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Application of otolith increment analysis to the study of maturation timing in female kokanee salmon

Yelin Xu, *The University of Western Ontario*

Supervisor: Dr. Yolanda Morbey, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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APPLICATION OF OTOLITH INCREMENT ANALYSIS TO THE STUDY OF MATURATION TIMING IN FEMALE KOKANEE SALMON

Thesis format: Monograph

by

Yelin Xu

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

I investigated the influence of growth history on the expression of female reproductive tactics in kokanee salmon (*Oncorhynchus nerka*) from Meadow Creek Spawning Channel, British Columbia, Canada. Female kokanee either arrive at the spawning area with red nuptial coloration, or less commonly, sexually immature with silver coloration. Silver- and red-arriving females may reflect different reproductive strategies in the population. I used otolith increment measurements to determine fish growth. In contrast to earlier studies, silver- and red-arriving females in 2013 did not differ in age at maturity (mostly were age 3+) or size at maturity (length from eye to tail, silver: 243.50 ± SE = 0.26 mm, red: 247.06 ± SE = 0.19 mm). In terms of females maturing at age 3+, silver- and red-arriving fish did not show a difference in any size-at-age or growth increment-at-age. This study indicates that growth is unlikely to influence the reproductive tactic adopted by spawning female kokanee salmon. Further research should focus on energy allocation differences during the pre-reproductive stages, and the heritability of the silver- and red-arriving tactics in female kokanee.

Keywords

kokanee, salmon, *Oncorhynchus nerka,* female reproductive tactics, maturation timing, age at maturity, size at maturity, growth, otolith

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Introduction

Reproductive tactics are traits or sets of traits that are adopted by individuals to maximize reproductive success in intra-specific competition. For example, male salmon and male beetles fight or sneak to approach mates, female wasps and bees, and female birds prepare nests or usurp other females' nests for broods (Yom-Tov 1980, Brockmann 1980, Gross 1985, Field 1992, Moczek 1998). Tactic expression can be determined by underlying genes, or can be dependent on an individual's condition or environment during development. Alternative reproductive tactics (i.e., fighting and sneaking behaviours) have been described in male Pacific salmon (*Oncorhynchus* spp.). The expression of reproductive tactics of these fish mainly depends on the body size at maturity, which is influenced by both their growth history and their genes (Gross 1991, Heath et al. 1994). In contrast, alternative reproductive tactics are less apparent in female salmon. Female kokanee salmon (*Oncorhynchus nerka*) have been found to adopt different reproductive tactics associated with maturation timing. Specifically, red-arriving females that are more mature at arrival spawn within a few days of arrival. On the contrary, the less mature silver-arriving females complete maturation on the spawning grounds and then spawn late in the season. Late-season spawning of females is proposed as a tactic to avoid nest superimposition by later-spawning fish (Morbey and Ydenberg 2003, Warren and Morbey 2011). However, the cause of this tactic expression remains unknown. The objective of my study is to investigate if growth history influences the expression of female reproductive tactics in a kokanee salmon population.

Evolutionary Stable Strategies and Alternative Reproductive Tactics

An evolutionary stable strategy (ESS) describes strategies that are maintained in equilibrium within a population under natural selection (Maynard Smith 1982). A strategy is considered to be an evolved rule that determines a set of distinct behaviours or phenotypes. These different behaviours or phenotypes, referred to as tactics, are used for achieving fitness under a strategy. Generally, an ESS is regulated by frequency-dependent selection: a tactic is more successful and increases in frequency when it is rare (Maynard Smith 1982, Gross 1984). An ESS can consist of alternative strategies, a mixed strategy, or a conditional strategy (Gross 1996). In a population with alternative strategies, individuals’ tactics have a genetic basis and do not switch (Gross 1996). In a mixed strategy and a conditional strategy, individuals adopt alternative tactics with a certain probability. A conditional strategy exists at the individual level. The expression of tactics relies on an individual’s condition (e.g., body size) or the external environment (Gross 1984, 1996, Tomkins and Hazel 2007). In a mixed strategy, an individual has the ability to adopt different tactics. The expression of a tactic depends on the environmental condition, and/or the incidence of other tactics in the population (Gross 1984, 1996).

Alternative reproductive tactics (ARTs) refer to individuals within a population adopting distinct ways to maximize reproductive fitness (Oliveira et al. 2008). ARTs are often subject to negative frequency-dependent selection, and coexist in an ESS (Oliveira et al. 2008, Taborsky and Brockmann 2010). ARTs can be expressed in forms of behavioural, morphological, and physiological differences (Gross 1996, Oliveira et al. 2008). For instance, male beetles and male salmon adopt either fighting or sneaking tactics during their reproductive stage. Dominant males and sneaking males also show differences in body size and secondary sexual characteristics (Gross 1985, Moczek 1998, Emlen and Nijhout 2000, Moczek et al. 2002). In vertebrates such as tree lizard, side-blotched lizard, and bluegill fish, males that adopt ARTs have different levels of hormones such as Testosterone (T) and/or 11-ketotestosterone (11-KT) that are involved in territorial and parental care behaviours (Kindler et al. 1989, Moore et al. 1998, Sinervo et al. 2000, Knapp and Neff 2007). Males with a higher hormone level express more active reproductive behaviours.

In an individual, ARTs can remain for life once adopted (i.e., fixed ARTs) or can be plastic in response to external conditions (i.e., conditional ARTs). The expression of fixed ARTs is usually caused by genetic polymorphism. When an individual experiences an ontogenetic change, reproductive tactics can also be irreversible (Taborsky 1998). In bluegill sunfish (*Lepomis macrochirus*), for example, males have distinct life history pathways and fixed reproductive tactics. Territorial males mature at about age seven and conduct dominant behaviours. Non-territorial males mature at much younger ages with smaller body sizes, they adopt sneaking or mimicking tactics, but never exhibit the dominant tactic in their life (Gross 1984, Henson and Warner 1997, Neff and Gross 2001). In other cases, the expression of tactics is more flexible, and an individual's tactic can depend on both environmental condition (e.g., refuge availability) and social status (e.g., population density and sex ratio, Oliveira et al. 2008). Individuals decide which tactic to use unconsciously based on their current physiological condition. The reversibility of tactics in conditional ARTs has been proposed to be associated with a late onset of tactic expression (e.g., in adulthood, Oliveira et al. 2008).

Alternative Reproductive Tactics in Male Salmonid Fish

ARTs have been widely reported in males in Pacific salmon (*O.* spp., Gross 1984) and Atlantic salmon (*Salmo salar*, Quinn 2005). When male salmon undergo sexual maturation, they develop extended secondary sexual characteristics such as large hooked snouts and exaggerated dorsal humps. Males with large body size and exaggerated secondary sexual characteristics are more likely to fight to obtain mates (Gross 1984, Mills 1991, Quinn 2005). Within a population, some male salmon have much smaller body size and less developed secondary sexual characteristics (known as jacks in Pacific salmon and parrs in Atlantic salmon). The small body size of these smaller males is not favoured in territorial behaviour, but is advantageous to sneaking. As a result, jacks and parrs usually sneak into nests in order to gain fertilizations as a female deposits her eggs (Gross 1985, 1991, Quinn and Foote 1994). In male salmon, the reproductive tactic is related to the body size at maturity: males with large body size fight during reproductive stage, whereas males with much smaller body size sneak. Fighting and sneaking behaviours are two alternative reproductive tactics in the population.

The expression of ARTs is associated with growth history and genetic variation in male salmon. Sneaking male salmon are often younger than dominant males, and are usually found to have experienced an accelerated growth earlier in life (Gross 1991, Hutchings and Myers 1994). The tactic expression is also heritable to a certain extent. A high proportion of sneaking males is usually seen in the offspring of non-dominant males compare to those of the dominant males (Iwamoto et al. 1984, Heath et al. 1994, Garant et al. 2003, Piche et al. 2008).

Female Alternative Reproductive Tactics

Less frequently reported than in males, ARTs also exist in females (Oliveira et al. 2008). In many damselfly species, females usually have distinct colouration morphs. Some female damselflies that have male-like colouration are less attractive to male damselflies during their reproductive stage. These females can avoid unnecessary male harassment, and thus save energy and maximize reproductive fitness (Cordero 1992, Cordero et al. 1998, Arnqvist and Nilsson 2000, Andrés et al. 2002). The colour polymorphism has been found to be genetically determined (Andrés and Cordero 1999).

Extra-pair paternity has been documented as an ART in socially monogamous female birds. Monogamous females can mate with more than one male in order to maximize reproductive success (Westneat and Stewart 2003, Arnqvist and Kirkpatrick 2005, Forstmeier et al. 2014). The expression of female ARTs can also be associated with parental care. Brood parasitism, where some females disperse eggs into the nests of other females to avoid parental care, is a commonly used ART in insects and birds (Yom-Tov 1980, 2001, Brockmann 1980, Field 1992, Arnold and Owens 2002). Theft of nests and prey have also been documented as an avoidance of nest digging and food hunting in female insects (Brockmann 1980, Field 1989, 1992).

Reproductive Tactics in Female Kokanee Salmon

In Meadow Creek Spawning Channel, British Columbia, female kokanee salmon (*O. nerka*) have been hypothesized to express reproductive tactics related to parental care (Morbey and Guglielmo 2006, Warren and Morbey 2011). Kokanee salmon are landlocked sockeye salmon, distributed along the west North America from California to Alaska, and in northeast Japan. Native kokanee populations are most widespread in British Columbia, and non-native populations have been introduced to lakes throughout North America. Kokanee salmon are semelparous. Adults spawn in creeks or rivers, the emergent fry migrate to lake and feed for 3-4 years, and then migrate back to natal areas to spawn using energy accumulated during their lake residency (Groot and Margolis 1991, Quinn 2005). In the salmon mating system, females conduct nest construction and parental care (i.e., post-mating defense of eggs and nests, Quinn and Foote 1994, Quinn

2005, Jonsson and Jonsson 2011). A high-quality spawning site is critical to fertilization success (Foote 1990, Quinn and Foote 1994, Fleming 1998). There is generally a lack of ideal nesting sites on spawning grounds, and as a result, female salmon need to compete for nesting sites, and need to defend their nests from later-nesting females until death.

Much work has been done characterizing the two types of female kokanee salmon from Meadow Creek Spawning Channel (Morbey and Ydenberg 2003, Morbey and Guglielmo 2006, Warren and Morbey 2011). During the breeding season, female kokanee arrive on the spawning ground with extreme variation in their degree of sexual maturation and red nuptial colouration (**Fig. 1**). These females also have different maturation timing. Females commonly arrive with red skin colouration in an advanced state of maturation (called red-arriving females). Red-arriving females build nests in the gravel substrate and spawn within a few days of reaching the spawning ground. A small proportion (2.2 % and 11.4 % in 2008 and 2009, respectively) of females arrives as silvery fish with scales still present on their bodies (called silver-arriving females, Warren and Morbey 2011). The colouration at arrival of female kokanee exhibited a bimodal distribution (silver vs. red) in the population (Warren and Morbey 2011). Silver-arriving females have a delayed state of maturation at arrival (i.e., less-developed secondary sexual characteristics). Silver-arriving fish usually arrive early in the spawning season and have a longer waiting period prior to nest settlement and spawning (about 3-4 weeks, Warren and Morbey 2011). Silver-arriving females eventually turn red and spawn in the late season once they complete sexual maturation. Although female kokanee showed a bimodal distribution in colouration, colour was found to be continuous. Thus, silver- and red-arriving phenotypes in female kokanee salmon cannot be defined as strict alternatives (Warren and Morbey 2011).

Silver- and red-arriving phenotypes of female kokanee salmon from Meadow Creek Spawning Channel seem to represent different means of conducting parental care. By spawning late, silver-arriving females have a reduced risk of nest superimposition since most females have already spawned (Morbey 2003, Morbey and Guglielmo 2006, Warren and Morbey 2011). The silvery colouration of silver-arriving fish also makes them more cryptic, and may benefit against predation from bears and birds and

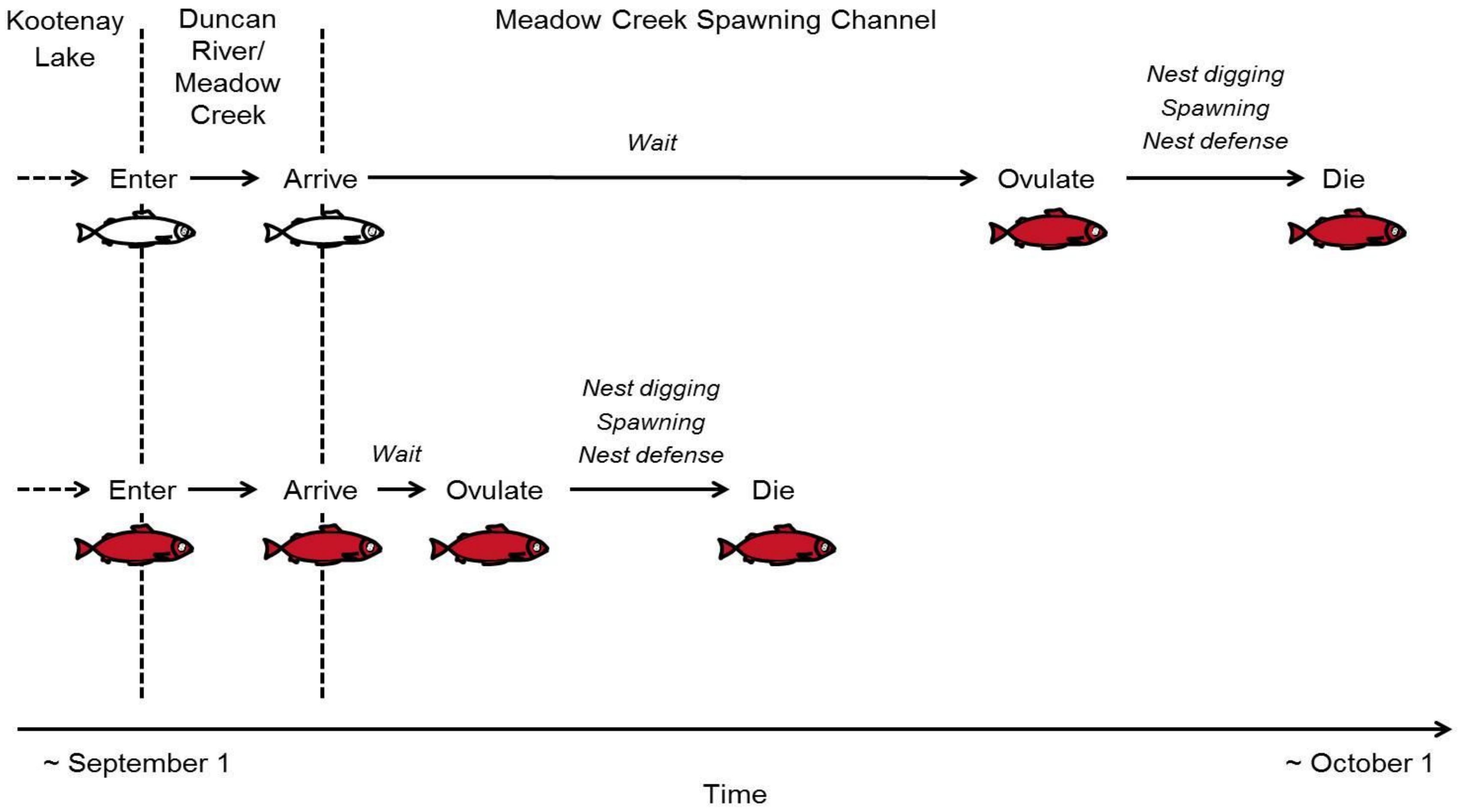

Figure 1 Life events during reproductive stage of silver- and red-arriving female kokanee salmon along time. The time of events were estimated based on Morbey and Ydenberg (2003), Warren and Morbey (2011), and Warren and Morbey (2012). All the female kokanee salmon have silver colouration during their lake residency and turn red during sexual maturation. Silver- and red-arriving phenotypes are two extremes in the kokanee population, females with intermediate colouration also exist in the population.

aggression from nesting females (Morbey and Guglielmo 2006, Warren and Morbey 2012). In comparison, red-arriving females spawn within a short time after arrival on the spawning ground but are subject to nest superimposition. As a result, they do not need to use limited energy to extend reproductive life span. Red females that arrive late in the season can also gain benefits. Late-arriving red fish feed longer in Kootenay Lake, therefore, they may obtain more energy for spawning.

