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SEASONAL VARIATION AND THE EFFECTS OF INBREEDING ON SPERM QUALITY IN LAKE TROUT (*Salvelinus namaycush*)

By

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23 May 2012

I hereby declare that this thesis incorporates material that is the result of joint research, as follows: my first data chapter was co-authored with my supervisor, Dr. Trevor Pitcher, and with Dr. Ian Butts and Dr. Chris Wilson. My second data chapter was co-authored with Dr. Trevor Pitcher and Dr. Chris Wilson. My collaborators provided valuable feedback, helped with project design and statistical analyses, and provided editorial input during the writing of each manuscript; however, primary contributions have been by the author. Chapter 2 was prepared as a manuscript, and has been submitted to the North American Journal of Aquaculture for publication. Chapter 3 has also been prepared as a manuscript for future submission to a peer-reviewed journal.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from my co-authors to include the above materials in my thesis.

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ABSTRACT

Inbreeding, the mating between relatives, has been reported to lead to inbreeding depression, which can influence male sperm quality. This thesis examined the effects of inbreeding on sperm quality in a captive population of lake trout (*Salvelinus namaycush*). First, I investigated seasonal variation in sperm quality (velocity, motility, linearity, longevity and density) across the natural spawning season of the species in order to determine when peak sperm quality occurs. My findings suggest that sperm quality tends to peak throughout the middle of the spawning season. Using this data, I then examined the effects of inbreeding depression on sperm quality. I found no significant difference in sperm traits between inbred (full sibling offspring), moderately inbred (maternal and paternal half sibling offspring) and outbred (unrelated offspring) males. Together, these results have implications for the optimization of fertilization protocols in hatchery populations as well as provide insight into experimental inbreeding in lake trout populations.

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Inbreeding Depression

The genetic diversity of a population is determined by several evolutionary factors including mutation, selection, random drift and migration (Wright, 1977). In addition, the mating system of a population can play a significant role on genetic diversity, both at the individual and population level. Generally, random mating is rare, instead individuals tend to base mating decisions on phenotypic similarities or dissimilarities, a process known as non-random mating (Waser 1993). For the most part, mating between close relatives is typically avoided, however with increased habitat fragmentation and population size decline, the probability of inbreeding increases (Keller & Waller 2002). Typically, inbreeding leads to inbreeding depression, which can be defined as a reduction in fitness (or phenotype value in practical terms) of offspring from matings between related individuals compared to the fitness of offspring from matings between unrelated individuals (Wright 1977). In order to understand the fitness associated costs of inbreeding, it is important to understand the genetic basis underlying inbreeding depression (Charlesworth & Willis 2009). Two hypotheses exist to explain the genetic mechanism of inbreeding depression: the dominance and overdominance hypotheses (Wright 1977). The dominance hypothesis posits that inbreeding results in an increase in homozygosity, which in turn leads to the unmasking of deleterious recessive alleles that reduce fitness (reviewed in Charlesworth & Charlesworth 1999). The overdominance hypothesis states that an increase in homozygosity leads to reductions in

fitness of inbred individuals at loci where heterozygotes had a selective advantage over homozygotes (reviewed in Charlesworth & Charlesworth 1999). Overall, a reduction in fitness is likely a combination of both of these mechanisms, which can be difficult to distinguish when studying inbreeding depression (Keller & Waller 2002).

Inbreeding depression is typically measured by comparing fitness related traits between inbred and outbred groups. Inbreeding levels can be calculated using the inbreeding coefficient, F, which is the probability that two alleles at a single locus will be identical by descent (Wright 1977). In order to calculate inbreeding coefficients, pedigree information is needed, however this is often difficult to obtain, particularly for wild populations. Nevertheless, pedigree construction is possible when studying controlled, laboratory-reared populations and estimates of F, for example in a single generation of full sibling offspring (F= 0.25) and half sibling offspring (F= 0.125) has become conventional knowledge (Pemberton 2004). A more recent method of measuring inbreeding levels has been to calculate individual heterozygosity using microsatellite markers (reviewed by Coltman & Slate 2003). This method is based on evidence that heterozygosity and fitness are correlated and assumes that individual inbreeding coefficients can be accurately estimated using 10 or more genetic markers (Balloux et al. 2004). Calculating average heterozygosity as a measure of inbreeding has come under recent scrutiny regarding the accuracy with which it can estimate inbreeding depression (Balloux et al. 2004) however, both pedigree and genetic methods present advantages and disadvantages and are both commonly used when studying the effects of inbreeding depression (Keller & Waller 2002). In order to standardize and compare fitness-related

traits affected by inbreeding depression across different species, a measure known as the coefficient of inbreeding depression (δ) can be calculated as follows:

$$\delta = 1 - (X_1 / X_o)$$

where X_1 = inbred trait value and X_o = outbred trait value (adapted from Crnokrak & Roff 1999).

 The negative effects of inbreeding depression have been documented across a wide variety of plant and animal species in both captive (Lacy et al. 1993) and wild settings (Crnokrak & Roff 1999; Keller & Waller 2002). Inbreeding depression has been shown to affect all aspects of life history traits from offspring survival to adult reproductive success (Crnokrak & Roff 1999). However, inbreeding depression has been primarily studied in survival-oriented metrics with the majority of research focusing on the effects on juvenile mortality including parasite susceptibility in young seals (Rijks et al. 2008), hatching success in birds (Van Noordwijk & Scharloo 1981) and fry mortality in salmonids (Kincaid 1976). These studies however may underestimate the severity of inbreeding, particularly when it is severe as individuals who express recessive alleles early in development are likely to die off, leaving behind those individuals with increased levels of heterozygosity (Keller & Waller 2002). An individual's fitness is contingent on both their survival to sexual maturity and their reproductive success once sexually mature (Stearns 1992), suggesting a need to also examine reproductive traits as they relate to inbreeding depression. Studies that have focused on reproductive traits have primarily looked at the effects on mating success (Dietz & Baker 1993) and fertility (Johnston 1992). More recently, however, inbreeding depression has been shown to affect fitness in

more cryptic ways, in particular through negative effects on sperm quality (Fitzpatrick &
Evans 2009).

Effects of Inbreeding on Sperm Quality

To date, much of the research regarding the effects of inbreeding depression on
sperm quality, which can be defined as the ability of a male's sperm to successfully
fertilize an egg as well as compete with other sperm (Rurangwa et al. 2004) has focused
on mammals, while studies on other taxa are rare (Table 1.1). A lack of literature
investigating male gamete quality can likely be attributed to the difficulties in measuring
sperm quality traits in many species. Nevertheless, studies that have done so have mainly
focused on factors such as reductions in sperm concentration (Margulis & Walsh 2002),
sperm competitiveness (Zajitschek et al. 2009) and sperm motility (Gomendio et al.
2000) as well as increased sperm abnormalities (Gage et al. 2006). Wildt et al. (1987)
reported significant increases in sperm abnormalities, along with decreased sperm
motility and concentration in an Asiatic lion (*Pantheo leo*) population that suffered from
decreased genetic variability. Similarly, sperm abnormalities, sperm motility and sperm
concentration were all significantly lower in an inbred Florida panther population (*Felis
concolor coryi*) when compared to outbred relatives (Barone et al. 1994). Evidence for
inbreeding affecting sperm quality in other taxa has thus far been limited.

It has been suggested that spermatozoa may be more susceptible to the effects of
inbreeding depression given the complex nature of spermatogenesis and the high
potential for mutational defects (Gage et al. 2006). In species where sperm quality is a

significant determinant of fertilization success (i.e. in fishes), effects of inbreeding depression on such traits ought to be further investigated as sperm quality can significantly contribute to the fitness of an individual. To date, studies that have investigated the effects of inbreeding depression on sperm quality in fish are limited. In the guppy (*Poecilia reticulata)*, both sperm concentration (Zajitschek & Brooks 2010) and sperm competitiveness (Zajitschek et al. 2009) were found to be significantly reduced in inbred compared to outbred males while in Nile tilapia (*Oreochromis niloticus*), male reproductive success (as a result of sperm competitiveness) was lower for inbred compared to outbred males (Fessehaye et al. 2009).

Lake Trout

Lake trout (*Salvelinus namaycush*) are an endemic North American member of the genus *Salvelinus*, also known as charr. Species of this genus are distinguished from other salmonids through a lack of dark spots, teeth on only the anterior portion of the vomer (bone making up the roof of the mouth), small sized scales and preference for cold, deep water (Behnke 2002). Lake trout are the largest of the charr species, occupying many northern North American inland and Great Lakes (Scott & Crossman 1998). The evolution of this species coincides with the Pleistocene glaciations, consequently restricting lake trout to the freshwater, closed-system lakes of their current range (Wilson & Mandrak 2004). As a result of their evolutionary history, lake trout are adapted to live in these low-productivity, glacial refugia lakes with low salinity tolerance and have thus evolved a life history strategy that includes long-lived (20-25 year old life span), late-maturing and large individuals (Gunn & Pitblado 2004).

Lake trout are iteroparous spawners, reaching sexual maturity around age 6 or 7 and spawning in the fall anywhere from September to December, depending on the lake (Scott & Crossman 1998). The spawning behaviour of lake trout differs drastically from that of their close relatives. Lake trout do not build nests (redds): rather, they broadcast gametes on open, clean, course gravel substrate (Gunn 1995). Sexual dimorphism is greatly reduced relative to other salmonids, and direct male-male competition appears to be absent (Martin & Oliver 1980). These differences can be attributed to the lake trout's propensity to spawn at night, another unique characteristic uncommon in other salmonids (Gunn 1995). Esteve et al. (2008) described lake trout spawning behaviour where multiple males were observed spawning with a single female. Instead of building nests, females of this species are known to swim around the spawning grounds accompanied by multiple males. Spawning initiates when both the female and nearby males begin to quiver, discontinue swimming and drop to the bottom of the lake. Here, gamete release occurs with both sexes raising their tails, quivering and gaping their jaws. As in all species of *Salvelinus,* spawning is very brief in lake trout, lasting only 1-2 seconds (Esteve et al. 2008). Eggs then develop with no parental care in the crevices of the rocky lake bottom for up to 4-5 months, eventually hatching in the spring (Scott & Crossman 1998). As lake trout spawning behaviour differs drastically from other salmonids, where mating success is highly dependent on sexual dimorphism as well as both male and female aggressive interactions (Fleming & Reynolds 2004), it may be possible that sexual selection in lake trout has shifted to the gametic level.

Over the past half-century, lake trout populations have drastically declined from their once abundant sizes. Declines in the Great Lakes have primarily been attributed to

overfishing and predation by the invasive sea lamprey (*Petromyzon marianus*) (Walters et al. 1980). Additional sources of decline in the Great Lakes and inland waters include contamination and eutrophication of lakes, habitat restructuring, and the stocking of domestic lake trout strains (Gunn & Pitblado 2004). The long-lived and late maturing nature of lake trout makes this species further susceptible to these threats (Gunn & Pitblado 2004). As lake trout are an important species both commercially and economically, re-stocking and rehabilitation programs have been set up to restore population abundances (Piller et al. 2005). Although these programs have been somewhat successful in restoring population numbers, previous lake trout abundance across the Great Lakes will likely never be achieved.

