Photoperiod and Growth Manipulation Reduces the Problem of Unwanted Sexual Maturation in Arctic Charr, *Salvelinus alpinus*

by

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Abstract

Farming diploid Arctic charr (*Salvelinus alpinus*; Labrador strain) in Atlantic Canada is greatly impeded by unwanted sexual maturation and associated loss of growth and meat quality. Up to 70% of fish in both sexes mature at age 2, due to accelerated growth from both a high energy diet and rearing in 'warm' 10°C well water. The goal to reduce maturity to <20% was achieved by manipulating photoperiod, rearing temperature and feeding in a series of five lab-based trials each lasting 12-18 months ending age 2. To explore the relationship between somatic growth and the physiological decision to mature, all fish were identified with a PIT-tag and measured monthly. Continuous light (LL) overwinter effectively reduced maturation. Histological analysis of germ cells revealed the change between natural daylength (LDN) and LL induced a dichotomous response, stimulating some fish and inhibiting others, dependent on the direction and timing of photoperiod change. Food deprivation and/or 5°C overwinter alone were less effective than LL at reducing maturation, but combining all three factors reduced maturity to <5%. Paradoxically, body weight, condition factor and lipid content were poor indicators of whether an individual would mature or not. Plasma melatonin monitoring indicated 50 lux at night was a sufficient intensity for effective LL treatment. Charr failed to exhibit a circannual rhythm of sexual maturation under LL and LD 8:16 suggesting the conventional thinking on the mechanism by which photoperiod controls sexual maturation among salmonids requires further investigation.

List of Abbreviations and Symbols Used

AIF	Atlantic Innovation Fund
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BPG	Brain-pituitary-gonad
BW	Body weight
CF	Condition factor
CV	Coefficient of variation
CZRI	Coastal Zones Research Institute
E2	17β-estradiol
ELISA	Enzyme-linked immunosorbent assay
FL	Fork length
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GSI	Gonadosomatic index
GTH	Gonadotropin hormone
IGF1	Insulin-like growth factor-1
IU/ml	International Unit per milliliter
LD	Light:dark cycle
LH	Luteinizing hormone
LL	24 h light
LDN	Simulated natural daylength
MIH	Maturation-inducing hormone
MS222	Tricaine methanesulfonate
MT	Metric tonnes
Na^+/K^+ ATPase	Sodium-potassium adenosine triphosphatase
pg/ml	Picograms per milliliter

PIT-tag	Passive integrated transponder tag
qPCR	Quantitative polymerase chain reaction
SGR	Specific growth rate
W/m^2	Watt per square meter
$\mu moles/sec/m^2$	Micromoles per second per square meter
°C	Degrees Celcius
°N	Degrees North
♀	Female
♂	Male

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Chapter 1: Introduction

1.1 Arctic charr aquaculture

Arctic charr (*Salvelinus alpinus*) has been a promising aquaculture species in Canada for the past three decades associated with a niche market demand. The global production of Arctic charr, about 6000-10,000 metric tonnes (MT), mainly occurs in the Nordic countries including Iceland, Sweden and Norway (Sæther et al., 2016). In Canada, Arctic charr farming remains small, about 500 MT per year compared with 120,000 MT of Atlantic salmon (*Salmo salar*; Statistics Canada, 2017). A major problem faced by the industry is early sexual maturation (Sæther et al., 2016; Liu and Duston, 2016). The maturation process results in the deterioration of flesh quality and reduced body size associated with the diversion of energy from somatic growth to development of gonads and secondary sexual characteristics. Unwanted sexual maturation among diploid Fraser River Arctic charr (Labrador strain) has been a serious impediment to farmers in Atlantic Canada, up to 80% reach maturity at age 2 prior to reaching market size (Liu and Duston, 2016). At an International Arctic charr meeting in 2011, the private sector emphasized the need to solve the problem which led to funding which made this thesis possible (Glebe, 2011).

The potential to exploit a profitable niche market remains, as does the advantage of a fish that can grow well at a high stocking density and in cold freshwater (Sæther et al., 2016). Canadian farmed stock has two genetically discrete lineages, *Salvelinus alpinus erythrinus* (Fraser River) and *Salvelinus alpinus alpinus* (Nauyuk and Tree Rivers, Glebe, 2011). A breeding program was established for the Fraser River population in 1996 in Shippagan, New Brunswick. The stock originates from wild eggs retrieved from the Fraser

River, Labrador in the 1980s by the Department of Fisheries and Oceans and the Huntsman Marine Science Centre (Glebe, 2011). The primary aim of the breeding program is to prevent inbreeding depression, a consequence of a small founding population, and provide sustainable commercial seed for the private sector (Pelletier, 2011). In 2012, the Atlantic Innovation Fund (AIF) awarded over $3 million to the Coastal Zones Research Institute (CZRI), recently named Valorēs, for a five-year research project entitled 'Aquaculture Development and Profitable Commercialization of Arctic charr in Canada'. The goal was to overcome the major obstacles that constrain the expansion of Arctic charr industry including the aspects of selective breeding program, husbandry practices and marketing. A portion of the research was contracted to Dr. James Duston at the Dalhousie University Faculty of Agriculture, with the goal to reduce the incidence of early sexual maturation among grow-out fish to < 20% at age 2 (ca. 1kg).

In Atlantic Canada, Arctic charr farming mainly occurs in freshwater land-based systems, and brackish fjords in Newfoundland. Sea-cage culture is not feasible since Fraser River Arctic charr have limited tolerance to seawater (Duston et al., 2007). The land-based facilities supplied with groundwater at 7-10 °C are preferred, since surface water in the Maritimes gets too warm in summer. The production cycle takes about 2 years from egg to over 1 kg market size. The accelerated growth and development, naturally, result in a reduced age at maturity. Increased maturity rate among Fraser River Arctic charr at age 2 contrasts markedly with wild fish that reach about 1 kg body size and first maturity between age 5 and 8 (Dempson and Green, 1985; Liu and Duston, 2016). The maturation process not only reduces somatic growth and flesh quality but also increases risk of disease associated with impaired immunity (Jobling et al., 1998). The problem of early maturation

also challenges the sustainability of other major aquaculture industries including Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*; Taranger et al., 2010; Carrillo et al., 2015).

Techniques to reduce the problem of early sexual maturation in the aquaculture industry mainly include monosex stocks, triploidy, photoperiod and growth manipulation. All-female diploids are used in the rainbow trout industry worldwide and a small proportion of Atlantic salmon producers (e.g. Tasmanian salmon industry; Lincoln and Scott, 1983; Thorpe, 2004). The adoption of this technique is because only males mature before the fish reach harvest size. This is not the case for diploid Fraser River Arctic charr since both sexes exhibit a high maturity rate at age 2 (Liu and Duston, 2016; 2018). Triploidy is an effective method to produce sterile fish and offers potential to eliminate the maturity problem (Chiasson et al., 2009). Triploid Fraser River charr, however, exhibited inferior growth and survival compared with diploid fish under the conditions of 10 °C and daily feeding (Chiasson et al., 2009; Duston, Unpubl. data). Icy Waters Ltd. has been selling 'Yukon Gold' triploid Arctic charr eggs (Nauyuk and Tree Rivers strain) for the past two decades, but triploid males still develop testes and secondary sexual characteristics, representing a costly problem (Lucas, J., 2015, Icy Waters Ltd., pers. comm.). The current project investigated two most common approaches: photoperiod and growth manipulation, with the goal to develop a reliable protocol to reduce the incidence of maturity to < 20% among Fraser River Arctic charr.

1.2 Research Scope and Aims of the Study

Sexual maturation for first time is the most important developmental stage post-embryogenesis, signifying the transition from juvenile stage to adulthood and the

acquisition of capacity to reproduce sexually, namely puberty (Okuzawa, 2002; Taranger et al., 2010). Puberty is a complex process that involves the integration of internal factors including nutrition, growth, and gonadal status and environmental factors including perception of time of year, temperature and food availability (Bromage et al., 2001; Taranger et al., 2010). This integration process that occurs in the brain-pituitary-gonadal axis leads to a 'go/no-go decision'. Once the decision to mature is taken, complex mechanisms trigger a hormonal cascade, gametogenesis and steroidogenesis, which ultimately result in the maturity of gametes (Schulz et al, 2010; Kagawa, 2013). Improving the understanding of the mechanism underlying puberty, especially the onset of puberty, is important for both basic science and the aquaculture industry.

Seasonality is the most pronounced characteristic of the reproductive cycle among most fish inhabiting temperate regions, including salmonids (Bromage et al., 2001). The timing of the maturation process is synchronized to the seasonal change in daylength. Use of photoperiod to decrease the incidence of sexual maturity was discovered during attempts to advance the timing of spawning of rainbow trout (Duston and Bromage, 1988) and increase the growth rate of Atlantic salmon (Hansen et al., 1992). Today, 24h light (LL) exposure is used extensively in the aquaculture industry to reduce early maturity among salmonids, sea bass and Atlantic cod (*Gadus morhua*, Carrillo et al., 2015). LL overwinter is applied globally in Atlantic salmon sea-cage farming to reduce the incidence of grilsing, namely maturation after one sea-winter (Taranger et al., 2010). The mechanism by which a photoperiod prevents sexual maturation, however, remains unclear. There are perplexing observations that 24h light induces sexual maturation in sea-cage and land-based farms when its application is delayed and/or environmental conditions for growth are optimized (Good and Davidson, 2016). In the present project, a series of long-term trials (each 12-16

months) were conducted to try to identify a reliable protocol to control early sexual maturation in the Fraser River stock. Since Arctic charr suffers from high incidence of early maturity in both sexes and tolerate high stocking densities under captivity, it also serves as a good laboratory model species to examine a controlling mechanism that has been generally assumed, perhaps wrongly, to be common to all salmonids (Wilkinson et al., 2010). The generation of seasonal reproductive cycles and its long-term rhythmicity is hypothesized to be controlled by an endogenous circannual timer, which can self-sustain under unchanging photoperiod and temperature (Duston and Bromage, 1991). The existence of an endogenous rhythm was tested here. In addition, light information is transduced by the hormone melatonin which elevates during the night and remains at the basal level during the day (Falcón et al., 2011). The project also evaluated different intensities of nighttime lighting on suppressing nocturnal plasma melatonin to understand the lighting requirement for the photoperiod protocol.

Increased maturity has been reported to be associated with the high growth rate or large body size promoted by daily feeding and high temperature (Fjelldal et al., 2011; Imsland et al., 2014) and a high lipid diet in Atlantic and Chinook salmon (*Oncorhynchus tshawytscha*; Shearer et al., 2006; Arge et al., 2012). The long-standing hypothesis is that the physiological 'decision' to commence maturation is dependent upon individual fish surpassing a genetically fixed threshold level of energy reserves during a critical time period during fall/winter (Thorpe et al., 1998; Thorpe, 2004). The 'decision period' is defined by the phase of photoperiod but the exact timing and duration are unclear. The energy reserves were a mix of inter-related factors: lipid, body size, growth rate and energy turnover (Rowe et al., 1991; Shearer et al., 2006; Trombley et al., 2014). Accordingly, a reduction in the maturity rate at a given year was achieved by decreasing rearing

temperature (Jonsson et al., 2013) and food rations/dietary lipid during the fall-winter 'decision period' (Duston and Saunders, 1999; Shearer et al., 2006). Hence, the factors of food availability and temperature were investigated in Arctic charr to better understand the interrelationship between photoperiod, somatic growth and sexual maturation and to what extent environmental factors influence the age at maturity. Food deprivation, although a sensitive welfare issue, can be a useful practice if maturation can be reduced because fish exhibit compensatory growth following the resumption of food making the technique economically viable (Savoie et al., 2017).

The objectives of this thesis were:

1. To review the biology of Arctic charr, photoperiod control of seasonal reproduction, and environmental regulation of somatic growth among salmonids (Chapter 2).

2. To better define the optimum start date and duration of 24h light (LL) treatment on preventing sexual maturation (Chapter 3).

3. To determine the synergistic effect of combining LL treatment with food deprivation and low temperature on suppressing maturity rate (Chapter 4).

4. To clarify the role of daylength by comparing LL and 18h light:6h dark (LD 18:6) and quantify the relative importance of photoperiod and food deprivation during the 'decision' period (Chapter 5).

5. To devise the relationship between light intensity and nocturnal plasma melatonin in Arctic charr (Chapter 6).

6. To understand the role of long and short photoperiod on sexual maturation by analyzing ovaries and testes histologically (Chapter 7).

7. To test the existence of an endogenous circannual rhythm (Chapter 8).

Chapter 2: Literature Review

2.1 Life-history of Arctic Charr

Arctic charr was originally described as *Salmo alpinus* by Carl Linnaeus in 1758. A new genus, *Salvelinus*, was established by John Richardson in 1836 for charrs (Klemetsen, 2010). '*Salvelinus*' is from German '*Saibling*', meaning little salmon. This genus along with *Salmo* and *Oncorhynchus*, the salmons and trouts of the Atlantic and Pacific regions, constitute the Salmonidae family (Klemetsen et al., 2003). Arctic charr is the northernmost cold freshwater fish indigenous to both lacustrine and riverine systems. They mainly distribute in the Holarctic regions across the basins of all Arctic seas, the north of the Pacific Ocean and the Atlantic Ocean (Esin and Markevich, 2018). They are also found in lakes at an altitude of more than 2000 m in the Alps and Pyrenees and to depths greater than 400 m in Norway (Klemetsen, 2010). Certain populations are anadromous, migrating from freshwater to coastal regions and seawater during summer. Fish used in the present study are indigenous to the Fraser River, Labrador (56˚N, 63˚W), and are an anadromous population (Dempson and Green, 1985).

Life-histories of Arctic charr are very diverse in terms of the range in size at maturity, phenotypes, behavior and ecological niches (Klemetsen, 2010). The high latitude freshwater environment is characterized by extreme seasonal fluctuation in climate and food abundance. The challenging living conditions include 6-7 months (November-May) of ice-covered winter periods, low temperature (0.5-2 °C) and food shortage (Mulder et al., 2018). To overcome the challenges, Arctic charr exhibit significant flexibility of life-history patterns. In general, the life-history can be divided into three main stages: freshwater stage, transition/migration and maturation.

In the Fraser River, spawning occurs in October and November, large yolky eggs (3.5-5 mm) are spread on/in the gravel substrate (Dempson and Green, 1985). Daylength is about 6 h and 50 mins on December 21 the winter solstice, and it increases to about 17 h and 45 mins on June 22 the summer solstice. The river begins to freeze in late October and is covered by ice until the following April and May, with a thermal range of 0.5-3°C during this period (Dempson and Green, 1985; Mulder et al., 2018). The eggs incubate in gravel redds overwinter and probably hatch out between March and April. The specific timeframe for the early development of the wild population is not clear because of the inaccessibility of the fish under the ice. The alevins live on the endogenous nutrients from the yolk-sac attached to the body. Fish enter the juvenile stage of fry and start to feed on exogenous prey following the absorption of the yolk-sac. The opportunity for somatic growth is concentrated to a short summer period between July and August when the water warms to 8-13 °C (Dempson and Green, 1985). Generally, growth rate of juveniles between age 0 and 4 is slow, gaining about 2.5-3.5 cm fork length (FL) annually (Dempson and Green, 1985). Juveniles grow in the freshwater until their first migration to the sea after reaching a certain body size. Some landlocked and resident populations, by comparison, remain in freshwater all their life (Rikardsen et al., 2004).

The anadromous life-history strategy allows for the avoidance of the adversity in freshwater, such as food scarcity and living space, and the pursuit of oceanic food abundance and accelerated growth and development. At the age of 3-6 year-old, Fraser River charr (FL: 9-20 cm) make their first seaward migration in May-June coinciding with spring runoff and ice breakup in the coastal rivers. The migratory population consists of

both first-time and repeat migrants (Dempson, 1995). They typically migrate from freshwater to coastal areas annually for a period of 30-60 days (Dempson and Green, 1985).

Species in the genus *Salvelinus* generally exhibit relatively low yet varying degrees of anadromy between populations, judged by the physiological and morphological changes, and the distance and duration of migration (Spares et al., 2015). For instance, Norwegian (Hammerfest) and Icelandic (Hólar) Arctic charr exhibited the seasonal adaptation to seawater by regulating the hydro-mineral ions (hypo-osmoregulation) prior to and during migration while still in freshwater (Jørgensen et al., 2007; Árnason et al., 2014). Fraser River Arctic charr, by contrast, through spring exhibited no increase in either hypo-osmoregulatory ability or gill Na^+/K^+ ATPase activity in freshwater, suggesting a population-specific physiological mechanism in controlling seawater adaptation (Duston, Unpubl. Data; Duston et al., 2007). This characteristic, however, is advantageous to my experimental design since smoltification sometimes is in a developmental conflict with sexual maturation (Thorpe et al., 1998), especially in photoperiod manipulation studies (Fjelldal et al., 2018). Other salmonid species in *Oncorhynchus* and *Salmo*, for instance, chum salmon (*Oncorhynchus keta*) and Atlantic salmon undergo prominent morphological and physiological changes (true smolting) and migrate to the ocean for up to several years; they return to freshwater with the attainment of maturity (McCormick, 2012). The physiological mechanisms triggering migration and seawater adaption are not fully understood. It is not impossible that Fraser River Arctic charr display negative rheotaxis associated downstream migration that carries them into estuaries which stimulate hypo-osmoregulatory mechanisms by acclimation. During the short duration of summer migration in local coastal regions, body size increases rapidly due to relatively warm sea

temperature of 8-13 °C and abundant prey (Michaud et al., 2010). Arctic charr are opportunistic feeders, mainly preying on capelin (*Mallotus villosus*), sand lance (*Ammodytes spp*.), hyperiid amphipods (*Parathemisto spp.*) and sculpins (*Triglops spp*.; Dempson et al., 2002). After the short summer of energy replenishment and somatic growth, Fraser River Arctic charr migrate back to freshwater systems from late July until September for overwintering and/or reproduction.