In previous studies, age and size at maturity differed between silver- and red-arriving females (Morbey and Guglielmo 2006, Warren and Morbey 2011). Silver-arriving females were generally younger than red-arriving females. Most of the silver-arriving females matured at age 3+, whereas most of the red-arriving females matured at age 4+. Silver-arriving females also had a smaller body size at arrival (1-3 cm shorter, on average) than red-arriving females did in three years of study. When comparing fish that matured at the same age, silver-arriving females usually had a smaller body size than red-arriving females did, although this size difference was only significant in some years (Morbey and Guglielmo 2006, Warren and Morbey 2011).

Warren and Morbey (2011) presented a graphical model to explain the younger age and smaller size of silver-arriving females than red-arriving females. The model includes two main assumptions. The first is that there is a size threshold for maturation (hereby, threshold 1) in a given year, and the second is a higher size threshold for adopting the red-arriving tactic (hereby, threshold 2). In salmonid fishes, precocious maturation has been reported as a consequence of the rapid growth (Hutchings and Jones 1998, Grover 2005, Morita et al. 2005, Hutchings 2011). As a result, fast growing female kokanee would achieve threshold 1 and mature at a younger age, but mostly not achieve threshold 2, and therefore adopt the silver-arriving tactic. If a female has extremely fast growth, she would also achieve threshold 2 at the younger maturation age and adopt the red-arriving tactic. In contrast, slow growing female kokanee would achieve threshold 1 at an older age and would mostly surpass threshold 2 due to an extra year of growth. Therefore, they would adopt the red-arriving tactic. An assumption is that smaller fish do better by adopting the silver-arriving tactic. Therefore, I hypothesize that variation in growth history during a female’s lifetime influences the expression of silver- and red-arriving reproductive tactics in kokanee salmon.

Growth and Maturation

The growth of a fish is highly influenced by ecological conditions. Food availability has been considered as the most important factor influencing fish growth. For example, salmonids grow faster in the ocean and in lakes than in streams owing to the high productivity of the former habitats (Gross et al. 1988, Keeley and Grant 2001). Similarly, juvenile salmon usually grow faster in warmer spring years owing to the greater abundance of prey (Farley and Trudel 2009, Kishi et al. 2010, Jonsson and Jonsson 2011). In addition to its effect on prey density, temperature can also influence fish growth through regulation of metabolism. The absorption rate of energy is temperature dependent in a fish. Consequently, the energy assimilated and used into growth varies under different temperatures (Wootton 1998, Saborido-Rey and Kjesbu 2009). Besides environmental factors, variation in the feeding ability of fish can also cause growth differences. Fish with advanced foraging ability are likely to have accelerated growth, because a higher consumption of prey means a higher energy intake (Gerking 1994).

For salmonid fishes, growth conditions during later life stages are thought to affect the onset of sexual maturation. Mechanistic models suggest that lipid stores in fall-spawning Atlantic salmon during each fall determines winter survival and the continuation of gonadal development in the next spring (Thorpe et al. 1998, Thorpe 2007). When lipid storage in fall is sufficiently high for both over-winter survival and the subsequent maturation process, Atlantic salmon will fully mature in the coming fall (Thorpe 1994). In contrast, when lipid storage in fall is sufficient for over-winter survival, but not for the initiation of maturation in following spring, gonadal development will be inhibited. In this case, maturation will be subsequently postponed for another year (Rowe and Thorpe 1990, Thorpe 1994, Thorpe et al. 1998). The condition of nursery environment in the spring is also critical for maturation. In a poor spring environment, fish are more likely to suspend maturation because of the lower probability of acquiring sufficient lipid for reproduction (Thorpe 2007).

Age and Size at Maturity

Growth is known to affect the optimal age at maturity of salmon. Optimal age at maturity is a decision subject to the trade-off between increasing body size and reducing generation time (Stearns and Koella 1986, Stearns 1989, Mangel and Stamps 2001). Large fish have larger eggs and higher fecundity in general (Hendry et al. 1999, Quinn et al. 2004, Campbell et al. 2006), and are also more competitive on the spawning ground due to their advanced abilities in fighting or nest digging (Foote 1990, Gross 1991, Beacham and Murray 1993). However, while fish extending their life span to achieve a larger body size, they also confront an increased mortality risk (Stearns and Koella 1986). The mortality risk could be caused by predation, parasitism, or starvation (Blanckenhorn 2000), or by fishery-related issues, such as size-selective fishing (Gross 1985, 1991). To balance benefits and costs, fast growing fish with larger body size in a certain year favour early sexual maturation in order reduce the mortality risk in the coming year. Conversely, slow growing fish with small body size favour delayed sexual maturation in order to grow larger and gain increased fecundity (Stearns and Koella 1986, Roff 1996, 2002). However, if fast growing fish experience a decreased mortality risk as body size increases, selection will favour a delayed sexual maturation (Hutchings and Jones 1998).

In life history studies, the maturation reaction norm represents the expression of maturation age in different environments. The maturation reaction norm is widely used to describe the potential age at maturity in response to different growth rates (Stearns and Koella 1986, Mangel and Stamps 2001, Hutchings 2004, 2011). The slope of maturation reaction norm can be positive or negative, which is shaped by the mortality rate of the fish (Hutchings 2011). For instance, early maturation was favoured after rapid growth and delayed maturity was favoured after slow growth in a brook trout (*Salvelinus fontinalis*) population from Cape Race, Newfoundland. In this case, the maturation reaction norm is reflected as a negatively sloped line. However, when mortality increased dramatically in whole populations after including environmental factors across three rivers, all individuals matured at an early age regardless of their growth. In this case, the slope of the maturation reaction norm is approximately vertical (Hutchings 1996). In salmonids, a negatively sloped maturation reaction norm has been frequently reported, with fast growers maturing at an earlier age in the laboratory and in the field (Hutchings 1993, 2004, Roff 2002), and also in a population of kokanee salmon (*O. nerka,* Grover 2005).

Body size and growth during certain life stages may have an influence on precocious maturation in salmon. An advanced initial size or growth has been widely found to be related to a younger age at maturity in both Pacific salmon (*O.* spp.) and Atlantic salmon (*S. salar*, **Table 1**). Meanwhile, a high recent growth (i.e., the growth increment in the year pre-spawning) in adults has been linked to an increased probability of maturation in chum salmon (*Oncorhynchus keta***, Table 1**, Morita and Fukuwaka 2006).

The growth and the age at maturity of a fish are related to the size at maturity. Size at maturity of a fish often will have a positive correlation with its age at maturity. Slow growing fish that delay maturation can grow for another year and achieve an increased size at maturity, and thereby enhanced fecundity. For example, in kokanee salmon from Bucks Lake, California, slow growing fish that matured at an older age had a larger body size than their size at previous age. These slow growing fish were also larger than other fast growing fish that had matured at a younger age in the same cohort (Grover 2005). In one cohort of chum salmon (*O. keta*) from Shari River, Japan, slow growing fish that matured at an older age were smaller at maturity than others that were growing faster and matured at a younger age (Morita et al. 2005). These differences in size at maturity vary among populations. When growth rates within a cohort are more similar, slow growing fish can compensate with future growth and would reach similar sizes as those fast growing fish with a younger age at maturity (Grover 2005). In this case, the difference in size at maturity between fish with different ages at maturity would be relatively small.

Lake Residency of Kokanee Salmon

Feeding strategies of Meadow Creek kokanee salmon in Kootenay Lake have

Table 1 Summary of potential critical size and growth period to salmonid precocious maturation.

Life Stage	Influential Factor	Species	Reference
Early Life	Smolt Size	coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), Atlantic salmon (*S. salar*)	Bilton et al. 1982, Hyatt and Stockner 1985, Nicieza and Braña 1993, Vøllestad et al. 2004
	First Summer Growth	Atlantic salmon (*S. salor*)	Berglund 1992, Friedland and Haas 1996
	Size after First Summer Growth	coho salmon (*O. kisutch*), Atlantic salmon (*S. salor*)	Berglund 1992, Beamish and Mahnken 2001
Late Life	Most Recent Growth	chum salmon (*O. keta*)	Morita and Fukuwaka 2006

been characterized (Thompson 1999). Kootenay Lake is a long, narrow lake, with the main lake constituting the North Arm and South Arm. In Kootenay Lake, kokanee smolts are likely to feed in schools. Unlike other fish smolts that only show a diel vertical migration at dawn and dusk, the foraging behaviour of kokanee smolts also occur during midday, especially between July and August. Adult kokanee salmon in Kootenay Lake also feed during most of the day, and they also potentially feed in schools. Kokanee salmon are planktivores, feeding on zooplankton such as mysis shrimp (*Mysis relicta*), *Cladocera* (mainly *Daphnia* spp.) and *Copepoda* (Thompson 1999). Nutrient level and prey availability along the lake have been proposed to affect the distribution of kokanee salmon in the lake (Thompson 1999, Schindler et al. 2010).

The growth rates of kokanee salmon in Kootenay Lake mainly depend on the feeding condition and the density of kokanee population. Since 1992, an experimental nutrient addition program has been ongoing in the North Arm of Kootenay Lake (Schindler et al. 2010). The aim of the nutrient addition program is to address the problem of nutrient deficiency due to the development of hydroelectric dams and the subsequent decrease in kokanee salmon (*O. nerka*) and Gerrard rainbow trout (*Oncorhynchus mykiss*) productivity (Ashley et al. 1997, Ericksen et al. 2009). Biomass of phytoplankton and zooplankton were successfully increased after the nutrient treatment. As a result, the abundance and body size of kokanee in the lake and spawning grounds showed an increase (Ashley et al. 1997). During years of high kokanee population density, a low growth rate was usually found as the fish had to encounter intense competition for food (Schindler et al. 2010). Overall, growth of kokanee salmon in Kootenay Lake is mainly influenced by food availability. Fast growth occurs during the period when there is sufficient food and less abundant fish in the lake, and vice versa (Schindler et al. 2010).

Altered growth conditions seem to influence the age at maturity of kokanee salmon in Meadow Creek Spawning Channel. Variation has been seen in the age of mature kokanee among years. Kokanee salmon usually migrate back to Meadow Creek Spawning Channel at age 3+ after lake residency. During years when Kootenay Lake has a rich rearing environment, as measured by nutrient and plankton conditions, a younger age at maturity (i.e., age 2+) is usually seen in the spawning kokanee salmon in Meadow Creek Spawning Channel (Thompson 1999, Schindler et al. 2010). The younger age at maturity can be explained by the maturation reaction norm, which states that accelerated growth is likely to result in early maturation (Stearns and Koella 1986, Hutchings 2004). Conversely, slow growth is likely to occur during poor lake conditions, and lead to a delayed age at maturity (i.e., age 4+, Thompson 1999, Schindler et al. 2010). It is not known how variations in growth and age at maturity in kokanee population influence the expression of silver- and red-arriving tactics.

Growth Measurement

The growth rate of fish can be determined by direct estimation or determined indirectly from certain calcified structures (Vigliola and Meekan 2009). Direct estimation refers to monitoring fish within various size classes of a population in a pen or a laboratory, or measuring the body length of marked or captive fish with known age (Campana 2001, Neilson and Campana 2008). However, direct estimation is difficult because of the demand for high sample size and the difficulty in fish collection at certain life stages (Morales-Nin 1992, Vigliola and Meekan 2009). As a result, structures that have recorded growth history (e.g., scales, otoliths, and bones) are usually used in studies of fish growth.

Otoliths are widely used in age and growth studies of fish (e.g., Francis 1990, Begg et al. 2005, Campana 2005). Otoliths are small calcified structures in the inner ear of a fish, and they grow continuously throughout a fish’s lifetime. Otoliths are easy to procure and store (Campana and Neilson 1985) and unlike scales, they cannot be absorbed during starvation periods (e.g., during homing migration of salmonid fish, Bilton 1974, Campana and Neilson 1985, Campana and Thorrold 2001). Otoliths form yearly rings (annuli) as they grow. For fish living in temperate climates, each annulus consists of a narrow zone, representing a period of slow growth, and a wide zone, representing a period of fast growth. The distance between two narrow zones is thereby a one year growth increment (Campana and Neilson 1985). The number of annuli (i.e., yearly rings) indicates the age of a fish, and the location of annuli can be used in the estimation of fish body size-at-age (Campana and Neilson 1985, Morales-Nin 1992). Therefore, the growth history of a fish can be reconstructed by back-calculating body size-at-age based on the corresponding otolith size (Vigliola and Meekan 2009).

A strong relationship between otolith size and fish body size is the principal basis of using otolith growth to represent fish somatic growth (Jones 1992). The relationship varies among species and populations (Campana 1990), and can be simply linear or allometric (e.g., exponential, Secor and Dean 1989, Vigliola and Meekan 2009). Using an inappropriate relationship can result in poor estimation of fish body size. Accordingly, the relationship between otolith size and body size needs to be validated, and the body size should be back-calculated with the best-fit regression model (Secor and Dean 1989, Campana 1990, Morita and Matsuishi 2001, Vigliola and Meekan 2009).

In Pacific salmon, otoliths have been used to back-calculate or to directly represent size and growth during certain life stages (e.g., smolt size and first summer sea growth). Otolith studies mostly aim to understand how size and growth of fish vary along temporal scales (West and Larkin 1987, Saito et al. 2009, Miller et al. 2013, Woodson et al. 2013) or spatial scales (Limm and Marchetti 2009, Utz et al. 2012, Chittaro et al. 2014). Otoliths have also been used to compare the life history pattern between cultured and wild chinook salmon (*O. tshawytscha*, Claiborne 2013), and between populations with different downstream migration timing (e.g., yearling migration vs. sub-yearling migration) in coho salmon (*O. kisutch*, Nordholm 2014). The relationship between otolith size and fish size has been tested in many different Pacific salmon species and populations. Coefficient of determination (R^2) values of the relationship in these studies range from 0.57 to 0.98 (West and Larkin 1987, Harvey et al. 2000, Saito et al. 2009, Black et al. 2011, Woodson et al. 2013, Chittaro et al. 2014). The relationship between otolith size and fish body size is likely to increase when a wide range of fish body sizes are included in the model (West and Larkin 1987, Meekan et al. 1998, Nordholm 2014).

In any otolith study, a suitable dimension should also be determined for otolith measurements. Since otolith thin cross sections highlight annuli, thin sections have usually been used in fish growth studies (Morales-Nin 1992). Otolith sizes measured along various planes may exhibit different relationships to fish body size. As a result, the proper plane needs to be chosen before otolith sectioning (Morales-Nin 1992, Hart 2005, Li et al. 2008). The most suitable plane can be determined by comparing the regression models of the relationship between different otolith measurements and fish body size. Fish with a range of sizes are required in the determination (Morales-Nin 1992).

Study Hypothesis and Predictions

My study tested for a correlation between growth and the expression of female reproductive tactics associated with maturation timing in kokanee salmon. I hypothesized that growth history would differ in female kokanee with different reproductive tactics. I predicted that (1) silver-arriving fish would generally have a younger age at maturity and smaller size at maturity than red-arriving fish, (2) fast growing fish would have a younger age at maturity and smaller size at maturity than slow growing fish, and (3) for fish maturing at the same age, fast growing fish would be more likely to adopt the red-arriving phenotype. I tested this hypothesis by conducting measurements on kokanee salmon otoliths from one population in Kootenay Lake. The validity of using otolith in kokanee growth study was tested and analyses and comparisons were carried out to test the hypothesis. In the validation study: (1) the relationships between different otolith dimensions and fish body size were compared using different regression models, (2) the dimension giving the best correlation to fish body size was selected for growth measurement, (3) a best-fit model was generated for fish body size back-calculation, and (4) the estimated otolith size-at-age and body size-at-age were compared to the directly measured otolith and body size-at-age of the same cohort. In the comparative study: (1) age and size at maturity were compared between silver- and red-arriving females, (2) otolith size-at-age (thereby body size-at-age) were compared between silver- and red-arriving females with the same age at maturity, and (3) otolith growth increments and back-calculated body growth increments were compared between silver- and red-arriving females with the same age at maturity.