The evolution, unique life history strategy and recent population declines makes the study of inbreeding depression on sperm quality an important issue in lake trout, particularly in inland lake populations. Habitat isolation along with declining population size is a common cause of inbreeding and consequently inbreeding depression in many populations (Keller & Waller 2002). In lake trout, both low genetic diversity of isolated populations (Ihssen et al. 1988) and previous population declines make inbreeding a probable occurrence in this species. Furthermore, effects of inbreeding depression on sperm quality have recently been reported for a number of species, including fish (Table 1.1). Given that gamete quality is likely an important factor in the mating success of lake trout, sperm quality may be highly susceptible to the effects of inbreeding depression.

Overview of the Thesis

The objective of my thesis was to investigate the effects of seasonal variation and inbreeding depression on sperm quality in a captively bred population of lake trout. Chapter Two examines the variation in sperm quality metrics (motility, velocity, linearity, longevity, and density) throughout the natural spawning season of lake trout by using both a repeated measures and regression approach. Sperm quality in both wild and captive fish populations have been shown to significantly vary throughout the reproductive season (Table 1.2), with no obvious pattern for a particular species. Variability in such studies highlights the importance of understanding when optimal sperm quality occurs from both an aquaculture standpoint, for the optimization of fertilization protocols as well as from a theoretical standpoint, in order to optimize sperm quality when studying external factors that may be affecting it (i.e. inbreeding depression). Using sperm obtained during the peak spawning season, as determined in Chapter Two, I then tested the inbreeding depression hypothesis by investigating the effects of inbreeding on sperm quality in Chapter Three. This experiment was conducted using experimentally inbred (full sibling offspring, maternal half sibling offspring, paternal half sibling offspring) and outbred (unrelated offspring) groups of male lake trout. Overall, this thesis has implications for improving our understanding of the reproductive biology for an important commercial and economic coldwater salmonid species.

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Table 1.1 Summary of the literature examining the effects of inbreeding on sperm quality (morphology, percent motility, concentration, velocity, and competitiveness). A negative sign (-) indicates the sperm trait significantly decreased as a result of inbreeding: brackets after a negative sign represent δ (coefficient of inbreeding depression), which was measured as [1- (mean inbred trait value/mean outbred trait value)], wherever means were provided. A zero (0) indicates no significant difference between the sperm trait and inbreeding. Blank spaces indicate that the sperm trait was not measured. Sperm traits were compared between inbred and outbred groups. Morphology is the percentage of abnormal sperm, % motility is the percentage of motile compared to non motile sperm cells, concentration is the number of sperm cells in a given sperm sample, velocity is the speed at which a sperm cell moves per distance traveled and competitiveness is the percentage of offspring sired by inbred compared to outbred sperm.

Common Name	Genus species	Measure of inbreeding	Sperm trait					References*
			Morphology	*% Motility*	*Concentration*	*Velocity*	*Competitiveness*	
Mammals								
Bull	*Bos taurus*	Pedigree	0	−	0			1
Dog	*Canis familiaris*	Pedigree		0	− (0.86)			2
Florida panther	*Felis concolor*	Average heterozygosity	− (0.70)	− (0.40)	− (0.72)			3

Gazelles	*Gazella cuvieri*	Pedigree	−	−				4
Golden hamsters	*Mesocricetus auratus*	Average heterozygosity		0	0	0	− (0.87)	5
Asiatic Lion	*Panthera leo*	Average heterozygosity	−	− (0.30)	− (0.56)			6
Mexican gray wolves	*Canis lupus*	Pedigree	−	−				7
Oldfield mice	*Peromyscus polionotus*	Pedigree			− (0.10)			8
Shetland ponies	*Equus caballus var.*	Pedigree	− (0.22)	− (0.07)	0			9
South African Cheetah	*Acinonyx jubatus*	Average heterozygosity	−	−	−			10
White-footed mice	*Peromyscus leucopus*	Pedigree	0	−				11
Wild rabbits	*Oryctoloagus cuniculus*	Average heterozygosity	−					12
Fishes								
Guppy	*Poecilia reticulata*	Pedigree			−		−	13,14

Common name	Scientific name	Method						Ref*
Nile tilapia	*Oreochromis niloticus*	Pedigree					−	15
Crustaceans								
Clam shrimp	*Eulimnadia texana*	Pedigree	− (0.52)					16
Insects								
Bulb mite	*Rhizoglyphus robini*	Pedigree					− (0.53)	17
Flour beetle	*Tribolium castaneum*	Pedigree					− (0.26)	18
Fruit fly	*Drosophila melanogaster*	Pedigree					− (0.25), (0.19)	19, 20

*1 (Maximini et al. 2011), 2 (Wildt et al. 1982), 3 (Barone et al. 1994), 4 (Gomendio et al. 2000), 5 (Fritzsche et al. 2006), 6 (Wildt et al. 1987), 7 (Asa et al. 2007), 8 (Margulis & Walsh 2002), 9 (van Eldik et al. 2006), 10 (Wildt et al. 1983), 11 (Malo et al. 2010), 12 (Gage et al. 2006), 13 (Zajitschek & Brooks 2009), 14 (Zajitschek et al. 2009), 15 (Fessehaye et al. 2009), 16 (Weeks et al. 2009), 17 (Konior et al. 2005), 18 (Michalczyk et al. 2010), 19 (Hughes 1997), 20 (Mack et al. 2002)

Table 1.2 Summary of the existing literature examining seasonal variation in sperm traits (D = density, V = velocity, M = motility L = longevity) throughout the reproductive season in fishes. Shape and direction refer to the pattern of sperm trait variation across the spawning season, where either linear or quadratic equations were fit to sperm data. A linear + (positive) refers to the straightline increase in sperm traits over the season, a linear - (negative) refers to the straightline decrease in sperm traits over the season, a quadratic positive refers to a U-shaped change in sperm traits over the season and a quadratic negative refers to a bell-shaped change in a sperm traits over the season. Blank spaces represent studies that found more than one shape or direction of seasonal change in a sperm trait.

Common Name	Genus species	Sperm trait	Shape	Direction	Reference
Atlantic cod	*Gadus morhua*	D, V, L	Linear	+	Butts et al. 2010
Atlantic cod	*Gadus morhua*	D	Linear	+	Rakitin et al. 1999
Atlantic cod	*Gadus morhua*	D, V	Quadratic	-	Rouxel et al. 2008
Atlantic halibut	*Hippoglossus hippoglossus*	D	Linear	+	Babiak et al. 2006
		V, M	Linear	_	

		L	Quadratic	-	
Atlantic salmon	*Salmo salar*	D	Linear	-	Aas et al. 1991
Atlantic salmon	*Salmo salar*	D	Linear	+	Piironen 1985
Barbel	*Barbus barbus*	D	Linear	-	Alavi et al. 2008
		V	Quadratic	-	
Brazilian flounder	*Paralichthys orbignyanus*	D	Quadratic	+	Lanes et al. 2010
		L	Linear	+	
Brown trout	*Salmo trutta*	D	Linear	-	Hajirezaee et al. 2010
Haddock	*Melanogrammus aeglefinus*	D	Linear	+	Rideout et al. 2004
European perch	*Perca fluviatilis*	D, V	Linear	-	Alavi et al. 2010

Ocean pout	*Zoarces americanus*	M	Quadratic	-	Wang & Crim 1997
Rainbow trout	*Oncorhynchus mykiss*	D	Linear	-	Büyükhatipoglu & Holtz 1984
		M, L	Quadratic	-	
Rainbow trout	*Oncorhynchus mykiss*	D, M	Linear	-	Munkittrick & Moccia 1987
Red porgy	*Pagrus pagrus*	D, M	Linear	+	Mylonas et al. 2003
		L	Quadratic	-	
Senegalese sole	*Solea senegalensis*	M	Linear	-	Beirão et al., 2011
Sharpsnout seabream	*Diplodus puntazzo*	D, L	Quadratic	-	Papadaki et al. 2008
Snow trout	*Schizothorax richardsonii*	D	Linear	-	Agarwal & Raghuvanshi 2009
Turbot	*Scophthalmus maximus*	D	Linear	+	Suquet et al. 1998

		M	Linear	-	
Yamú	*Brycon amazonicus*	D	Quadratic	-	Cruz-Casallas et al. 2007

Synopsis

The objective of this study was to investigate variation in sperm quality metrics (motility, velocity, linearity, longevity, and density) of hatchery-reared lake trout *Salvelinus namaycush* throughout the spawning season. Seasonal variation in sperm quality was investigated using both a regression and repeated-measures approach. Sperm was collected from the same 16 individuals over four sampling periods, separated by three-week intervals. Regression analyses showed that 7 to 27% of the variation in sperm traits could be explained by seasonal variation, indicating that seasonality can have a significant impact on the quality of sperm. Significant positive linear relationships were found for percent motility and linearity at 5 s post-activation. Significant negative quadratic relationships were found for velocity at 5 s post-activation, longevity and density, while a positive quadratic relationship was found for linearity at 10 s post-activation. Repeated measures ANOVAs showed a significant effect of season for percent motility and linearity at 5 and 10 s post-activation, velocity at 10 s post-activation and longevity. The present study is important for optimizing fertilization protocols for hatchery production and can also be used to understand reproductive biology and ecology of wild lake trout stocks.

[1] This chapter is the product of joint research with Dr. Trevor Pitcher, Dr. Ian Butts and Dr. Chris Wilson

Introduction

Lake trout *Salvelinus namaycush* have historically been a significant commercial and economic species important to North American inland lakes and the Great Lakes (Behnke 2002). Over the past half-century, declines in a number of populations largely owing to sea lamprey *Petromyzon marinus* predation and overfishing (Jensen 1994; Walters et al. 1980), have resulted in the establishment of re-stocking and rehabilitation programs (Piller et al. 2005). Although captive breeding of this species has occurred for several decades, remnant populations remain in only some of the Great Lakes, including Lake Huron and Lake Superior (Guinand et al. 2003). Today, hatchery populations are used primarily to stock lakes in need of rehabilitation (Evans & Willox 1991). As restoration efforts have become increasingly relevant, investigating gamete quality under a captive environment is important for the successful propagation of this species.

Sperm quality, which includes measures of sperm motility, velocity, longevity and density, are considered the primary determinants of fertilization success (Casselman et al. 2006; Tuset et al. 2007). Essentially, these sperm quality indices can be used to optimize fertilization protocols, which become important for producing large numbers of progeny from a limited number of eggs. Sperm quality has been found to be highly dependent on season (Babiak et al. 2006; Billard 1986; Munkittrick & Moccia 1987). For example, in Atlantic halibut *Hippoglossus hippoglossus*, sperm density, percentage of motile cells, velocity and linearity were found to vary significantly throughout the course of the spawning season (Babiak et al. 2006). The most common pattern of seasonal change in sperm quality across species is linear or quadratic. Linear patterns generally increase or decrease steadily throughout the spawning season (e.g. Butts et al. 2010),

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whereas quadratic patterns generally represent bell-shaped curves in which sperm quality increases at the start of the season, peaks during the middle, and decreases towards the end of the season (e.g. Papadaki et al. 2008). It is therefore important to optimize reproductive protocols to reflect the natural biology of propagated species.