Arctic charr are iteroparous, spawning repeatedly during their 18-20 year life span. Many Pacific salmon (*Oncorhynchus*), by contrast, are semelparous only spawning once then dying (Sloat et al., 2014). It is usually assumed that iteroparous fishes spawn annually after reaching maturity (Rideout et al., 2005). Wild anadromous Arctic charr, however, tend to reproduce with the interval of 2-3 years due to the energetic constraints associated with a short summer feeding period and low productivity in their habitats (Dutil, 1986; Beddow et al., 1998). The omission of spawning among the wild populations represents a trade-off between reproductive success and survival, an adaptive trait in long-lived fishes (Rideout et al., 2005). Surveys of other wild fish such Atlantic cod and winter flounder (*Pseudopleuronectes americanus*) in the north Atlantic suggested they 'skip' a spawning season when nutritional or temperature is not suitable for gamete development (Rideout et al., 2005).

Maturation among Arctic charr is group-synchronous culminating in a single spawning event in the fall or winter, the culmination of a maturation process that can take up to one year from the initiation of gametogenesis to final ovulation/spermiation (Sloat et al., 2014). Fraser River Arctic charr typically spawn in October during a span of a three-week period at the temperature of 1-3°C (Dempson and Green, 1985). The age at first

maturity among wild Fraser River charr varies within and between sexes. In males, mean age at 50% maturity is 5 years with FL about 25 cm. Females reach their 50% maturity at an older age of 7 years, coupled with larger mean body size of 38 cm (Dempson and Green, 1985). The earliest maturity was indicated at age 3 among males and age 5 among females (Dempson and Green, 1985). The sexual dimorphism is attributed to the higher energetic cost of producing eggs compared to sperm (Fleming, 1998). This difference of energetic costs between male and female also can be described by the gonadosomatic index, which is the ratio of fish gonad weight to body mass (GSI=gonad weight/body weight*100%). The GSI of wild Fraser River charr ranges from < 0.2% among immature fish up to 36% among mature females and up to 6% among mature males (Beddow et al., 1998). After spawning, they search for suitable habitats with large volumes of deep water within the river or lake system for overwintering (Beddow et al., 1998). Reduced activity or movement under ice-covered lakes is evident and considered as a survival strategy to minimize energy expenditure (Mulder et al., 2018).

The life-history of Arctic charr is changed dramatically in captivity. With the goal of maximizing production in the shortest time period, optimum rearing conditions are provided by farmers. Using the Fraser River Arctic charr, the breeding program in Shippagan produces eggs in the fall between October and November, a similar timeframe to wild fish. Fertilized eggs are incubated at about 5-6 °C in darkness. Increased temperature reduces the incubation period by about 3 months compared to wild conditions. After hatching, the alevins take about one month or 150 °C-days (days spent at temperature) for the yolk sac nutrients to be absorbed, then they swim up and start feeding. At this point, the temperature is increased to about 10 °C and photoperiod is 24h light (LL) to promote

feeding activities and rapid growth (Pelletier, C., 2018, Valorēs, pers. comm.). Under the conditions of 10°C and daily feeding, it takes about two years to reach market size of 1 kg, a body size that typically takes 5-8 years to achieve in the wild. Unfortunately, the environmental conditions for optimum fish growth accelerate development and induce early maturity at age 2 among farmed Fraser River charr. The physiological mechanisms linking individual conditions such as somatic growth and body size and the process of maturation are not fully understood, hence are suitable subject matter for this thesis. The following section reviews the factors that contribute to the plasticity of life-history among salmonids under captivity.

2.2 Framework for Understanding Sexual Maturation in Salmonid Fish

Salmonids inhabit environments in which developmental stages are linked to an annual cycle. As for sexual maturation, the 'decision' to commence the process requires a coincidence between a specific entraining cue and an energetic physiological 'threshold' to which both body size and energy reserves contribute (Thorpe et al., 1998; Sloat et al., 2014). The term 'decision' does not imply a cognitive process, but instead a developmental switch among alternate physiological pathways, for example, maturing vs. immature (Mangel and Satterthwaite, 2016). Seasonal photoperiod acting as an entrainment cue defines the 'decision period' which seems to occur one year before final maturation. Among salmonids, the working hypothesis is that sexual maturation proceeds when individuals attain a specific genetically fixed threshold, perhaps body size, lipid reserves or rate of acquisition of energy stores during the critical 'decision window' (Thorpe et al., 1986, 1998; Duston and Saunders, 1992; Mangel and Satterthwaite, 2016). The model was built based on the empirical data from Atlantic salmon. Among one-year-old male parr, a

rapid increase in mean condition factor (CF=100*(body weight/FL^3; an indicator of body 'fatness') and significantly higher mesenteric fat levels in April-May were linked to maturation (Rowe and Thorpe, 1990; Rowe et al., 1991). The need to attain energetic thresholds was also indicated by maturing Atlantic salmon after one sea-winter (grilse); maturing individuals showed higher growth rate and CF than immature fish about one year before final maturation (Kadri et al., 1996; Duston and Saunders, 1999). Whether increases in somatic tissue and lipid reserves are a cause or effect of maturation is unclear. Reducing dietary lipid contents or using dietary supplement to suppress body lipid stores also reduced the maturity rate among parr, post-smolts or grilse (Alne et al., 2009; Arge et al., 2012; Jonsson et al., 2013; Trombley et al., 2014). Searching for the adiposity signal that potentially triggers puberty in fish remains difficult. The circulating levels of the hormone leptin is proportional to the size of stored body fat and has a permissive role in puberty in mammals, but does not appear to serve a similar function in Atlantic salmon and other fishes (Trombley et al., 2014).

Modeling of the life-history of Atlantic salmon implicated two switch points during the early sexual maturation process. Once the body size or lipid reserve of an individual exceeded the genetically fixed threshold in the first fall around October-November, the switch to maturation is activated. The second switch is in the following spring around April-May which allows the continuation of maturation, or halts the process if energy or lipid reserves diminish overwinter below a critical threshold (Thorpe et al., 1998). The second switch also gives the opportunity to 'correct' the physiological 'decision' made during the fall based on individual fitness, so that maturation doesn't exhaust the body reserves and compromise survival of the fish. The model was mainly supported by the

winter food deprivation experiments. Exposure of Atlantic salmon to periodic food deprivation during winter and/or spring significantly reduced the incidence of sexual maturation in the following fall, which implies that the accumulation of body reserves during this 'decision window' is critical for commencing the maturation process (Thorpe et al., 1990; Duston and Saunders, 1999).

The Atlantic salmon model was suggested to be applicable to Arctic charr in culture (Adams and Huntingford, 1997), and was adjusted for this species based on proximate consideration of life-histories (Rikardsen et al., 2004). Studying a single cohort of a non-anadromous population showed that maturing fish were heavier and exhibited higher growth rate from April onwards compared with immature charr (Adams and Huntingford, 1997). Among Hammerfest Arctic charr, an anadromous population, maturing fish exhibited rapid growth and higher body weight between March and July compared with individuals that remained immature in the fall, but the difference was not significant due to the large variation in body size (Tveiten et al., 1996). The period of accelerated growth coincided with a slight increase in sex steroids, suggesting it was an effect of maturation rather than a cause (Tveiten et al., 1996). Other studies using the same strain, by contrast, revealed no difference in mean body weight and CF between maturing and immature fish until close to the completion of gonadal development when mature fish showed significantly reduced body size (Jobling and Baardvik, 1991; Sæther et al., 1996; Damsgård et al., 1999). The contradictory somatic growth patterns between maturing and immature charr between studies was suggested to be due to genetic background/family differences and rearing environment (Jobling and Baardvik, 1991). The considerable variation of body size between individual Arctic charr of all strains also reduces the

chances of detecting statistically significant differences when comparing means (Tveiten et al., 1996).

Among individually identified Fraser River Arctic charr, at 10 °C, age 2 maturing fish grew rapidly under both simulated natural daylength (LDN) and constant 18h light/6h dark (LD 18:6); they were significantly heavier and had a higher CF from March onwards compared with the immature charr (Duston et al., 2003). The accelerated growth among maturing fish was interpreted as an effect of sexual maturation since the physiological 'decision' was probably already made at that time based on the sex steroid profiles (Imsland and Gunnarsson, 2011). When photoperiod was LD 18:6 for 45 days from February 3 to March 16 followed by short photoperiod LD 8:16 or LDN, maturing and immature fish shared nearly identical growth trajectories (Duston et al., 2003). For this thesis, I assessed if Arctic charr exhibit a dimorphism in body weight/size between immature and maturing fish, and whether rapid growth or better condition is the cause of the earlier age at maturity. Parameters of FL, body weight and CF of individually tagged Arctic charr were monitored from early fall until the final maturation season for 12-16 months for all trials (2012-2017). Lipid content was also measured in the 2016-17 trial using a non-destructive instrument (fatmeter, Model FM 692, Distell, Scotland).

The onset and completion of reproduction is regulated by the neuroendocrine system through the brain-pituitary-gonadal (BPG) axis. The initial functional competence of the BPG axis starts with the onset of germ cell maturation and culminates in the first spermiation or ovulation (Okuzawa, 2002). The function of the BPG axis mainly relies on the interaction of three major groups of signals, generated at the three major levels of the axis. Firstly, the gonadotropin-releasing hormone (GnRH) neurons located at the

hypothalamus directly innervate gonadotropin-producing cells (gonadotropes) in the pituitary. The GnRH neurons stimulate the synthesis and release of two distinct but chemically related gonadotropins from the pituitary into the blood stream. Finally, the hormone gonadotropins interact with the germ cells and stimulate the development of gametes and production of sex steroids (Zohar et al., 2010). In teleosts, the activation of GnRH at the hypothalamus is the key event during the onset of puberty. What leads to the activation and its communication with different groups of neurons that integrate the external (e.g. photoperiod, temperature) and internal (e.g. biological clocks, nutritional status) stimuli are not fully understood. Kisspeptins, dopamine, thyroid-stimulating hormone, steroid receptors and gonadotropin-inhibitory hormone are all implicated to be associated with the control of GnRH neurons but their decisive roles remain to be elucidated (Taranger et al., 2010; Amano, 2010; Muñoz-Cueto et al., 2017).

Fish, like mammals, have two gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), playing central roles in modulating gonadal development and the secretion of sex steroids (Swanson et al., 2003). They are relatively large glycoproteins produced by gonadotropic cells in the anterior pituitary. FSH and LH consist of two subunits: a common α-subunit and a hormone specific β-subunit that determines their biological function (Swanson et al., 2003). Their biological activity is exerted by binding to receptors, FSHR and LHR, located in the gonads. FSH-β and LH-β are expressed in the pituitary throughout the maturation process but the specific role of each differs. In salmonids, an increase of plasma FSH occurs one year prior to completion of maturation associated with rapid spermatogonial proliferation in males and pre-vitellogenesis in females, whereas LH is mainly up-regulated during the final maturation

of gametes (Tyler et al., 1997; Campbell et al., 2006). The elevation of FSH-β transcript levels occurred in November at the initiation of gonadal maturation among female Atlantic salmon, preceding the increase of sex steroids and GSI by 1-2 months (Andersson et al., 2013). Importantly, photoperiod-induced changes in sexual maturation are highly associated with elevated FSH and LH transcript levels in the pituitary (Andersson et al., 2013). In Arctic charr, published information of gonadotropin secretion is limited to LH, and long photoperiod exposure during the final maturation stage in the fall delayed the increase of plasma LH and timing of ovulation (Gillet and Breton, 2009).

In male fish, the testis synthesizes and releases a number of sex steroids responsible for spermatogenesis, secondary sexual characters and reproductive behavior. The major androgens are testosterone and 11-ketotestosterone (Schulz et al., 2010). Both androgens develop biological function via the testicular somatic cells and influence testicular development. Among Arctic charr, the levels of testosterone and 11-ketotestosterone remain low during the immature stage and increase gradually as spermatogenesis starts in April or May and peak in August few months before the final maturation season (Mayer et al., 1992; Frantzen et al., 2004). 11-ketotestosterone appears to be more important than testosterone at the onset of the pubertal stage as it stimulates the proliferation of Sertoli cells which nourish germ cells and promote spermatogonial proliferation leading to meiosis and the later phases of spermatogenesis (Miura et al., 1991; Schulz et al., 2010). Estrogens such as 17β-estradiol (E2) are technically 'female' hormones but also are produced in male vertebrates. Estrogens may be involved in early stage of spermatogenesis but their exact role is not clear (Schulz et al., 2010).

Female Arctic charr exhibit group-synchronous ovarian development. After receiving stimulatory gonadotropin signaling, the theca and granulosa cells of the ovarian follicle start to produce a range of sex steroids including the androgens, estrogens and progestrogens (Kagawa, 2013). In Arctic charr, the key estrogen, E2, remains very low in the blood at the primary oocyte stage (perinucleolous stage) but increases with the onset of vitellogenesis between April and May (Mayer et al., 1992; Frantzen et al., 2004). E2 rises rapidly and peaks during the rapid vitellogenic growth phase in July-August prior to the final maturation season (Frantzen et al., 1997). E2 stimulates the hepatic synthesis of vitellogenin which is subsequently taken up by the rapid growing oocytes (Tyler et al., 1997). A major androgen produced by female teleosts is testosterone, acting as precursor for the synthesis of E2. It starts to increase in conjunction with E2, but peaks one to two months after E2 (Frantzen et al., 1997; 2004). The seasonal change of sex steroids has only been described in the Hammerfest Arctic charr. Fish usually grow slowly during the winter and spring period due to the low ambient water temperature exposure (0-5 °C) and the endocrine system and oocyte sizes do not show any significant changes until late spring in May and June (Mayer et al., 1992; Frantzen et al., 1997).

The timing of the initiation of gonadal development among wild Fraser River Arctic charr has not been documented, since the fish are inaccessible under thick ice and snow. Elsewhere, the chronology of maturation differs between studies. In the lab, among male Fraser River charr held at 2-4°C overwinter, mitotic proliferation of spermatogonia commenced in September-October, one year prior to completion of maturation, associated with a relatively high CF (Rice, 1999). Among the Hammerfest strain, the commencement of spermatogenesis was not investigated but among females the onset of vitellogenesis

was evident in March when the temperature was at 5-6°C (Frantzen et al., 1997). A divergence in size between primary and secondary oocytes was apparent in June, within a few months of spawning (Frantzen et al., 1997). Integrating knowledge from Norwegian and Canadian studies on the chronology of gametogenesis in Arctic charr is difficult, because the relatively warm winter climate in coastal Norway compared to Eastern Canada. Among cultured Arctic charr, well water at 10 °C year-round drives rapid somatic growth and offers little restriction on sexual maturation, possibly altering the timing of gametogenesis. Histological analysis of gonadal development from early fall to late spring was an important part of my research to define the timing of commencement of sexual maturation among Fraser River Arctic charr under farmed conditions. My histological analysis of gonadal development of both sexes of Arctic charr is novel, especially the perspective of gametogenesis under the influence of photoperiod manipulation. Previous studies followed either males (Rice, 1999) or females (Frantzen et al., 1997) under LDN. Aligning the gonad histology data with body size and similar data from other salmonid species is needed to refine the life-history model linking body 'state' with the decision to mature. To better understand to what extent sexual maturation is modulated by photoperiod, food availability and temperature, I included combinations of all three factors in my experiments.

2.3 Photoperiod Manipulation to Suppress Sexual Maturation

In salmonids, the seasonal change in daylength synchronizes the reproductive cycle, possibly by entraining some kind of endogenous circannual physiological rhythm (circa: about; annual: one year; Bromage et al., 2001). Using artificial photoperiod cycles to alter the timing of spawning in salmonids began with Hoover and Hubbard (1937), and is now

commonly used to supply gametes year-round (Migaud et al., 2013). In culture, the timing of spawning can be manipulated by condensing or extending the seasonal light cycle or using 'square-wave' photoperiod regimes and altering the timing of the switch between 'long' and 'short' daylength (Bromage et al., 2001). Although most investigators embrace this entrainment hypothesis (e.g. Frantzen et al., 2004), evidence of a free-running maturation rhythm that underpins it is restricted to female rainbow trout (Duston and Bromage, 1991). Arctic charr are seemingly good candidates to study long-term rhythms, because they are iteroparous, reproducing multiple times throughout a long life span, and can be easily cultured at high stocking densities. The entrainment of seasonal reproduction in response to photoperiod manipulation has been demonstrated in charr and it is similar with other salmonids (Frantzen et al., 2004; Liu and Duston, 2018). The existence of an endogenous circannual rhythm has been frequently speculated for Arctic charr in controlling seasonal feeding, locomotion and reproduction (Jørgensen and Johnsen, 2014; Striberny et al., 2015; Hawley et al., 2017). Producing evidence of a circannual rhythm of reproduction in Arctic charr was an important objective of this thesis.

The general theory of the entrainment of a circannual rhythm of maturation in salmonids satisfactorily explains the alterations in the timing of spawning to photoperiod manipulation, in female rainbow trout at least. Artificially long days in the early spring and/or short days in the early summer can advance the timing of ovulation since the circannual clock is perceived as running 'behind time' (Randall et al., 1998). By contrast, short days in the late spring and long days in the late summer lead to corrective delays in the completion of maturation because the circannual clock is perceived as running 'ahead of time' (Randall et al., 1998). Photoperiodic history and the direction of change between

a long and short photoperiod is far more important on the timing of sexual maturation than the absolute length of the photoperiod or the magnitude of the change in photoperiod (Bromage et al., 2001). For example, female rainbow trout exposed to 'long' photoperiods of between 12 and 22h from January to May followed by shorter photoperiods of between 3.5 and 13.5h all exhibited a similar degree of advancement in the spawning time (Duston and Bromage, 1987; Randall and Bromage, 1998).

The extent by which spawning can be advanced or delayed has its limits. Attempts to advance ovulation in rainbow trout by 6 months by subjecting them to a supposedly stimulatory LD 18:6 in early January followed by further stimulatory 'long-to-short' change in photoperiod on March 1, resulted in most of the fish remaining immature (Duston and Bromage, 1988). This result was explained in terms of a gating mechanism in which a switch in photoperiod to LD 18:6 initiated a 'GO' decision to commence sexual maturation only among individuals that had reached a 'threshold', *sensu* Thorpe 1986 (Duston and Bromage, 1988). The 'gate' was then closed by the long-to-short photoperiod cue, preventing fish from reaching the threshold level and embarking on sexual maturation (Duston and Bromage, 1988). The weakness of this study was the body size of individual fish was not recorded, so the influence of somatic growth on the decision to mature was unknown. To better define the nature of the 'threshold' by monthly measurement of individually identified female rainbow trout, Taylor et al. (2008) reported growth, body size and CF were not significantly different between maturing and immature fish during early reproductive cycle, leaving the putative role of somatic 'factors' in the decision to mature unclear. Similarly, I tested the hypothesis that the gating mechanism is based on body size and somatic growth using Arctic charr as the salmonid model. In the experiments

reported in this thesis, maturing and immature Arctic charr of mixed parentage were of similar body size and CF during the early reproductive stages. The physiological nature of this 'threshold' and gating mechanism remains to be defined despite many studies on the photoperiod control of sexual maturation in salmonids (e.g. Taranger et al., 1999; Taylor et al., 2008).