Methods

Study Area

Kokanee salmon (*O. nerka*) were studied at the Meadow Creek Spawning Channel (50° 15.4’ N, 116° 59.8’ W, **Fig. 2**), 14 km upstream to the north end of Kootenay Lake. Kootenay Lake is located in southwestern British Columbia, with its drainage entering the Columbia River. The Meadow Creek Spawning Channel was constructed in 1967 to compensate for kokanee spawning habitat lost during the construction of the Duncan Dam. The spawning channel is 2.9 km long, oriented parallel to Meadow Creek with 8 winding legs, and supplied with water from Meadow Creek and John Creek. The gravel substrate and water flow are maintained as consistent as possible to provide a high quality spawning habitat for kokanee (Warren and Morbey 2011). During late August to late September, kokanee migrate upstream to spawn. The start of spawning season varies among years, with the spawning season running for three-four weeks (Morbey and Ydenberg 2003, Warren and Morbey 2011). Fish enter the spawning channel through an enumeration fence located at the downstream end. Fish that pass the enumeration fence are visually counted by fisheries staff from BC Ministry of Forests, Lands and Natural Resource Operations. Every year there are from 200,000 to 500,000 kokanee salmon returning to the spawning channel. The eggs are fertilized and the embryos develop in the channel over winter. The fry then migrate downstream to Kootenay Lake in the following spring (approximately in April). During the spawning season, bears (*Ursus americanus* and *Ursus arctos*) and ravens (*Corvus corax*) are the major predators of kokanee.

Fish Collection

Newly-arriving female kokanee salmon (n = 65) were collected early in the fall spawning season of 2013 (September 5-10) when the difference in colouration between silver- and red-arriving females was the most obvious based on previous research (Morbey and Guglielmo 2006, Warren and Morbey 2011). The early-season fish collection was also to ensure the acquirement of extremely immature females (i.e., silver-

Figure 2 Location of Meadow Creek Spawning Channel and trawl survey sampling sites in Kootenay Lake, British Columbia. KLF1, KLF2, KLF4, KLF5 and KLF7 are five different sampling sites of Kootenay Lake fertilization program (Schindler et al. 2010). Map source: Google earth satellite map.

arriving females), as there is a decline in the number of females that arrive in a less mature status over the season. Fish were captured with a dip net at the enumeration fence. Thirty-four silver- and 31 red-arriving female kokanee were collected. Initially, colour was assessed subjectively as silver vs. red. Fish with intermediate colouration were not collected. All fish were killed by anaesthetic overdose with prolonged submerging in a clove oil solution (4 mg/litre of water). For each fish, fork length (mm, from the tip of the snout to the fork of the caudal fin), standard length (mm, from the tip of the snout to the posterior end of caudal peduncle) and snout length (mm, from the tip of the snout to the middle of eye) were measured with a dial caliper (150 mm/0.1 mm, SPI 2000), and the wet body mass (g) was measured with an electronic scale (× 0.1 g, capacity 4000 g). Maturity status was checked after dissection. Egg diameter (mm, average of 20 eggs) was measured as a continuous estimation of maturity status since it is strongly correlated with ovary maturation. Fecundity of each fish was measured by counting the total number of eggs. Fish otoliths were extracted for age and growth analyses. After slicing off the top of the skull with a sharp fillet knife, fish otoliths were exposed in the inner ear canals and removed using stainless steel forceps (Magna Stainless M10-0200). Both the left and right sagittal otoliths were extracted, cleaned of adhering tissue, and stored dry in vials.

Colour Evaluation

Standard digital photographs were taken of fish sampled at the Meadow Creek Spawning Channel enumeration fence to evaluate the subjective assignment of colour (silver vs. red, Stevens et al. 2007, Warren and Morbey 2011). Photos were collected from 63 of 65 fish (photos of 2 fish were not usable). Pictures were taken of each fish immediately after anaesthetization to avoid colouration loss due to death. A no-light box (61 × 40 × 41.9 cm) with two fixed flashlights was used to provide an environment with consistent light. A metric ruler and two colour standards (redness gradient standard C·I·L colour card 2L1, pure white standard Martha Stewart colour card MSL253) were placed at the bottom of the box for further colour analyses. Pictures were taken with a Canon G10 12MP digital camera (resolution 4416 × 3312 pixels) through a small hole on the top of the box. The digital camera was held at a consistent height to ensure all pictures were taken at the same scale. Fish were placed on the bottom of the box with left side facing up. All pictures were taken with a consistent white balance (pure white standard Martha Stewart colour card MSL253) to avoid colour variation among photos.

The CIELAB (1976) colour space was used to evaluate the colouration of fish in silver and red colour categories. Three coordinates in the CIELAB (1976) colour space describe the lightness (L^*, ranging from 0 to 100) from black to white, the redness (a^*) from green to red, and the yellowness (b^*) from blue to yellow. The CIELAB (1976) colour notation precisely represents the range of human perceived colouration and has been used to describe the carotenoid-associated colour variance of salmon skin (Craig and Foote 2001, Miller et al. 2007, Warren and Morbey 2011). Colour information of both silver- and red-arriving fish was extracted with Image-J software (version 1.47). On each fish, four glareless locations (2 mm × 2 mm = 4 mm^2/location) showing variances in colouration among the collected fish were selected (**Fig. 3**). R, G and B values, indicating the intensity of light in the red, green and blue spectrum respectively, were measured from each location. Colour attributes were calculated from each pixel and the mean value of all pixels within each 4 mm^2 location was calculated.

R, G and B values from each 4 mm^2 were then transformed into L^*, a^* and b^* values in the CIELAB (1976) colour space using R statistical computing software (version 3.0.1). The L^*, a^* and b^* values were calculated with following equations (Ohta and Robertson 2005).

$$\begin{pmatrix} X \\ Y \\ Z \end{pmatrix} = \begin{pmatrix} 2.7689 & 1.7517 & 1.1302 \\ 1.0000 & 4.5907 & 0.0601 \\ 0.0000 & 0.0565 & 5.5943 \end{pmatrix} \begin{pmatrix} R \\ G \\ B \end{pmatrix}$$

$$L^* = 116(Y/100)^{1/3} - 16$$

$$a^* = 500\left[(X/100)^{1/3} - (Y/100)^{1/3}\right]$$

$$b^* = 200\left[(Y/100)^{1/3} - (Z/100)^{1/3}\right]$$

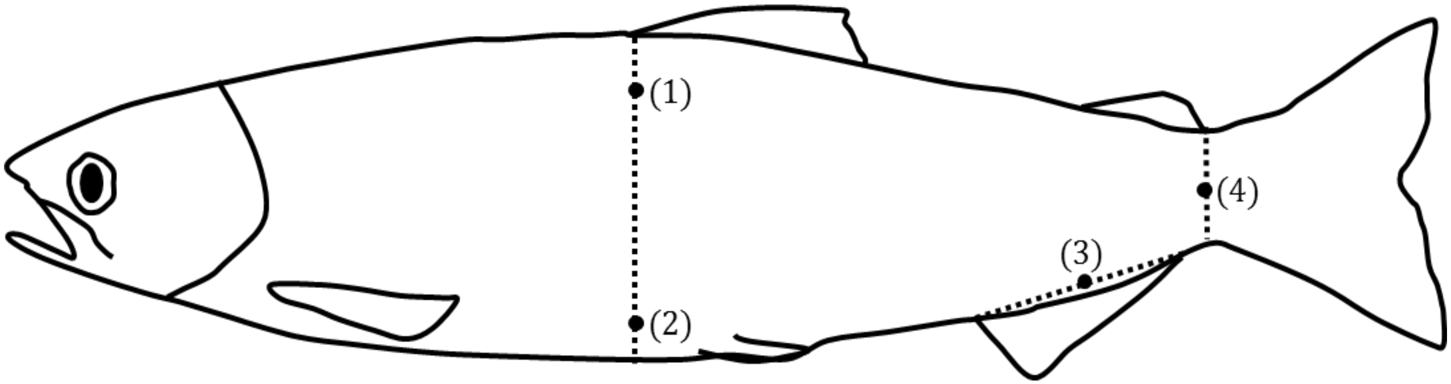

Figure 3 Four 4 mm^2 locations where colour information was extracted from each photograph of female kokanee salmon.

Where *X*, *Y*, and *Z* are the tristimulus values from the CIE XYZ colour space. CIE XYZ colour space has been commonly used as a standard reference in the conversion between colour spaces (Wyman et al. 2013).

Otolith Size–Body Size Relationship Validation

Archived kokanee salmon from the Kootenay Lake trawl survey were collected to validate the positive correlation between otolith size and fish body size of Kootenay Lake kokanee. Fish were captured by the British Columbia Ministry of Forests, Lands and Natural Resource Operations for their annual kokanee stock assessment. In the assessment program, fish were collected from five different sites along the main area of Kootenay Lake (**Fig. 2**: KLF1, KLF2, KLF4 in the North Arm, KLF5 and KLF7 in the South Arm) during the new moon period in September. Biologists used an echosounder to find the fish. Stepped-oblique trawls were used to fish each 5 m layer from 20 to 40 m depth for 8 minutes per layer (Schindler et al. 2010). Kokanee from different trawl surveys were bagged whole and frozen, but sometimes had incomplete labels. I was able to acquire carcasses (both sexes) of a variety of sizes from 51-177 mm (n = 80) from 2010, 2011 and 2012. Fork length, standard length and snout length were measured from each fish, otoliths were removed, cleaned and stored dry in vials.

Intact otolith samples from fish with a range of sizes were used to validate the relationship between otolith size and body size of kokanee salmon (n = 173). The whole section of the left otolith was used in the validation study. Otoliths collected during previous studies in the Meadow Creek Spawning Channel (2008: n = 11, 2009: n = 18, Warren and Morbey 2011) were included in the validation study together with the otolith samples obtained in 2013 (lake trawl survey: n = 80, spawning channel fish collection: n = 64, one of 65 samples was lost). The whole otolith was observed under a dissecting microscope (Meiji EMZ 13TR) at a 25 × objective setting with a dark background. The light source from above was provided by a microscopy illuminator (Meiji Techno FL150). Two pictures were taken from each otolith using a Lumenera INFINITY camera (vision 5.0.3), with the core (i.e., the area surrounding the primordium) or the sulcus (i.e., a groove along one surface of the otolith) facing up. The core of each otolith was focused on by rotating the polarizing filter of the dissecting microscope. A photograph of an objective micrometer (× 0.1 mm) was taken under the same magnification and was used as a standard for the subsequent otolith morphological measurements.

Photos of otolith whole sections were analyzed using Image-J software (version 1.47) and different dimensions were measured to validate the otolith size to body size relationship. The clarity of annuli on otolith whole sections was not sufficient for age determination and growth measurement. As a result, otolith sectioning was needed to procure thin sections for an improved measurement. Measurements of length, width, and radius dimensions have been used in otolith studies (Anderson et al. 1992, Secor et al. 1992, Harvey et al. 2000, Moreno and Morales-Nin 2003). Kokanee salmon otoliths grow curvilinearly over time along the longitudinal plane, and the cutting process along the length axis cannot accurately estimate size-at-age (**Fig. 4**). As a result, nine otolith dimensions, including six radius dimensions and three width dimensions (combination of suitable radius measurements, **Fig. 5**) were measured on each otolith. All the measured dimensions were used to validate the relationship between otolith size and fish body size and to determine the best dimension for subsequent growth measurement.

Linear regression, power regression, and quadratic regression analyses were assessed in the validation of the relationship between otolith size and fish fork length. Linear and power regressions were included in the validation study, because both linear and allometric relationships have been observed between otolith size and fish body size (Francis 1990, Morita and Matsuishi 2001). Quadratic regression analysis was included since it provided a good fit of the relationship. For each otolith dimension, three regression models were generated.

The linear regression equation was:

$$FL = a + b \times O$$

The power regression equation was:

$$FL = c + d \times O^{f}$$

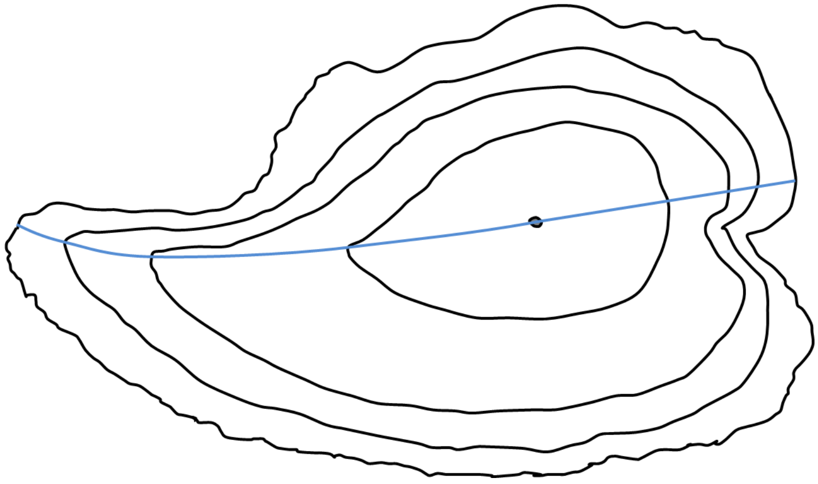

Figure 4 Kokanee salmon otoliths grow curvilinearly along the longitudinal axis over time.

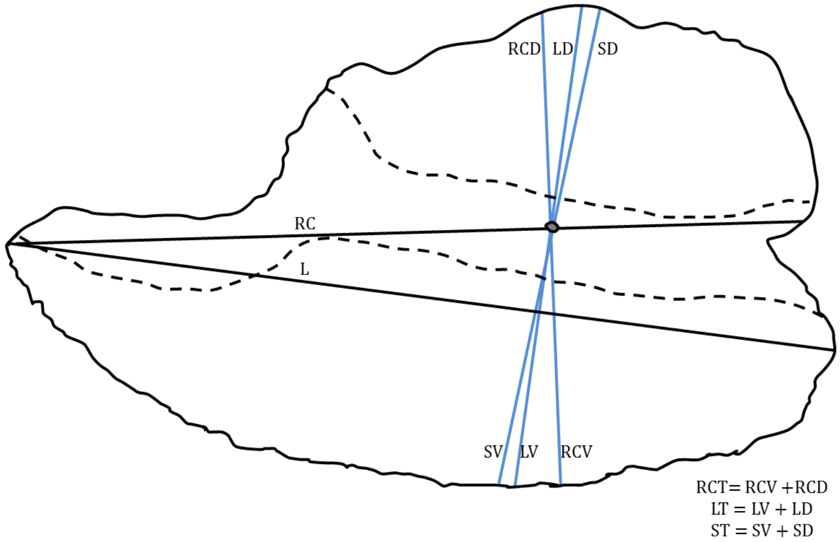

Figure 5 Nine morphological dimensions on the otolith whole section of kokanee salmon. Dashed lines: outline of otolith sulcus. Landmarks: RC: the length from the rostrum through the core; L: the length from the rostrum to the post-rostrum. Radius dimensions: RCV: the radius perpendicular to RC from the core to the ventral edge; LV: as in RCV but perpendicular to L; SV: as in RCV but perpendicular to the sulcus; RCD: as in RCV but to the dorsal edge; LD: as in RCD but perpendicular to L; SD: as in RCD but perpendicular to the sulcus. Width dimensions: RCT: the width perpendicular to RC, passing through the core; LT: as in RCT but perpendicular to L, and ST: as in RCT but perpendicular to the sulcus.

The quadratic regression equation was:

$$FL = g + h_1 \times O + h_2 \times (O)^2$$

Where FL is fork length, O is otolith size, and a, b, c, d, f, g, h_1 and h_2 are constants.

Six data points located distantly away from the line of best-fit in residual plots were regarded as outliers and removed from the dataset. Mean squared error (MSE) and coefficient of determination (R^2) were calculated to evaluate the accuracy and precision of each regression model (Power 1993). A model with high accuracy (i.e., low MSE) means good fit. A high precision (i.e., high R^2) indicates low spread around the regression trendline. For each otolith dimension, models of linear, power and quadratic regression models were ranked based on the MSE and R^2 outcomes. The top ranked otolith dimension was chosen for the growth comparison study and the corresponding plane was used for otolith sectioning. The top ranked regression model was also used for body size back-calculation.

The relationship between the top ranked otolith dimension and fish fork length was compared among cohorts to test for differences among cohorts in the otolith size to body size relationship. Annuli were clearer on the otoliths of lake-captured fish than on mature fish, which is probably due to lower number of formed annuli. Consequently, age of lake-captured kokanee could be determined from whole otolith sections. Fish were aged based on the number of annuli on the otolith (Melvin and Campana 2010). Kokanee salmon from Kootenay Lake are fall spawning fish. As a result, I counted opaque rings (annuli) that formed during winter to represent the age of fish. The birth year was estimated by using the sampling year minus the age of a fish. The birth year was regarded as the year when parental fish spawned and the eggs were fertilized.