An understanding of seasonal changes in sperm quality can be used to optimize fertilization success for hatchery production (Suquet et al. 1998), improve gamete storage techniques (Rideout et al. 2004), increase the efficiency of selective breeding programs (Butts et al. 2010), and assess the impacts of sperm aging (Alavi et al. 2008). In this study, I assessed variation in sperm quality of hatchery-reared lake trout over a two-month interval that spanned the natural spawning season.

Methods

Broodstock and Sperm Sampling

Variations in sperm quality traits were assessed for seven-year old hatchery-reared lake trout from the Ontario Ministry of Natural Resources Codrington Fisheries Research Facility, located in Codrington, Ontario (Latitude: 44.1468; Longitude: -77.8045). Fish originated from wild spawn collections in 1983 (two generations earlier), from native lake trout populations in two interconnected lakes in Haliburton, Ontario (Clean and Macdonald Lake, Latitude: 45.2501, Longitude: -78.5329). All fish were housed in two 20ft x 5ft fiberglass raceway tanks (working depth of 26 in) fed by untreated water from a local stream source and were kept under a natural photoperiod and temperature regime. Fish were fed AquaBrood feed (7.5 mm pellet; Corey Nutrition Company, Fredericton, NB, Canada) at 0.5% body weight per day. At three-week

intervals in 2010 (October 28, November 16, December 7 and December 27) milt was collected from the same 16 individuals in the context of a repeated measures experimental design. The mean ± SEM total length and weight of the broodstock at the onset of spawning were 582.94 ± 6.73 mm and 1894.12 ± 79.22 g, respectively. Fish were anaesthetized using 40 to 50 ppm solution of MS-222 (Syndel International, Vancouver, BC, Canada). Milt samples were collected, using slight pressure to the abdomen and massaging towards the urogenital pore, in 532 mL Whirl-Pak© plastic bags (Nasco, Newmarket, ON, Canada) and stored in a cooler. Extra care was taken to ensure that urine, feces, blood or water did not contaminate the milt samples.

Sperm Activity

Sperm were video-recorded using a CCD black and white video camera (XC-ST50, Sony, Japan) module at 50 Hz vertical frequency, mounted on an external-phase contrast microscope (CX41 Olympus, Melville, NY, USA) with a 10× negative-phase magnification objective (Pitcher et al. 2009). Sperm metrics (motility, velocity, longevity and linearity) were assessed by activating an aliquot (< 0.2 µL) of milt with 10 µL of stream water. A bionomic controller (model BC-110) and heat exchanger (model HEC-400, 20/20 Technology Inc., Wilmington, NC, USA) were used to maintain water temperature at $8.6 \pm 0.1°C$ (the temperature of the activation water during the first sampling date). Once recordings were taken, sperm traits were analyzed using the HTM-CEROS sperm analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, photometer = 55-65, minimum cell size = 3 pixels. Sperm motility,

velocity, and linearity were analyzed at 5 and 10 s post-activation. In salmonids, it has been shown that 80% of fertilizations occur within the first 5 seconds of sperm activation along with the closing of the micropyle occurring shortly thereafter (Hoysak & Liley 2001), therefore examining sperm at a post activation time of both 5 and 10 seconds was the most biologically relevant as it corresponds to conditions found in the wild. Sperm motility was calculated as the percentage of motile cells divided by the total number of cells. Sperm velocity was measured as the average velocity measured over the actual point-to-track followed by the cell. Sperm path linearity was measured as the departure of the cell track from a straight line. Linearity is the straightness with which a sperm cell moves per unit of distance traveled. Straighter swimming sperm will have a larger linearity value. Longevity was estimated as the time for ~95% of the sperm cells to become immotile (Gage et al. 2004). For each male, the mean value of all sperm cells per each activation was used for statistical analysis.

Sperm Density

Sperm density was estimated by adding 1.5 µL of milt to 500 µL of Cortland's saline solution (7.25 g/L NaCl; 0.38 g/L KCl; 0.47 g/L $MgSO_4$: $7H_2O$; 0.4 g/L Na_2HPO_4: H_2O; 1.0 g/L $NaHCO_3$; 0.22 g/L $MgCl_2$; 1.0 g/L $C_6H_{12}0_6$) to prevent activation. The sperm suspension was then gently mixed using a wide-bore transfer pipette and 10 µL was loaded onto a Neubauer-improved haemocytometer. Sperm cells were counted in 5 of the 25 (1 mm^2) squares on the haemocytometer (4 corner squares and 1 middle square). Sperm density was then estimated by taking the mean number of sperm cells in the 5 squares, multiplying by 25, and then finally by 10 (the depth of each chamber in the

haemocytometer). This number was then multiplied by the initial volume of the sample to obtain the total number of sperm cells in 1 mL of milt (Pitcher et al. 2009).

Statistical Analyses

To examine sperm quality of lake trout throughout the spawning season I used two statistical approaches. In the first approach, I examined seasonal variation in sperm motility, velocity, linearity, longevity and density by fitting either linear or quadratic equations to the data (PROC REG; SAS Institute, 2003). This allowed me to create predictive models to explore the shape (positive or negative) of seasonal variation. Linear and quadratic equations were chosen *a priori* to fit the data based on the available literature (e.g. Butts et al. 2010; Lahnsteiner et al. 1996, 1998). Final equation selection (linear or quadratic) was based on an *F*-statistic: $df_j \times (r^2_j - r^2_i)/(1 - r^2_j)$ where: r^2_i is the r^2 for the i_{th} order; r^2_j is the r^2 for the next higher order; df_j is the degrees of freedom for the higher-order equation with *j* degrees of freedom in the numerator and $df_j = n - j - 1$ degrees of freedom in the denominator (McDonald 2009).

In the second approach I analyzed the data using a series of repeated measures mixed-model ANOVAs (PROC MIXED; SAS Institute 2003). By using this approach I was able to determine how the four sampling periods differed throughout the spawning season (i.e. Oct 28 vs. Dec 7). Repeated measures mixed-model ANOVAs, for sperm related variables, were run at each post-activation time. Akaike's (AIC) and Bayesian (BIC) information criteria were used to assess which co-variance structure (compound symmetry, autoregressive order, or unstructured) was most appropriate (Littell et al. 1996). Sampling date was considered fixed whereas male identity was considered random

and included as the subject in the repeated statement. Tukey post-hoc analyses were used to compare least square means between treatments.

All data were analyzed using SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC, USA). Residuals were tested for normality using the Shapiro-Wilk test and homogeneity of variances were tested using plot of residuals vs. fit values. Sperm velocity, density, and longevity were $\log_{10}$ transformed while sperm motility and linearity were arcsine square root transformed when data deviated from normality and homoscedasticity (Zar 1996). Data were expressed as mean $\pm$ SEM.

Results

Motility

There was a significant positive linear relationship between sampling date and sperm motility at 5 s post-activation ($r^2 = 0.18$, $F_{1,63} = 14.0$, $P < 0.001$, y = 8.34x + 39.28), however there was no significant relationship at 10 s post-activation ($r^2 = 0.07$, $F_{2,63} = 2.5$, $P = 0.09$, y = 21.51x + 3.41x^2 + 17.32) (Figure 2.1a). Sampling date had a significant effect on sperm motility at 5 ($F_{3,35.1} = 6.43$, $P = 0.0014$) and 10 s post-activation ($F_{3,33.6} = 7.82$, $P = 0.0004$) (Figure 2.1b).

Velocity

There was a significant negative quadratic relationship between sampling date and sperm velocity at 5 s post-activation ($r^2 = 0.10$, $F_{2,63} = 3.23$, $P = 0.046$, y = 40.93x − 8.27x^2 + 70.0) and a marginally significant negative quadratic relationship at 10 s post-activation ($r^2 = 0.09$, $F_{2,63} = 3.05$, $P = 0.05$, y = 23.64x - 4.88x^2 + 49.73) (Figure 2.1c).

Sampling date had a significant effect on sperm velocity at 10 s post-activation ($F_{3,32.9} = 3.81$, $P = 0.02$), but not at 5 s post-activation ($F_{3,44.9} = 2.04$, $P = 0.12$ (Figure 2.1d).

Linearity

There was a significant positive linear relationship between sampling date and sperm path linearity at 5 s post-activation ($r^2 = 0.27$, $F = 22.99_{1,63}$, $P < 0.0001$, $y = 6.77x + 44.56$), while a significant positive quadratic relationship was found at 10 s post-activation ($r^2 = 0.21$, $F = 8.31_{2,63}$, $P < 0.001$, $y = -18.75x + 4.55x^2 + 79.92$) (Figure 2.1e). Sampling date had a significant effect on sperm path linearity at 5 ($F = 11.2_{3,34.1}$, $P < 0.0001$) and 10 ($F = 9.2_{3,33.4}$, $P = 0.001$) s post-activation (Figure 2.1f).

Longevity

A significant negative quadratic relationship was found between sampling date and sperm longevity ($r^2 = 0.13$, $F_{2,63} = 4.40$, $P = 0.02$, $y = 7.30x - 1.69x^2 + 17.66$) (Figure 2.2a). Running the ANOVA showed that sampling date had a significant effect on sperm longevity ($F_{3,35} = 3.2$, $P = 0.036$) (Figure 2.2b).

Density

There was a significant negative quadratic relationship between sampling date and sperm density ($r^2 = 0.12$, $F_{2,63} = 4.10$, $P = 0.02$, $y = 1.00x - 0.18x^2 + 0.13$) (Figure 2.2c). Sampling date had a marginally significant effect on sperm density ($F_{3,35.7} = 2.8$, $P = 0.05$) (Figure 2.2d).

Correcting for Multiple Tests

Using a conservative Bonferroni correction for multiple tests, I divided the alpha value (0.05) by the number of tests (n=5) per grouping (5 and 10 seconds post-activation for both linear and quadratic equations) thus rendering the new alpha value 0.01. In this case, 4 of the significant results would become non-significant for the different groupings. However, Nakagawa (2004) points out that the use of Bonferroni corrections reduces statistical power, thus increasing type II errors, which may lead to bias in data interpretation.