The photoperiod signals to prevent sexual maturation can be sub-divided into three components, the timing of the increase from LDN to a long photoperiod, the duration of long photoperiod, and the timing of decrease from long back to LDN or a short photoperiod. Among Atlantic salmon, rainbow trout, and Arctic charr, the incidence of sexual maturation can be altered by the combined effect of an abrupt increase from LDN to a long photoperiod then subsequent return to LDN. While both LD 18:6 and LL have been used as a long day signal in photoperiod regimes, whether or not they have same/different physiological effect on the sexual maturation was questioned in two studies on Atlantic salmon (Fjelldal et al., 2011; Good et al., 2016). To clarify, I tested the response of Arctic charr by exposing them to both LD 18:6 and LL from mid-October to February with LDN before and after the long day treatment in the Chapter 5. The rest of the experiments in this thesis used LL as a long day signal to conform with industry practice and pertinent Norwegian studies.

Among farmed Atlantic salmon, LL from winter to the summer solstice is used to suppress early sexual maturation. In Norway, LL from either January to July or March to July in a sea-cage system reduced the incidence of maturation to 9 and 67% among female Atlantic salmon compared to 91% in controls (LDN; Taranger et al., 1999). The reduction in the incidence of maturity was explained by an advancement of the timing of a 'critical

period' during winter and spring due to the early arrival of LL, which resulted in fewer fish meeting some 'criteria' (undefined) during the critical decision period to commence sexual maturation (Taranger et al., 1999). Similarly, in the Bay of Fundy, LL exposure from November 3 until May 31 reduced the maturity to <1% in both sexes compared with 50% of males and 7% of females maturing under natural daylength (Peterson and Harmon, 2005). Delaying the start date to mid-February impaired the efficacy of treatment resulting in 17% of males and 4% of females maturing (Peterson and Harmon, 2005).

Among Fraser River Arctic charr, both 'Long-to-short' and 'Short-to-Long' photoperiod cues can influence the incidence of sexual maturation. The maturity rate among charr reared under LD 18:6 for 42 days starting either Dec 21, Feb 1 or Mar 15, followed by LDN exhibited a stepwise increase from 43 to 57 and 68%, compared to 77% in controls under LDN (sexes pooled, MacPherson, 2012). Clearly, the timing of long photoperiod treatment was critical to delaying maturation, but which component of the photoperiod treatment caused the arrest or delay needed to be clarified. The approach to determining the relative importance of starting and ending LL, and the duration of LL photoperiod regime in stopping sexual maturation required experiments with several 'nested' photoperiod regimes. Repeating the most effective photoperiod regimes to ensure their efficacy was necessary before making recommendations on industry practice.

A paradox resulting from photoperiod manipulation is that a switch from LDN to LL on February 1 stimulated gonadal development in some Atlantic salmon but arrested maturation in other individuals, termed a 'dichotomous' response (Schulz et al., 2006; Andersson et al., 2013). Among the 60% of males that matured under this photoperiod regime, their GSI in June was significantly greater than LDN controls (1.25 vs. 0.4% GSI;

Schulz et al., 2006). By contrast, among the 40% of males that remained immature under LL, testis development stopped at the spermatogonia stage in mid-February after 20 days of LL exposure (Schulz et al., 2006). In the same experiment, among females, the 'dichotomized' response was observed in June, with 3.1% GSI among LL-accelerated fish, significantly higher than 2.3 % GSI in controls (Andersson et al., 2013). By comparison, among those females that remained immature, the LL signal was associated with the arrest of vitellogenesis that had already started and GSI was maintained less than 0.6% consistently between February and June (Andersson et al., 2013). The arrest of oocyte development was associated with a significant decrease in the gene expression of pituitary FSH-β and reduced sex steroid E2 in blood plasma 6-14 weeks after LL had started, suggesting the signal via the BPG axis changed from GO to STOP. By contrast, among females that matured, the LL signal remained stimulatory throughout as indicated by elevated LH-β transcript and E2 during vitellogenesis (Andersson et al., 2013). The regulation of salmonid maturation clearly involves integration between photoperiod cues and the gonadotropic axis. To date, gonadotropic activity has been demonstrated by detecting the gene expression of pituitary gonadotropins subunits in Atlantic salmon, coho salmon (*Oncorhynchus kisutch*) and masu salmon (*Oncorhynchus masou*; Campbell et al., 2006; Furukuma et al., 2008; Andersson et al., 2013). In Arctic charr, there is no published information on photoperiod-induced changes in gonadotropic hormones. Measuring the pituitary FSH-β and LH-β transcript levels can be achieved by using qPCR and they generally follow the similar patterns of plasma FSH and LH. Nevertheless, the gene expression change of the pituitary gonadotropins was investigated outside of this thesis to reveal the interaction between the endocrine system and photoperiod manipulation during the initiation period of sexual maturation.

Knowledge of how an animal transduces daylength information in the brain has only been recognized recently (Migaud et al., 2010). Teleosts perceive light by both the eyes and pineal gland, similar to other vertebrates. The pineal gland is considered the main organ transducing light information into neuroendocrine signals that dictate physiological responses (Migaud et al., 2010). Photoreceptor cells on the epithelial layer of the pineal react directly to the light change resulting in high circulating melatonin levels at night and low levels during the day. The daily rhythm of melatonin secretion is hypothesized to synchronize both diurnal and seasonal physiological rhythms in teleosts, but a role for melatonin in the reproductive axis has not been found (Mayer et al., 1997; Migaud et al., 2010). In mammals, by contrast, the role of melatonin in seasonal reproduction is established. The light information is perceived by the eyes, and then transduced to the pineal gland via the master circadian clock located in the suprachiasmatic nucleus. Melatonin secreted from the pineal gland synchronizes the endogenous clock associated with the BPG axis, and manipulating melatonin administration can induce the photoperiodic responses (Dardente, et al., 2016). In salmonids, it is proposed that the alteration of the melatonin profile due to artificial photoperiod manipulation interferes with the perception of the seasonal daylength which alters the endocrine patterns of reproduction (Porter et al., 1999). This, however, remains conjecture due to lack of evidence.

Measurement of melatonin proved a useful tool to verify the efficacy of photoperiod regimes to prevent sexual maturation among farmed Atlantic salmon in the Bay of Fundy (Manning et al., 2010). In land-based farms, photoperiod treatment is usually effective in suppressing maturation because the combination of light-proof tanks and bright artificial lighting is sufficient to suppress melatonin secretion. In marine net-pens, however, the

lighting sometimes fails to reduce the incidence of sexual maturation, possibly because the light intensity is insufficient to override the natural light (Taranger et al., 2006). For instance, sexual maturation in Atlantic cod can be delayed for up to three years when fish are exposed to LL in light-proof tanks (Davie et al., 2007), whereas similar regimes in open net pens failed to prevent sexual maturation, only delaying the spawning time by 3-5 months (Taranger et al., 2006). Arctic charr are very sensitive to light; a clear melatonin profile was produced by fish under thick ice and snow in an Arctic lake and a threshold level of light intensity in suppressing melatonin secretion appeared to be between 0.01 and 0.001 W/m^2 (Strand et al., 2008). Hence, the lighting requirements for Arctic charr to suppress maturation may differ from Atlantic salmon. In Chaper 6, tank-based trials were conducted to identify the threshold of light intensity to suppress the night-time melatonin rise among Fraser River Arctic charr. Samples of blood were collected and the plasma sent to our collaborator, Dr. Manning (Research and Productivity Council, Fredericton, NB) for melatonin analysis by an enzyme-linked immunosorbent assay (ELISA).

Photic information perceived through photoreceptors located in the hypothalamus purportedly stimulates GnRH via some kind of undefined pathway that initiates the reproductive cycle through the activation of the BPG axis (Migaud et al., 2010). Even though the axis has been well described in fish, the incorporation of the photic information pathway in this working model is still missing as melatonin may not necessarily act as the initiating messenger in reproduction.

2.4 Control of Somatic Growth Using Food Availability and Temperature

Food availability and temperature affect metabolism and surplus energy, which in turn have consequences for the energy allocation to somatic growth and reproduction. A

major cause of early sexual maturation among farmed fish is because they are fed a highly nutritious diet to satiation daily with high lipid content which leads to increased fat storage in the body (Thorpe, 2004). The main effects of food restriction are a decrease of lipid storage in carcass and muscle coupled with an increase in the levels of moisture and ash content in the tissues (Shearer et al., 2006). Moreover, fish are poikilotherms, therefore water temperature controls metabolic rate. Change of temperature dictates appetite and metabolic rate and suboptimal temperature could reduce energy investment in growth and reproduction indirectly by a reduction in food consumption (Gunnarsson et al., 2011). In Atlantic salmon, manipulation of temperature and food restriction during winter and spring suppressed the proportion of mature fish in autumn associated with the reduction of growth rate during the critical 'decision window' in the spring (Adams and Thorpe, 1989; Herbinger and Friars, 1992; Duston and Saunders, 1999). Experiments described in this thesis extended the state of knowledge by combining photoperiod manipulation with food deprivation and temperature to reduce the incidence of sexual maturation in Arctic charr.

Arctic charr have a high tolerance to food deprivation, a valuable adaptation in their challenging habitat. In captivity, Arctic charr (Nauyuk strain, ca. 185 g) offered no food for 101 days only resulted in about 5 g weight loss at 5.5 °C (Cassidy et al., 2018). Similarly, at 5 °C, three months of food deprivation among Hammerfest strain (ca. 146 g) had no effect on weight change, but the whole-body fat content was decreased from 5% to 1.2% (Jørgensen et al., 2013). The whole-body protein content or concentration, however, remained unchanged in response to food deprivation in both studies. It indicates that a true phase of starvation was not entered since protein was not catabolized as the fuel (McCue, 2010). Food deprivation, however, was ineffective at suppressing sexual maturation in

Arctic charr. Fraser River Arctic charr deprived of food for six weeks starting in mid-September slightly reduced the male maturation rate to 45% compared with 56% in control fed group, whereas alternating the feeding (2 weeks) and no food (2 weeks) cycle for 16 weeks between November and February had no effect on maturity rate, 60-70% among males (Rice, 1999). The lack of response of Fraser River Arctic charr to food deprivation was in contrast to findings for Atlantic salmon (Duston and Saunders, 1999; Trombley et al., 2014).

In response to food restriction or a period of growth depression, compensatory growth, a phase of accelerated growth, is expected once feeding is resumed. Compensatory growth is widely reported in the literature among many species, but the nature of the underlying feedback mechanisms which adjust growth rates to achieve a target trajectory remains a mystery (Ali et al., 2003; Bar, 2014). Two forms of compensatory growth: partial and full, are commonly observed in fish studies. Full compensation indicates that, following the resumption of feeding, the deprived animals eventually reach the same body size at the same age compared to the animals fed to satiation throughout. In partial compensation, by contrast, the deprived animals fail to achieve the same size at the same age compared with the fed fish when favorable conditions return, but do show relatively rapid growth rates and may have better food conversion ratios (Ali et al., 2003). The degree of compensation depends on several factors, including the duration of the food deprivation period, age and sex of fish, diets and rearing temperature. For instance, after 101 days without food, Nauyuk Arctic charr (initial av. BW 185 g) exhibited partial compensatory growth during a re-feeding period of 126 days reaching a final mean weight of about 470 g compared with control charr at 580 g (5.5-7.7 °C; Cassidy et al., 2018). Among Icelandic

Arctic charr (ca. 21 g), by contrast, reducing the feeding ration to 50% for two six-week periods (a. Sep.-Nov.; b. Dec.-Feb.) decreased the growth temporarily; following the return to full ration, fish caught up and showed no significant differences in mean body weight after a further four months of rearing (Imsland and Gunnarsson, 2011). Overall, the strong compensatory growth observed among Arctic charr indicates that food deprivation can be a cost-effective means for fish farmers to reduce the problem of unwanted maturation.

Growth rates of juvenile Arctic charr increase with increasing temperature, reaching a maximum around 12-16 °C (Siikavuopio et al., 2013; Sæther et al., 2016). The growth efficiency or food utilization seems to be better at a lower temperature, around 10-12 °C (Sæther et al., 2016). In Atlantic Canada, Arctic charr farms are typically supplied with ground water with a stable temperature range between 8-10 °C, comparable with the optimum temperature for good growth efficiency. Among Icelandic charr, high temperature of 12-15 °C increased the growth of fish but also promoted gonadal development (Gunnarsson et al., 2011). In August, mean GSI of yearling charr was > 6% among females at temperature of 12 or 15 °C but < 3% at 9 °C, although the incidence of maturity was not reported (Gunnarsson et al., 2011). By contrast, yearling Arctic charr reared at Dal-AC at constant 5 °C resulted in < 20% mature fish at age 2 compared with over 80% among fish reared at 10 °C (MacPherson, 2012). The relatively low growth rate at constant 5 °C, however, is not compatible with fish farming, where reaching harvest as soon as possible is the general goal (MacPherson, 2012). Hence, to investigate whether low temperature can reduce maturity rate among Fraser River Arctic charr, 5 °C water was

applied during the 'critical period' (November - April) and then switched back to 10 °C water so that compensatory growth can be made in the following grow-out months.

Knowledge of the interaction effect between photoperiod, temperature and food control to reduce the incidence of sexual maturation in Arctic charr is lacking. Previously, LL combined with increased temperature increased the incidence of male sexual maturation among post-smolt Atlantic salmon (Fjelldal et al., 2011; Imsland et al., 2014). For instance, underyearling *Salmo salar* (ca. 83 g) subjected to 5, 10 or 16 °C in combination with LL, after 6 weeks (Dec.-Jan.), 47% males matured under the exposure of 16 °C and LL combined compared with 0% in other treatments (Fjelldal et al., 2011). Together, photoperiod plays a directive role on the onset of sexual maturation, whereas temperature or food availability acts as a rate controlling factor on the physiological responses to the changes in photoperiod. In Chapter 4, I tested the hypothesis that lower temperature or food deprivation combined with 24h light can produce synergistic effects to further eliminate the incidence of sexual maturation in Arctic charr.

Chapter 3: Efficacy of 24h Light to Reduce Maturation in Arctic Charr (*Salvelinus alpinus*) is Dependent on Both the Start Date and Duration

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3.1 Abstract

Two lab-based trials quantified the effect of a range of continuous light (LL) treatments overwinter on the somatic growth and incidence of sexual maturation in the fall of age 2 individually identified underyearling Arctic charr (Labrador strain) at constant 10 °C. Controls were maintained under simulated natural photoperiod (LDN; Latitude 45 °N). In trial 1 (n=120/treatment, mean 54 g initial body weight), LL starting the 1st day of either October, November, December, January or February (all returned to LDN on April l) resulted in a step-wise reduction in maturity of 43, 55, 59, 67 and 73% compared with 77% in the control (sexes pooled). In trial 2 (n=90/trt, mean 24 g initial body weight), LL starting either August 13 or October 1 and ending April 1 reduced maturity to <10%, compared to 33% (LL Dec1-Apr1) and 50% in the LDN controls. LL starting October 1 and ending either February 1, April 1 or June 1 were equally effective, resulting in <15% maturity. The importance of returning from LL to LDN in spring to reduce maturity was demonstrated by the LL constant (Oct-Oct) treatment which resulted in relatively high maturity, 31%. The reduction of maturity rate was independent of both somatic growth and condition factor during winter in all treatment groups. Among fish that matured, the timing of completion of sexual maturation was affected by both the LL start and end date.

3.2 Introduction

The high incidence of sexual maturity at age 2 among diploid Arctic charr (Labrador strain) has greatly constrained the productivity of farming this fish in Atlantic Canada (Liu and Duston, 2016). Early maturation is an undesirable trait because energy derived from fish feed is diverted from somatic growth into worthless gonads, resulting in poor meat yield and quality (Hatlen et al., 1996). Continuous light (LL) for two months or more can be a cost-effective method to reduce unwanted maturation among farmed salmonids, gadoids and percids (Taranger et al., 2010; Karlsen et al., 2014; Carrillo et al., 2015). It appears to inhibit gonadal development by suppressing the secretion of gonadotropin-releasing hormone by the hypothalamus, perhaps by disrupting a circannual clock entrained to the annual daylength cycle (Bromage et al., 2001; Taranger et al., 2010). Among Labrador Arctic charr reared in well water (10 °C), a 50% reduction in maturity in autumn was achieved either following LL from mid-November to April (Liu and Duston, 2016) or 18h light:6h dark (LD 18:6) from February to mid-March (Duston et al., 2003). Aiming to further reduce the serious maturation problem in this strain, our objective was to define the optimum start date and duration for LL treatment.

An effective start date for LL seems to be around the time of year when gonadal maturation commences. Among Atlantic salmon (*Salmo salar*) in sea-cages, the incidence of maturation in autumn was 9% following LL from January to July, but 67% when the LL started in March (Taranger et al., 1999). Similarly, among cage reared *S. salar* in Atlantic Canada, delaying the start date of the LL treatment from November to February greatly reduced its efficacy in preventing grilsing (Peterson and Harmon, 2005). Among Atlantic cod (*Gadus morhua*) exhibiting initial elevations in sex steroids in July, an LL

start date of October reduced the incidence of maturity the following spring to 13%, compared to 98% under simulated natural daylength (LDN; Davie et al., 2007). In other studies, by contrast, the response to LL was equivocal: the maturity rate of male Atlantic salmon was either increased or unaffected following a range of LL start dates and durations between November and July (Endal et al., 2000). That an LL signal in winter could stimulate gonadal maturation among some individuals but inhibit maturation in others confirmed the complex 'dichotomous' nature of the response, in Atlantic salmon at least (Schulz et al., 2006). This contrasting response is proposed to be influenced by differences in body size or energy reserves relative to a genetically determined threshold among individual fish (Thorpe et al., 1998; Bromage et al., 2001; Taranger et al., 2010). Fraser River Arctic charr failed to support this hypothesis: the 50% decrease in maturity rate due to either LL or LD 18:6 in winter was independent of somatic growth (Duston et al., 2003; Liu and Duston, 2016). By expanding the number of LL treatments and their duration, following the approach of Davie et al. (2007), the present study was our most thorough attempt yet to identify a connection between the timing of the LL treatment, somatic growth and incidence of maturation in Arctic charr.