Growth Estimation

Thin cross sections of otoliths were used in growth estimation. A cross-section was obtained by cutting the otolith along the plane with the best dimension. Otoliths of kokanee salmon captured in Meadow Creek Spawning Channel in 2013 were used in growth estimation (n = 65). For consistency, the left otolith that had been used in the validation study was used for sectioning. Otolith embedding and sectioning were conducted by fisheries ageing biologists Susan Mann and Wendy Irvine from Ontario Ministry of Natural Resources. Briefly, otoliths were embedded in mixed epoxy (weight ratio, Araldite GY 502 resin: Aradur 956-2US hardener = 5:1) for sectioning. The embedded otolith block was sectioned perpendicular to the sulcus through the core area along the plane using an Isomet saw. A pair of diamond wafer blades was used for otolith sectioning and a plastic spacer (500 μm) was set up between two blades. After the cut, the transverse otolith section was glued onto a slide with a small amount of Araldite epoxy. All transverse otolith sections were placed in the same orientation on each slide, with the otolith ventral side oriented towards the left.

The surface of each thin section was further exposed to increase the clarity of annuli by evenly grinding with a mechanized grinding wheel or by etching with 1 % HCl (30-60 s). During the exposing process, the surface of the otolith section was examined under a dissecting microscope (Meiji EMZ 13TR). The exposing ended when the primordium (i.e., the initial structure of an otolith) and annuli of otolith could be clearly seen under the dissecting microscope. The etched otolith surface was carefully washed with acetone and distilled water, and dried with small tissue wipe. Thin sections were observed under a stereomicroscope (Nikon SMZ 1500) at an 80 × objective setting with direct light from a microscopy illuminator (Fiber-Lite MI-150). A small amount of mineral oil was added with a syringe to improve the surface clarity of the thin section when necessary. Photos of thin sections and an objective micrometer (× 0.1 mm, as measurement standard) were taken with Nikon NIS-Elements software (vision 3.2) with a dark background.

Photos of otolith thin sections were analyzed using Image-J software (version 1.47). All the photos were relabeled by a third party and analyzed blindly to prevent biased measurements (with no information on body size or colour). The age of fish was determined by counting the number of winter-formed annuli on the otolith thin section (**Fig. 6**). The expression of age consisted of a number of completed annuli, and a “+” symbol indicating growth after the last annulus. For instance, age 3+ means a fish experienced 3 years of growth and spawned in its fourth year. Otolith size-at-age was

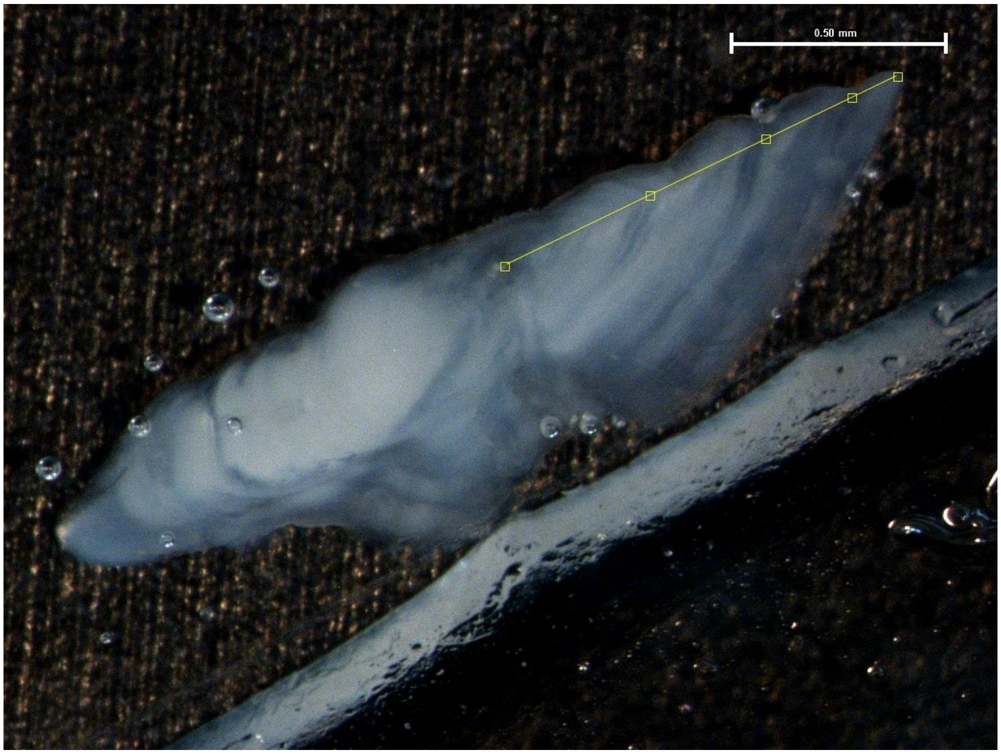

Figure 6 An otolith thin section of Kootenay Lake kokanee salmon. Empty squares are the locations of winter-formed annuli referred as aging standard and the ventral edge of ventral axis. Otolith radius measurements run from the primordium to each annulus and the ventral edge of the otolith along the ventral axis.

measured as the distance from the primordium to the winter annulus, and the final radius (i.e., otolith size-at-capture) was measured from the primordium to the ventral edge of the otolith (**Fig. 6**).

Fish fork length-at-age was back-calculated from the otolith thin section measurement. The absolute growth increment was calculated for both otolith size and fork length. Absolute annual growth increment was reflected by the difference between sizes at two consecutive ages.

Otolith Analysis Validation

The otolith measurement analyses were validated from two different perspectives. First, the otolith size-at-capture measured from the otolith thin sections (growth measurements) was compared to the otolith size-at-capture measured from the otolith whole section (otolith size to body size relationship validation). This would reflect the precision of the measurements on the same dimension generated from thin sections and whole sections. Second, the otolith size-at-age estimated on the otolith thin section was compared to the otolith size at the same age measured on the otolith whole section of the same cohort. Meanwhile, the back-calculated fork length-at-age was compared to the directly measured body size at the same age of the same cohort. These comparisons were conducted on the 2009 cohort, but not the 2008 cohort owing to the small number of available samples of the latter cohort. The comparisons would reflect the accuracy of otolith size-at-age estimation and body size-at-age back-calculation based on otolith thin section measurements.

Statistical Analysis

R statistical computing software (version 3.0.1) was used for all statistical analyses. Differences were considered as significant under $\alpha < 0.05$. Assumptions were evaluated before each analysis. Residuals were considered as normally distributed when data points located on the 45° line in Q-Q plot. Variances were considered as homogenous when plotted points scattered evenly around the line of best-fit in residual plots. To satisfy assumptions, data were transformed when needed.

Colour Evaluation

Multivariate analysis of variance (MANOVA) was carried out to confirm the colour differences between silver and red fish. Twelve variables (3 attributes × 4 locations = 12 variables) were included in the statistical model. Each variable consisted of a colour attribute (L^*, a^*, or b^*) and a number that indicated location on the fish (1-4). Seven variables (L^*1, L^*3, L^*4, a^*1, a^*2, a^*3, and b^*1) that exhibited a lack of normality in residuals were *ln*-transformed. The Wilks comparison test was used in the MANOVA model.

Discriminant function analysis (DFA) was used to classify colour groups based on the colouration variables. A linear discriminate score (LD1) was derived, with the highest LD1 score indicating silver colouration and lowest LD1 score indicating red colouration. The re-substitution method of cross-validation was used to assess the classification accuracy of predicted categories to the subjective colour assignment.

Age and Body Condition at Maturity Comparison

A difference in age distribution between silver- (n = 34) and red-arriving fish (n = 31) was tested with Fisher's exact test to cope with the small number of age 2+ (n = 1) and age 4+ fish (n = 4).

Fork length, snout length, length from eye to tail, wet body mass, egg size, and fecundity were compared separately to test for differences between silver- and red-arriving females. Snout size, a secondary sexual characteristic of female salmon, increases during sexual maturation (Groot and Margolis 1991, Quinn 2005). To control the influence of the snout length variation due to the difference in maturity status between silver- and red-arriving females, length from eye to tail was included as an indicator of body size in the comparison. Comparisons were based on the subjective colour category and the assigned LD1 score from DFA model. Comparisons were first conducted between all the silver- and red-arriving females regardless of the age at maturity (hereby, mixed age females, silver: n = 34, red: n = 31), and then, only between females that matured at age 3+ (hereby, age 3+ females, silver: n = 33, red: n = 27). One-way

ANOVA model was used for the comparisons of fork length and length from eye to tail in mixed age females, and snout length, mass, and egg size in both mixed age and age 3+ females. Due to non-normally distributed residuals, non-parametric ANOVA model (Kruskal-Wallis comparison test) was used to compare fork length and length from eye to tail in age 3+ females. The relationship between fecundity and body size was tested with linear regression model. When there was no correlation between body size and fecundity, non-parametric ANOVA model (Kruskal-Wallis comparison test) was used in the comparison of fecundity between silver- and red-arriving females.

Growth Comparison

Sample sizes were insufficient for growth comparisons with age 2+ (n = 1) and age 4+ fish (n = 4), and data was not available for seven individuals due to the low clarity of annuli. Growth was compared between silver- (n = 31) and red-arriving females (n = 23) that matured at age 3+. Comparisons were based on the subjective colour category and the assigned LD1 score from DFA model. Growth was analyzed as (1) otolith size-at-age, (2) otolith absolute growth increment, and (3) body size absolute growth increment.

Otolith radius was first compared between silver- and red-arriving females using repeated measures ANOVA: age was used as the repeated, within subject factor and colour as the between subject factor. The interaction between factors was tested, and removed from the model if not significant. Otolith radius at each age was *ln*-transformed to improve the normality assumption in the repeated measures ANOVA model. The magnitude of difference in size-at-age between silver- and red-arriving females was estimated using the estimated coefficient (β) ± standard error of the colour effect (colour category, dummy variables: 0-red, 1-silver) in the multiple regression model generated from the repeated measures ANOVA test. Subsequently, otolith radius at each age was compared between silver- and red-arriving kokanee with one-way ANOVA. Size at age 1, age 3 and age 3+ were *ln*-transformed in the comparisons.

Growth increment for otolith size and body size were compared between silver- and red-arriving kokanee with repeated measures ANOVA model. This model contained the same factors as the otolith radius repeated measures ANOVA model. The interaction between factors was tested and non-significant interaction was dropped from the model. The magnitudes of otolith and body growth differences between silver and red fish were estimated in the same way as in the otolith radius repeated measures ANOVA model. Following this test, annual growth increment during each age period (i.e., age 0-1, age 1-2, age 2-3, age 3-3+) were compared between silver- and red-arriving females to examine any differences in annual growth increment. Growth increments were compared using one-way ANOVA model in terms of otolith size and body size, respectively. Growth increment during age 3-3+ was *ln*-transformed in one-way ANOVA model.

The statistical power of the repeated measures ANOVAs conducted on growth increments was examined to determine the probability of correctly rejecting the null hypothesis when growth differences exist. Power was estimated under various sample sizes. New datasets of growth increment with increased sample sizes were simulated based on the effect size of growth in this study. Specifically, the simulation was conducted with the mean of each annual growth increment of silver- and red-arriving fish, respectively, and the standard deviation of each annual growth increment of all individuals. The standard deviation was defined in the simulation to satisfy the sphericity assumption of repeated measures ANOVA. Simulations were conducted for 500 iterations. With a given effect size (i.e., specified mean, standard deviation) and sample size, the power of repeated measures ANOVA on the simulated growth increments was subsequently calculated.

Otolith Analysis Validation

Different analyses were conducted to validate the otolith measurements. (1) Analysis of covariance (ANCOVA) was used to test for a cohort effect in the otolith size to fish body size relationship. (2) Linear regression model was used to test for a correlation between otolith radius measurements from thin sections and from whole sections using mature fish samples. (3) Two-sample hypothesis test was used to compare the estimated otolith and body size-at-age of mature fish samples with the directly measured otolith and body size-at-age of lake sampled fish in the same cohort. A Wilcoxon rank sum test was used because it can handle the non-normal distribution and unequal sample size in the tested data. (4) To determine if otolith increments were sufficiently variable to detect natural variability in growth within the population, the silver- and red-arriving kokanee captured in 2013 were regrouped into two categories based on body size at arrival (i.e., small and large females), and the sizes-at-age were compared between regrouped small and large fish. The assumption is that fish with larger body size at arrival also were larger at previous ages. *Ln*-transformed otolith radius-at-age was used in the comparison using repeated measures ANOVA model: age was used as the repeated, within subject factor and size as the between subject factor. The interaction between age and size was tested and dropped if not significant. Otolith radius at each age was subsequently compared between small and large kokanee using one-way ANOVA model. Sizes at age 1, age 2 and age 3 were *ln*-transformed to satisfy the normality assumption.

Results

Colour Evaluation

The MANOVA model showed that the silver- and red-arriving kokanee salmon differed in objective colour attributes ($F_{12, 50} = 36.04$, $p < 0.0001$). In the DFA model, the LD1 scores of subjectively categorized silver and red fish were highly discriminant (**Fig. 7**). Silver fish were mostly assigned with a high LD1 score and red fish were all assigned with a low LD1 score (**Fig. 7**). The DFA model gave an overall classification accuracy of 96.8 %, indicating a very high reliability of my subjective colour assignment. Specifically, all 31 red fish were correctly classified as red in the DFA model, whereas two (6.3 %) of 32 silver fish were misclassified as red in the DFA model.

Otolith Size–Body Size Relationship Validation

Fish fork length showed a positive relationship with otolith size in all 27 regression models. Otolith width and otolith ventral radius (i.e., RCT, LT, ST, RCV, LV, and SV) produced highly accurate ($458.42 < MSE < 571.01$) and highly precise ($0.856 < R^2 < 0.884$) models in linear regression, power regression and quadratic regression models (**Table 2**). This indicated a strong otolith width to fish body size relationship and otolith ventral radius to fish body size relationship over a wide range of sizes. The annuli on the otolith thin section were clear along the ventral axis, but not along the dorsal axis. This added difficulties in the measurement of otolith width, a dimension from the ventral edge to the dorsal edge on otolith. Therefore, otolith ventral radius dimensions (i.e., RCV, LV, and SV) were considered for otolith sectioning and growth measurements on thin sections. Under the three dimensions, models of otolith ventral radius perpendicular to the sulcus (i.e., SV) had the top rank in both accuracy and precision ($MSE < 535.21$, $R^2 > 0.865$, **Table 2**). Thus, the transverse plane perpendicular to the sulcus was chosen for otolith sectioning and growth measurements. The plane perpendicular to the sulcus has been widely used in otolith sectioning for the purpose of both age determination and growth measurement (Morales-Nin 1992, Campana et al. 2008).