Discussion

Quantifying sperm quality throughout the spawning season is important for estimating a stock's reproductive potential (Trippel 1999, 2003), timing of optimal fertilization for hatchery production (Rana 1995) and improvement of short-term and long-term storage (cryopreservation) techniques for many captivity-bred and endangered species (Rideout et al. 2004). Variation in sperm quality across the spawning season has been previously reported for a number of freshwater and marine fishes (e.g. Beirão et al. 2011; Billard 1986; Munkittrick & Moccia 1987; see Chapter 1, Table 1.2). Results have shown that within and across species, seasonal changes in sperm quality can differ. For example, one study on turbot *Scophthalmus maximus* (Suquet et al. 1998) demonstrated that motility decreased linearly as the spawning season progressed, while a significant linear increase in sperm motility was shown for red porgy *Pagrus pagrus* (Mylonas et al. 2003). For sperm velocity, negative quadratic relationships were found in barbel *Barbus barbus* (Alavi et al. 2008), and Atlantic cod *Gadus morhua* (Rouxel et al. 2008). Other

studies have shown that velocity gradually decreased throughout the spawning season, such as in European perch *Perca fluviatilis* (Alavi et al. 2010), while a linear increase in velocity was found in Atlantic cod (Butts et al. 2010). In terms of longevity, studies on rainbow trout *Oncorhynchus mykiss* (Büyükhatipoylu & Holt 1984) and sharpsnout seabream *Diplodus puntozzo* (Papadaki et al. 2008) found negative quadratic relationships. In contrast, a study on Brazilian flounder *Paralichthys orbignyanus* (Lanes et al. 2010), found that sperm longevity increased as the spawning season progressed.

For lake trout I found significant linear increases for sperm motility and linearity at 5 s post-activation. A negative quadratic relationship was found for longevity and velocity at 5 s post-activation. These results suggest the highest sperm longevity and velocity occurs during the middle portion of the spawning season. In my results, sperm quality was generally lower at the onset of the spawning season, in terms of each of the metrics that I measured. It is probable that sperm may be less motile at this time as they may not have gained the capacity for forward movement (Billard 1986; Mylonas et al. 2003). Essentially, this may be linked to the seasonal variation in progestin, 17α,20ß-dihydroxy-4-pregnen-3-one, which induces spermatozoa maturation in salmonids (Nagahama 1994).

Understanding how sperm density changes throughout the season is necessary for determination of optimal sperm to egg ratios to essentially maximize fertilization success (Butts et al. 2009; Casselman et al. 2006). Quantifying density can also be useful for estimating the biochemical properties of a known volume of milt (i.e. ATP, protein concentration; Boryshpolets et al. 2009). In the present study, we found a negative quadratic relationship for sperm density, with the densest sperm samples found on Dec 7

(or middle of the season). Studies on Atlantic cod (Rouxel et al. 2008) and yamú *Brycon amazonicus* (Cruz-Casallas et al. 2007) found similar relationships. Increases in sperm density throughout the spawning season have been found in Atlantic salmon *Salmo salar* (Piironen 1985), and Atlantic cod (Butts et al. 2010; Rakitin et al. 1999). In contrast, studies on rainbow trout (Büyükhatipoylu & Holt 1984), snow trout *Schizothorax richardsonii* (Agarwal & Raghuvanshi 2009), brown trout *Salmo trutta* (Hajirezaee et al. 2010) and Atlantic salmon (Aas et al. 1991) found that sperm density decreased throughout the season.

In salmonids, gametogenesis is a discontinuous process where sperm is released from the sperm ducts over several months, ageing throughout the spawning period (Billard 1986). During this timeframe, declines in ATP levels (Dreanno et al. 1999), hormonal activity of the sperm duct (Koldras et al. 1996; Shangguan & Crim 1999) and seminal plasma contents (i.e. ions, proteins, antioxidants; Ciereszko & Dabrowski 1995; Hajirezaee et al. 2010) have been reported. All of these biochemical and physiological milt indices have been linked to sperm activity (Lahnsteiner et al. 1996, 1998). Therefore, further work should be undertaken to explore how these indices are affecting sperm quality in lake trout. In addition, researchers should investigate how diet, stage of maturation, environmental conditions (e.g. temperature, photoperiod), and broodstock stress can affect sperm quality in captivity.

In conclusion, I found that 7 to 27 % of the variation in sperm traits can be explained by seasonal variation, indicating that seasonality can have a significant impact on the quality of sperm motility, velocity, linearity, longevity, and density. Understanding this seasonal variation in sperm quality is important for quantifying paternal effects on

fertilization success. For example, based on the available literature for another salmonid (Tuset et al. 2008), seasonal differences in sperm velocity that I have shown would result in ~ 10% difference in fertilization success. Overall, these findings have relevance for the aquaculture industry, salmonid rehabilitation programs as well as for estimation of a male's reproductive potential in the wild.

Acknowledgements

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Figure Captions

Figure 2.1 The relationship and effect of sampling date on motility (A and B), velocity (C and D) and linearity (E and F) at 5 (dashed lines) and 10 s (solid lines) post-activation. Filled circles in plots A, C, and E represent mean values ± SEM for each of the 16 male lake trout at 5 s post-activation; open circles represent mean individual values for 10 s post-activation. Means with shared letters in plots B, D, and F did not differ significantly from one another based on Tukey post-hoc tests.

Figure 2.2 The relationship and effect of sampling date on longevity (A and B) and density (C and D). Means with shared letters in plots B and D did not differ significantly from one another based on Tukey post-hoc tests.

Figure 2.1
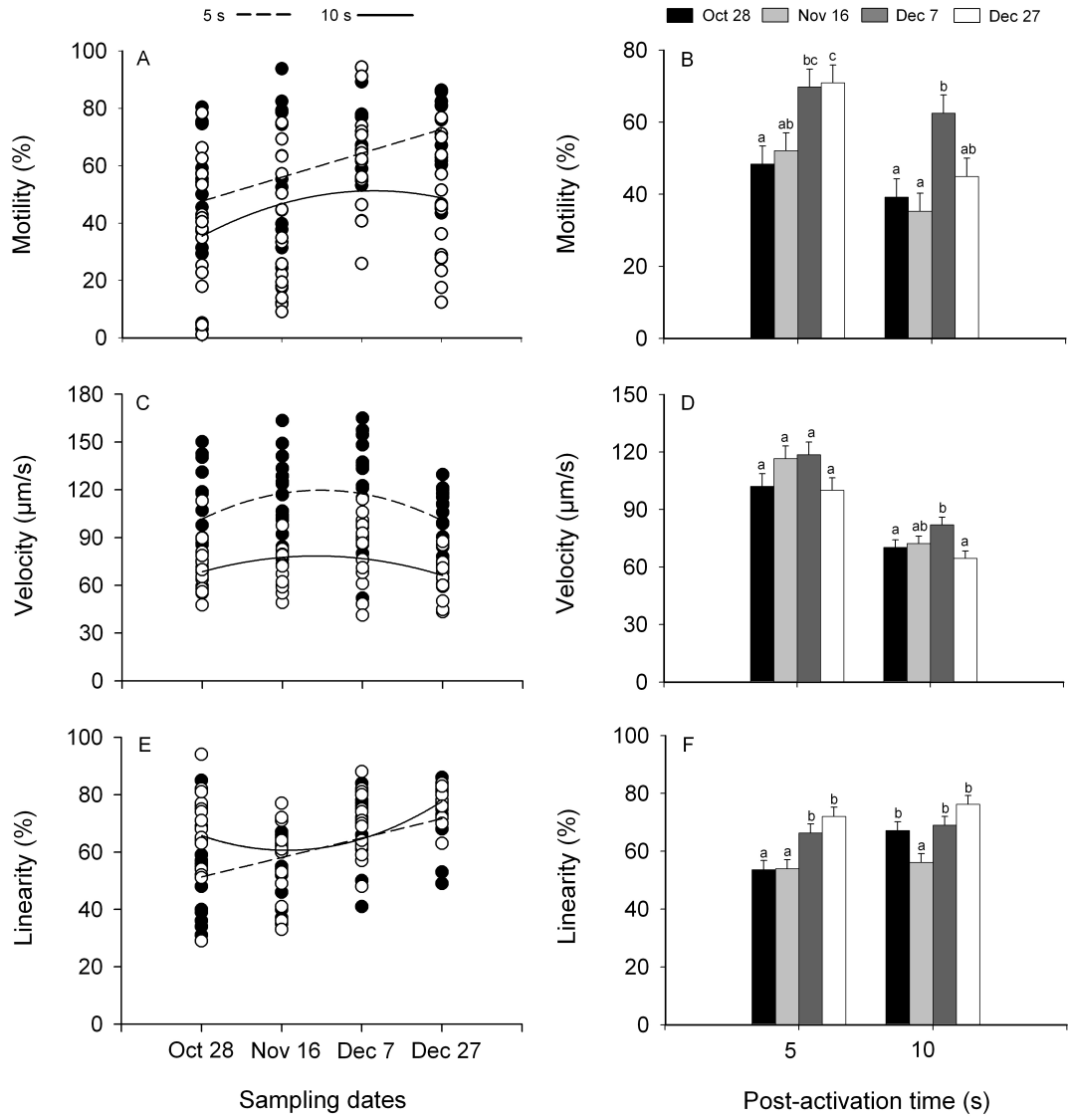
Figure 2.2
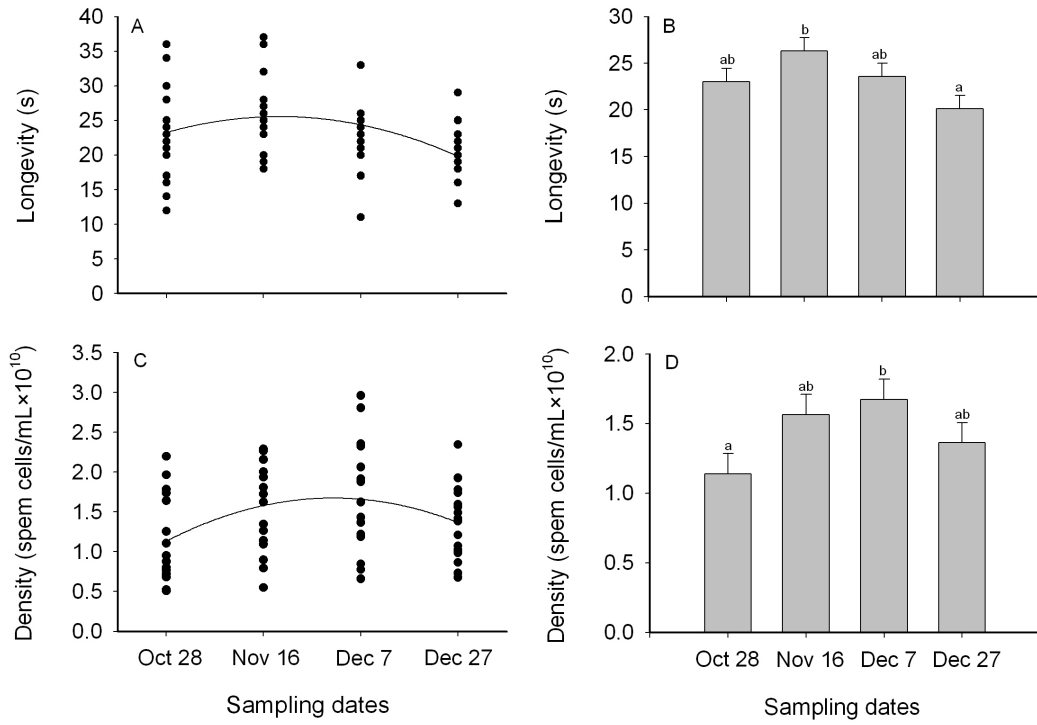
CHAPTER 3: THE EFFECTS OF INBREEDING ON SPERM QUALITY IN LAKE TROUT, *SALVELINUS NAMAYCUSH*[2]

Synopsis

The effects of inbreeding, the mating between relatives, in both captive and wild species have been reported to negatively affect a wide variety of life history traits related to survivorship, known as inbreeding depression. More recently however, the effects of inbreeding on reproductive traits such as male sperm quality have become of particular interest (primarily in mammals while studies on other taxa are lacking) because of their direct role in competitive fertilization success. The objective of this study was to test the inbreeding depression hypothesis as it relates to sperm quality metrics using sexually mature males from a captive population of experimentally inbred and outbred lake trout, *Salvelinus namaycush.* I found that sperm quality metrics (including velocity, motility, linearity, longevity and concentration) of moderately (whose parents were maternal and paternal half siblings) and highly (whose parents were full siblings) inbred males, did not differ significantly from outbred males, providing no support for the predictions of the inbreeding depression hypothesis. The lack of differences in sperm quality traits can likely be explained by several factors including no inbreeding depression in the population (due to purging, low levels of inbreeding or no detection at the gamete level), or the captive conditions of the individuals used in the experiment. The findings in this study suggest that inbreeding does not necessarily lead to inbreeding depression in captive salmonid populations.