3.3 Materials and Methods

3.3.1 Rearing conditions and photoperiod treatments

Two trials were conducted, each lasting just over one year (October, 2012 to November, 2013; August, 2014 to October, 2015). Arctic charr (Fraser River population, Labrador strain) were produced by a pedigreed breeding program at the Coastal Zones Research Institute (Shippagan, New Brunswick). Fish were raised under 24h light (LL) from first feeding in February to 10 g body weight (June), followed by 18h light and 6h

dark. In August, 2012 and 2014, about 800 underyearlings (ca. 20 g), a mix from 10-15 full-sib families, were trucked from Shippagan to Truro. There, until the start of the trials, they were reared in a single tank (1200 L) supplied with well water (9.5-10°C; oxygen saturation > 80%, total alkalinity of 100mg/L; total hardness of 188mg/L) under a simulated natural photoperiod (LDN, Latitude 45˚N) and hand-fed a commercial salmon diet (protein 44-45%, fat 24-26%, fibre 1.3%, Corey Feed Mills Ltd., New Brunswick). To allow individual fish to be the experimental unit, each was identified with an electronic Passive Integrated Transponder (PIT) tag inserted into the body cavity prior to the start of each trial.

Trial 1 started September 20, 2012, when each of six identical lightproof tanks (1.15 m diameter, 310 L) was stocked at random with 120 fish (mean 54 g, range: 33-95 g). Each tank was illuminated by an overhead fluorescent light providing between 700-800 lux at the water surface, and supplied with flow-through well water (8-10 L/min, 10 °C). Initially, all six tanks were maintained on a simulated natural daylength, with no twilight, regulated by a photoperiod controller (SunMatch; Aquabiotech, Quebec). To test the effect of LL start date on the incidence of maturation, the photoperiod was increased from LDN to LL in sequence in five of the tanks on October 1, November 1, December 1, January 1 and February 1 respectively. The mean body weight at the LL start date ranged from 70 g (October) to 351 g (February). As a control, one tank remained on LDN throughout the trial (Fig. 1; Left panel). On April 1, the photoperiod of the five tanks on LL was returned to LDN. Also, to provide more rearing space, all the fish were randomly distributed among three larger tanks (1200 L) with the same flow-through well water supply, in which they remained until the end of the trial in November 2013.

Trial 2 started August 9, 2014, when 720 underyearlings (mean 24 g, range: 10-46 g) were distributed at random among eight identical light-proof tanks (1 m diameter, 500 L; 90 fish per tank) in a recirculation system (total volume 5000 L) with about three turnovers of water per day. Temperature was constant 10 °C. Each tank was lit by an incandescent bulb (40 W) providing about 80 lux at water surface. Seven LL treatments were tested, together with a control (LDN throughout). The duration of LL ranged from four to twelve months by adjusting the LL start-date from mid-August to December, and LL end-date from February to October (Figure 2, left panel). The mean body weight at the start of the LL treatments was about 24 g (August), 70 g (October) and 210 g (December). To provide more rearing space, in June, half of the fish from each tank were split to an identical eight-tank recirculation system and photoperiod remained the same within each treatment. The trial ended in October 2015.

3.3.2 Data sampling and analysis

In both trials, at monthly intervals, each fish was anaesthetized (MS222, 0.1 g/L) to record fork length (FL; to 1 mm), body weight (BW; to 1 g) and PIT-tag number. Condition factor (CF) was calculated as $CF=100\ (BW/FL^3)$. Fish displaying secondary sexual characteristics (orange colored flanks, hook jaw) were noted and gentle hand pressure was applied to their abdomen to determine if either milt or eggs could be expressed. At the end of each trial, fish were euthanized and dissected to reveal sex and maturity status. Individuals were classified as 'immature' if the female gonads comprised only of primary oocytes, and if male gonads were thread-like. Fish that were either maturing or freely expressed gametes were classified as sexually mature. Fish that released

gametes during the summer but had regressed gonads in November were also classified as 'mature' because they retained an unmarketable small body size and poor condition factor.

3.3.3 Statistics

The experimental unit was an individually identified fish that represents the experimental error. Maturity rate was analyzed using the CATMOD procedure of SAS with the generalized response function and contrast statement (SAS 9.4, 2013). Body weight, fork length and condition factor were analyzed as repeated measures using the MIXED procedure of SAS as a four-factor factorial (SAS 9.4, 2013). The four factors were photoperiod treatment (trial 1: six; trial 2: eight), maturity (mature or immature), sex (male or female), and date (repeated factor). The most appropriate covariance structure for MIXED procedure for BW and CF was autoregressive order 1 determined by Akaike's Information Criterion and Schwarz's Bayesian Criterion. The Kenward-Roger correction was used to prevent Type I error rates from over-inflation (Littell et al. 2006). To satisfy the assumptions of normality and homogeneity of variance for ANOVA, a square root transformation was applied on body weight data in both trials. Treatment combinations from the highest order significant interaction effects were computed. For all analyses, significance was declared at $P < 0.05$.

All procedures were approved by the local Animal Care and Use Committee (File # 2012-079 and 2014-064).

3.4 Results

3.4.1 Incidence of sexual maturation

The overall incidence of maturation in trial 1 increased in a step-wise manner from 43% among charr under LL from October 1, to 73% among fish exposed to LL from February 1 to April 1, and 77% in the control (Fig. 3.1; left panel). The maturity rate was significantly affected by the interaction between photoperiod and sex ($P<0.01$). The female maturity rate was reduced significantly in all five LL treatments compared to the control (88%), with LL starting October 1 the most effective (44%; Fig. 3.1 females). LL starting November, December or January was equally effective, female maturity rate ranging from 62% to 66% (Fig. 3.1 females). Among males, LL starting either January 1 or February 1 was ineffective, the maturation rate was 69-70% (Fig. 3.1 males). By comparison, LL starting October 1 to December 1 significantly reduced the male maturation rate to between 41 and 52% (Fig. 3.1 males).

In trial 2, the overall maturity rate in the LDN control was lower than trial 1 (50 vs. 77%). Among the seven LL treatments, the overall maturity rate ranged from 7 to 33% (Fig. 3.2; left panel). The maturity rate was significantly affected by the interaction between photoperiod and sex ($P<0.01$), like trial 1. Among females, the four LL treatments starting in either August or October and ending either February or April were equally effective, the maturity rate ranged from 3 to 16% compared to 66% in the control (Fig. 3.2; females August-February, August-April, October-February, October-April). Delaying the LL start date to December significantly reduced its efficacy; 24% of females matured in the December-April treatment compared to 7% in the October-April treatment (Fig. 3.2 females). Extending LL for the full year also significantly reduced its efficacy, 42%

maturing (Fig. 3.2; females Oct-Oct). Among males, the six LL treatments starting either August or October and ending between February and October were equally effective, significantly reducing the maturity rate to 8-19% (Fig. 3.2; males August-February, August-April, October-February, October-April, October-June, October-October). Delaying the LL start date to December rendered the treatment ineffective, 42% of males matured, a similar rate to the control (Fig 3.2; males December-April, LDN).

3.4.2 Timing of spermiation and ovulation

Among fish in trial 1 that matured, the timing of the completion of this process was advanced in a step-wise manner dependent on LL start date. Males were more responsive than females, over 20% expressing milt on April 30 among charr exposed to LL starting either October 1 (28%, 8/29) or November 1(24%, 7/29; Fig. 3.3 left panel). By comparison, LL from February 1 to April 1 resulted in the initial detection of ripe males on July 3 (38%, 18/47). Under LDN, ripe males were first detected in mid-September (Fig. 3.3 left panel). By November, most of the early maturing males were no longer producing milt (after expressing milt for 2-5 months). Females ovulated in sequence dependent on the LL start date, but the response time to completion of maturation was slower than males, beginning May 28 in the group exposed to LL from October 1 (Fig. 3.3 right panel). By November, 50-70% of maturing females had ovulated in the five groups exposed to LL the previous winter, compared to <30% among females under LDN (Fig. 3.3 right panel). Trial 2 confirmed LL in winter advanced the completion of maturation in a step-wise manner, but LL end date was a more dominant cue compared to start date. Among males, the median date for spermiation was April 21, May 14 and July 15 when the LL end date was February 1, April 1 and June 1 respectively (LL start date October 1; data not shown).

On May 14, 19 out of 21 mature fish in the LL December-April group expressed milt. By contrast, among males exposed to constant LL throughout, only 1 out of 11 maturing fish produced milt. In the LDN group the maturing males (n=15) expressed milt between September and October. In the LL August groups (August-February, August-April), there were too few maturing males (N<5) for analysis. Similarly, in trial 2 there were too few maturing females for analysis.

3.4.3 Somatic growth

Body size data for males and females was pooled in both trials, as the effect of sex was not significant. Comparing maturing and immature fish, a significant difference in somatic growth and condition factor was evident only towards the end of each trial (Day*Treatment*Maturity, $P<0.01$). In trial 1, the charr grew rapidly overwinter independent of both photoperiod and future maturity status. Between September and April their mean body weight increased from 54 to 450 g and condition factor from 1.1 to 1.35 (Fig. 3.4). A significant divergence in mean body weight developed between maturing and immature fish from April onwards that contrasted markedly between the LDN and LL treatments. In the LDN group, sexually maturing fish were significantly heavier than immature fish from May through August, their relationship reversing only at the completion of maturation from September onwards (Fig. 3.4a). By contrast, among all LL groups, sexually maturing fish grew poorly through the summer associated with the temporal advance in maturation, and by November were about 50% smaller than immature fish (Fig. 3.4b to f). Condition factor further emphasized the poor growth performance of maturing fish in the LL treatments, becoming significantly lower than immature fish through the summer. By contrast, in the control group, mean condition factor was

relatively good for most of the trial independent of maturation status, decreasing only in November among maturing fish (Fig. 3.4).

In trial 2, the somatic growth patterns were similar to trial 1. All fish grew rapidly during the first half of the trial, mean body weight increasing from about 25 to 470g between August and March (Fig. 3.5). During winter, the putative decision period for commencing maturation, the mean body size of maturing and immature fish was similar. Immature fish in all eight treatments maintained this rapid growth throughout, exceeding 1 kg by October (Fig. 3.5). Maturing fish in the LDN group maintained a similar growth pattern to immature fish except for a small loss in mean weight in October at the completion of maturity (Fig. 3.5a). By contrast, maturing fish in five of the seven LL treatments grew poorly through summer and, similar to trial 1, their final mean weight was about 50% smaller than immature fish, and their condition factor was significantly lower (Fig. 3.5d to h). The other two continuous light treatments, constant LL and LL from August to February, exhibited growth trajectories similar to the LDN control (compare Fig. 3.5a, b, c).

3.5 Discussion

An abrupt increase in photoperiod to 24 h light (LL) before the winter solstice prompted individual Arctic charr to make a physiological decision to mature or remain immature, the outcome independent of their body size and condition factor. To minimize the problem of maturation age 2, a start date for LL between August and October is recommended. Delaying the LL start date from November onwards increased the incidence of maturity. The end date of the LL treatment was less critical, between February and June was equally effective provided the start date was October. The abrupt increase in

photoperiod in the fall stimulated gonadal development among the few fish that could respond confirming its dichotomous influence on the brain-pituitary-gonadal axis.

An LL start date in the autumn was of major importance in reducing maturity rate in Arctic charr in both trials, supporting findings on Atlantic salmon in sea-cages in both Norway and Atlantic Canada (Taranger et al., 1999; Peterson and Harmon, 2005). In Atlantic salmon, the arrest in gonadal maturation was evident one month after the onset of LL based on histological analysis of gonads and reduced levels of pituitary follicle-stimulating hormone (Taranger et al. 1999; Schulz et al. 2006; Andersson et al. 2013). The inhibitory effect of LL during early stages of gonadal development in Atlantic salmon is matched by cultured Fraser River Arctic charr, which also commence spermatogenesis in the autumn based on gonadal histology (Rice, 1999). The response of the salmonid brain-pituitary-gonad axis to a switch from LDN to LL changes progressively through winter from being inhibited to stimulated. This 'dichotomy' of a zeitgeber stimulus, LL here, was first clearly recognized by Schulz et al. (2006) among male Atlantic salmon, and later reported for females (Andersson et al., 2013). The established hypothesis to explain the dichotomy of the response to LL is the permissive role of body size or energy reserves that dictates whether an individual matures or not (Oppedal et al., 2006; Taranger et al., 2010). In sea bass (*Dicentrarchus labrax*), body size has been indicated to be a limiting factor for the activation of the brain-pituitary-gonadal axis and onset of gametogenesis (Rodríguez et al., 2012; Espigares et al., 2017). Also, body size of female Atlantic salmon contributed to the dichotomous response of gonadal development under LL exposure, with large fish being stimulated and small fish being inhibited (Andersson et al., 2013). Among Arctic charr, by contrast, no differences in mean body size was evident between maturing and

immature individuals exposed to LL overwinter (present study; Liu and Duston, 2016). Undoubtedly, body size and energy reserves can dictate whether or not an Arctic charr commences maturation, as evidenced by food deprivation reducing the incidence of maturation (Liu and Duston, 2016). But, the influence of somatic growth on decision to mature appears to be discrete from the influence of LL. Consequently, we proposed a two-step gating model (Liu and Duston, 2016). In the first step, prevention of sexual maturation is solely affected by photoperiod, a growth independent factor, which defines a period of time during which individuals have an opportunity to commence gonadal maturation. The second step is only eligible to those succeeding the first step and have faster growth rate and larger body size.

The importance of the end date of LL, or long photoperiod, on the incidence of maturation differs between studies and species. In the present study, a return from LL to LDN between February and June was equally effective at reducing maturation following an October start date. Similarly, among Atlantic salmon in sea-cages, ending the LL exposure in either mid-April, May or June following a January start did not alter the incidence of maturation (Leclercq et al., 2010). Contrasting results, however, were produced among female rainbow trout (*Oncorhynchus mykiss*). Following the start of a long photoperiod treatment in early January, the timing of a switch from LD 18:6 to LD 6:18 in either March, April, May or June led to a step-wise increase in the incidence of maturation (Duston and Bromage, 1988). Despite the flexibility of the timing of ending LL among Arctic charr, the long-to-short photoperiod switch appears to be essential to arrest the maturation process because constant LL or LD 18:6 exposure resulted in modest to high maturity rate (present study; Duston et al., 2003). By comparison, among Chinook

salmon (*Oncorhynchus tshawytscha*), a single switch in photoperiod from LDN to LL during the austral autumn effectively reduced the maturity rate to 0% among females and < 10% among males (control: Female: 25%; Male: 90%; Unwin et al., 2005). Overall, these different responses suggest the role of photoperiod in controlling age at maturity is species-specific, even among salmonids, and may defy attempts to establish a unified model.

The 'coupled' response to LL of a reduction in the incidence of maturation in some charr with an advance in the timing of gamete releasing in others conforms with studies on rainbow trout (Duston and Bromage, 1988; Randall et al., 1998) and Atlantic salmon (Taranger et al., 1998; 1999). This consistent response supports the hypothesis the same endogenous timing mechanism controls both the physiological decision to mature and the rate of gametogenesis. The autumnal decrease in photoperiod appears to be the major entrainment cue to the endogenous reproductive cycle for Arctic charr, since overriding it by LL blocked gonadal growth. The chronology of this physiological decision period seems to be earlier compared with rainbow trout and Atlantic salmon (Taylor et al., 2008; Andersson et al., 2013). These differences can be reconciled if considering the extreme overwintering conditions in subarctic regions for Arctic charr, signified by long period of darkness and prolonged scarcity of feeding opportunities (Jørgensen and Johnsen, 2014).

The sexually dimorphic growth patterns in Arctic charr reported here were mainly a consequence of sexual maturation, rather than a cause, confirming previous studies (Jobling and Baardvik, 1991; Tveiten et al., 1996). The deterioration in both somatic growth and condition factor among maturing charr was clearly evident following the decrease in photoperiod from LL to LDN because the fish became anorexic and significant

energy was diverted into gonadal development (Tveiten et al., 1996). Among immature charr, LL did not enhance somatic growth confirming previous studies (Mortensen and Damsgård, 1993; Liu and Duston, 2016). Among Atlantic salmon and rainbow trout, by comparison, LL stimulated somatic growth and enhanced muscle fiber recruitment through a direct stimulation of the somatotropic axis or alteration of the endogenous growth rhythms (Stefansson et al., 1991; Endal et al., 2000; Johnston et al., 2003; Taylor et al., 2006).

The nearly full suppression in maturation age 2 by use of continuous light during fall-winter period over three year-classes demonstrates the method can be reliably used by farmers growing Fraser River Arctic charr, with the optimal start date being between August and October and an end date between February and June (Liu and Duston, 2016). In Atlantic Canada, Arctic charr production occurs in indoor tank facilities supplied with well water (8-10 °C). The likelihood of interference from seasonal ambient illumination is relatively low provided sufficient underwater lighting. Assessment of light sensitivity by measuring nocturnal melatonin levels, a hormone which communicates photic signal, would be useful for implementing the technique in commercial setting (Porter et al., 1999).

In conclusion, the physiological decision to mature among intensively reared Arctic charr can be changed from maturing to immature by the means of continuous light exposure during the fall-winter period. The similar body size and condition factor between maturing and immature Arctic charr confirms that the suppression of maturation can be influenced by a growth-independent factor.