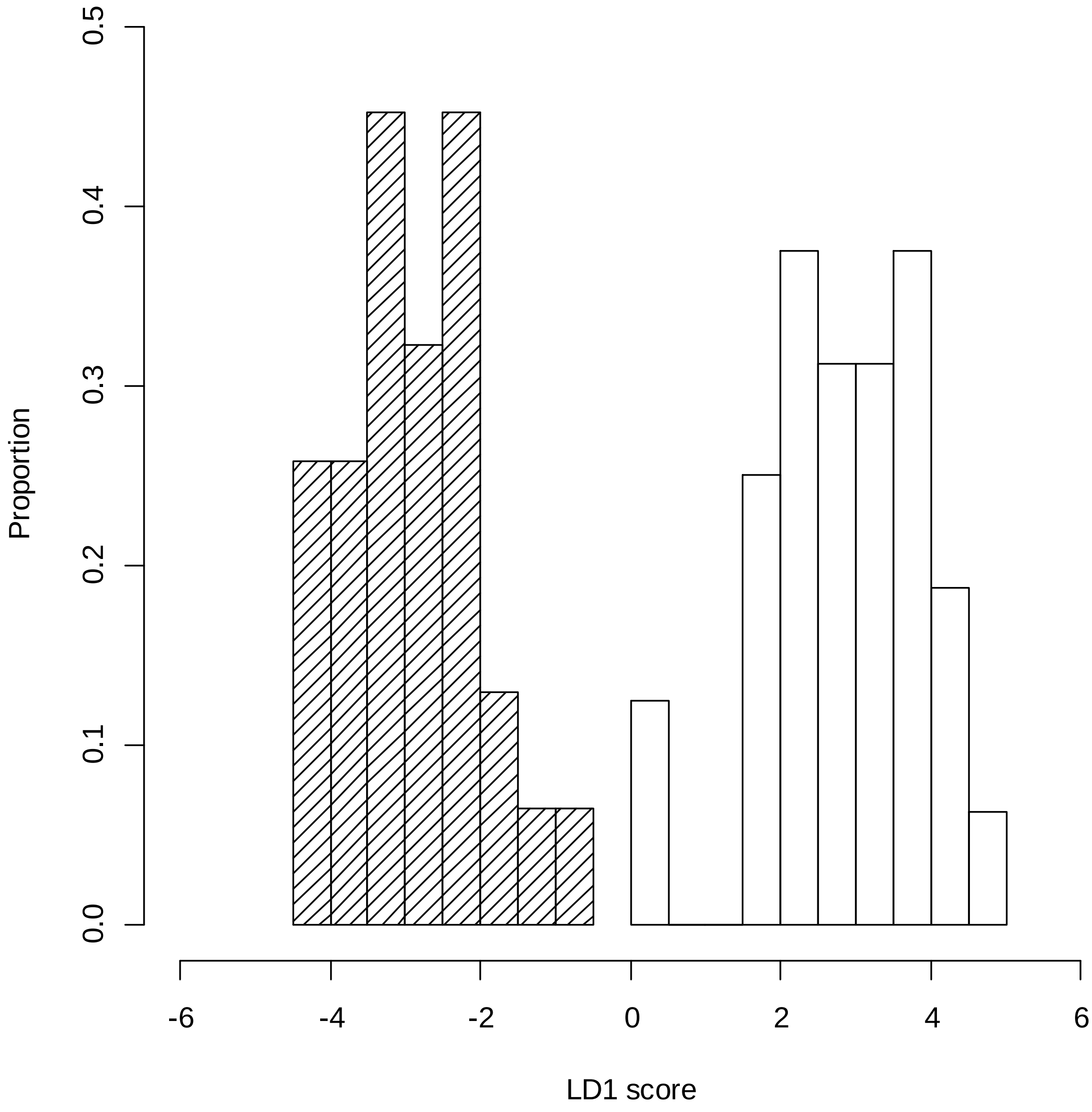

Figure 7 Proportion of linear discriminant (LD1) scores for silver- (open bars) and red- arriving female kokanee salmon (dashed bars). A low LD1 score represents more red in objective colouration and a high LD1 score represents more silver in objective colouration.

Table 2 Summary of regression models of otolith size versus fish size of kokanee salmon. All nine dimensions are different otolith measurements as shown in Figure 5. For all regression models, the accuracy is represented by mean standard error (MSE) and the precision is represented by coefficient of determination (R^2).

Measurement	Dimension	Model	Accuracy		Precision	
			MSE	Rank	R^2	Rank
Ventral Radius	RCV	Linear Function	550.12	9	0.861	9
		Power Function	544.04	6	0.863	6
		Quadratic Function	544.58	7	0.862	7
	LV	Linear Function	547.86	8	0.862	8
		Power Function	542.44	4	0.863	4
		Quadratic Function	542.86	5	0.863	5
	SV	Linear Function	535.21	3	0.865	3
		Power Function	531.26	1	0.866	1
		Quadratic Function	531.37	2	0.866	1
Dorsal Radius	RCD	Linear Function	1316.26	8	0.668	8
		Power Function	1337.56	9	0.662	9
		Quadratic Function	1314.34	7	0.668	7
	LD	Linear Function	1099.61	3	0.722	3
		Power Function	1093.80	2	0.724	2
		Quadratic Function	1092.55	1	0.724	1
	SD	Linear Function	1107.61	5	0.720	5
		Power Function	1108.83	6	0.720	6
		Quadratic Function	1104.96	4	0.721	4
Width	RCT	Linear Function	571.01	9	0.856	9
		Power Function	519.84	7	0.869	6
		Quadratic Function	513.64	5	0.870	5
	LT	Linear Function	527.34	8	0.867	8
		Power Function	469.14	3	0.882	3
		Quadratic Function	458.42	1	0.884	1
	ST	Linear Function	518.44	6	0.869	6
		Power Function	469.77	4	0.882	4
		Quadratic Function	462.81	2	0.883	2

Among the three models of otolith ventral radius perpendicular to the sulcus (i.e., SV), the one generated from power regression analysis exhibited the highest accuracy (MSE = 531.26, **Table 2**) and highest precision ($R^2 = 0.866$, **Table 2**). Consequently, the equation of power regression was used to back-calculate size-at-age with ventral radius perpendicular to the sulcus (i.e., SV, **Fig. 8**).

$$FL = 207.54 \times SV^{1.38}$$

Where *FL* is the fork length, *SV* is the otolith ventral radius perpendicular to the sulcus.

The ANCOVA model did not show a significant difference between 2008 and 2009 cohorts (n = 35 and n = 18, respectively) in terms of the otolith ventral radius to fish fork length relationship (**Fig. 9**, $F_{1, 50} = 0.17$, $p = 0.68$). This means that the otolith size to fish body size relationship was consistent among cohorts.

Age and Body Condition at Arrival Comparison

Silver- and red-arriving females were not different in age at maturity in 2013 (p = 0.17, two-tailed Fisher's exact test). The majority of silver- and red-arriving females were age 3+ (90 % and 97 %, respectively, **Table 3**).

At arrival, silver-arriving females had significantly shorter fork length and snout length, and had smaller eggs at arrival than red-arriving females (**Table 4**). However, there was no significant difference in terms of the length from eye to tail, wet mass, or fecundity between silver and red fish (**Table 4**). The results were consistent when excluding the fish that matured at age 2+ and age 4+ (**Table 4**).

Growth Comparison

For fish that matured at age 3+, the repeated measures ANOVA model showed no significant colour effect or colour × age interaction, indicating that silver- and red-arriving females did not differ in otolith radius (thereby body size) or age-specific growth (**Table 5**, **Fig. 10**). In general, otolith size-at-age of silver-arriving fish was 0.027 ± 0.012 mm smaller than that of red-arriving females. The overall age effect was significant. When compared at each age, otolith radius (thereby body size) did not differ between

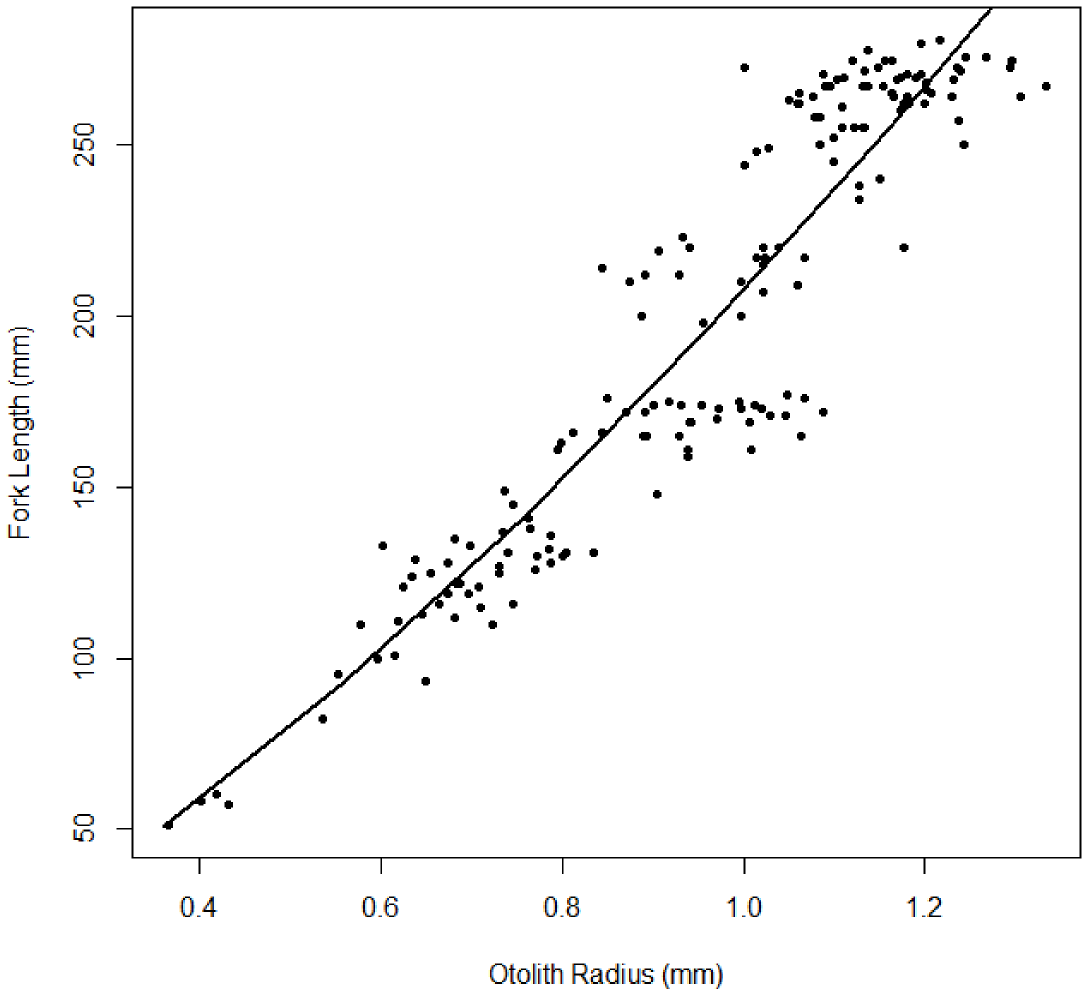

Figure 8 Best-fit line of otolith size to body size relationship of kokanee salmon. The relationship is generated with power regression (Fork Length = c + d × [Otolith Size]^f) between otolith ventral radius (SV) and fish fork length.

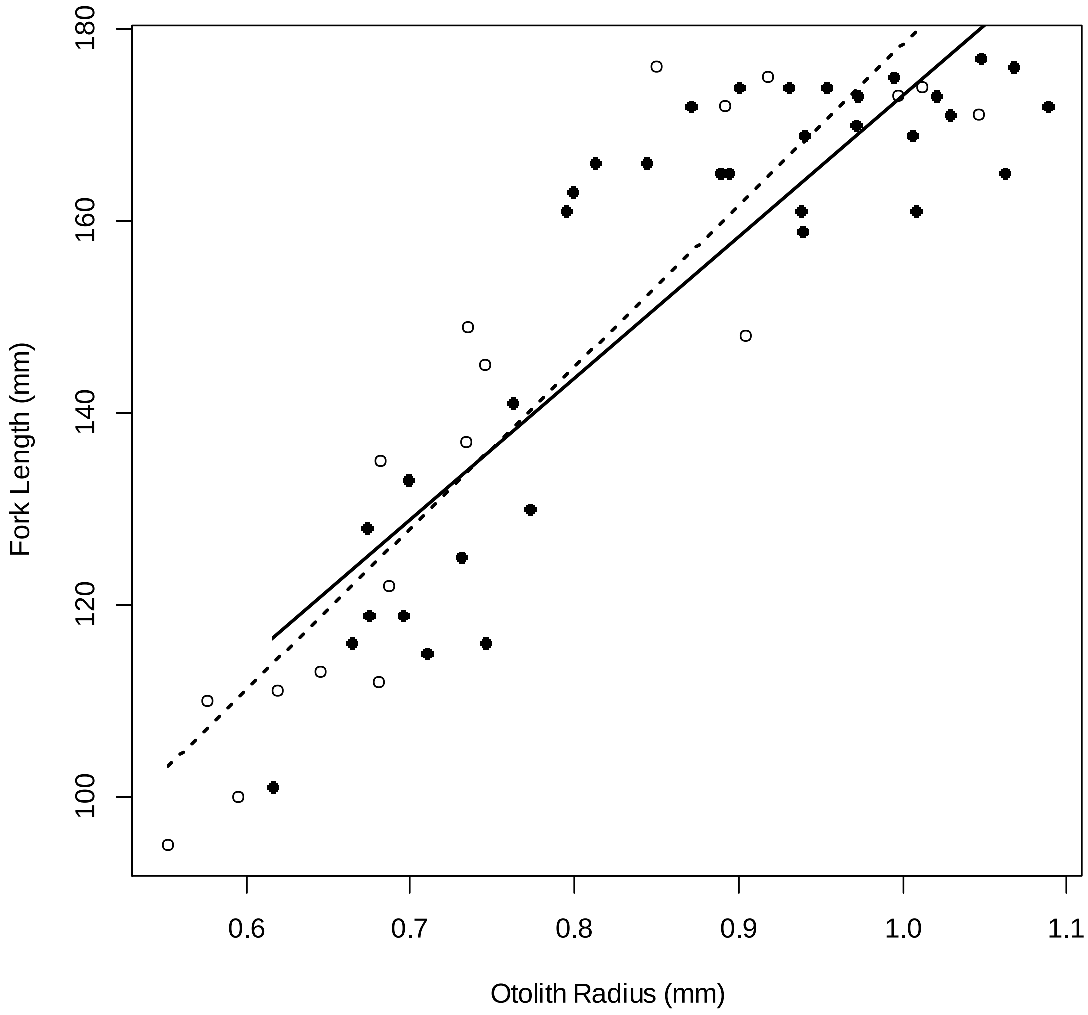

Figure 9 Linear regression between otolith radius and fish fork length in two cohorts of kokanee salmon. Solid circles (n = 35) and solid line represent the 2008 cohort and empty circles (n = 18) and dashed line represent the 2009 cohort.

Table 3 Summary of age at maturity of silver- and red-arriving female kokanee salmon in 2013. Age at maturity is presented as the number of individuals and the corresponding proportion within the population.

Sampling Year	Sample Type	Age 2+	Age 3+	Age 4+
2013	Silver-arriving	1 (3 %)	33 (97 %)	
	Red-arriving	1 (3 %)	27 (87 %)	3 (10 %)

Table 4 Body condition comparisons between silver- and red-arriving female kokanee salmon. Comparisons were conducted regardless of and considering of age influence (mixed age vs. age 3+), and were conducted based on the subjective colour category and the assigned LD1 score, respectively. Measurements were shown as mean ± standard error, n (group sample size). ‘*’ $P < 0.05$, ‘**’ $P < 0.01$, ‘***’ $P < 0.001$. Comparisons were not conducted based on LD1 score in fecundity, and fork length and length from eye to tail in age 3+ females, owing to the inapplicability of non-parametric model with numeric variables as factor.

Age	Traits	Silver		Red	Effect of Phenotype	
					Colour Category	LD1
Mixed	Fork Length (mm)	263.53 ± 0.28, 34	<	268.97 ± 0.20, 31	$F_{1, 63} = 7.42^{**}$	$F_{1, 61} = 5.13^{*}$
	Snout Length (mm)	20.03 ± 0.04, 34	<	21.91 ± 0.04, 31	$F_{1, 63} = 38.55^{***}$	$F_{1, 61} = 44.33^{***}$
	$\text{Length}_{\text{eye to tail}}$ (mm)	243.50 ± 0.26, 34	=	247.06 ± 0.19, 31	$F_{1, 63} = 3.60$	$F_{1, 61} = 2.00$
	Mass (g)	211.59 ± 0.75, 34	=	216.14 ± 0.59, 31	$F_{1, 63} = 0.68$	$F_{1, 61} = 0.25$
	Egg Size (mm)	4.79 ± 0.01, 33	<	5.21 ± 0.01, 31	$F_{1, 62} = 37.72^{***}$	$F_{1, 60} = 30.83^{***}$
	Fecundity	249.64 ± 1.36, 33	=	268.77 ± 1.60, 31	Chi squared = 1.27	NA
3+	Fork Length (mm)	263.58 ± 0.29, 33	<	269.33 ± 0.20, 27	Chi squared = 5.74^{**}	NA
	Snout Length (mm)	20.08 ± 0.04, 33	<	22.09 ± 0.04, 27	$F_{1, 58}= 41.86^{***}$	$F_{1, 57} = 49.44^{***}$
	$\text{Length}_{\text{eye to tail}}$ (mm)	243.49 ± 0.27, 33	=	247.25 ± 0.18, 27	Chi squared = 2.04	NA
	Mass (g)	212.06 ± 0.77, 33	=	217.71 ± 0.59, 27	$F_{1, 58} = 1.00$	$F_{1, 57} = 0.68$
	Egg Size (mm)	4.79 ± 0.01, 32	<	5.23 ± 0.01, 26	$F_{1, 57} = 35.45^{***}$	$F_{1, 56} = 29.91^{***}$
	Fecundity	250.12 ± 1.43, 32	=	271.44 ± 1.94, 26	Chi squared = 1.39	NA

* Kruskal-Wallis comparison test was used for the comparisons of fecundity, and fork length and length from eye to tail in age 3+ matured females.

