =		21			12						10			50			41	
X^2 =		0.43			1.33			N/A			2.00			0.72			5.15	
d.f. =		1			1			N/A			3			3			3	
P =		*0.51*			*0.25*			*N/A*			*0.57*			*0.87*			*0.16*	

Appendix A14 cont'd.

CH1	F228xM234			F228xM240			F228xM252			F230xM243			F231xM233			F231xM247		
OtsG83b	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	165 185	5	5.25	185 185	6	3	177 185	3	2	173 201	6	3	185 201	11	12.25	197 201	13	10.5
	185 193	6	5.25	185 221	1	3	185 185	2	2	173 205	3	3	201 221	15	12.25	201 225	8	10.5
	165 229	4	5.25	185 229	5	3	177 229	1	2	201 225	0	3	185 225	8	12.25	197 225	12	10.5
	193 229	6	5.25	221 229	0	3	185 229	2	2	205 225	3	3	221 225	15	12.25	225 225	9	10.5
N =		21			12			8			12			49			42	
X^2 =		0.52			8.67			1.00			6.00			2.84			1.62	
d.f. =		3			3			3			3			3			3	
P =		*0.91*			***0.03***			*0.80*			*0.11*			*0.42*			*0.66*	
OtsG432	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E
	111 123	5	5.25	111 123	2	3	123 123	4	2.25	111 111	4	3	107 107	13	12	107 111	6	10.75
	123 131	6	5.25	123 135	1	3	123 135	3	2.25	111 159	2	3	107 127	15	12	107 127	10	10.75
	111 127	3	5.25	111 127	4	3	123 127	1	2.25	111 123	1	3	107 159	5	12	111 159	10	10.75
	127 131	7	5.25	127 135	5	3	127 135	1	2.25	123 159	5	3	127 159	15	12	127 159	17	10.75
N =		21			12			9			12			48			43	
X^2 =		1.67			3.33			3.00			3.33			5.67			5.84	
d.f. =		3			3			3			3			3			3	
P =		*0.64*			*0.34*			*0.39*			*0.34*			*0.13*			*0.12*	
Ots13	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E	Genotype	O	E	Genotype	O	E
	90 90			90 90			82 90	3	4	90 90	3	5.5	90 90	15	19.5	90 90	6	9
							90 90	5	4	90 92	8	5.5	90 98	24	19.5	90 100	10	9
																90 98	4	9
																98 100	16	9
N =								8			11			39			36	
X^2 =		N/A			N/A			0.50			2.27			2.08			9.33	
d.f. =		N/A			N/A			1			1			1			3	
P =		*N/A*			*N/A*			*0.48*			*0.13*			*0.15*			***0.03***	

Appendix A15. Chi-squared tests for the six major families in CH3 for all 12 loci genotyped. O: observed number of individuals with a particular genotype. E: expected number of individuals with a particular genotype. N: number of individuals assigned to that family genotyped for a particular locus. X^2: chi-squared value. d.f.: degrees of freedom. *P*: probability associated to the X^2 value. (*) indicate families with a low number of assigned offspring which had expected frequency cells lower than 5. No tests resulted significant.

Appendix A15 cont'd.