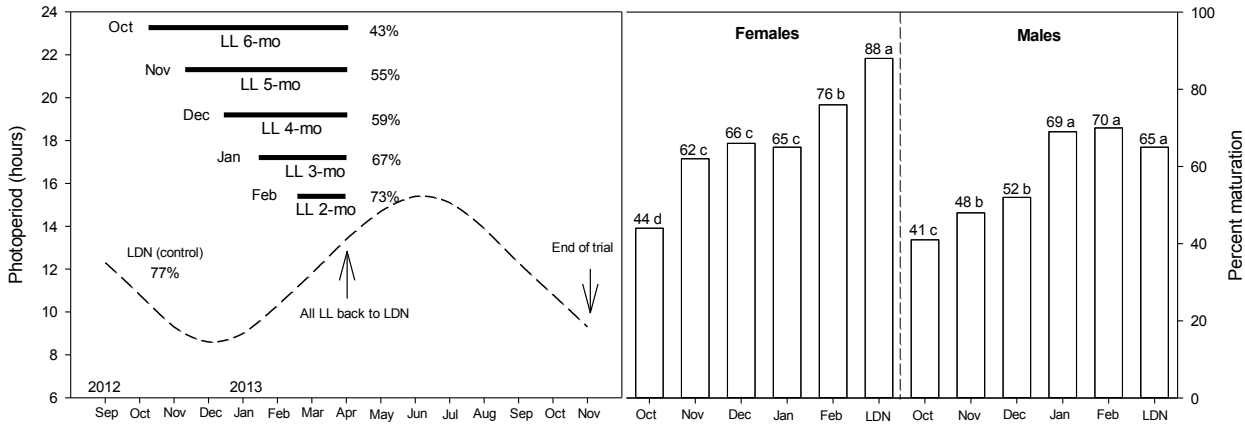

Figure 3.1. Trial 1. Left panel: Photoperiod regimes and overall incidence (%) of sexual maturity among Arctic charr at 2 years of age (November 2013). 24h light (LL) treatments started on the 1st day of October, November, December, January or February, with the same end date, April 1. The control group received a simulated natural daylength cycle (LDN). Right panel: Incidence of maturity subdivided by sex. Within each sex, maturity rates sharing the same letter are not significantly different at the 5% level. On the x-axis, the month label indicates the LL start date. Number of fish per treatment: females 63 to 73, males 45 to 51.

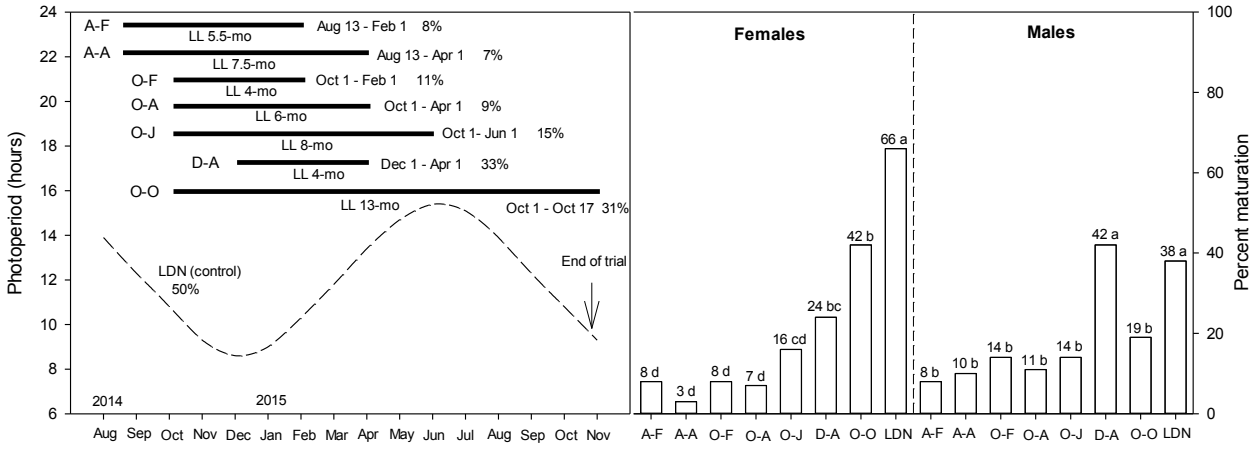

Figure 3.2. Trial 2. Left panel: Eight photoperiod regimes and overall incidence of sexual maturity among Arctic charr at 2 years of age (October 2015). 24h light (LL) start dates ranged from August to December 2014, and end dates from February to October 2015. The control received a simulated natural daylength cycle (LDN). Right panel: Incidence of maturity subdivided by sex. Within each sex, maturity rates sharing the same letter are not significantly different at the 5% level. On the x-axis, the letter codes for month indicate the LL start and end dates. Number of fish per treatment: females 38 to 51, males 38 to 51.

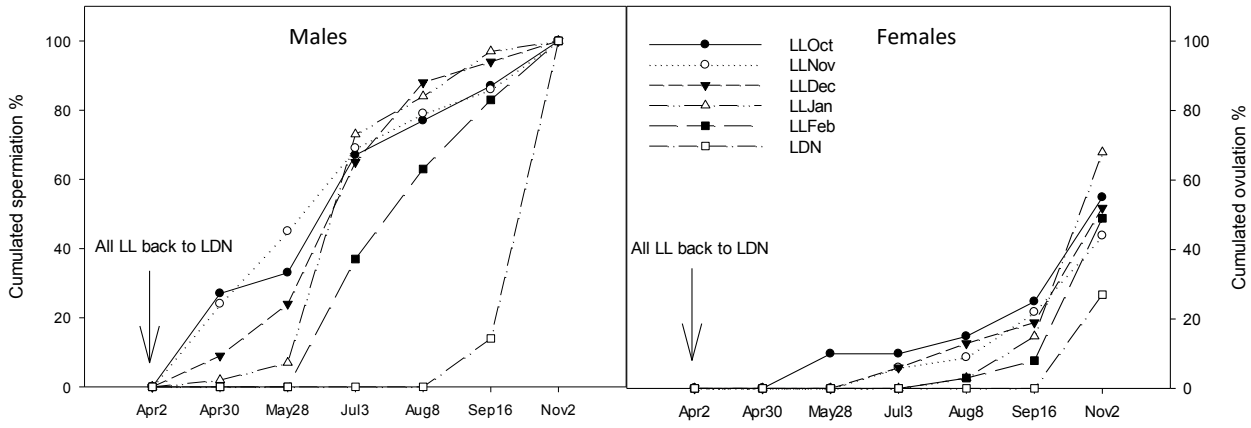

Figure 3.3. Trial 1. Cumulative spermiation (Left) and ovulation (Right) among matured Arctic charr from April to November, 2013, following five 24 h light (LL) treatments, started from 1st day of either October, November, December, January or February and returning to LDN on April 1. The control received a simulated natural daylength cycle (LDN). The total number of mature fish from which the percentages are derived in LLOct, LLNov, LLDec, LLJan, LLDec and LDN: males: 29, 29, 34, 45, 47, 41; females: 20, 32, 31, 34, 37, 45, respectively.

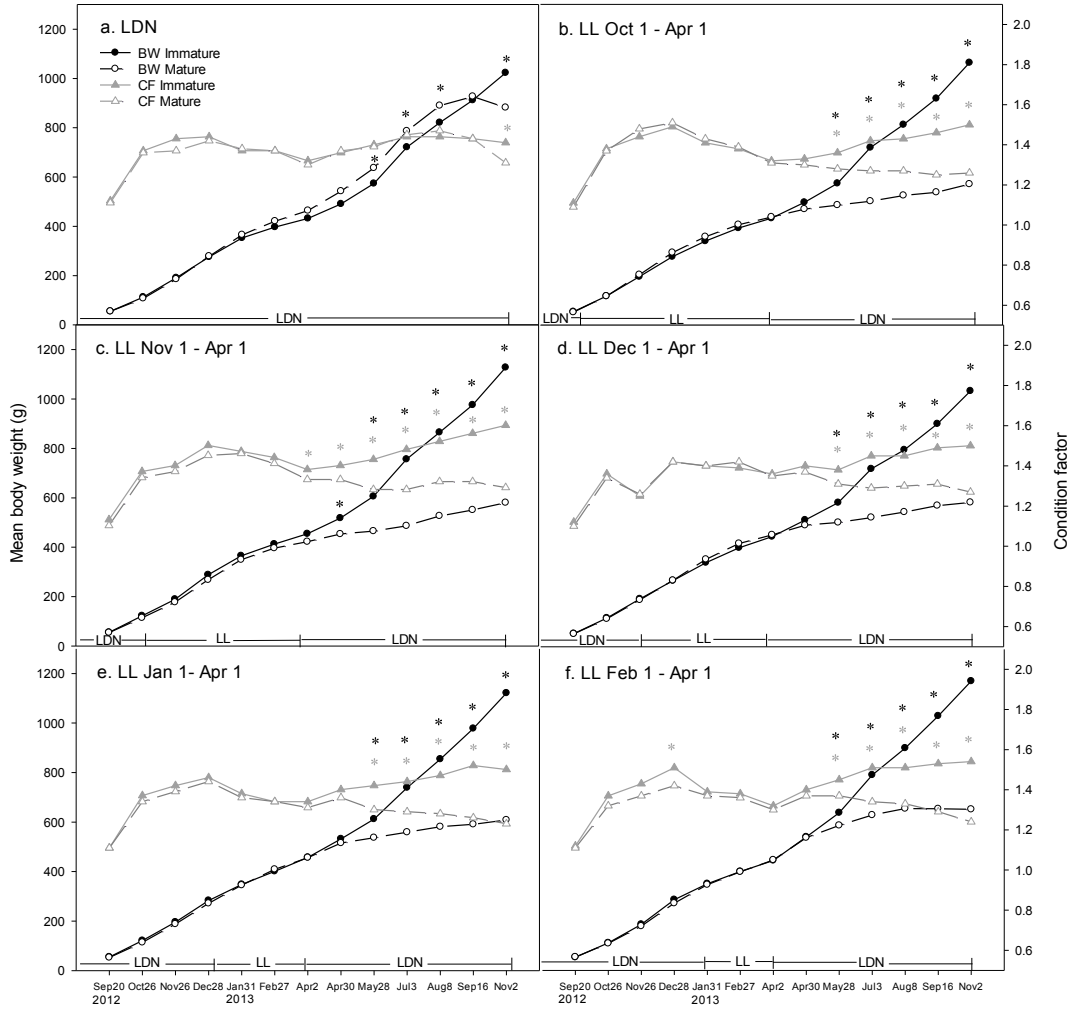

Figure. 3.4. Trial 1. Mean body weight (BW, g) and condition factor ($CF=100*BW/FL^3$) of immature and mature Arctic charr (sexes pooled). 24h light (LL) started either on the 1st day of October, November, December, January or February, all returning on April 1 to a simulated natural daylength cycle (LDN). The control was maintained under LDN throughout the trial. Within each treatment at each date, black and grey asterisks indicate significant differences ($P<0.05$) in mean BW and CF between maturing and immature fish respectively.

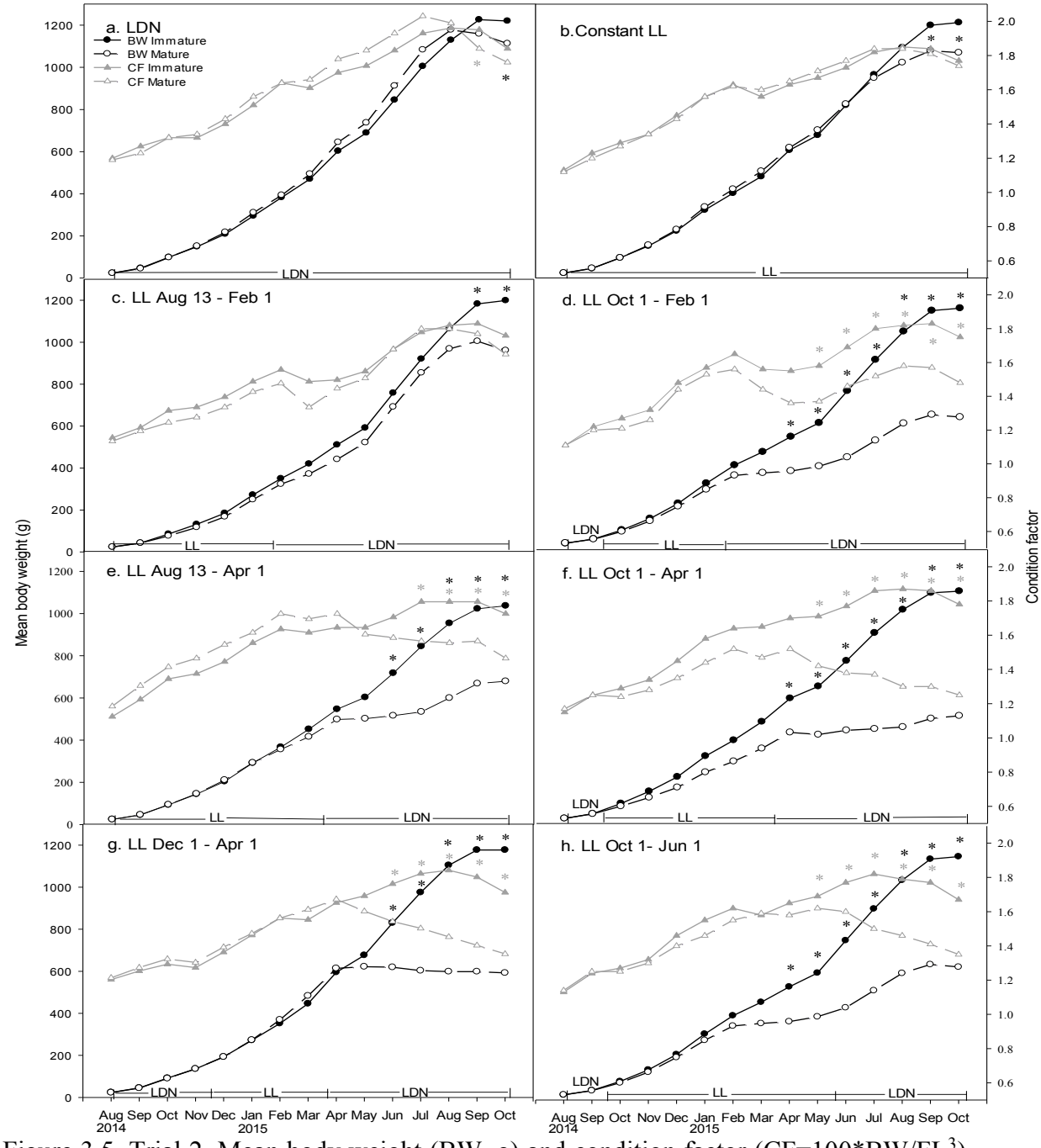

Figure 3.5. Trial 2. Mean body weight (BW, g) and condition factor (CF=100*BW/FL^3) of immature and mature Arctic charr (sexes pooled) reared under eight photoperiod treatments. Six 24h light (LL) treatments were derived from combinations of three start dates: August 13, October 1, or December 1, and three end dates: February 1, April 1 or June 1. The seventh treatment was constant LL from October 1, 2014 onwards. The control received a simulated natural daylength cycle (LDN). Within each treatment at each date, black and grey asterisks indicate significant differences ($P<0.05$) in mean BW and CF between maturing and immature fish respectively.

Chapter 4: Preventing Sexual Maturation in Arctic Charr by 24h Light Overwinter and Suppressing Somatic Growth

Status: Published, Aquaculture 464: 537-544 (2016).

4.1 Abstract

To address the problem of high maturity at 2 years of age among *Salvelinus alpinus* (Labrador strain), PIT-tagged yearlings (ca. 110 g) were reared for 18 weeks overwinter (Nov. 16 to Apr. 1) under six treatments (90 fish per treatment) that combined three factors: photoperiod (natural daylength, LDN or 24h light, LL), temperature (10 or 5 °C), and food (fed daily or no food). On April 1 all fish were returned to LDN, 10 °C and fed daily for a further six months. In November, the maturity rate was very high among fish reared the previous winter under LDN and fed daily at both 10 °C (♀ 94%, ♂ 43%) and 5 °C (♀ 87%, ♂ 45%). Replacing LDN with LL overwinter, at 10 °C halved the maturity rate (♀ 49%, ♂ 19%), and at 5 °C eliminated maturity among females, and male maturity was 6%. Food deprivation for 18 weeks posed no health problems, and combined with LL also was highly effective at preventing maturation, both at 10 °C (♀ 0%, ♂ 2%) and 5 °C (♀ 0%, ♂ 7%). Compensatory growth following the suppression of growth overwinter indicates this approach to reducing maturity can be a commercially viable means to produce 1 kg immature product around 2 years old. A new two-step gating mechanism is proposed to explain the photoperiod prevention of maturation, one independent of somatic growth, the other dependent.

4.2 Introduction

In Atlantic Canada, profiting from farming diploid Fraser River Arctic charr in 10 °C well water has been difficult due to the high incidence of sexual maturation of both sexes (Duston et al., 2003). Unwanted maturation afflicts numerous species of farmed fish globally (Taranger et al., 2010), with high grilse rates among cage-farmed Atlantic salmon (*Salmo salar*) particularly costly (e.g. McClure et al., 2007). 24h light (LL) for several months overwinter is a standard industry practice to reduce grilse rates (Taranger et al., 1999; Leclercq et al., 2010). By comparison, among both Arctic charr and rainbow trout (*Oncorhynchus mykiss*), a long photoperiod in winter was less effective, at best only halving the incidence of maturation in land-based facilities (Duston et al., 2003; Taylor et al., 2008). The mechanism underlying the photoperiod induced arrest of sexual maturation in salmonids remains unclear, but is proposed to be due to an advance in the timing of a 'decision period' to which small or slow growing individuals cannot respond, and remain immature (Duston and Bromage, 1988; Thorpe et al., 1998; Taranger et al., 2010). To test this hypothesis, the present study combined photoperiod treatment with food deprivation and/or low temperature overwinter, aiming to reduce the incidence of maturation at 2 years of age, thereby improving the commercial viability of farming diploid Fraser River Arctic charr.

Sexual maturation among salmonids is considered a threshold trait which proceeds only when an individual exceeds a 'threshold' that remains ill-defined, but includes factors such as body size, lipid reserves, growth rate, and time of the year (Thorpe et al., 1998; Sloat et al., 2014). Maturation is synchronized by the annual photoperiod cycle and is initiated about 1 year prior to spawning as evidenced by signs of gametogenesis, sexual dimorphic body size, elevated fat and sex steroid levels (Shearer and Swanson, 2000;

Campbell et al., 2006; Andersson et al., 2013). Accordingly, the incidence of maturation among various salmonids was decreased by reducing somatic growth and/or lipid reserves during this purported 'decision period' (Rowe et al., 1991; Duston and Saunders, 1999; Shearer et al., 2006). Among Labrador Arctic charr, by contrast, the maturity rate was unaffected by reducing somatic growth in winter (Rice, 1999; MacPherson, 2012). Nevertheless, we hypothesized growth suppression could be effective if extended over a longer time period and might work in synergy with LL treatment. To date, only four studies on salmonids have combined photoperiod treatment with manipulation of growth to alter the incidence of maturation, with mixed results (Adams and Thorpe, 1989; Wilkinson et al., 2010; Fjelldal et al., 2011; Imsland et al., 2014).