Table 5 Summary of repeated measures ANOVA results on size-at-age and growth increments of female kokanee salmon. Comparisons were conducted based on the subjective colour category and the assigned LD1 score, respectively. ‘***’ $P < 0.0001$. Otolith radius-at-age was *ln*-transformed in analyses.

Measurement	Method	Colour	Colour × Age	Age
Size-at-age	Colour Category	$F_{1, 52} = 1.81$	$F_{3,156} = 0.18$	$F_{3, 159} = 2905$***
	LD1	$F_{1, 51} = 1.65$	$F_{3,153} = 0.07$	$F_{3, 156} = 2815$***
Otolith Growth Increment	Colour Category	$F_{1, 52} = 1.73$	$F_{3,156} = 0.44$	$F_{3, 159} = 447.6$***
	LD1	$F_{1, 51} = 1.16$	$F_{3,153} = 0.24$	$F_{3, 156} = 436.5$***
Body Growth Increment	Colour Category	$F_{1, 52} = 1.69$	$F_{3,156} = 0.51$	$F_{3, 159} = 120.6$***
	LD1	$F_{1, 51} = 1.13$	$F_{3,153} = 0.22$	$F_{3, 156} = 118.6$***

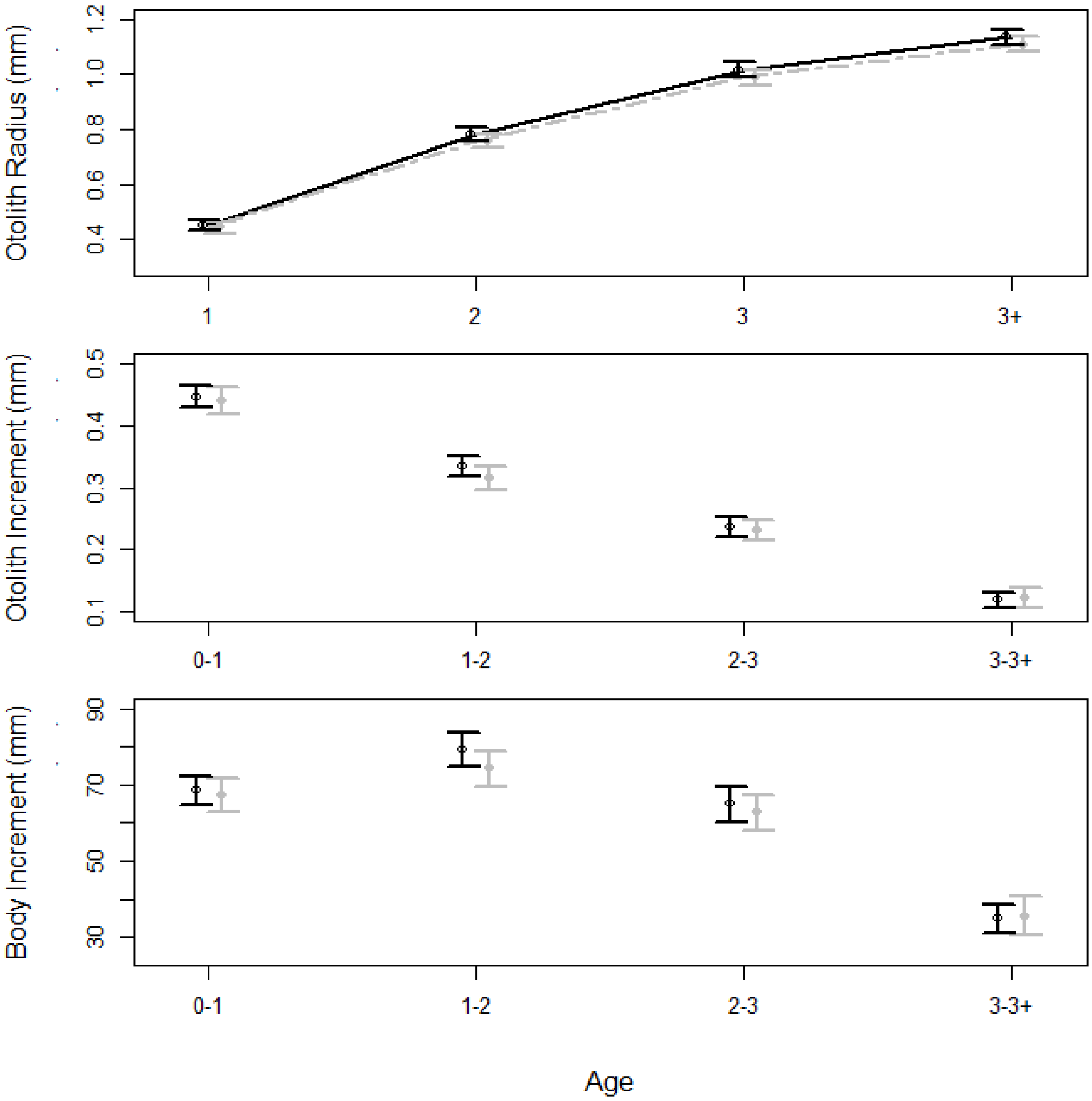

Figure 10 Otolith radius (upper panel), otolith size absolute increment (middle panel), and body size absolute increment (lower panel) at each age of red- (black dots and bars) and silver- arriving female kokanee salmon (grey dots and bars). Dots are the mean values of otolith radius (upper panel), otolith size absolute annual increment (middle panel), and body size absolute annual increment (lower panel). Bars are the 95 % confidence intervals, the black solid line shows the growth trend of red-arriving females, and the grey dashed line shows the growth trend of silver-arriving females. Otolith radius at age 1, age 3 and age 3+ were *ln*-transformed in analyses.

silver- and red-arriving fish (**Table 6, Fig. 10**).

No significant colour effect was detected in the repeated measures ANOVA of otolith growth increment or body size growth increment (**Table 5**, **Fig. 10**). Therefore, silver- and red-arriving females did not grow differently in their lifetime. In silver-arriving females, the otolith size annual increment was 0.0067 ± 0.0062 mm smaller, and the body size annual increment was 1.97 ± 1.61 mm smaller in general. The one-way ANOVA model also showed no significant differences in otolith size increment or body size increment during each age period (**Table 6**, **Fig. 10**).

Repeated measures ANOVA had a low statistical power to detect growth differences in otolith size and body size between silver- and red-arriving female kokanee. The low power could be caused by a low sample size of fish and/or a small effect size of growth difference. The differences in the otolith and body size annual growth increments between silver- and red-arriving females were 0.0067 ± 0.0062 mm and 1.97 ± 1.61 mm, respectively, and the effect sizes of size/growth difference-at-age were also regarded as small, ranging from 0.05-0.43 (**Table 6**, Sullivan and Feinn 2012). When the sample size of each phenotype was between 20 and 30, the power of otolith growth increment comparison from repeated measures ANOVA was between 0.176-0.292, and that of the body growth comparison was between 0.198-0.316. To achieve a power of 0.8 from repeated measures ANOVA, 100-110 samples from each phenotype would be needed for the otolith growth comparison, and 80-90 samples from each phenotype would be needed for the body growth comparison.

Otolith Analysis Validation

The same otolith measurements from thin sections and whole sections were not highly correlated. Linear regression showed a $R^2 = 0.60$ between otolith radius measured from different perspectives (i.e., thin section vs. whole section).

The estimated otolith size and body size mostly showed a difference from the same measurement generated directly from fish in the same cohort. At age 2, the

Table 6 Summary of differences in otolith sizes at previous ages, otolith size at maturity, and absolute annual growth increments between age 3+ silver- and red-arriving female kokanee salmon. Comparisons were conducted based on the subjective colour category and the assigned LD1 score, respectively. Size and growth measurements were shown as mean ± standard error, n (group sample size). All the comparison have a $P > 0.05$. Otolith radius at age 3, age 3+ and otolith increment during age 3-3+ were *ln*-transformed in analyses. Effect size (d) were calculated as the absolute mean difference between silver- and red-arriving females divided by the pooled standard deviation of silver- and red-arriving females (the square root of the average of variances in silver- and red-arriving females).

Traits		Age	Measurements (mm)			Effect of Phenotype		Effect Size
			Silver		Red	Colour Category	LD1	
Size	Otolith Radius	1	0.44 ± 0.01, 31	=	0.45 ± 0.01, 23	$F_{1, 52} = 0.35$	$F_{1, 51} = 0.63$	0.13
		2	0.76 ± 0.01, 31	=	0.78 ± 0.01, 23	$F_{1, 52} = 1.77$	$F_{1, 51} = 1.35$	0.37
		3	0.99 ± 0.01, 31	=	1.02 ± 0.02, 23	$F_{1, 52} = 2.40$	$F_{1, 51} = 1.63$	0.43
		3+	1.11 ± 0.01, 31	=	1.14 ± 0.02, 23	$F_{1, 52} = 1.82$	$F_{1, 51} = 1.25$	0.37
Increment	Absolute Otolith Increment	0-1	0.44 ± 0.01, 31	=	0.45 ± 0.01, 23	$F_{1, 52} = 0.21$	$F_{1, 51} = 0.63$	0.13
		1-2	0.32 ± 0.01, 31	=	0.33 ± 0.01, 23	$F_{1, 52} = 2.11$	$F_{1, 51} = 0.63$	0.41
		2-3	0.23 ± 0.01, 31	=	0.24 ± 0.01, 23	$F_{1, 52} = 0.20$	$F_{1, 51} = 0.06$	0.13
		3-3+	0.12 ± 0.01, 31	=	0.12 ± 0.01, 23	$F_{1, 52} = 0.003$	$F_{1, 51} = 0.01$	0.08
	Absolute Fork Length Increment	0-1	67.19 ± 2.22, 31	=	68.43 ± 1.86, 23	$F_{1, 52} = 0.17$	$F_{1, 51} = 0.57$	0.12
		1-2	74.19 ± 2.31, 31	=	79.19 ± 2.17, 23	$F_{1, 52} = 2.34$	$F_{1, 51} = 0.92$	0.43
		2-3	62.69 ± 2.31, 31	=	64.94 ± 2.22, 23	$F_{1, 52} = 0.47$	$F_{1, 51} = 0.19$	0.19
		3-3+	35.50 ± 2.52, 31	=	34.90 ± 1.79, 23	$F_{1, 52} = 0.03$	$F_{1, 51} = 0.03$	0.05

estimated otolith size and body size were larger than those measured from lake-captured kokanee in the same cohort (otolith radius: W = 147, p = 0.002, fork length: W = 173, p = 0.006, **Fig. 11**). At age 3, the estimated otolith size was similar to that measured from the lake-captured kokanee in the same cohort (W = 121, p = 0.208, **Fig. 11**), whereas the back-calculated body size was larger than the body size directly measured from lake-captured kokanee in the same cohort (W = 22, p = 0.0005, **Fig. 11**).

A significant difference was found in size-at-age between regrouped large and small kokanee. Repeated measures ANOVA of otolith size showed an overall size difference between large and small kokanee ($F_{1, 52} = 4.03$, $p = 0.05$, **Fig. 12**) and an overall age effect ($F_{3, 159} = 2905$, $p < 0.0001$, **Fig. 12**). The results indicate that otolith size-at-age of large fish was significantly larger than that of small fish, and also indicate that otolith size differed significantly among ages. Since no significant interaction was found between size and age ($F_{3, 156} = 1.90$, $p = 0.13$), small fish had a smaller otolith radius than large fish did throughout their lifetime. One-way ANOVA showed that small fish had significantly smaller otolith radius than that of large fish at age 1 ($F_{1, 52} = 4.51$, $p = 0.04$), but not at age 2, age 3, or age 3+ (**Fig. 12**).

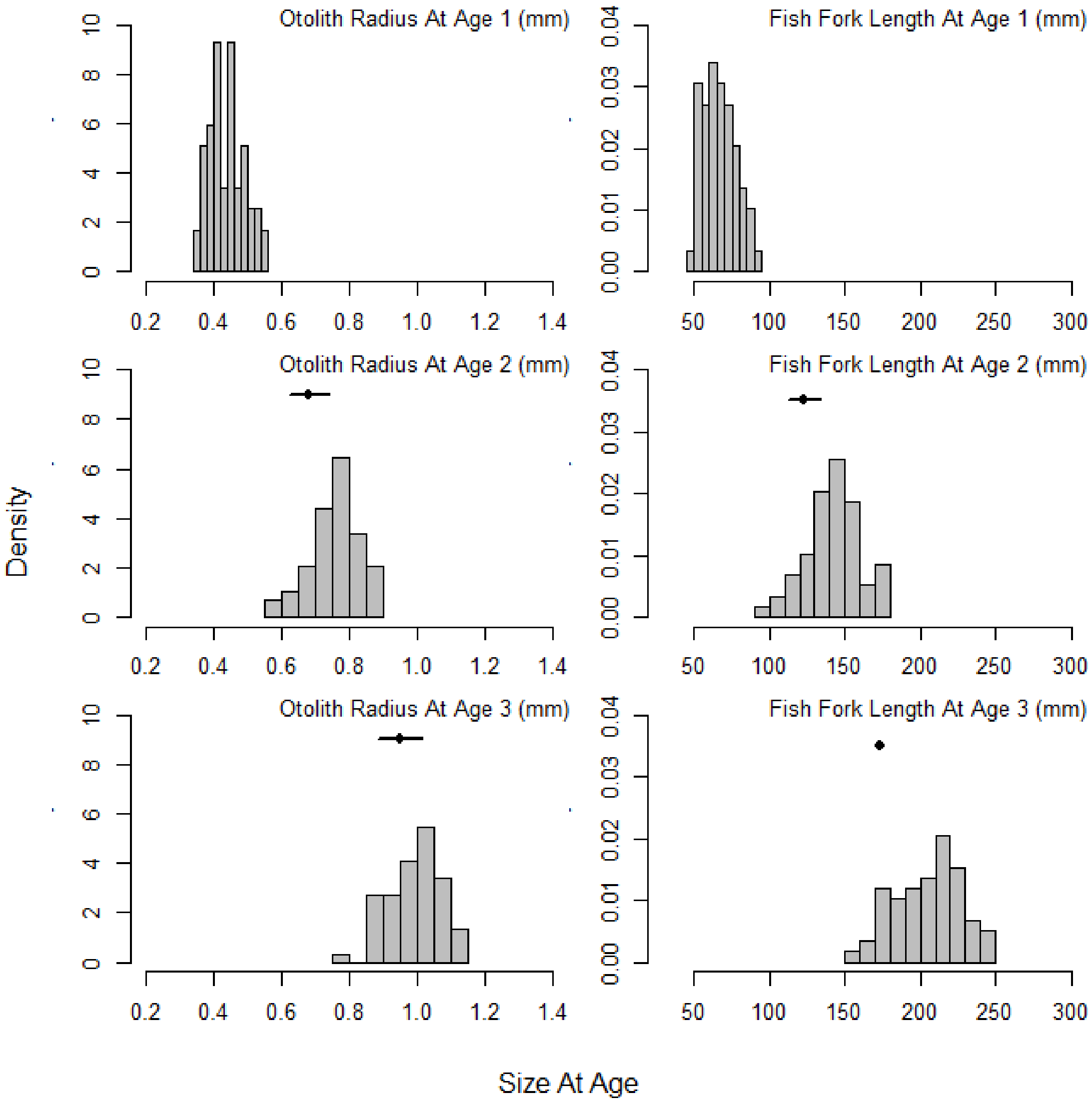

Figure 11 Multi-panel scatterplot of otolith size-at-age (left column panels) and fish body size-at-age (right column panels) for the 2009 kokanee salmon cohort. Histograms represent the estimated thin section otolith radius (left) and the back-calculated fish fork length (right) at age 1, age 2, and age 3 of spawning females, black dots and line segments represent mean and confidence interval of whole section otolith radius (left) and fish fork length (right) of age 2 (n = 12) and age 3 (n = 6) lake-captured fish. Note that sizes at age 1, age 2, and age 3 show the estimated sizes during winter, sizes represented by dots and line segments show the otolith sizes and body sizes in September.