CH3	F267xM258			F267xM276			F270xM275			F270xM276			F281xM271		
OtsG253b	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E
	164 208	12	12.5	164 188	5	5.75	164 164	17	12.25	164 188	5	4.25	208 216	11	9
	208 208	13	12.5	164 208	6	5.75	164 204	19	24.5	164 208	4	4.25	208 236	7	9
				188 208	6	5.75	204 204	13	12.25	188 204	5	4.25			
				208 208	6	5.75				204 208	3	4.25			
N =		25			23			49			17			18	
X^2 =		0.04			0.13			3.12			0.65			0.89	
d.f. =		1			3			2			3			1	
P =		*0.84*			*0.99*			*0.21*			*0.89*			*0.35*	
OtsG249	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	211 219	8	6.5	163 211	11	7	219 243	12	12.75	163 219	4	5	215 219	2	4.5
	211 243	3	6.5	211 259	4	7	219 259	12	12.75	219 259	5	5	219 251	6	4.5
	219 235	10	6.5	163 235	7	7	235 243	10	12.75	163 235	9	5	215 231	5	4.5
	235 243	5	6.5	235 259	6	7	235 259	17	12.75	235 259	2	5	231 251	5	4.5
N =		26			28			51			20			18	
X^2 =		4.46			3.71			2.10			5.20			2.00	
d.f. =		3			3			3			3			3	
P =		*0.22*			*0.29*			*0.55*			*0.16*			*0.57*	
Ots3	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	84 90	12	13	90 90			88 90	9	13.75	88 90	10	9.5	84 90	6	4.75
	90 90	14	13				88 92	20	13.75	90 90	9	9.5	84 92	3	4.75
							90 90	11	13.75				90 100	6	4.75
							90 92	15	13.75				92 100	4	4.75
N =		26						55			19			19	
X^2 =		0.15			N/A			5.15			0.05			1.42	
d.f. =		1			N/A			3			1			3	
P =		*0.69*			*N/A*			*0.16*			*0.82*			*0.70*	

Appendix A15 cont'd.

CH3	F267xM258			F267xM276			F270xM275			F270xM276			F281xM271		
Ots4	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E
	146 148	5	6.5	146 146	5	7	146 146	31	26.5	146 146	10	10	146 146	9	9.5
	146 150	7	6.5	146 150	7	7	146 150	22	26.5	146 150	10	10	146 150	10	9.5
	148 156	5	6.5	146 156	11	7									
	150 156	9	6.5	150 156	5	7									
N =		26			28			53			20			19	
X^2 =		1.69			3.43			1.53			0.00			0.05	
d.f. =		3			3			1			1			1	
P =		*0.64*			*0.33*			*0.22*			*1.00*			*0.82*	
Omy325	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	88 90	5	6.5	88 94	5	7	84 88	8	13	84 94	6	5	90 90	3	4.75
	88 94	7	6.5	88 100	9	7	84 104	17	13	84 100	5	5	90 94	12	9.5
	90 104	9	6.5	94 104	6	7	88 100	11	13	94 100	2	5	94 94	4	4.75
	94 104	5	6.5	100 104	8	7	100 104	16	13	100 100	7	5			
N =		26			28			52			20			19	
X^2 =		1.69			1.43			4.15			2.80			1.42	
d.f. =		3			3			3			3			2	
P =		*0.64*			*0.70*			*0.25*			*0.42*			*0.49*	
Ots104	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	217 221	6	6.5	217 229	10	14	209 217	25	26	217 229			213 217	3	4.5
	217 237	8	6.5	229 253	18	14	217 217	27	26				217 229	5	4.5
	221 253	6	6.5										213 229	6	4.5
	237 253	6	6.5										229 229	4	4.5
N =		26			28			52						18	
X^2 =		0.46			2.29			0.08			N/A			1.11	
d.f. =		3			1			1			N/A			3	
P =		*0.93*			*0.13*			*0.78*			*N/A*			*0.77*	

Appendix A15 cont'd.