4.3 Materials and Methods

4.3.1 Fish stock and rearing conditions

Underyearling Arctic charr (Fraser River population, Labrador strain) were supplied by a pedigreed breeding program at the Coastal Zones Research Institute (CZRI, Shippagan, New Brunswick). Fish were raised under 24h light (LL) from first feeding to 10 g body weight, then 18h light and 6h dark (LD 18:6) from 10 to 30 g until trucked to Truro in August 2013. About 600 fish, a mix of 20 full-sib families were held in a single tank (1000 L) supplied with 10 °C well water under simulated natural daylength (LDN, latitude 45 °N) and hand-fed twice daily a commercial salmonid diet (protein 44-45%, fat 24-26%, fibre 1.3%, Corey Feed Mills Ltd., New Brunswick). In October, each fish was anesthetized (MS222, 0.1 g/L) and identified by a Passive Integrated Transponder (PIT) tag inserted into the body cavity. In early November, fish were distributed randomly among six identical lightproof tanks (1.15 m diameter, 310 L volume) with 90 fish/tank.

Each tank had a flow-through supply (8-10 L/min) of well water (9.5-10 °C; oxygen saturation > 80%; total alkalinity 100 mg/L; total hardness 188 mg/L). A chilled (5 °C) well water supply was also available to each tank when needed. Each tank was illuminated by an overhead light consisting of six fluorescent tubes (54 W/tube) with shade cloth providing about 700-800 lux at the water surface. Simulated natural daylength, with no twilight, was regulated by a timer clock (Astral photoperiod controller, Sunmatch). On November 16, 2013, six treatment combinations commenced and ran until April 1 (18 weeks): 1) Control: LDN, 10 °C, fed to satiation (LDN10Fed); 2) LL, 10 °C, fed to satiation (LL10Fed); 3) LDN, 5 °C, fed to satiation (LDN5Fed); 4) LL, 5 °C, fed to satiation (LL5Fed); 5) LL, 10 °C, not fed (LL10Unfed); 6) LL, 5 °C, not fed (LL5Unfed). Water temperature was adjusted to 5 °C by gradually reducing the 10 °C supply and increasing the 5 °C supply over three days. On April 1, 2014, all the treatments ended and all six tanks of fish were returned to LDN photoperiod, 10 °C and daily feeding. Fish were hand-fed to apparent satiation two times daily. Pellet size was increased from 3 to 5.5 mm as the fish grew. On August 5, to provide more space, the fish were transferred to six larger identical light-proof tanks (1 m diameter, 500 L) in a recirculation system (total volume 5000 L) while maintaining the LDN photoperiod and 10 °C. Each tank was lit by an incandescent bulb (40 W) providing about 80 lux at the water surface.

4.3.2 Data sampling and analysis

Every month, each fish was anaesthetized (MS222, 0.1 g/L) to record fork length (FL; to 1 mm), body weight (BW; to 1 g) and PIT-tag. At the end of the trial in November, fish were euthanized (MS222 overdose), then maturity status and sex were determined following dissection. Fish that were either maturing or ‘running gametes’ were classified

as sexually mature. If the gonads were thread-like (males), or comprised only primary oocytes (females), fish were classified as immature.

Proximate analysis was conducted on whole fish sampled both at the start of the experiment in November (n=10 fish) and the following April (n=5 from each treatment). Samples were frozen (-20 °C) for subsequent analysis. Each fish was ground and moisture content determined (80-100 g sample) by freeze drying for 24h (Modulyod-115; AOAC, 2011). Lipid was determined using a high temperature solvent extraction system (ANKOM XT15), protein by measuring the nitrogen content using the combustion method (Leco FP-528), and ash following 12h in a 550 °C oven (Isotemp Muffle Furnace; AOAC, 2011).

4.4 Statistical methods

The experimental unit was a single fish that represents variability (experimental error). Maturity rate was analyzed using the CATMOD procedure of SAS with the generalized response function and contrast statement (SAS Institute, 2013). Body weight, fork length and condition factor (CF=100 (BW/FL^3)) were analyzed as repeated measures using the MIXED procedure of SAS as a four-factor factorial (SAS Institute, 2013). The four factors were treatment (six combinations), maturity (mature or immature), sex (male or female), and date. Measurements were repeated on November 26 and December 30 of 2013, and in 2014 on February 3, March 7, March 31, May 2, May 30, June 30, August 1, September 2, October 4 and November 1. Since all treatments stopped on April 1, 2014 and switched to the same rearing conditions of 10 °C, LDN and daily feeding, the growth data was analyzed by the MIXED procedure in two phases: phase 1, November 26, 2013 to March 31, 2014; phase 2, March 31 to November 1, 2014. The lsmeans statement of

Proc Mixed with the pdiff option was used to produce p-values for all pairwise comparisons (SAS Institute, 2013). Because of the large number of treatment combinations in the repeated measures analysis, alpha = 0.01 was used in the letter groupings to protect against the over-inflation of Type I experiment-wise error. For all repeated measures responses, the most appropriate covariance structure was determined based on the Akaike's Information Criterion and Schwarz's Bayesian Criterion (Littell et al., 1998). Square root transformation was applied to body weight data from both phase 1 and phase 2 to satisfy the assumptions of normality and constant variance. The coefficient of variation (%CV=100 (standard deviation/mean) was used to express variability in final body weight.

All procedures were approved by the local Animal Care and Use Committee (File # 2013-084).

4.5 Results

4.5.1 Incidence of sexual maturation

The incidence of maturation at 2 years of age was significantly affected by the following interactions: photoperiod*temperature ($p<0.001$), photoperiod*sex ($p<0.001$), and feeding*temperature*sex ($p=0.011$). In the control group (LDN10Fed), the overall maturity rate was 67%, females 94% and males 43% (Fig. 4.1). Reducing the rearing temperature to 5 °C overwinter, but maintaining feeding and the LDN photoperiod failed to affect the maturity rate (♀ 87%, ♂ 45%; Fig. 4.1). Substituting LL for LDN overwinter and maintaining 10 °C and feeding reduced the maturity rate by about half (♀ 49%, ♂ 19%; Fig. 4.1). Colder 5 °C water combined with LL greatly reduced the maturity rate,

despite daily feeding; all 49 females were immature, and only 6% of males matured (Fig. 4.1). Similarly, very low maturation rates resulted from 18 weeks of food deprivation combined with LL, independent of rearing temperature (Fig. 4.1).

4.5.2 Somatic growth and condition factor

Mean body weight was significantly affected by a four-way interaction (Treatment*Maturity*Sex*Date, P=0.001) from November 26, 2013 to March 31, 2014 (phase 1) and a three-way interaction (Treatment*Maturity*Date, P=0.001) from March 31 to November 1, 2014 (phase 2). Within each treatment, the growth patterns of male and female Arctic charr were similar as all two- and three-way interactions with sex were not significant (Table 4.1). Hence, general growth patterns with pooled sexes were summarized graphically to emphasize the interaction effect between treatment, maturity and date.

Analysis of somatic growth between maturing and immature fish was possible only within three treatment groups, LDN10Fed, LDN5Fed and LL10Fed, the others had insufficient mature individuals. Throughout phase 1 within these three groups, the mean body weight of maturing and immature fish was not significantly different, increasing from about ca. 110 g in November to about 500-600 g at 10 °C, and 350-400 g at 5 °C (Fig. 4.2 both panels). Growth overwinter was independent of photoperiod. The two groups of charr not fed for 18 weeks (LL10Unfed, LL5Unfed) maintained good health, they remained inactive in a tight school and showed no signs of aggression. Their mean body weight decreased significantly by <10% during the food deprivation period, independent of rearing temperature (104 vs.107 g in March; Fig. 4.2 left panel). At the end of March, the mean body weight of the unfed fish was four- to five-fold lower than those fed daily. They

regained full appetite within a week of the resumption of feeding on April 1.

During phase 2, within the LDN10Fed and LDN5Fed treatments, the mean body weight of immature and mature was not significantly different through to August. Thereafter the immature fish continued to grow, whereas maturing fish began losing weight and were significantly smaller than immature fish from September onwards (Fig. 4.2 both panels). In the LL10Fed treatment, by comparison, the divergence in mean body weight between immature and maturing fish was evident from May onwards soon after the decrease in photoperiod from LL to LDN on April 1.

Full compensatory growth was exhibited by both immature and maturing fish fed throughout following the increase from 5 to 10 °C in April (LDN5Fed, LL5Fed). From June-August onwards, their mean body weight was similar to controls reared at constant 10 °C (Fig. 4.2 both panels). By comparison, partial compensatory growth was evident during summer among immature charr following 18 weeks without food (LL10Unfed, LL5Unfed). Their mean body weight increased to around 750 g at harvest, significantly lower than immature fish fed to satiation throughout (820-1000 g; Fig. 4.2 left panel).

Mean condition factor (CF) was significantly affected by a four-way interaction during both phase 1 and 2 (Treatment*Maturity*Sex*Date; P=0.001), but was a poor predictor of which individuals would become sexually mature. Initial mean CF was independent of maturation status at 2 years of age (P=0.131). A significant difference in mean CF between maturing and immature fish during winter was evident only among females in the LL10Fed treatment from late December to early March, when maturing fish were ‘fatter’ (Fig. 4.3b). By comparison, among males in the LL10Fed treatment, the changes in mean CF between immature and maturing males overwinter were not significantly different (Fig. 4.3b). Similarly, in the LDN10Fed and LDN5Fed groups, the

changes in mean CF overwinter between immature and maturing fish within each sex were similar ($P>0.05$), increasing from about 1.1 in November to between 1.4 and 1.5 in March (Phase 1, Fig. 4.3a, c). Similar increases in mean CF were exhibited among the high number of immature fish of both sexes in the LL5Fed treatment (Fig. 4.3d). Withholding food for 18 weeks resulted in mean CF decreasing from 1.0 to about 0.8 among fish remaining immature, with too few maturing fish to analyze (Fig. 4.3e, f).

Between April and November, mean CF among the four treatment groups that were fed overwinter decreased gradually, the rate of decline greatest among maturing males, followed by maturing females (Fig. 4.3a, b, c, d). By comparison, among the two treatment groups not fed overwinter, mean CF increased quickly following the resumption of feeding from 0.8 to 1.2 in about one month, stabilizing around 1.3 from June through to November among both immature males and females (Fig. 4.3e, f).

The greatest yield of 'high-value' sexually immature charr >1kg body weight was from the LL5Fed treatment, producing almost 27 kg (n=24 fish out of 80; Fig. 4.4b). By contrast, the control group yielded only 4.8 kg (4 out of 78 fish) of high-value product due to the high incidence of sexual maturation (Fig. 4.4a). Withholding feed overwinter limited the yield of high-value product to between 10.9 and 13.7 kg (Fig. 4.4e, f). However, there were about 65% of immature fish between 600 to 900 g in these groups that were growing fast and we estimate would have reached 1 kg within about three months.

Final body weight ranged greatly among all six treatments from 200 to 1600 g, with coefficient of variation between 26 to 35% (Fig. 4.4). In the two treatment groups reared on LDN overwinter, both mature and immature fish were broadly distributed between 400 and 1400 g (Fig. 4.4a, b). In the other four treatments, by contrast, the final

body weight of mature individuals was mostly <600 g, with mature males the smallest (Fig. 4.4c, d, e, f).

4.5.3 Whole-body composition

Whole body composition of charr in the four treatment groups fed to satiation overwinter remained stable despite large increases in both body weight and condition factor (Table 4.2). In April, protein was around 17%, whole body lipid 14%, moisture 66% and ash 1.6% (Table 4.2). Among charr not fed overwinter, the decrease in condition factor from 1.12 to 0.83 was associated with a three-fold decrease in whole body lipid to around 3.7%, moisture increased to 77%, whereas protein remained stable at around 16% (Table 4.2). Condition factor and lipid content among charr fed to satiation were highly correlated in both November and April ($r=0.73$, $P<0.01$).

4.6 Discussion

The problem of sexual maturation among farmed diploid Fraser River Arctic charr can be largely eliminated, it appears from this study, by a winter treatment of 24h light combined with a suppression of somatic growth either by lowering the temperature or withholding feed. The impressive reduction in maturation suggests this combined treatment approach may be equally effective on other salmonid species, since they are believed to share a common mechanism (Taranger et al., 2010), and deserves to be tested on non-salmonids.

The 50% reduction in the incidence of maturation among Arctic charr reared under LL from November to April and fed to satiation (LL10Fed) was similar to the response observed in the same strain of charr reared under LD 18:6 for six weeks starting early

February also at 10 °C and fed to satiation (Duston et al., 2003). That both LL and LD 18:6 caused a similar response confirms the magnitude of photoperiod change is of minor importance (Randall and Bromage, 1998), whereas the timing of photoperiod manipulation is typically of major importance. For example, the efficacy of LL during winter to reduce the incidence of maturity among Atlantic salmon in sea-cages was severely reduced when the start-date was delayed by two or three months, but the end-date remained the same (Taranger et al., 1999; Peterson and Harmon, 2005). Similarly, among Arctic charr, delaying the start of the photoperiod treatment beyond December reduced the efficacy of LL in reducing maturation, prompting us to adopt a November start-date in the present study. Also, extending the end-date of LD 18:6 (start early Jan.) reduced the efficacy of preventing maturation in rainbow trout (Duston and Bromage, 1988). By contrast, comparing the present results with Duston et al. (2003) distorts the picture since the similar 50% reduction in maturity in Arctic charr occurred despite large differences in the timing of the respective long photoperiod treatments. Nevertheless, a general hypothesis to explain these responses, first proposed by Thorpe (1986), is that certain individuals failed to mature because their somatic growth rate and/or energy reserves were below a genetically determined threshold at a specific time of year. The duration and timing of this 'decision period' is shifted by photoperiod manipulation, which ultimately alters the incidence of maturation (Duston and Bromage, 1988; reviewed by Bromage et al., 2001; Taranger et al., 2010). This hypothesis is supported by the present data, since suppression of somatic growth worked in synergy with photoperiod treatment to reduce maturation.

Reducing the maturity rate of Arctic charr from 90 to 0% in females and <10% in males was the best demonstration yet of the utility of manipulating both photoperiod and somatic growth together to delay puberty in salmonids. The previous four studies using long photoperiod in winter all altered temperature to manipulate somatic growth. Maturation among male Atlantic salmon parr reared 5 °C above ambient was 7.5% under LDN, but under an advanced photoperiod cycle starting February, analogous to LL used here, was 0% (Adams and Thorpe, 1989). Similarly, an advanced photoperiod cycle starting in fall reduced maturation among female rainbow trout to 18 and 40% at either ambient or elevated temperature, compared to >70% under LDN (Wilkinson et al., 2010). Both papers support the hypothesis that photoperiod advanced a cue, or decision period, to which only relatively few individuals were in a suitable physiological state to respond and commence gonadal maturation (Thorpe, 1986). Two studies on 'jacking' among male Atlantic salmon post-smolts also supported the hypothesis that temperature, via its effect on growth, was an enabling factor affecting the magnitude of the response to photoperiod signal, although in both cases LL increased the maturation rate (Fjelldal et al., 2011; Imsland et al., 2014). The 'duality' of a long photoperiod cue in winter, that can either inhibit or stimulate sexual maturation (Schulz et al., 2006), has been reported previously, and can be explained by considering photoperiod history and ontogenetic state (Bromage et al., 2001). Unravelling the neuroendocrine pathways to explain why the same LL photoperiod treatment arrests gonadal development in some individuals, but stimulates it among others remains a challenge (Andersson et al., 2013). Insulin-like growth factor-1, leptin and ghrelin are all implicated connecting somatic growth and gonadal development, but the mechanism remains obscure (Taranger et al., 2010). Our results emphasize the complexities. The seemingly solid link between the suppression of somatic growth and the

decision to commence maturation between treatments, was not evident within treatments, where maturing and immature fish exhibited similar somatic growth during the putative winter 'decision period'.

Initial mean body weight and condition factor were poor predictors of which individual Arctic charr would remain immature in response to the long photoperiod treatment, confirming Duston et al. (2003). Moreover, among the two groups reared at 10 °C and fed to satiation daily (LDN10Fed and LL10Fed), mean body weight increased between four- and five-fold overwinter, and condition factor increased from around 1.0 to 1.4, irrespective of maturity status in the fall. Under these highly favorable growing conditions, the 50% reduction in maturation due to the LL treatment surely cannot be attributed to deficiencies in somatic growth according to Thorpe's model. Similarly, reducing the incidence of maturation in rainbow trout by photoperiod was associated with similar growth trajectories among immature and maturing individuals (Taylor et al., 2008; Wilkinson et al., 2010). We suggest a new two-step gating mechanism to account for the observed results (Fig. 4.5). Step 1 involves the part of the brain that entrains salmonids to the annual or corrective daylength cycle to ensure spawning occurs at a time of year to optimize fitness of the offspring. Photoperiod manipulation can disrupt this entrained system, altering the incidence of maturation. The criteria for passing step 1 are independent of body size, and rather loose, since the 'pass' rate can be 50% or so, for Arctic charr at least. Step 2 is more stringent and can prevent sexual maturation if criteria for body size, food intake, growth rate are not met. Both food deprivation and reducing the rearing temperature from 10 to 5 °C were sufficiently strong suppressors of growth to cause all but a few males to fail step 2. That four months without food largely eliminated sexual

maturation, may seem unsurprising, yet subjecting Fraser River charr to either 6 or 12 weeks of food deprivation overwinter had no effect on the incidence of maturity (Rice, 1999; MacPherson, 2012). More unexpected was that 5 °C overwinter was as effective as food deprivation at eliminating sexual maturation despite the fish being fed to satiation and body weight increasing three-fold overwinter and condition factor increasing from 1 to 1.4, similar to controls.