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[2] This chapter is the result of joint research with Dr. Trevor Pitcher and Dr. Chris Wilson

Introduction

Inbreeding, defined as the mating between relatives, has been demonstrated to reduce the fitness of species in a wide variety of taxa. A reduction in fitness (or phenotype value in practical terms) of offspring from matings between related individuals compared to the fitness of offspring from randomly mated individuals is known as inbreeding depression (Wright 1977). The reduction in fitness arising from inbreeding is commonly believed to be due to the combination of effects originating from dominance and overdominance (Charlesworth & Charlesworth 1999; Wright 1977). Fitness loss as a result of inbreeding has been reported in both captive (Lacy et al. 1993) and wild (Keller & Waller 2002; Crnokrak & Roff 1999) populations. Fitness losses due to reduced survivorship caused by inbreeding are related to a number of factors including for example, increased parasite susceptibility (Rijks et al. 2008), growth deformities (Grant & Grant 1995), and decreased juvenile survivorship (Jiménez et al. 1994). Most research to date has focused on aspects of survivorship, however an individual's fitness is contingent on both their survival to sexual maturity and their reproductive success once sexually mature (Stearns 1992), suggesting a need to examine reproductive traits as they relate to inbreeding depression.

Inbreeding depression has been primarily studied in survival-oriented metrics however, reproduction is closely linked to fitness and can therefore be significantly reduced as a result of inbreeding depression (Saccheri et al. 2005; Crnokrak & Roff, 1995). The focus of such studies has been to investigate reproductive traits such as mating success (Joron & Brakefield, 2003) and fertility (Johnston 1992), although, inbreeding depression has also been shown to affect fitness in more cryptic ways such as

having detrimental effects on sperm quality (reviewed by Fitzpatrick & Evans 2009). For example, in both wild rabbits (*Oryctoloagus cuniculus*) and lions (*Panthera leo*), decreased levels of heterozygosity (resulting in the expression of increased levels of deleterious recessive alleles) were associated with an increase in sperm abnormalities (Gage et al. 2006; Wildt et al. 1987). Additionally, inbreeding depression has been shown to affect sperm quality traits such as sperm motility, sperm velocity and sperm concentration, which have been determined to have significant effects on fertilization success (Fitzpatrick & Evans 2009). In white-footed mice (*Peromyscus leucopus*) and Mexican gray wolves (*Canis lupus*) an increase in inbreeding led to significant reductions in sperm motility (Malo et al. 2010; Asa et al. 2007) while in the Florida panther (*Felis concolor coryi*), sperm motility and sperm concentration were significantly lower when compared to non-inbred panther populations (Barone et al. 1994). It is thought that spermatozoa may be more susceptible to the effects of inbreeding depression given the complex nature of spermatogenesis and the high potential for mutational defects (Gage et al. 2006). As sperm quality can significantly contribute to the fitness of an individual, it becomes an important mechanism influencing reproductive success. The majority of studies that have investigated the effects of inbreeding on sperm quality have focused on mammals, meanwhile studies on other taxa have been largely ignored (but see Weeks et al. 2009; Zajitschek & Brooks 2010; Zajitschek et al. 2009).

Lake trout (*Salvelinus namaycush*) are an interesting species to study the effects of inbreeding on sperm quality metrics due to their unique life history. Unlike most salmonids, lake trout are a long-lived (average life span of 20 years), iteroparous species that have a tendency to breed nocturnally (Gunn 1995). As a result, lake trout lack sexual

dimorphism and exhibit little to no pre-spawning male-male competition (Esteve et al. 2008; Gunn 1995). Given these unique traits, it is possible that sexual selection operates predominantly at the gamete level in this species.

Lake trout are also of conservation and management concern, due to population declines and losses in the Great Lakes over the past half-century (McDermid et al. 2010). Overfishing and predation by the invasive sea lamprey (*Petromyzon marinus*) caused major declines in lake trout populations throughout the 1940s and 1950s, culminating in the loss of native populations from several of the Great Lakes (Walters et al. 1980; Selgeby et al. 1995 and references therein). The long-term persistence of many inland populations is similarly of concern, with many jurisdictions employing re-stocking and rehabilitation efforts to help restore natural populations (Piller et al. 2005). As these programs are focused on ways to improving the success of this species in the wild, assessing gamete quality is an important element of anticipating reproductive success or failure.

In this study I tested the inbreeding depression hypothesis by examining the effects of varying levels of inbreeding on sperm quality metrics in a captively bred population of lake trout. I predict that inbred offspring would produce poor quality sperm (including lower velocity, motility, linearity, longevity and concentration) compared to offspring arising from unrelated parents. I examined sperm traits from individuals from four different levels of inbreeding, including: (i) offspring arising from full siblings, (ii) offspring arising from maternal half siblings, (iii) offspring arising from paternal half siblings and (iv) offspring arising from unrelated individuals.

Methods

Creation of Family Lines

      Lake trout originated from wild-caught populations in two interconnected lakes in Haliburton, Ontario (Clean and Macdonald Lake, Latitude: 45.2501; Longitude: -78.5329). In 1983, wild lake trout were manually spawned at the Ontario Ministry of Natural Resources Codrington Fisheries Research Facility, located in Codrington, Ontario (Latitude: 44.1468; Longitude: -77.8045). Single-pair crosses (10 males and 10 females) from this collection were used to create 10 presumably unrelated families. All fish at the hatchery were freeze-branded and fin clipped to allow for individual and family identification. In 1994 (when males and females were sexually mature), four unrelated offspring from the single-pair crosses (2 males and 2 females) were chosen haphazardly and used to set up a 2 x 2 factorial cross, producing four unrelated families (see Figure 3.1a). Individuals chosen for the 2 x 2 factorial cross were unrelated based on allozyme data (Wilson C., personal communication). In 2003, the mature adults from these four families were used to set up experimental inbreeding crosses. A factorial cross with 8 females and 4 males (each female mated to the same four males) was used to create families (n = 32), each exhibiting four levels of inbreeding; (i) full siblings, (ii) maternal half siblings, (iii) paternal half siblings and (iv) unrelated individuals (see Figure 3.1b). On November 9 and 10, 2009, 102 males from the 32 families were sampled for milt. Previous research has shown that sperm quality traits (e.g. velocity, longevity and density) in captive lake trout tends to peak in the middle of the spawning season (Chapter 2), therefore the data we used in this study was collected during that timeframe.

Fish Husbandry and Sperm Collection

Individuals were housed in 5 circular tanks (5' diameter with a working depth of 24") fed by untreated water from the headwaters of Marsh Creek (Latitude: 44.1834; Longitude: -77.7828) and were kept under a natural photoperiod and temperature regime. Individuals from all four inbreeding treatments were distributed randomly throughout the 5 tanks. Males and females in each tank were prevented from mating by a lack of natural substrate on the bottom (Yeates et al. 2007). Fish were fed AquaBrood feed (7.5 mm pellet; Corey Nutrition Company, Fredericton, NB, Canada) at 0.5% body weight per day. Total length and weight (mean ± SEM) of the males at the onset of spawning were 539.87 ± 3.83 mm and 1697.08 ± 36.74 g, respectively. Males were anaesthetized using 40 to 50 ppm solution of MS-222 (Syndel International, Vancouver, BC, Canada). Milt samples were collected, using slight pressure to the abdomen and massaging towards the urogenital pore, in 532 mL Whirl-Pak© plastic bags (Nasco, Newmarket, ON, Canada) and stored in a cooler (~ 8˚C). Extra care was taken to ensure that urine, feces, blood, or water did not contaminate the milt samples.

Sperm Activity

Sperm were video-recorded using a CCD black and white video camera (XC-ST50, Sony, Japan) module at 50 Hz vertical frequency, mounted on an external-phase contrast microscope (CX41 Olympus, Melville, NY, USA) with a 10 × negative-phase magnification objective (Pitcher et al. 2009). Sperm quality traits (velocity, motility, linearity, and longevity) were assessed within 3 hours of collection, by activating an

aliquot (< 0.2 µL) of milt with 10 µL of source water circulating through the hatchery. Water temperature was maintained by holding water samples in a cooler at ~8°C (temperature of the water on November 9 and 10, 2009). Once recordings were taken, sperm quality traits were analyzed, using the HTM-CEROS sperm analysis system (version 12, CEROS, Hamilton Thorne Biosciences, Beverly MA, USA) set at the following parameters: number of frames = 60, minimum contrast = 11, photometer = 55-65, minimum cell size = 3 pixels. Sperm velocity, motility and linearity were recorded at 5 s post-activation. It has been shown in salmonids that once sperm is released and activated, there is ~ 5 s to fertilize eggs (Hoysak & Liley 2001). Therefore, a post-activation time of 5 s is the most biologically relevant as it corresponds to conditions found in the wild. Sperm velocity was estimated as the average velocity measured over the actual point-to-track followed by the cell. We also examined sperm velocity in terms of VAP (average path velocity) and VSL (straightline velocity) and found qualitatively similar relationships, so we do not present them here. Sperm motility was calculated as the percentage of motile cells divided by the total number of cells. Sperm path linearity, which is the straightness with which a sperm cell moves per unit of distance traveled, was measured as the departure of the cell track from a straight line. Straighter swimming sperm will have a larger linearity value. Longevity was estimated as the time for ~95% of the sperm cells to become immotile (Gage et al. 2004). The sperm analysis system uses the average number of sperm per recorded track to estimate sperm quality traits. Each sperm track was manually checked for quality assurance (e.g. no sperm were simply moving due to flow of the water added for activation).