CH3	F267xM258			F267xM276			F270xM275			F270xM276			F281xM271		
Ots107	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	228 240	9	6	228 240	5	7	228 240	13	13.25	228 240	7	5	228 244	3	4.5
	240 244	3	6	240 244	11	7	240 244	17	13.25	240 244	4	5	236 244	6	4.5
	228 248	8	6	228 248	3	7	228 244	7	13.25	228 244	5	5	228 248	5	4.5
	244 248	4	6	244 248	9	7	244 244	16	13.25	244 244	4	5	236 248	4	4.5
N =		24			28			53			20			18	
X^2 =		4.33			5.71			4.58			1.20			1.11	
d.f. =		3			3			3			3			3.00	
P =		*0.23*			*0.13*			*0.20*			*0.75*			*0.77*	
Ots311	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)
	298 310	10	6	298 358	10	6.75	294 310	12	12.5	310 358	5	4.75	290 314	3	4.75
	298 330	5	6	298 374	8	6.75	298 310	14	12.5	310 374	5	4.75	290 346	5	4.75
	310 330	5	6	330 358	5	6.75	294 374	11	12.5	358 374	5	4.75	310 314	8	4.75
	330 330	4	6	330 374	4	6.75	298 374	13	12.5	374 374	4	4.75	310 346	3	4.75
N =		24			27			50			19			19	
X^2 =		3.67			3.37			0.40			0.16			3.53	
d.f. =		3			3			3			3			3	
P =		*0.30*			*0.34*			*0.94*			*0.98*			*0.32*	
OtsG68	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	207 227	5	6	227 235	5	6.75	235 235	15	12	235 235	3	5	175 235	4	3.75
	227 267	8	6	227 239	5	6.75	235 243	22	24	235 239	6	5	219 235	4	3.75
	207 243	7	6	235 243	8	6.75	243 243	11	12	235 243	3	5	175 255	5	3.75
	243 267	4	6	239 243	9	6.75				239 243	8	5	219 255	2	3.75
N =		24			27			48			20			15	
X^2 =		1.67			1.89			1.00			3.60			1.27	
d.f. =		3			3			2			3			3	
P =		*0.64*			*0.60*			*0.61*			*0.31*			*0.74*	

Appendix A15 cont'd.

CH3	F267xM258			F267xM276			F270xM275			F270xM276			F281xM271		
OtsG83b	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)
	201 221	5	6	165 221	8	6.75	173 197	15	12.25	165 173	2	4.25	165 197	5	3.75
	209 221	8	6	205 221	5	6.75	173 201	17	12.25	173 205	8	4.25	165 201	3	3.75
	201 225	5	6	165 225	6	6.75	185 197	8	12.25	165 185	6	4.25	197 225	4	3.75
	209 225	6	6	205 225	8	6.75	185 201	9	12.25	185 205	1	4.25	201 225	3	3.75
N =		24			27			49			17			15	
X^2 =		1.00			1.00			4.80			7.71			0.73	
d.f. =		3			3			3			3			3	
P =		*0.80*			*0.80*			*0.19*			*0.05*			*0.87*	
OtsG432	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)	Genotype	O	E(*)
	107 107	9	6.25	107 111	6	6.75	107 111	11	12.25	111 111	6	4.5	123 127	4	4.5
	107 163	7	6.25	107 159	11	6.75	111 111	16	12.25	111 159	2	4.5	127 155	7	4.5
	107 135	5	6.25	111 135	4	6.75	107 135	10	12.25	111 135	4	4.5	123 135	4	4.5
	135 163	4	6.25	135 159	6	6.75	111 135	12	12.25	135 159	6	4.5	135 155	3	4.5
N =		25			27			49			18			18	
X^2 =		2.36			3.96			1.69			2.44			2.00	
d.f. =		3			3			3			3			3	
P =		*0.50*			*0.27*			*0.64*			*0.49*			*0.57*	
Ots13	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E	Genotype	O	E(*)
	84 90	12	12.5	90 90			88 90	11	13.25	88 90	10	9.5	84 90	6	4.75
	90 90	13	12.5				88 92	17	13.25	90 90	9	9.5	84 92	3	4.75
							90 90	12	13.25				90 100	6	4.75
							90 92	13	13.25				92 100	4	4.75
N =		25						53			19			19	
X^2 =		0.04			N/A			1.57			0.05			1.42	
d.f. =		1			N/A			3			1			3	
P =		*0.84*			*N/A*			*0.64*			*0.82*			*0.70*	