Food deprivation for 18 weeks at either 5 or 10 °C posed no health threat to the Fraser River Arctic charr likely because initial lipid stores were high (> 10%). Whole body fat at the end of the food deprivation period, 3.6 to 3.8%, was still about 50% higher than wild anadromous charr (>400 g body weight) at the end of winter (Jørgensen et al., 1997), and over three-fold higher than the 1% fat in small lake dwelling charr that suffer considerable overwinter mortality (Rikardsen and Elliott, 2000). The similar whole body protein levels in both fed and fasted groups in April confirm the fish were not exhausted, since protein is catabolized only when lipid depletion is severe (Bar, 2014). Among the unfed fish, the rate of decrease in mean body weight, condition factor and lipid content of unfed fish in the present study was similar to Svalbard Arctic charr around 150 g body weight not fed for 11 weeks at 5 °C (Jørgensen et al., 2013). The tendency of the Labrador strain in our tanks to form an immobile tight school when not being fed, and their passive nature would help conserve energy. This behavior seems to be in marked contrast to the more aggressive Hammerfest strain (Christiansen and Jobling, 1990). Also, the appetite of the Labrador strain in culture was consistent through winter, and returned immediately when they were returned to full ration, whereas the Hammerfest strain exhibited a strong seasonal feeding cycle (Sæther et al., 1996). The compensatory growth following the

return of all groups to 10 °C and full ration in April was predictable based on previous studies (Rikardsen et al., 2000; Imsland and Gunnarsson, 2011). Full compensation by November among immature fish reared at 5 °C and fed overwinter coupled with LL indicates this is the best treatment for many farms to maximize yield. However, LL10Unfed treatment and partial compensation would be the best compromise for farmers in Atlantic Canada supplied with groundwater at 10 °C year-round, as chilling to 5 °C is not cost effective. The partial compensatory growth means delaying time to harvest by a few months. Regardless to what extent these regimes described here can eliminate early sexual maturation, the large variation in growth rate between individual Arctic charr remains a problem for farmers (Duston et al., 2003).

In conclusion, continuous light overwinter combined with a suppression of somatic growth resulted in the most effective combination to date to eliminate the problem of sexual maturation in Arctic charr. We predict other species of salmonid will respond similarly and could be a useful tool to eliminate grilse from land-based culture of Atlantic salmon (Good et al., 2016). Moreover, the interaction between photoperiod manipulation and growth suppression to control maturation shows great promise and deserves closer scrutiny among non-salmonids.

Table 4.1. P values of the main and interaction effects of treatment, maturity state and sex from repeated measures analysis on mean body weight (BW), fork length (FL) and condition factor (CF) of Arctic charr during phase 1: November 16, 2013 to April 1, 2014, and phase 2: April 1 to November, 2014.

Source of variation	Phase 1			Phase 2		
	BW	FL	CF	BW	FL	CF
Trt	0.001	0.001	0.001	0.001	0.001	0.001
Maturity	0.203	0.427	0.305	0.001	0.001	0.021
Trt*Maturity	0.025	0.062	0.571	0.007	0.001	0.263
Sex	0.001	0.001	0.12	0.494	0.004	0.001
Trt*Sex	0.558	0.523	0.971	0.983	0.943	0.901
Maturity*Sex	0.474	0.632	0.927	0.670	0.573	0.700
Trt*Maturity*Sex	0.075	0.542	0.027	0.120	0.407	0.007
Date	0.001	0.001	0.001	0.001	0.001	0.001
Trt*Date	0.001	0.001	0.001	0.001	0.001	0.001
Maturity*Date	0.571	0.593	0.971	0.001	0.001	0.001
Trt*Maturity*Date	0.549	0.760	0.252	0.001	0.001	0.001
Sex*Date	0.179	0.147	0.001	0.076	0.937	0.001
Trt*Sex*Date	0.931	1.000	0.324	0.224	0.001	0.092
Maturity*Sex*Date	0.581	0.424	0.931	0.141	0.106	0.157
Trt*Maturity*Sex*Date	0.001	0.951	0.001	0.181	0.066	0.001

Table 4.2. Arctic charr body weight (BW), condition factor (CF) and whole-body composition both initially (November 1, 2013) and April 1, 2014 after completion of six treatment combinations derived from three factors: photoperiod: natural daylength (LDN) or 24h light (LL); rearing temperature: 10 or 5 °C; feeding: fed to satiation daily or unfed (Mean ± S.E. are shown; n=5).

Treatment	BW (g)	CF	%Body composition (wet weight basis)			
			Protein	Fat	Moisture	Ash
Initial sample	132 ± 5.5	1.12±0.02	16.7±0.27	11.6±0.36	69.9±0.35	1.8±0.06
LDN10Fed	485±91.3	1.53±0.09	17.7±0.93	13.8±0.67	66.6±0.91	1.6±0.15
LL10Fed	539±57.9	1.37±0.02	17.1±0.23	14.2±0.65	66.1±0.62	1.6±0.13
LDN5Fed	395±62.7	1.46±0.06	16.7±0.09	13.5±1.01	67.1±1.15	1.6±0.11
LL5Fed	369±36.8	1.61±0.14	16.3±0.32	14.6±0.74	66.4±0.73	1.6±0.23
LL10Unfed	120±11.7	0.88±0.03	16.6±0.39	3.8±0.87	77.0±0.83	2.2±0.13
LL5Unfed	112±12.1	0.83±0.02	16.3±0.21	3.6±0.57	77.7±0.48	3.6±0.57

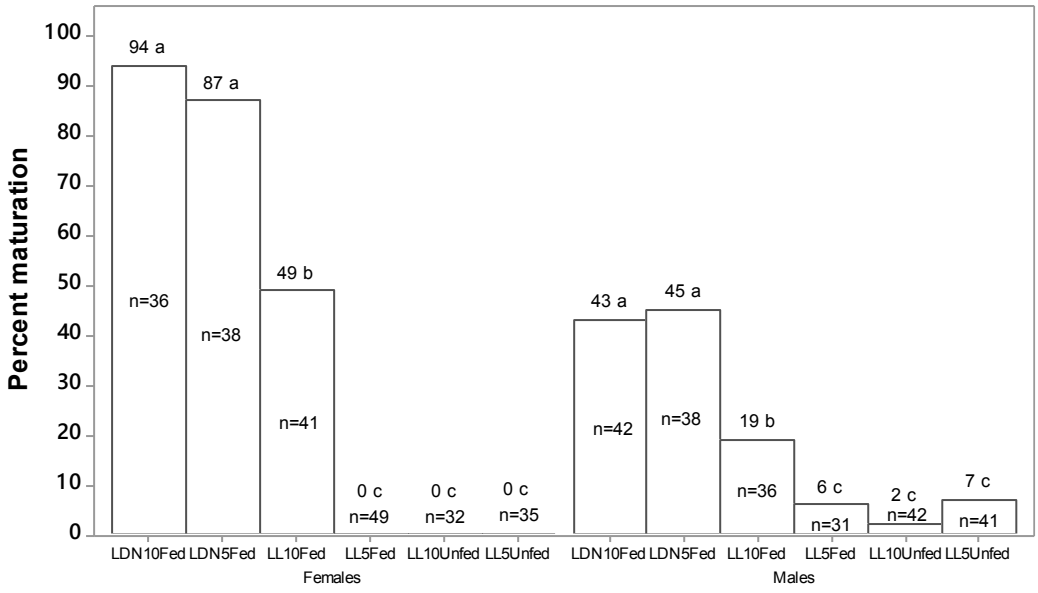

Figure 4.1. Incidence of sexual maturation among Arctic charr in November at 2 years of age following six treatment combinations the previous winter (Nov. 16 to April 1) derived from three factors: photoperiod: natural daylength (LDN) or 24h light (LL); temperature: 10 or 5 °C; feeding regimes: fed to satiation or no food. From April 1 onwards all groups were reared under LDN, 10 °C and fed to satiation. Within each sex, treatments sharing the same letter are not significantly different at the 5% level.

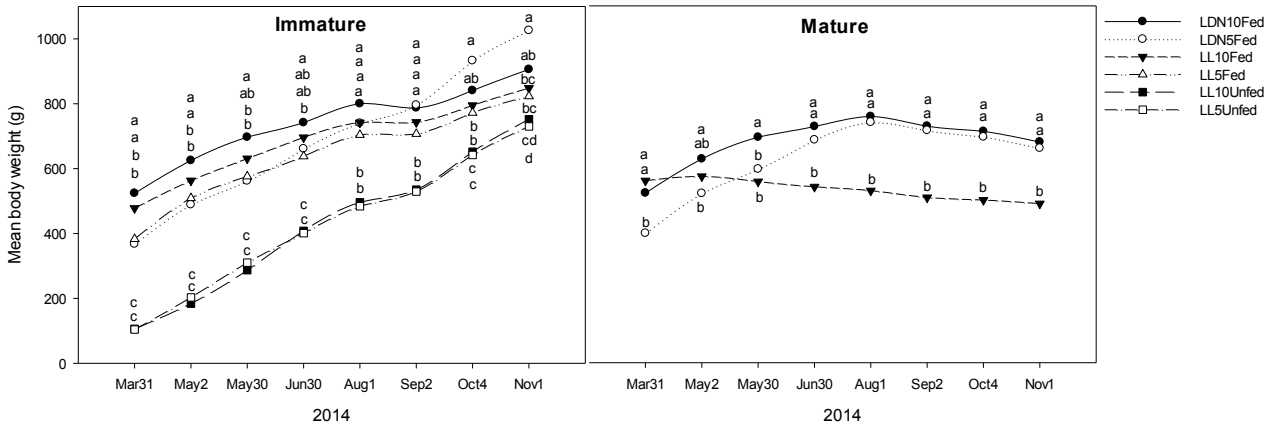

Figure 4.2. Mean body weight of immature (left) and maturing (right) Arctic charr from March 31 to November 1, 2014 (phase 2) under natural daylength (LDN), 10 °C and fed to satiation. Previously from November 16, 2013 to March 31, 2014, six treatment combinations were derived from three factors: photoperiod (LDN or 24h light, LL); temperature (10 or 5 °C); feeding regime (Fed to satiation daily or no food). In the right panel, only three datasets (LDN/10°C/Fed, LL/10°C/Fed and LDN/5°C/Fed) had presented due to insufficient number of maturing fish to be analyzed in other three treatments. Sexes are pooled because its effect was not significant (P>0.05). Means sharing the same letter within each date are not significantly different at the 1% level within each panel.

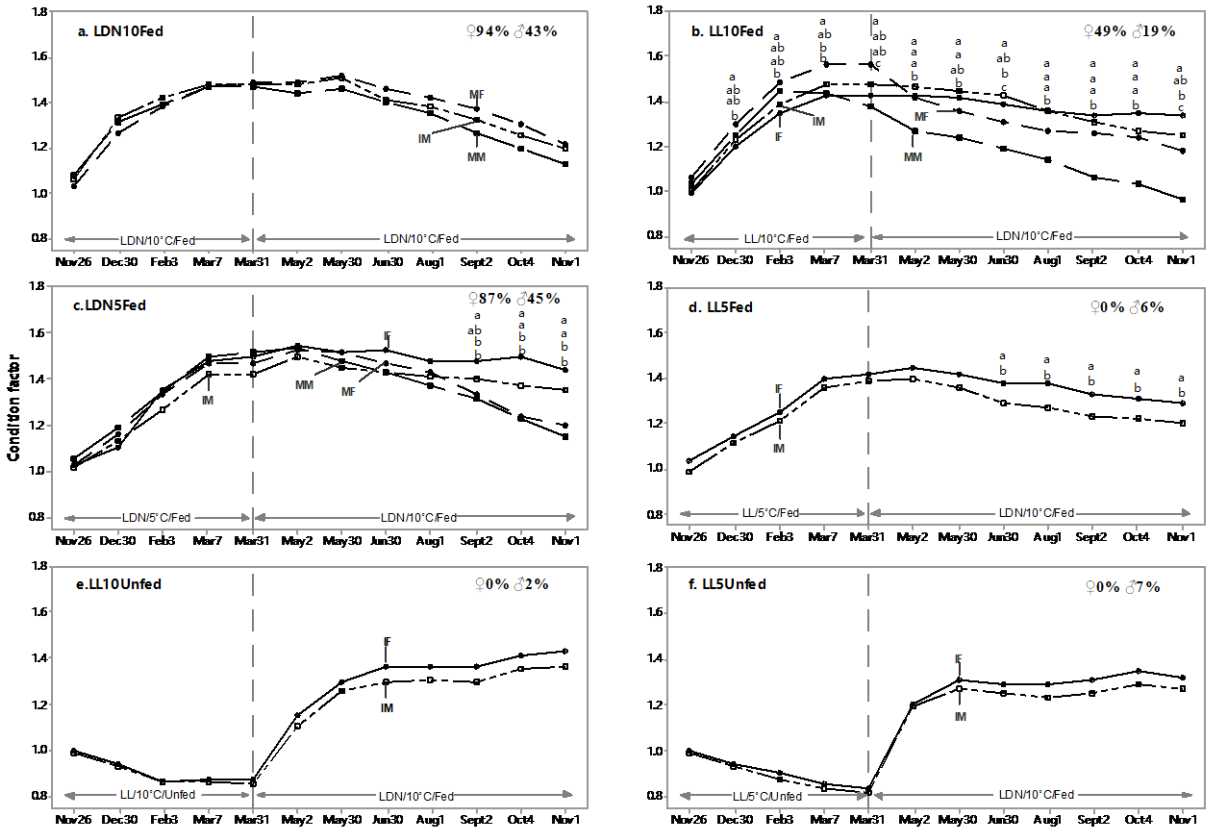

Figure 4.3. Mean condition factor of Arctic charr sub-divided sex (M, F) and maturity (I, M) at 2 years of age. Six treatment combinations were derived from three factors: photoperiod (Natural daylength, LDN or 24h light, LL); temperature (10 or 5 °C); feeding regime (Fed to satiation daily or no food). Treatments started November 16, 2013 and ended on March 31, 2014. Thereafter, all fish reared under LDN, 10 °C and fed to satiation daily. The maturity rate is stated at top-right in each box. Insufficient number of maturing fish in treatments LL5Fed, LL10Unfed and LL5Unfed, and immature female in treatment LDN10Fed to be plotted. Within each treatment, means sharing the same letter at each date are not significantly different at the 1% level. Where letters are absent, means are not significantly different.

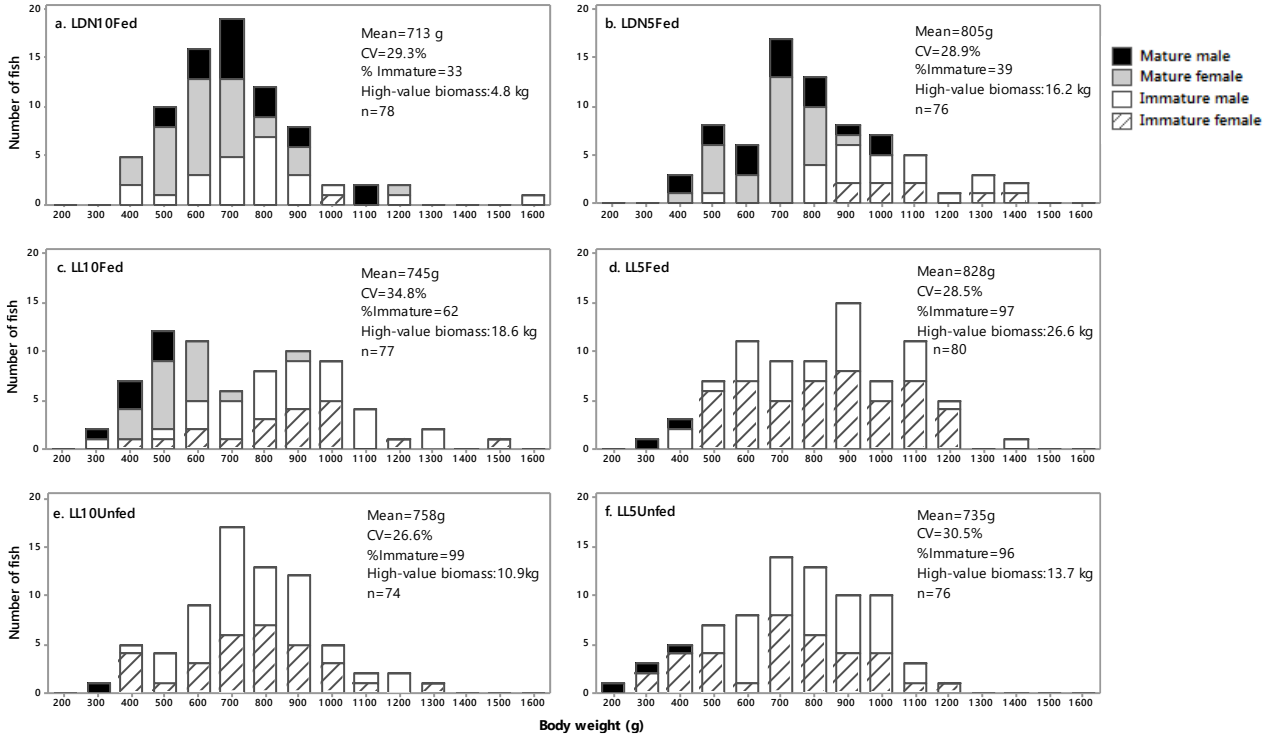

Figure 4.4. Final body weight distribution of Arctic charr at 2 years of age sub-divided by treatment, sex and maturity. Six treatment combinations were derived from three factors: photoperiod (Natural daylength, LDN or 24h light, LL); temperature (10 or 5 °C); feeding regime (Fed to satiation daily or no food). Treatments started November 16, 2013 and ended March 31, 2014. Thereafter, all fish were reared under LDN, 10 °C and fed to satiation daily until November 2014. Displayed within each panel, mean body weight, coefficient of variation (%CV), percent immature fish (pooled sexes), and high-value biomass (sum of >1 kg immature fish weight) are presented.