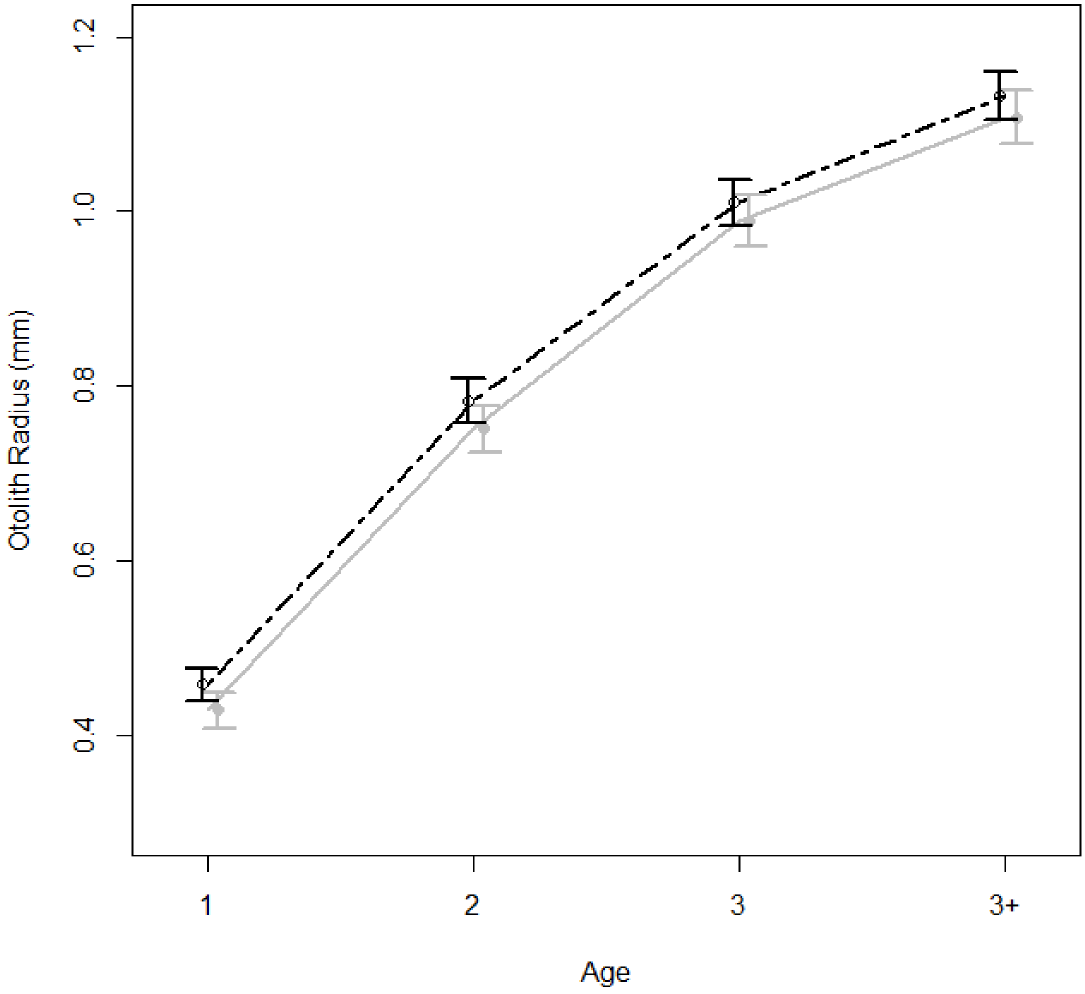

Figure 12 Otolith radius at each age of large (black dots and bars) and small female kokanee salmon (grey dots and bars). Dots are the mean values of otolith radius, bars are 95 % confidence interval, black solid line shows the growth trend of large females, grey dashed line shows the growth trend of small females. Otolith radius at age 1, age 3 and age 3 were *ln*-transformed in analyses.

Discussion

I investigated the effect of growth history on the expression of different female reproductive tactics in kokanee salmon. I compared the age- and size-at-maturity, two important characteristics in the salmon life history, between female kokanee that adopt silver- and red-arriving phenotypes. I also compared body sizes at previous ages and the annual growth increments, as proxies of growth history, between silver- and red-arriving fish. The results imply that the growth history does not appear to have a correlation to the expression of female reproductive tactics in kokanee salmon.

Age at Maturity

Silver- and red-arriving female kokanee did not have a difference in age at maturity in 2013. Age at maturity of silver- and red-arriving females in 2013 was not consistent with previous findings showing that silver-arriving females were generally younger than red-arriving females in the population. Age information was collected from early-arriving silver and red female kokanee salmon in 2003, 2008, and 2009. The age distribution of silver- arriving females was always shifted to a younger age (**Table 7**, Morbey and Guglielmo 2006, Warren and Morbey 2011). Based on these earlier studies, a younger age at maturity seemed to be a major characteristic associated with the expression of the silver-arriving phenotype. However, the same age at maturity of silver- and red-arriving females (97 % and 87 % matured at age 3+, respectively) in 2013 does not support this hypothesis. Nevertheless, it may be premature to negate an age difference between silver- and red-arriving females based on one year’s result. It is necessary to continue the collection of age information in order to understand if the lack of age difference between silver- and red-arriving females in 2013 is an exception.

Size at Maturity

Within the same age at maturity, body size from eye to tail or body mass at arrival were not significantly different between silver- and red-arriving females in 2013, indicating that silver-arriving females did not have a smaller size at maturity than red-

Table 7 Summary of age at maturity of silver- and red-arriving female kokanee salmon in 2003, 2008, 2009 and 2013. Data of each age group is shown as the number of individuals and the corresponding proportion within the population. Age information in 2003 was summarized in Morbey and Guglielmo (2006), age information in 2008 and 2009 were summarized in Warren and Morbey (2011).

Sampling Year	Sample Type	Age 2+	Age 3+	Age 4+
2003	Silver-arriving		10 (48 %)	11 (52 %)
	Red-arriving		2 (11 %)	16 (89 %)
2008	Silver-arriving		14 (93 %)	1 (7 %)
	Red-arriving		7 (47 %)	8 (53 %)
2009	Silver-arriving		11 (85 %)	2 (15 %)
	Red-arriving	2 (12 %)	7 (41 %)	8 (47 %)
2013	Silver-arriving	1 (3 %)	33 (97 %)	
	Red-arriving	1 (3 %)	27 (87 %)	3 (10 %)

arriving females. Differences in size at maturity between silver- and red-arriving females did not show a consistent trend among study years. In 2008 and 2009, body size at maturity was significantly smaller in silver-arriving females in fish that matured at age 3+, but not in fish that matured at age 4+ (**Table 8**, Warren and Morbey 2011). In 2003, the difference in body size at maturity was not significant between silver- and red-arriving fish that matured at age 3+ or age 4+ (**Table 8**, Morbey and Guglielmo 2006). Possibly, the low sample size of age 3+ red-arriving females in 2003 and age 4+ red-arriving females in 2008 and 2009 could have reduced the statistical power of the comparisons (**Table 8**).

With age at maturity controlled for, fork length at arrival of silver-arriving females was significantly shorter than that of red-arriving females in 2013 and previous years (Morbey and Guglielmo 2006, Warren and Morbey 2011). Fork length consists of snout length and length from eye to tail of the fish. Snout length is a secondary sexual characteristic of female kokanee salmon. Red-arriving fish that are in an advanced maturity status have a well-developed snout, whereas silver-arriving fish with a delayed maturity status have a shorter snout. Thus, the difference in fork length is likely due to the significant difference in snout size, which is influenced by the state of maturation of female kokanee at arrival. Consequently, for silver- and red-arriving females collected at arrival, the length from eye to tail can better represent overall body size at maturity.

The expression of different reproductive tactics is thought to arise from a size threshold for adopting the red-arriving tactic (Morbey and Guglielmo 2006). If a smaller size at maturity is related to the incidence of adopting the silver-arriving tactic, I expect the proportion of silver-arriving females to be larger when spawning fish are smaller in Meadow Creek Spawning Channel. I also expect that within a year, silver-arriving females will be smaller than red-arriving females. The proportion of silver-arriving females has only been measured in 2008 and 2009. Population assessment showed that there was a higher proportion of silver-arriving female kokanee in the population in 2009 (11.4 %) than in 2008 (2.2 %). Consistent with my expectation, spawning fish in 2009 were significantly smaller than those in 2008 (Warren and Morbey 2011). However, a

Table 8 Body size comparison between silver- and red- arriving female kokanee salmon with age as a factor. Fish from 2008 and 2009 were pooled to increase the sample size. Body size was measured as length from eye to tail and shown as mean ± standard error, n (group sample size). '*' means significant difference. Body size data in 2003 was extracted from Morbey and Guglielmo (2006), body size data in 2008 and 2009 was extracted from Warren and Morbey (2011).

Sampling Year	Age	Silver-arriving (mm)		Red-arriving (mm)	P
2003	3+	19.1 ± 0.2, 10	=	19.8 ± 0.4, 2	0.19
	4+	19.8 ± 0.2, 11	=	20.3 ± 0.2, 16	0.11
2008 and 2009	3+	20.0 ± 0.3, 25	<	21.5 ± 0.5, 14	0.002 *
	4+	20.9 ± 0.3, 3	=	22.3 ± 0.7, 16	P > 0.50
2013	3+	24.3 ± 0.3, 33	=	24.7 ± 0.2, 27	0.06

* Kruskal-Wallis comparison test was used for the body size comparison in 2013.

significant difference in size at maturity between silver- and red-arriving females was only seen in 2008. Thus, the proposed size thresholds may be too simple as the primary mechanism of reproductive tactic expression in female kokanee salmon. The relationship between size at maturity and the incidence of silver-arriving tactic needs further examination.

Variation in age at maturity of female kokanee did not have a strong influence on the size at maturity comparison. When fish that matured at age 2+ and age 4+ were included, the results of body size comparisons between silver- and red-arriving females in 2013 remained the same (**Table 4**). The consistent results may be due to the small sample size of age 2+ and age 4+ mature fish in 2013. However, in 2008 and 2009, the body size difference between silver- and red-arriving females also remained consistent when sizes were compared with and without controlled age (Warren and Morbey 2011). Body size at a younger age has been found to be smaller than that at an older age in salmonid fishes (Stearns and Koella 1986, Morita and Morita 2002, Morita and Fukuwaka 2006), including kokanee salmon (Grover 2005, Warren and Morbey 2011). Accordingly, it would be expected that an inclusion of younger or older fish may influence the difference in size at maturity between silver- and red-arriving females. How the maturation age and colouration (therefore the maturity status at arrival) influence the size at maturity of female kokanee needs to be studied.

Growth

The consistent results from my different growth comparisons indicate that silver- and red-arriving fish did not grow differently during their life. Both silver- and red-arriving females showed an increase in otolith size, thereby body size, with age. However, silver- and red-arriving fish did not have a significant difference in otolith size, and thereby body size, over any ages in life. When the otolith size-at-age was compared between silver- and red-arriving females, no growth difference was found. The absence of a statistical interaction between colour and age implies a similar rate of growth in silver- and red-arriving females. The annual growth increment at all ages of silver- and red-arriving fish was also compared to test differences in growth. The comparison did not show a significant difference in the otolith growth increment or the body growth increment over any age periods between silver- and red-arriving fish. Because silver- and red-arriving female kokanee did not show differences in age at maturity or size at maturity in 2013, perhaps it is not surprising to find that these females experienced a similar growth history. Meanwhile, it remains unclear if a younger age at maturity in female kokanee salmon is correlated to a faster growth. Relevant growth comparisons could not be carried out due to the limited samples of age 2+ and age 4+ mature fish.

Different methods of growth comparison (i.e., the subjective colour category and the objectively assigned LD1 score) gave the same outcome. The comparable results may be due to the small amount of fish (n = 2) with different colour assignments from the subjective and the objective colour classification. LD1 score representing the colouration of a fish seems to be a potential variable to replace subjective colour categories in future studies. In this situation, growth history or other conditions of the fish can be compared based on the assigned LD1 score.

The expression of female reproductive tactics in kokanee salmon does not appear to be related to growth history. This finding contradicts the frequently stated hypothesis that growth history affects the expression of reproductive tactics. In male salmon, sneaking males have grown faster than males that mainly adopt dominant reproductive tactic, especially in their early life (Silverstein et al. 1998, Vøllestad et al. 2004, Koseki and Fleming 2006, Koseki and Fleming 2007). The fast initial growth and/or larger initial body size causes a younger age at maturity, at a smaller body size. The small body size subsequently influences the reproductive tactic expression (Gross 1985, Thomaz et al. 1997, Fleming 1998). Differences in life history have also been seen in female seed beetles with brood parasitic reproductive tactic. In seed beetle *Acanthoscelides obtectus*, females with shorter longevity have higher incidence in egg dumping behaviour (Seslija et al. 2009). Similarly, in seed beetles *Callosobruchus maculatus*, dumpers have a significantly short longevity, and also a shorter larvae (i.e., pre-maturation) life span (Messina et al. 2007). It is hypothesized that non-dumpers benefit from the longevity, whereas dumpers benefit from an enhanced offspring quantity (Seslija et al. 2009).

However, growth has not been directly compared between female seed beetles with alternative reproductive tactics.

Maturity Status and Energy Allocation

Silver-arriving females arrived at the spawning channel in a less mature stage with smaller eggs. In the past studies, eggs of silver-arriving fish were consistently smaller than those of red-arriving fish at arrival (Morbey and Guglielmo 2006, Warren and Morbey 2011). Smaller eggs at arrival represent the delayed state of sexual maturation of silver-arriving females, as do the lack of red nuptial colouration and the shorter snout length, Silver-arriving fish completed red nuptial colouration changes before settling and spawning on the ground (Warren and Morbey 2011). Further ovary development occurs in silver-arriving fish, with the egg size increasing with time (Thorn, unpublished data). In 2013, silver-arriving females had the same egg size at ovulation as red-arriving females (Thorn, unpublished data).

Silver-arriving females do not seem to pay costs in fecundity for their earlier arrival and extended reproductive life span. The fecundity of silver- and red-arriving females did not differ in 2013, which was consistent with previous findings in 2003, 2008 and 2009 (Morbey and Guglielmo 2006, Warren and Morbey 2011). Fecundity of fish is usually related to the size at maturity, and can influence the reproductive fitness (Groot and Margolis 1991, Hendry et al. 1999, Campbell et al. 2006). Since the size difference between silver- and red-arriving females was usually small, it is not unexpected to find a similar fecundity. If silver-arriving females also experience less nest dig-up, it is not clear why all females do not adopt the silver-arriving tactic.

Silver-arriving females likely allocated more energy in soma to extend reproductive life span. During their reproductive stage, energy invested in soma is used to maintain reproductive behaviours and reproductive life span, whereas energy invested in gonads can influence reproduction success (Hendry et al. 1999, Briggs 2008). The energy used to maintain the longer pre-spawning period in silver-arriving females may be allocated from the energy for ongoing egg development, or from an extra energy intake during life. In the former situation, silver-arriving females would have low quality eggs, as the fecundity and the egg size at maturity (Thorn, unpublished data) were not different from those of red-arriving females. The energy allocation in soma and gonad can be evaluated by measuring energy contents in muscle and eggs (i.e., protein, fat, and water). A higher level of protein and fat means higher energy (Williams et al. 1986, Jonsson et al. 1991, 1997, Hendry and Berg 1999, Hendry et al. 1999). In sockeye salmon (*O. nerka*) from Pick Creek, different energy allocation strategies were used by early- and late-breeding females with different reproductive life span. Early-breeding females that lived considerably longer had higher energy storage at the onset of breeding, whereas late-breeding females with a shorter reproductive life span invested more energy in reproduction (Hendry et al. 1999). In future study, it is necessary to record the energy storage in somatic tissue and gonads at multiple time points in silver- and red-arriving females (e.g., arrival and spawning) to understand the energy allocation strategy in response to different tactics.