Sperm Concentration

Sperm concentration was estimated by adding between 250 µL to 1000 µL milt (depending on how much milt was available) for individual males into separate 1500µL Eppendorf microcentrifuge tubes. Eppendorf tubes were then centrifuged for 10 min at 7500 x g (accuSpin Micro 17, Fisher Scientific, Fair Lawn, New Jersey, USA). Centrifuging milt separates sperm and seminal plasma into opaque white and clear solutions, respectively (Hoysak & Liley, 2001). To calculate sperm concentration, the volume of the opaque layer was recoded for each sample and then divided by total milt volume and expressed as a percentage.

Statistical Analyses

JMP (v.8.0.2; SAS Institute Inc., Cary, NC, U.S.A) statistical software was used to analyze data. One-way ANOVA models were used to compare sperm quality traits and Fulton's condition factor [K= total mass/(total length)^3 x 100000] between the levels of inbreeding. Sperm velocity, motility and linearity were analyzed at 5 s post-activation. All traits were tested for normality using the Shapiro-Wilk test and homogeneity of variances were tested using Levene's test. Motility data were arcsine square root transformed to satisfy statistical assumptions. The Kruskal-Wallis test was used to analyze longevity, sperm concentration and Fulton's K after attempting transformation which was not successful. Potential tank effects were tested for before running one-way ANOVAs. All p-values were greater than 0.05, therefore individual tanks were not included in the analyses.

Results

Sperm velocity (full-sibling offspring (FS): 111 ± 21.7; maternal half-sibling offspring (MHS): 102.8 ± 26.4; paternal half-sibling offspring (PHS): 106.7 ± 25.3; unrelated offspring (UN): 118.8 ± 25.2; $F_{3,98}$ = 2.03, P = 0.12), motility (FS: 61.8 ± 19.2; MHS: 59.6 ± 18.8; PHS: 59.9 ± 23.6; UN: 62.6 ± 21.9; $F_{3,98}$ = 0.17, P = 0.92), and linearity (FS: 69.4 ± 10; MHS: 68.2 ± 9.1; PHS: 73.1 ± 10.2; UN: 71.5 ± 8.7; $F_{3,98}$ = 1.30, P = 0.28) did not significantly differ between the levels of inbreeding (Figure 3.2).

For traits that were analyzed using the Kruskal-Wallis test [sperm longevity (FS: 24.3 ± 7.6; MHS: 24.9 ± 8.9; PHS: 25.8 ± 10.3; UN: 27.6 ± 8.1; χ^2= 2.86, df = 3, P = 0.41), sperm concentration (FS: 48.2 ± 22.8; MHS: 51.4 ± 16; PHS: 43.5 ± 26.1; UN: 44 ± 23.4; χ^2 = 4.60, df = 3, P = 0.20) and Fulton's K (FS: 1.07 ± 0.11; MHS: 1.06 ± 0.07; PHS: 1.05 ± 0.10; UN: 1.06 ± 0.06; χ^2 = 1.35, df = 3, P = 0.72)], no significant difference was found between the levels of inbreeding (Figure 3.3).

Because no significant relationship was found between sperm quality metrics and inbreeding, a post-hoc power analysis test was conducted to examine the statistical power of our tests (GPower, http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/). I found on average, the statistical power for sperm traits was 76% (percent motility: 0.55; velocity: 0.93; linearity: 0.85; longevity: 0.74; concentration: 0.74).

Discussion

My findings did not provide support for the hypothesis that inbreeding depression affects sperm quality in a captive population of lake trout. I found that for moderately

inbred (whose parents were maternal and paternal half siblings) to highly inbred males (whose parents were full siblings), sperm quality showed no apparent signs of inbreeding depression. These results do not follow the general pattern found in the literature; inbreeding causes detrimental effects on sperm quality in many species (see Chapter 1, Table 1.1). For example, in a recent comparative analysis across 20 endangered mammal species, decreased levels of heterozygosity (as a result of increased levels of inbreeding) significantly reduced sperm quality metrics across both wild and captive populations (Fitzpatrick & Evans 2009). Here, I provide possible explanations as to why inbreeding did not lead to inbreeding depression in my study.

The lake trout's unique life history strategy along with their natural distribution may provide support for a lack of detection. The retreat of glaciers following the Pleistocene has resulted in the existence of lake trout inhabiting small, inland, freshwater lakes, which act as isolated, closed systems (Wilson & Hebert 1998). Lake trout, unlike most salmonids, are a long-lived iteroparous species adapted to live in small populations throughout these isolated lakes (Wilson & Mandrak 2004). Given this, it is possible that generations of inbreeding may have occurred and consequently the purging of lethal alleles (Larsen et al. 2011). Purging is a mechanism that reduces the fitness related losses of inbreeding through the removal of deleterious recessive alleles via natural selection (Hedrick 1994; Thornhill 1993). Purging is more likely to occur in small populations with a long history of inbreeding and consequently an accumulation of these lethal alleles (Crnokrak & Barrett 2002). Once populations have been purged of deleterious recessive alleles, further inbreeding would in theory have no effect on the fitness of the population. Evidence for purging has been shown in both plants and animals (reviewed in Crnokrak

& Barrett 2002). For example, in guppies (*Poecilia reticulata*), purging was demonstrated in a captive population that had been experimentally reared for 10 generations (Larsen et al. 2011). Inbreeding depression in offspring survival and clutch size increased throughout the first 4 to 6 generations of inbreeding followed by a recovery in fitness thereafter, despite further generations of inbreeding (Larsen et al. 2011). Overall, it is difficult to determine whether deleterious recessive alleles were previously lost in this population due to the conditions and assumptions underlying purging as a mechanism for reducing inbreeding depression. An understanding of the genetic mechanisms responsible for inbreeding i.e. dominance (necessary for purging) or overdominance, strength of the deleterious recessive alleles, and strength of selection on those alleles in the wild is needed to provide support for purging (Wang et al. 2002).

Another possible explanation for my results may be that inbreeding depression only affects early life-history traits. There is a possibility that if inbreeding depression was severe enough, individuals who expressed recessive alleles early in development would have likely died off, for example at the hatching stage, leaving behind those individuals with increased levels of heterozygosity and thus increased fitness (Keller & Waller 2002). Once these remaining males reached sexual maturity, they would in theory be unaffected by further inbreeding and possible inbreeding depression, which would otherwise reduce sperm quality. In salmonids, there is evidence for inbreeding depression affecting early life history traits such as fry mortality in rainbow trout (*Oncorhynchus mykiss*) (Kincaid 1976), while no effect was found for fertility or egg hatching success (Su et al. 1996). In order to conclude that inbreeding depression in this population had no

effect at the gamete level, I would need more information on the wild-origin ancestors of the population.

A lack of inbreeding depression in this lake trout population could also be attributed to having only one generation of experimental inbreeding in my study. In wild salmonids, inbreeding is expected to be a gradual process that can take several generations to demonstrate any negative effects (Wang et al. 2002). Several studies have found evidence for inbreeding depression in species that have experienced multiple generations of inbreeding (Crnokrak & Barrett 2002). In a study on guppies by Zajitschek et al. (2009), 4 generations of brother-sister matings ($F = 0.59$) were required to detect any evidence of inbreeding depression on sperm competitiveness, while moderately inbred males ($F = 0.25$) showed no reduction in the trait. With one generation of inbreeding in my study, full sibling offspring had an F value of 0.25, while maternal and paternal half sibling offspring had an F value of 0.125. My results are potentially consistent with those found in the guppy where an inbreeding coefficient of 0.25 was not strong enough to detect negative effects on sperm quality. In rainbow trout, it was suggested that a lack of detection of inbreeding depression on male fertilization success could be attributed to low levels of inbreeding in the study (Su et al. 1996). Although I did not find evidence for inbreeding depression in this population, we cannot exclude the long-term effects i.e. successive generations of inbreeding as a potential source of inbreeding depression.

Soft selection due to captive conditions has more recently been shown to mask the effects of inbreeding depression (Armbruster & Reed 2005; Crnokrak & Roff 1999). Differences in the severity of inbreeding depression between captive and wild

populations have been documented in a variety of species. Benign living conditions often result in an underestimate of the effects of inbreeding due to a lack of environmental pressure (e.g. Armbruster & Reed 2005; Crnokrak & Roff 1999; Hedrick & Kalinowski 2000; Keller & Waller 2002). These environmental pressures range from unpredictable climate conditions, disease, and resource limitation to the absence of competition for mates (Crnokrak & Roff 1999; Joron & Brakefield 2003). Studies that involve the introduction of captively bred individuals into their wild environments often show an increase in the negative effects due to inbreeding. For example, in white-footed mice (*Peromyscus leucopus*), survivorship rates were lower for inbred individuals reintroduced into the wild, compared to those living under laboratory conditions (Jiménez et al. 1994). In butterflies, male mating success was lower when inbred individuals experienced a natural free flight environment compared to a caged environment (Joron & Brakefield 2003). As male-male competition is generally absent in captive populations, negative effects of inbreeding depression are frequently exposed when males directly face competition for mates (Meagher et al. 2000). For example, in Nile tilapia (*Oreochromis niloticus*), the proportion of offspring sired in a single spawning event by inbred males was decreased when there were an increased number of males competing for copulations (Fessehaye et al. 2009). Male-male competition at the gamete level has also elucidated similar negative effects at the gamete level. In the flour beetle (*Tribolium castaneum*), male fertility was not significantly different between inbred and outbred individuals, however under competition, inbred males were found to suffer from decreased sperm competitiveness (Michalczyk et al. 2010). In guppies (*Poecilia reticulata)*, sperm number was significantly reduced in inbred compared to outbred males however this effect was

not observed under laboratory conditions (Zajitschek & Brooks 2010). Lake trout in this study had been living in a captive environment for seven years. Although conditions were semi-natural, with no manipulation of water quality (i.e. water was provided by a natural stream) or temperature, competitive interactions for both resources and mates were absent. Evidence for relaxed selection on lake trout from the same hatchery I used in this study has been demonstrated. McDermid et al. (2010) found that wild-origin lake trout outcompeted hatchery-reared lake trout in a number of life history traits, including fertilization success (an indicator of sperm quality). Wild lake trout, compared to captive, have an opportunity for post-copulatory sexual selection as multiple males have been observed accompanying a spawning female (Esteve et al. 2008). The loss of this selective pressure on sperm competition in captively bred males may help to explain why both inbred and outbred individuals did not differ in sperm quality metrics.

Finally, it is possible that we did not have enough statistical power to detect any effects of inbreeding depression on sperm quality (Thomas & Juanes 1996). However, using a post-hoc power analysis test suggests that this is unlikely. Statistical power for sperm traits ranged from 55-92%, with an average of 76%. Compared to other studies that have investigated the effects of inbreeding on sperm quality, a sample size of 102 males in our study is large. Therefore, it is expected that if inbreeding depression had any detrimental affects on sperm quality, it would have been statistically detected in my study.

In summary, sperm quality appears to be unaffected by experimental levels of inbreeding in a captive population of lake trout. Coefficients of inbreeding depression (δ), where $\delta = 1 - (X_1 / X_o)$ (adapted from Crnokrak & Roff 1999) in my study were

extremely low, ranging from 0.12 for sperm longevity to -0.09 for sperm concentration (where the inbred mean trait value, X_1, was higher then the outbred mean trait value, X_o). Compared to inbreeding coefficients calculated for sperm traits in other fish species, along with other species in general (Table 1.1 in Chapter 1), I assume with a high level of certainty that these fish did not suffer from the effects of inbreeding depression on sperm quality. Although my findings are inconsistent with those found in the literature, the lack of differences in sperm quality traits might be explained by no inbreeding depression in the population (due to purging, low levels of inbreeding or no detection at the gamete level), or the captive conditions of the experiment. My study highlights the importance of the conditions and assumptions needed to detect inbreeding depression at the gamete level and provides important insight into inbreeding in hatchery-reared salmonid populations.

Acknowledgements

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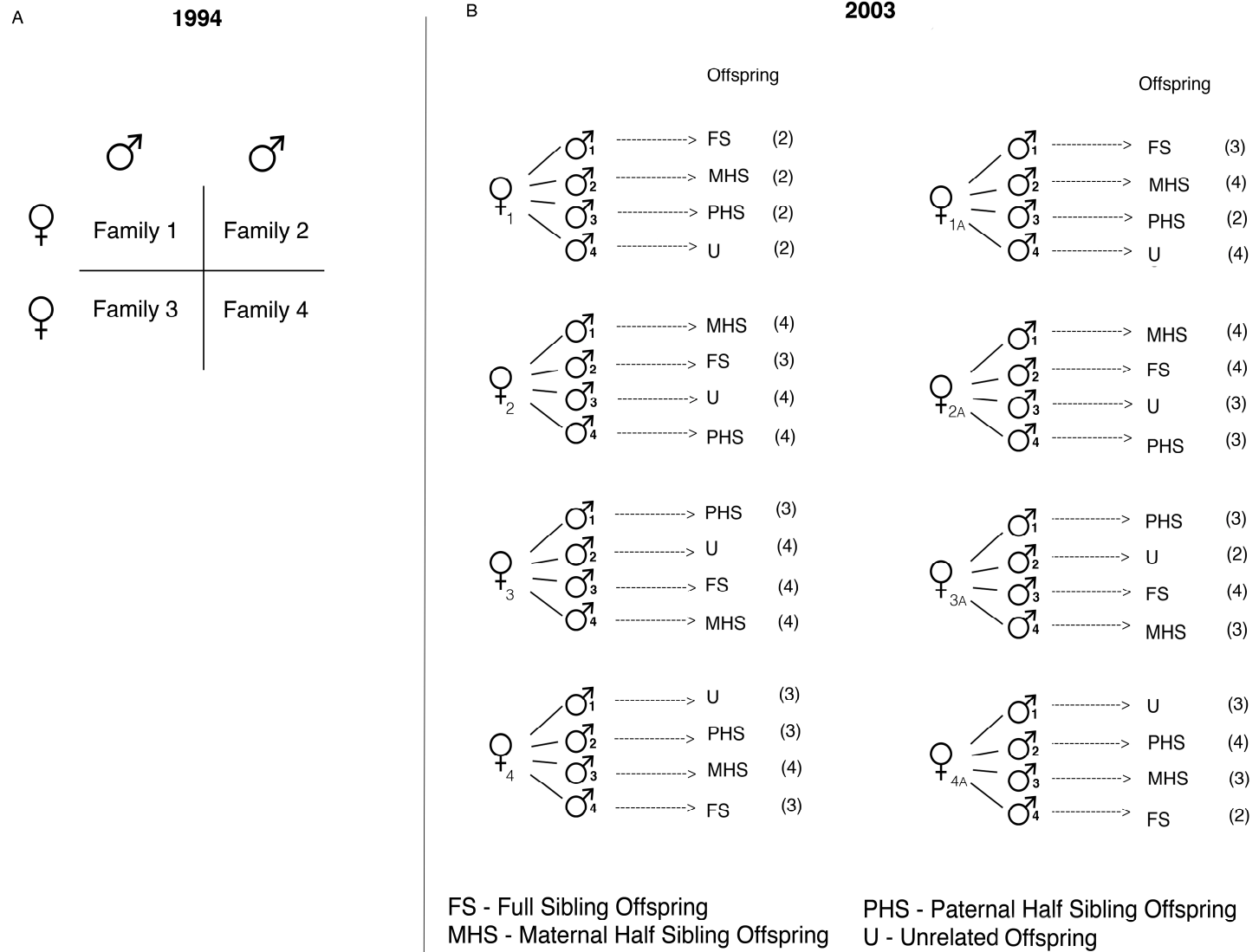
Figure Captions

Figure 3.1 Breeding scheme, representing the creation of four levels of experimentally inbred and outbred lake trout. In 1994, four fish (2 males and 2 females) were used to set up a 2 x 2 factorial cross, producing four families (A). Sexually matured offspring from the 2 x 2 cross were then used to set up experimental inbreeding crosses in 2003 (B). Eight individual females were mated to the same four same males to produce full sibs, maternal half sibs, paternal half sibs and unrelated individuals. Subscript numbers indicate female family origin (family 1, 2, 3 or 4) and subscript letters indicate different females from the same family.

Figure 3.2 The relationship between sperm velocity (A), percent motility (B) and linearity (C) at five seconds post-activation. These sperm performance metrics did not significantly differ between inbred and outbred males (see text for details). The line in the center of each box represents the median. The upper and lower bounds of the box represent the upper and lower quartiles respectively and the extended lines represent the maximum and minimum values excluding outliers. Raw data are presented for the sake of clarity.

Figure 3.3 The relationship between inbreeding level and measures of sperm longevity (A), sperm concentration (B) and individual Fulton's condition (C). These sperm metrics and body condition did not significantly differ between inbred and outbred males (see text for details). The line in the center of each box represents the median. The upper and lower bounds of the box represent the upper and lower quartiles respectively and the

extended lines represent the maximum and minimum values excluding outliers. Raw data

is presented.

Figure 3.1

A — 1994

B — 2003

FS - Full Sibling Offspring
MHS - Maternal Half Sibling Offspring
PHS - Paternal Half Sibling Offspring
U - Unrelated Offspring

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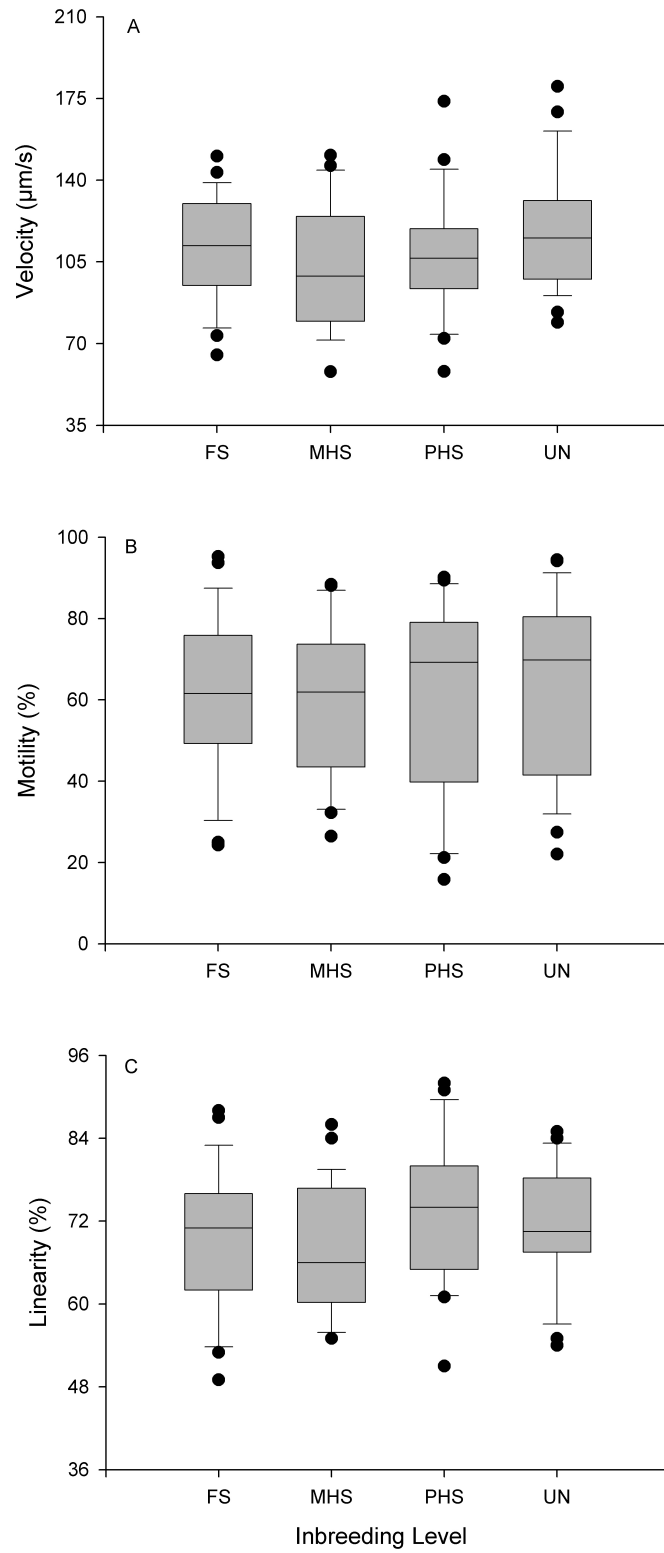
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Figure 3.3
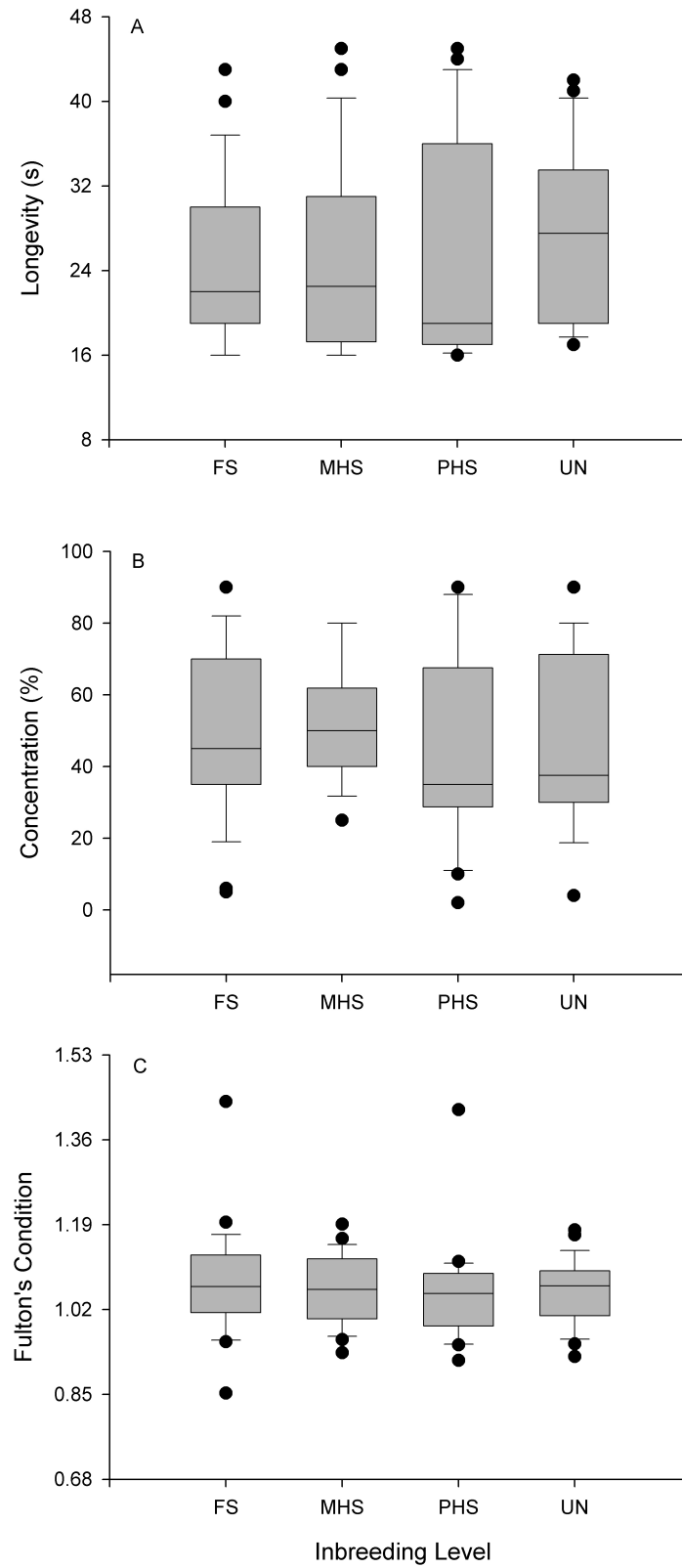
Summary