Other Potential Proximate Mechanisms

Genetic differences may influence the expression of female reproductive tactics in kokanee salmon. For instance, tactic expression can be heritable. In full-sibling and half-sibling crossing experiments, the longevity and the tendency of brood parasitism significantly differed in the offspring of dumpers and non-dumpers in seed beetle (*C. maculatus*, Messina and Fry 2003, Messina et al. 2007). Genetic polymorphism can also cause different expressions of female reproductive tactics. For example, the discrete reproductive colourations of female damselfly (*Ceriagrion tenellum*) are due to the expression of one of three alleles on an autosomal locus (Andrés and Cordero 1999). Differences in circadian-clock genes have also been proposed to cause the different maturation timing in silver- and red- arriving female kokanee salmon (Morbey et al. 2014). Circadian genes have been found influence the timing of migration and spawning in Pacific salmon (*O.* spp.) and Atlantic salmon (*S. salar*, Hansen and Jonsson 1991, Gharrett and Smoker 1993, Quinn et al. 2000, O’Malley et al. 2010), including kokanee salmon (*O. nerka*, Lemay and Russello 2014). However, no differences were found in *Clock1b* gene, other circadian-linked loci, or presumably neutral loci between silver- and red-arriving female kokanee (Morbey et al. 2014). Thus, the expression of reproductive tactics in female kokanee does not appear to be caused by a genetic polymorphism in circadian genes. Future study can test the heritability of silver- and red-arriving tactics, and other potential genes that are involved in maturation progress, because the different tactic expressions in female kokanee salmon may be determined by some underlying genetic variances.

Secondly, current energy condition may influence the expression of silver- and red-arriving reproductive tactics in female kokanee salmon. In birds, females in a low energetic condition that could not obtain nests may use brood parasitism to salvage reproductive success [redhead *Aythya Americana* (Sorenson 1991), American coots *Fulica Americana* (Lyon 1993, 2003), moorhens *Gallinula chloropus* (McRae 1998)]. In contrast, it has also been found that some females in a high energetic condition use intraspecific parasitism as a tactic to enhance their fecundity (Sorenson 1991, Brown and Brown 1998, Åhlund and Andersson 2001, Lyon 2003). In my study, the energy storage during a certain life stage in the body of a fish could not be estimated from otoliths. Energy condition is very difficult to measure, and the relationship between otolith growth and energy stores has not been validated. In salmon, the energy storage may influence the onset of sexual maturation in a given year (Thorpe et al. 1998, Thorpe 2007), and therefore, the maturation timing. In cultured growth hormone (GH) transgenic Atlantic salmon, it has been found that the cumulated energy may have been reduced due to an accelerated growth in structural tissues, and the sexual maturation in these fish was consequently inhibited (Moreau and Fleming 2012). Further research needs to include measurements on the energy status of kokanee salmon, especially during the months of reproductive maturation, perhaps by tracking individuals through time.

Finally, hormone regulation may influence the expression of silver- and red-arriving tactics in female kokanee salmon. Hormone levels regulate sexual maturation and can influence reproductive behaviours of an individual. The gonadal maturation of fish is controlled through the brain–pituitary–gonad (BPG) chain. Gonadotropin-releasing hormone (GnRH) in the brain can stimulate the pituitary gland to release gonadotropin (GTH). GTH subsequently causes steroidogenesis in gonads, and therefore, sexual maturation (Briggs 2008, Ueda 2012). In salmon, testosterone (T) influences the secondary sexual characteristic development, breeding colouration switch, and territory behaviours (Briggs 2008). T is also highly involved with 17β-estrogen (E_2) in vitellogenesis in females, and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is active in the final stage of maturation (Nagahama 1997, Ueda 2012). The elevation of associated hormone levels is likely to affect the maturation timing, and subsequently cause the different colourations and behaviours in silver- and red-arriving females. Progesterone level was shown to have an influence on the alternative reproductive tactics (i.e., throat colouration) displayed by female side-blotched lizards (*Uta stansburiana*, Cooper and Greenberg 1992). In side-blotched lizards, females have orange or yellow throat colour, exhibit different degrees of aggression during reproductive stage, and achieve reproductive fitness differently by adjusting the quantity and quality of offspring (Sinervo and Zamudio 2001, Oliveira et al. 2008). It has been proposed that mutation in the endocrine gene caused the differentiation in hormone regulation, and subsequently, the variance in throat colour and aggressiveness (Sinervo et al. 2000, Sinervo and Zamudio 2001). The hormonal regulation of maturation in female kokanee needs more study, as it may differ between silver- and red-arriving females.

Otolith Measurement and Analysis

Validation of otolith size to body size relationship

Otolith size was highly correlated to fish body size when including a wide range of fish body sizes. The otolith ventral radius to the fish fork length relationship showed a high R^2 value (0.87). The R^2 value is close to the findings in other Pacific salmon species and populations (R^2 ranging from 0.57 to 0.98). However, when the same relationship was tested using fish with similar body size (i.e., with only spawning fish), the R^2 value showed a dramatic decrease (unpublished data). The same trend has also been seen in coho salmon (Nordholm 2014) and sockeye salmon (*O. nerka*, West and Larkin 1987, Meekan et al. 1998). For instance, the R^2 value of the regression between otolith size and fry body size was 0.46 in a population of coho salmon (*O. kisutch*). When juvenile salmon with a wide range of sizes were included in the test, the R^2 value increased to 0.92 (Nordholm 2014). Similarly, the R^2 value changed from 0.26 to 0.64 as the range of included body sizes increasing in a sockeye salmon population (*O. nerka*, West and Larkin 1987, Meekan et al. 1998). Thus, the precision of using otolith size to represent fish body size can be influenced by the range of body sizes, thereby the scale of the time interval in a fish's life history (Aubin-Horth and Dodson 2002).

The sampling procedure for validation study has certain issues and can be improved. A relatively high variation in otolith size was seen at the same body size during the later life stage (**Fig. 8**). The variation was not due to the inclusion of different fish cohorts, since various cohorts did not show a significant difference in the relationship between otolith size and fish size (**Fig. 9**). Otolith growth is a process of calcium carbonate concretion (Payan et al. 1997, Campana 1999). Factors such as the metabolic rate of a fish (Wright et al. 1990, Yamamoto et al. 1998) and the temperature (Bradford and Geen 1992, Lombarte and Lleonart 1993, Clarke and Friedland 2004, Song et al. 2009) can have an influence on the otolith size to fish body size relationship. The increased variation in otolith size during later life may be due to an accumulation of individual differences in otolith growth with time. The sample collection of lake-captured kokanee was not designed for my validation study. To ensure fish with a wide range of body sizes were included, the sampling year and sampling site could not be kept consistent due to the limited number of archived fish. The obtained fish may have experienced different rearing environments, which would add individual variation in otolith growth. As well, since the archived fish were kept frozen in bags, the defrosting process of kokanee samples may have also influenced the size of fish body or otolith.

The best-fit model and most suitable otolith dimension were used to cope with the individual variation in the otolith size to fish body size relationship. Power regression on otolith ventral radius was used to back-calculate fish body size, because the corresponding regression model and dimension gave the highest accuracy and precision. The biological intercept model (BI) has been frequently used in the body size back-calculation to adjust an effect of various otolith growth within a population (Campana 1990). The model uses a biological intercept (i.e., the otolith size and fish body size at hatching) instead of a statistical intercept. BI model does not require any parameters estimated from other fish samples in the population, therefore, can be applied to body size back-calculation at an individual level. However, the BI model is built on a linear relationship between otolith size and fish body size (Campana 1990). Consequently, the BI model is not suitable for this study. In the studies that involve fish body size back-calculations, the linearity of the relationship between otolith size and fish body size should be examined prior to the use of BI model. When the assumption of linearity is violated, other regression models should be considered to replace the BI model in back-calculation.

Sources of Error

The application of thin section measurements in a back-calculation model generated from whole section measurements could have induced additional variation in the back-calculated body size and growth. In the validation study, the otolith ventral radius was measured on whole otolith sections. In the growth comparison, growth increments of the chosen otolith dimension was measured on otolith thin sections in order to get an accurate assignment of annuli (Morales-Nin 1992). The measurements on the same dimension from otolith thin sections were not highly correlated to those obtained from otolith whole sections. It would be ideal to validate the otolith size to fish size relationship and generate the back-calculation model based on only thin section measurements. However, otolith sectioning is difficult on small otoliths. Nevertheless, data used in the comparisons of otolith size-at-age and otolith growth increment did not require the use of back-calculation model. As a result, otolith measurements from thin sections would not bring in errors in the statistical analyses. Although the body growth increment had a potential induced error, the corresponding comparison showed a consistent result as other growth comparisons that were conducted on the size-at-age and the otolith growth increment.

The existence of certain indeterminacy of annuli assignment on otoliths was the major challenge in this study. Otolith sectioning has enabled a better determination of annuli on otolith thin sections. As has been stated in literature (Morales-Nin 1992, Beamish and McFarlane 2000), the annuli on otolith thin sections have an improved clarity than those observed from otolith whole sections. However, owing to the potential error in the assigned annuli, measurements of otolith radius are still not highly accurate. As a result, the back-calculated body size can be influenced. The estimated otolith size at age 2 was biased towards a larger size compared to the same dimension measured directly on the whole otolith of lake-captured fish in the same cohort. The same trend was seen in back-calculated body sizes at age 2 and age 3, when the estimated body sizes were compared to the sizes directly measured from fish in the same cohort. However, the difference between estimated size-at-age and directly measured size may be due to the inconsistent fish sizes they represent. Lake-captured kokanee salmon were collected in September. Therefore, the directly measured sizes represent fish size in fall. The assigned annuli were formed during the winter. Thus, the estimated sizes-at-age represent fish size after a period of continuous growth compared to their size in fall.

Statistical Power

The power analysis of the growth comparison implies that a large sample size (approximate 200 female kokanee) would be needed to achieve a high statistical power to detect a small growth difference between silver- and red-arriving females. In the estimation of statistical power, both the sample size and the effect size can influence the statistical power of a comparison. An increase in the number of collected fish may increase the statistical power of the growth comparison. Nevertheless, the demand of large sample size in the detection of a significant difference implies that the biological difference (i.e., growth difference between silver- and red-arriving females) is probably small in the kokanee population (otolith size annual growth difference: 0.0067 ± 0.0062 mm, body size annual growth difference: 1.97 ± 1.61 mm).

A difference has been seen in otolith size at age 1 between the rebuilt groups based on the body size at maturity of collected kokanee, indicating that otolith measurements seem to be able to detect existing size differences. However, it is impossible to validate the significant result, because the actual body size at age 1 of the collected fish is unknown. In the comparison of regrouped large- and small-fish, otolith size at age 3+ was not different between large and small fish, although there was a difference in the actual fish body size. This again implies that the application of otolith measurements in size and growth study of kokanee salmon is restricted in later life.

Otoliths should be used cautiously when studying age and growth of fish. Otolith made it possible to estimate fish sizes at previous ages and annual growth increments, which both play an important role to understand the growth history of silver- and red-arriving females. However, it should be kept in mind that growth measurements on otolith have a number of restrictions (Campana 1990). Interpretations and conclusions need to be made with caution.

Colour Analysis

The subjective sample collection based on colouration showed a high accuracy. During sexual maturation, sockeye and kokanee salmon transport the carotenoid pigments (mainly astaxanthin) from flesh to skin (Craig and Foote 2001). A silvery colouration with skin covered by scales is associated with an early stage in sexual maturation. The assignments of colouration based on digital photograph analysis were highly consistent with subjective colour categories in 2013. In 2008 and 2009, the subjective and objective colour assignments also showed a high consistency (Warren and Morbey 2011). During the kokanee spawning season, the appearance of a large proportion of fish with intermediate colour adds difficulties in sample collection. Also, different light conditions among sampling dates can cause a misclassification of fish in the colour-based sample collection. Nevertheless, the result of colour category validation indicates that visual classification based on fish colouration is a convincing sampling process.

The colour analysis methodology in 2013 was refined based on that in 2008 and 2009. In my study, colour attributes from four locations in the photos of kokanee salmon were used in the DFA model, whereas those from nine locations were used in the previous study in 2008 and 2009 (Warren and Morbey 2011). The reduction of selected locations was aimed to avoid the influence of glare on the colour attributes. Notwithstanding, four selected locations showed the major variances in colouration among kokanee females and may well represent the stage of maturity of the fish. Besides, three colour attributes, L^* (lightness), a^* (redness), and b^* (yellowness) were included in the DFA model. In contrast, L^* and H^*_{ab} (hue) were used in the former study (Warren and Morbey 2011). H^*_{ab} describes the closeness of a measured colour to a landmark colour (e.g., red, green, blue, and yellow). H^*_{ab} is calculated as arctan (b^*/a^*), and ranges from 0° to 360°. Values that are close to 0° or 360° both represent a reddish colouration, however the actual values used in statistical comparison would have a significant difference. As a result, a^* and b^* were used instead of H^*_{ab}. The selection of candidate colour attributes would influence the assigned LD1 score of each fish from the DFA model, and subsequently, affect the growth comparison based on LD1 score.

Conclusion

I investigated differences in age and size at maturity and in growth history between silver- and red-arriving female kokanee salmon in 2013. This is the first study that examined the influence of growth history on the expression of different female reproductive tactics. It filled in the gaps of studies in the proximate mechanism underlying female alternative reproductive tactics. The study showed that age and size at maturity were not different between silver- and red-arriving females. The sizes at previous ages and the growth increments did not show a noticeable difference between silver- and red-arriving fish, thus the study proved that somatic growth condition does not appear to influence the expression of distinct female reproductive tactics in kokanee salmon. Besides the somatic growth condition, other conditions such as energy storage condition and hormone levels have not been measured in this study. In female salmon, as an example, energy accumulation and hormone regulation are critical to the maturation and reproductive behaviours, and the energy allocation during females' reproductive stage is associated with reproductive life span and timing of spawning. Accordingly, in the studies on the potential influences of female reproductive tactics, physiological conditions that are closely related with individuals' reproductive phenotypes and behaviours should be taken into consideration. Although genetic difference has not been found between silver- and red-arriving females in circadian gene, the tactics could still be heritable. In future studies on kokanee salmon, the heritability of reproductive tactics also needs to be tested.

Otolith increment measurements were used in the test of growth difference between silver- and red-arriving fish. I validated the relationship between otolith size and fish body size of the kokanee population in Kootenay Lake. Thin section measurement was chosen in coping with the lack of clarity of annuli on otoliths of kokanee salmon. A best-fit model was generated for fish body size back-calculation after comparing multiple regression models. In other studies that involve growth measurements, assumptions of widely used models should not be neglected, as a poorly selected model may have a considerable negative impact on the results and conclusion.

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Appendices

Appendix A: Animal Use Protocol

AUP Number: 2013-033

PI Name: Morbey, Yolanda

AUP Title: Evolutionary Biology And Ecology Of Salmon

Approval Date: 08/07/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Evolutionary Biology And Ecology Of Salmon" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2013-033::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Curriculum Vitae

Yelin Xu

Post-secondary Education and Degrees:

Ningbo University Ningbo, Zhejiang, China B.Sc (Hons) Marine Biological Resources and Environment	2008-2012

Honours and Awards:

Western Graduate Research Scholarship (UWO)	2012-2014
Graduation Award: Zhejiang Province Ministry of Education Letter of Distinction (Zhejiang Province, China)	2012
In-course Scholarship (NBU, China)	2009-2011

Related Work Experience:

Teaching Assistant (Introductory Biology)	Fall 2012/2013
Teaching Assistant (Introductory Biology)	Winter 2013/2014
Teaching Assistant (Statistics for Science) The University of Western Ontario, Canada	Fall 2014
Volunteer Research Assistant Benthic Ecosystem Lab, Ningbo University, China	2009-2012
Intern Ningbo Environmental Monitoring Center, Ningbo, China	May-July, 2011

Presentations:

Xu, Y., Morbey, Y.E., “*The Effect of Growth on Reproductive Tactics in Female Kokanee Salmon*” Oral presentation at Western’s 5th Biology Graduate Research Forum, Oct, 2014, London, Ontario.

Xu, Y., Morbey, Y.E., “*Are Female Reproductive Tactics Growth Dependent in Kokanee Salmon?*” Oral presentation at GENOMES TO/AUX BIOMES Canadian Society of Ecology & Evolution joint meeting, May, 2014, Montreal, Quebec.

Xu, Y., Morbey, Y.E., “*Female Reproductive Tactics in Kootenay Lake Kokanee Salmon*” Poster presentation at Western’s 11th Earth Day Colloquium, April, 2014, London, Ontario.

Xu, Y. and Morbey, Y.E., “*Validation of Otolith Analysis to Study The Effect of Growth on Reproductive Tactics in Female Kokanee Salmon (Oncorhynchus nerka).*” Poster presentation at Ontario Ecology, Ethology, and Evolution Colloquium, May, 2013, London, Ontario.

Xu, Y., Lv, X., and Lin, X., “*The Effect of Thermal Discharge from A Power Plant on the Community Structure of Meiofauna in Xiangshan Bay*.” Oral Presentation at Challenge Cup Science and Technology Colloquium of Ningbo University, Nov, 2010, Ningbo, China.