In this thesis I examined seasonal variation and the effects of inbreeding on sperm quality in a captive population of lake trout. The purpose of this final chapter is to summarize the findings of my research and provide implications and future directions that could expand on my results. I found that sperm quality tends to peak throughout the middle of the reproductive season in lake trout. Overall, I was able to demonstrate that 7 to 27% of the variation in sperm quality traits can be explained by seasonality. Using these data, sperm from experimentally inbred and outbred lake trout were sampled during the peak of the reproductive season in order to test the inbreeding depression hypothesis, which predicts that inbreeding will have detrimental effects on sperm quality. I found no significant relationship between inbreeding level (full sibling offspring, maternal half sibling offspring, paternal half sibling offspring, and unrelated offspring) and sperm quality suggesting there was no inbreeding depression. Together, these results provide important implications for studying sperm quality in captively bred salmonid populations.

Chapter Two

Sperm quality, which includes measures of motility, velocity, longevity and density are considered the primary determinants of fertilization success in various externally fertilizing fish species, including salmonids (e.g. Casselman et al. 2006; Gage

et al. 2004; Tuset et al. 2007). As sperm quality plays a vital role in fertilization success, it is important to understand the environmental factors that can affect sperm quality for example, temperature and photoperiodic conditions (Chemineau et al. 2007), which ultimately depend on the timing of the reproductive season. It has been demonstrated that sperm quality is highly dependent on the timing of the spawning season, both in wild and captive fish species (Babiak et al. 2006; Butts et al. 2010; Munkittrick & Moccia 1987). Therefore, understanding when peak sperm quality occurs has important implications for both the aquaculture industry and studies that examine sperm quality (in order to rule out seasonality as possible consequence of poor performance). Thus, in Chapter Two, I examined the seasonal variation in sperm quality of a captively bred population of lake trout using both a regression and a repeated measures approach.

I found significant positive linear relationships for percent motility and linearity at 5 seconds post activation, significant negative quadratic relationships for velocity at 5 seconds post activation, longevity and density and finally a significant positive quadratic relationship for linearity at 10 seconds post activation. Using the repeated measures approach, I found a significant effect of season on percent motility, velocity, linearity and longevity. Overall, sperm quality was highest during the middle two sampling dates, corresponding with the middle of the reproductive season for wild populations of lake trout in Ontario.

Sperm quality was generally low at the start of the reproductive season for all sperm traits measured except linearity at 10 seconds post activation. It has been suggested that sperm may be less motile at the onset of the season as they may not yet have gained the capacity for forward movement (Billard 1986; Mylonas et al. 2003). Following the

onset of the spawning season, sperm quality in this experiment either increased continually until the end of the season or peaked in the middle, decreasing thereafter. This decrease in sperm quality towards the end of the spawning season for traits where negative quadratic relationships were found can likely be attributed to sperm ageing. In salmonids, spermatogenesis is a discontinuous process in which sperm is released from the sperm ducts over several months, ageing throughout the spawning period (Billard 1986). During this timeframe, declines in ATP levels (Dreanno et al. 1999), hormonal activity of the sperm duct (Koldras et al. 1996; Shangguan & Crim 1999) and seminal plasma contents (i.e. ions, proteins, antioxidants; Ciereszko & Dabrowski 1995; Hajirezaee et al. 2010) have been reported. These biochemical and physiological metrics have been linked to differences in sperm quality (Lahnsteiner et al. 1996, 1998).

In order to further understand mechanisms underlying seasonal changes in each of the sperm quality metrics, future work should investigate whether biochemical (i.e. ions, proteins, antioxidants) and physiological (i.e. ATP, hormonal activity) metrics in milt differ across the reproductive season as well as and whether these differences correlate which the observed differences in sperm quality demonstrated in lake trout. Future studies should also measure the most biologically relevant time frame to measure sperm quality in lake trout. This could be achieved through fertilization success experiments, where percent fertilization is measured for batches of eggs fertilized using sperm activated at different post activation times. Although studies in other salmonids have found that eggs are fertilized within 5 seconds of sperm activation (Hoysak & Liley, 2001), time to fertilization has yet to be determined in lake trout. Finally, it would be useful to study the effects of seasonality on sperm quality in a microenvironment that

more closely mimics that found in the wild. For example, female ovarian fluid, which is released along with eggs during a spawning event, has been shown to increase sperm velocity in a number of salmonids (eg. Rosengrave et al. 2009; Turner & Montgomerie 2002; Wojtczak et al. 2007). Future studies could look at the effects of seasonality on sperm quality by activating sperm with female ovarian fluid rather than water throughout the reproductive period.

Chapter Three

Using sperm obtained from the peak of the reproductive season, as determined in Chapter 2, I tested the hypothesis that inbreeding has detrimental effects on male sperm quality in lake trout by comparing sperm quality from highly inbred, moderately inbred and unrelated males. It has been previously demonstrated that mating between relatives can lead to significant reductions in sperm quality and consequently, fertility and reproductive success (Gomendio et al. 2000; Fritzsche et al. 2006). The effects of inbreeding on cryptic life history traits such as sperm quality have most often been demonstrated in mammals (Fitzpatrick & Evans 2009), while studies on fish are rare.

Overall, I found no significant differences in sperm quality between highly inbred (full sibling offspring), moderately inbred (maternal and paternal half sibling offspring) and unrelated offspring. I have proposed four possible explanations as to why inbreeding depression did not lead to reductions in sperm quality in a captive population of lake trout. First, it is possible that the life history of this species (large, long-lived, late-maturing) and distribution (small, isolated freshwater lakes) led to purging of deleterious

recessive alleles via natural selection. In order to determine if purging occurred in our population of lake trout, an idea of ancestral inbreeding patterns in the wild as well as the strength of selection on detrimental alleles is necessary. Second, it is possible that inbreeding depression had no effect at the gamete level in our population of lake trout. Again, this explanation relies on the assumption that the wild ancestral population was inbred however, those individuals that were inbred would have expressed deleterious recessive alleles early in development, dying off before reaching sexual maturity. The remaining males would in theory be unaffected by further inbreeding depression and thus would not express any detrimental effects on sperm quality due to inbreeding. Third, a lack of inbreeding depression could be attributed to limiting our study to only first generation inbred males. Previous studies have shown that multiple generations of inbreeding in both wild and captive environments are required for the effects of inbreeding depression on sperm quality to take effect (Crnokrak & Roff 1999). The final explanation for a lack of inbreeding depression on sperm quality involves "soft" selection on reproductive traits due to captive conditions. Relaxation on the detrimental effects of inbreeding depression between captive and wild populations has been extensively demonstrated (Armbruster & Reed 2005; Crnokrak & Roff 1999). Harsh conditions in the wild often result in greater selective pressure on life history traits and therefore an increase in the effects of inbreeding depression (Crnokrak & Roff 1999; Joron & Brakefield 2003).

Future studies investigating inbreeding depression in lake trout should address these four factors that may explain the lack of observed inbreeding depression in sperm related traits. Understanding such mechanisms will aid in the development of future

inbreeding studies as well as provide insight into relative contributions of genetic versus environmental factors leading to inbreeding depression, or, a lack thereof in lake trout. Additionally, it would be useful to look at inbreeding depression at the level of reproductive success as it may be possible that although no difference in sperm quality traits were observed, inbred males may suffer from decreased fertilization success, as was found in the golden hamster (Fritzsche et al. 2006), where no apparent differences in sperm traits were observed between inbred and outbred males however, significant reductions in reproductive success were demonstrated.

Conclusion

In my thesis, I have determined that sperm quality in a captive population of lake trout peaks in the middle of the reproductive season, while remaining unaffected by inbreeding depression. These findings have implications for the aquaculture industry and provide insight into sperm quality of a captive population of lake trout, which, has yet to be explored. For example, based on an index of fertilization success as determined by sperm velocity in another salmonid species (Tuset et al. 2008), the seasonal differences in sperm velocity demonstrated in Chapter 2 would result in a 10% increase in fertilization success between the greatest (peak spawning season) and lowest recorded sperm velocities. This information is important for artificial breeding programs as well as estimation of a male's reproductive potential in the wild. In terms of inbreeding depression, our results are inconsistent relative to other taxa as demonstrated by comparing the coefficient of inbreeding depression, δ (Crnokrak & Roff 1999) for sperm

traits. For example, in terms of sperm motility, my results translate into an δ value of

0.01, while the average value determined in other studies is 0.26 (Barone et al. 1994; van

Eldik et al. 2006; Wildt et al. 1987; see Chapter 1, Table 1.1). My findings suggest that

inbreeding does not necessarily lead to inbreeding depression in captive salmonid

populations, however other fitness traits such as fertilization success should be

investigated in the future.

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