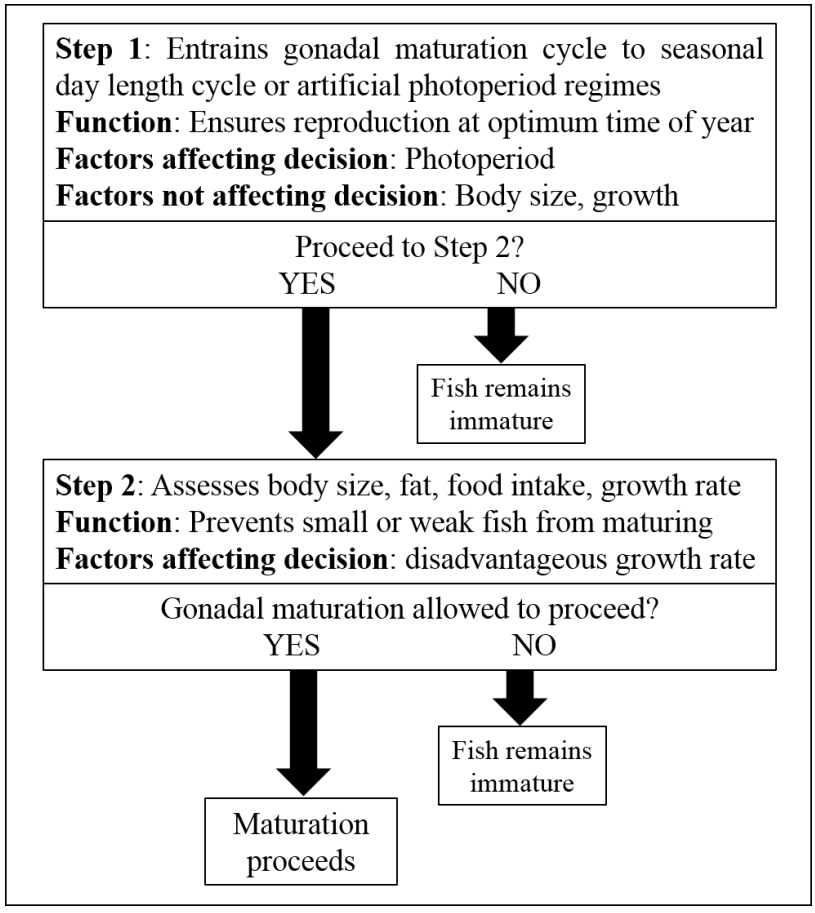

Figure 4.5. A two-step gating mechanism to explain the effects of photoperiod and growth manipulation on the 'decision' to mature among Arctic charr. In step 1, photoperiod controls the window of opportunity for initiating maturation development and optimizing the reproductive schedule, independent of body size and growth; in step 2, fish further assess their nutritional reserves within the 'decision window' as reproduction is energetically gated, and this process is affected by body size, growth, food availability and lipid reserves.

Chapter 5: Long Photoperiod in Winter is More Effective Than Food Deprivation in Stopping Unwanted Sexual Maturation in Arctic Charr

Status: Published, Aquaculture 501: 213-218 (2019).

5.1 Abstract

Yearling *Salvelinus alpinus* (ca. 30 g body weight) at constant 10 °C were subjected to one of four food deprivation regimes: Fall (7 weeks no food: Oct. 10 to Nov. 30), Winter (8 weeks: Dec. 1 to Jan. 31), Fall + Winter (15 weeks, Oct. 10 to Jan. 31) or fed to satiation daily (control). Each feeding regime was sub-divided between three photoperiod treatments (n=70 fish/treatment) each lasting 15 weeks (Oct. 10 to Feb. 1): either 24 h light (LL), 18 h light:6 h dark (LD 18:6) or simulated natural daylength (LDN). On February 1, all fish were returned to daily feeding and LDN for a further 8 months. The incidence of maturation in October age 2 was reduced seven-fold to < 6%, (sexes pooled) by both LL and LD 18:6 overwinter compared to 43% in the control (LDN and daily feeding), independent of the feeding regime. Fall + Winter food deprivation under LDN reduced female maturity six-fold to 11%, but male maturity was unaffected (♂:27%). Food deprivation under LDN in fall had no significant effect on maturation (♀:52%; ♂:42%) but in winter was more effective, significantly reducing maturity in both sexes (♀:32%; ♂:15%). The changes in mean body weight and condition factor between incipient maturing and immature charr within treatments through fall and winter were similar. From March onwards, however, mean condition factor decreased significantly following the decrease in photoperiod from LL or LD 18:6 to LDN associated with a low incidence of maturation age 2. Neither food deprivation nor LL raised welfare concerns

based on survival, behavior and growth, and the reduction in the maturity was a health benefit.

5.2 Introduction

Photoperiod manipulation and food deprivation are two approaches to address the problem of sexual maturation among pre-market farmed salmonids. Among cage-farmed Atlantic salmon (*Salmo salar*), 24 h light (LL) for several months during their first winter in seawater is used throughout the industry to reduce grilse rates (Iversen et al., 2016). The efficacy of winter photoperiod to reduce maturation in Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) and Arctic charr is strongly dependent on the start and end date (Duston and Bromage, 1988; Taranger et al., 1999; Liu and Duston, 2018). The magnitude of photoperiod change, by contrast, was considered unimportant as a *Zeitgeber* for salmonids (e.g. Randall and Bromage, 1998) until a comparison of LL and LD 18:6 resulted in significant differences in the maturity rate among male post-smolt and adult Atlantic salmon (Fjelldal et al., 2011; Good et al., 2016). Among Arctic charr, LD 18:6 and LL were both effective in reducing the maturity rate, but the two photoperiods were not compared directly (Duston et al., 2003; Liu and Duston, 2018). Animal welfare is potentially threatened when livestock are reared under continuous illumination (Vera and Migaud, 2009), further strengthening the rationale for comparing the response of Arctic charr to LD 18:6 and LL from mid-October to February, an effective timeframe for applying long photoperiod to prevent sexual maturation (Liu and Duston, 2018).

Food deprivation can reduce unwanted sexual maturation among farmed salmonids, but has raised animal welfare concerns (Liu and Duston, 2016; O'Halloran, 2017; Rollin, 2018). A physiological link between body size/lipid reserves during fall-winter and the

decision by an individual to commence gonadal development has been demonstrated by manipulating food intake and dietary lipid content in both Atlantic and Chinook salmon (*Oncorhynchus tshawytscha*; Hopkins and Unwin, 1997; Duston and Saunders, 1999; Shearer et al., 2006; Trombley et al., 2014). Arctic charr, by comparison, were less responsive. Food deprivation in winter (Rice, 1999; MacPherson, 2012) or 50% ration (Imsland and Gunnarsson, 2011) under a natural photoperiod failed to reduce the incidence of maturation. Longer-term food deprivation combined with LL was highly effective, reducing the maturity rate to < 5%, but the relative importance of each factor could not be quantified (Liu and Duston, 2016). Here, the objective was to quantify the relative efficacy of food deprivation and a long photoperiod in winter to reduce the incidence of maturity at age 2, and assess the welfare of the fish in terms of survival, behavior and growth.

5.3 Materials and methods

5.3.1 Fish stock and rearing conditions

Arctic charr (Fraser River population, Labrador strain) were produced by a pedigreed breeding program at Shippagan, New Brunswick (Valorēs). Due to a founding population of only 19 families, the principal objective of the breeding program is to prevent inbreeding depression, with limited scope for selection for late maturity. Fish were raised under LL from first feeding to 10 g body weight (February to June), followed by LD 18:6. On July 27, 2015, about 850 underyearlings (ca. 18 g; a mix of 10-15 full-sib families) were trucked from Shippagan to the Aquaculture Centre in Truro, and reared in a 1200L tank supplied with flow-through well water (9.5-10 °C; oxygen saturation > 80%, total alkalinity of 100mg/L; total hardness of 188mg/L). Photoperiod was simulated natural daylength with no dawn or dusk (LDN, Latitude 45°N), controlled by a computer.

The duration of daylight ranged from 8 h and 43 minutes on December 21 to 15 h and 40 minutes on June 21. Fish were hand-fed to satiation twice daily a commercial salmonid diet (protein 44-45%, fat 24-26%, fibre 1.3%, Corey Feed Mills Ltd., New Brunswick). On August 20, each fish was identified with a Passive Integrated Transponder (PIT) tag inserted into the body cavity. On October 9, 70 fish were randomly distributed among each of 12 lightproof tanks (each 500 L) in two identical recirculation systems. Light intensity was 70-80 lux provided by an incandescent bulb (40 W) positioned about 1 m above the water surface. Temperature was constant 10 °C, maintained by a heater/chiller unit and a high turnover rate of well water to ensure good water quality.

Twelve treatments began October 10, 2015, by combining one of three photoperiods: simulated natural daylength (LDN), 18 h light and 6 h dark (LD 18:6) or 24 h light (LL), with one of four feeding regimes: a. fed daily (control); b. Fall food deprivation (no food October 10 to November 30, 7 weeks); c. Winter food deprivation (no food December 1 to January 31, 8 weeks); d. Fall + Winter food deprivation (no food October 10 to January 31, 15 weeks; Table 5.1). The behavior of the fish was monitored daily during the food deprivation period, seeking signs of distress. All fish were returned to daily feeding and LDN on February 1, 2016.

At monthly intervals between September 2015 and October 2016, each fish was anaesthetized (MS222, 0.15 g/L) to record body weight (BW; to 1 g) and fork length (FL; to 1 mm). During monthly measurement, each fish was also assessed for any gross abnormalities including fin and eye damage, operculum and skin erosion. Finally, in October, all fish were euthanized and dissected to identify sex and maturity status. Fish were classified as either mature or immature based on gonadal morphology and

gonadosomatic index (GSI% = 100 x Gonad weight/Body weight), with a threshold of 1% for females and 0.2% for males similar to the criteria of Adams and Huntingford (1997).

5.3.2 Statistical analysis

The experimental unit was each individually identified fish. Incidence of maturity was analyzed using the CATMOD procedure with the generalized logits response function (SAS 9.4, 2013). When the treatment effect was significant, the proportions were further compared using the contrast statement of the CATMOD procedure. The effect of food deprivation on somatic growth among the four LDN treatments was analyzed using repeated measures (MIXED procedure; SAS 9.4, 2013) as a four-factor factorial: food ration (four levels), maturity (mature or immature), sex (male or female) and date (repeated factor). Within each feeding regime, the effect of photoperiod on body weight and condition factor was analyzed as a three-factor (photoperiod, sex, date) factorial using repeated measures (MIXED procedure; SAS 9.4, 2013). The most appropriate covariance structure for the MIXED procedure was either autoregressive order 1 or compound symmetric structure determined by Akaike's Information Criterion and Schwarz's Bayesian Criterion (Littell et al., 2006). The Kenward-Roger correction was used to prevent Type I error rates from over-inflation (Littell et al., 2006). Significance of least square means pair-wise comparison was claimed at the 5% level. To satisfy the assumptions of normality and constant variance, a cubic root transformation was applied to the body size data.

All procedures were approved by the local animal care and use committee (File # 2015-066).

5.4 Results

5.4.1 Incidence of sexual maturation

The maturity rate was significantly affected by a three-way interaction between Photoperiod* Feeding Regime*Sex ($P<0.01$). Under LDN, among fish fed to satiation, the overall maturation rate was 43%, with female maturity over double that of males (♀72%, ♂27%; $P<0.01$; Group 1, Table 5.1). Fall food deprivation under LDN failed to reduce the maturity rate (Group 2, 46% overall, ♀52%, ♂42%; $P>0.05$; Table 5.1). Winter food deprivation and LDN, by contrast, significantly reduced the female maturity rate from 72 to 32% (Group 1 vs. 3, Table 5.1). Male maturity was reduced from 27 to 15 %, but the difference was not statistically significant ($P=0.21$, Group 1 vs. 3). Fall + Winter food deprivation and LDN (Group 4) further reduced the female maturity rate to 11%, but male maturity was 27%, the same as males fed to satiation daily (Table 5.1).

Both LL and LD 18:6 from October 10 to February 1 were equally highly effective at reducing the incidence of maturation to <10%, independent of food deprivation (Group 5-12; Table 5.1). Within each sex, the number of Arctic charr reaching maturity in Group 5-12 was between zero to four among totals of 29 to 39 individuals (Table 5.1).

5.4.2 Somatic growth and food deprivation (LDN)

A comparison of the somatic growth history between maturing and immature individuals was possible only among charr reared under simulated natural daylength (LDN; Group 1-4, Table 5.1). All eight long photoperiod treatments contained too few mature fish for analysis (Group 5-12, Table 5.1). Under LDN, within each sex, mean body weight (BW) and condition factor (CF) changes between maturing and immature charr were not

significantly different throughout the 12-month trial, hence data were pooled. The highest order effect of food deprivation on mean BW and CF was a three-way interaction, Day*Feed Regime*Sex ($P<0.01$), shown in Figure 5.1. Somatic growth was significantly suppressed by all food deprivation treatments during the fall/winter period. The significant influence of sex on somatic growth was relatively minor, and evident only at the end of the trial (August-October; Fig. 5.1). Following the start of the trial in October the fish fed daily grew fast, mean BW increased from about 30 g to over 200 g by February, and CF increased from 1.06 to 1.30 (Fig. 5.1 left panel). Charr deprived of food for 15 weeks (October 10 to January 31) maintained a mean BW of 32 g, but CF decreased from 1.10 to 0.80 due to mean fork length increasing nearly 2 cm to 16 cm (Fig. 5.1 left panel). Fall food deprivation for 7 weeks (October 10 to November 30) also had no significant effect on mean BW, but CF decreased significantly to 0.87 on December 1. Following the resumption of feeding on December 1, mean BW increased four-fold in two months and CF increased to 1.25 on February 1 (Fig. 5.1 left panel). Winter food deprivation for 8 weeks (December 1 to January 31) resulted in a decrease in mean body weight from 93 to 86 g, the difference was not significant ($P>0.05$), but CF decreased significantly from 1.19 to 0.94 between December and February (Fig. 5.1 left panel).

Food deprivation resulted in no mortality, and no signs of aggression were observed. Skin, eyes and fins all remained in perfect condition. During food deprivation the charr remained in a tight school at the mid-water level, holding stationary by slightly beating of their caudal fins. The fish remained alert and responded in a normal vigorous manner to netting and capture for monthly weighing and examination. When feeding resumed, all fish immediately exhibited normal feeding behavior, they pursued and

ingested pellets in mid-water and at the surface. Among females, full compensatory growth followed 7 or 8 weeks of food deprivation in either fall or winter; by October their mean BW was similar to the controls fed to satiation throughout, between 750 and 800 g (Fig. 5.1 left panel). Females deprived of food for 15 weeks exhibited partial compensatory growth, by the end of the trial they were about 250 g smaller than the fully fed control group ($P<0.01$; Fig. 5.1 left panel). Males exhibited only partial compensatory growth following food deprivation, and by the end of the trial, the mean BW of the four groups were all significantly different ($P<0.01$; controls 903 g > Fall food deprivation 796 g > Winter food deprivation 662 g > Fall + Winter food deprivation 550 g; Fig. 5.1 right panel).

5.4.3 Somatic growth and photoperiod effects

The effect of photoperiod on somatic growth was evident as a significant interaction of Day*Photoperiod among all of the feeding regimes ($P<0.05$; Fig. 5.2). Somatic growth was independent of sexual maturation in the control group. The abrupt increase in photoperiod in October, from LDN to either LD 18:6 or LL, had no effect on mean BW and CF compared to LDN controls. By contrast, the abrupt decrease in photoperiod from LL or LD 18:6 to LDN on February 1 suppressed somatic growth and significantly decreased CF from March onwards among daily feeding and fall food deprivation treatment groups (Fig. 5.2a, b, c and d). This effect was one month delayed in the Winter food deprivation treatment (Fig. 5.2f). The magnitude of the reduction in daylength also influenced the changes of CF. Charr subjected to a switch from LL to LDN on February 1 had slightly lower mean CF over summer months compared with the treatment group switching the photoperiod from LD 18:6 to LDN (Fig. 5.2b, f).

5.5 Discussion

The reduction in unwanted sexual maturation was far greater using photoperiod manipulation than food deprivation among diploid Fraser River Arctic charr. The efficacy of photoperiod was independent of the magnitude change of daylength, LD 18:6 vs. LL, confirming previous studies on rainbow trout. The lower maturity rate among female charr deprived of food overwinter reaffirmed the importance of body size or energy stores on the physiological 'decision' to commence sexual maturation. But, paradoxically, the mean somatic growth of maturing and immature charr within a treatment group was very similar, confirming our previous studies. Concerns that animal welfare was compromised by prolonged food deprivation and constant illumination were not evident, but Arctic charr maybe a special case. The health benefits of preventing sexual maturation should be factored into any consideration of welfare issues when rearing salmonids.

LL or LD 18:6 overwinter were equally effective at reducing the maturity rate among age 2 Arctic charr. Similarly, the timing of ovulation of rainbow trout was independent of the magnitude of the change in photoperiod (Randall and Bromage, 1998). Both studies support the hypothesis that the timing of seasonal events in salmonids do not operate on a critical daylength mechanism (Duston and Bromage, 1986). Among Atlantic salmon, the maturity rate exposed to either LL or LD 18:6 was not consistent between post-smolts (LL: 47% vs. LD 18:6: 0%, Fjelldal et al., 2011) and adults (LL: 7.5% vs. LD 20:4 25%, Berg et al., 1996; LL: 27% vs. LD 18:6: 41%, Good et al., 2016). LL-induced high maturity rate among post-smolts possibly resulted from the interaction with a high rearing temperature (16 °C), which significantly accelerated growth and increased the risk of early maturation (Fjelldal et al., 2011). Warm freshwater (15 °C) and constant

photoperiod may also contribute to the variation of maturity rate (Duston and Saunders, 1997; Good et al., 2016). By contrast, no difference was observed between two photoperiod regimes at 5 or 10 °C, in line with the present study (Fjelldal et al., 2011). In our experience, the timing and directional change of photoperiod is key to altering the chronology of sexual maturation and is of great importance in reducing the incidence of sexual maturation (Liu and Duston, 2018).

The efficacy of food deprivation on reducing the incidence of maturity was dependent on its duration and sex of the fish. Among female charr, the age at first maturity was highly dependent on somatic growth between October and February. Fall, Winter and Fall + Winter food deprivation treatments resulted in a step-wise reduction in the proportion of mature females. Long-term food deprivation through fall and winter suppressed somatic growth and changed the maturation 'decision' from maturing to remain immature at that given year among most females. Among coho salmon (*Oncorhynchus kisutch*), food deprivation for 17 weeks in spring (Mar.-Jul.) suppressed both body and ovary weight and increased the incidence of atretic follicles during vitellogenesis (Yamamoto et al., 2011). The arrest of ovarian development was associated with the reduced pituitary gonadotropin content, plasma insulin-like growth factor-1 (IGF-1) and follicular steroid production (Yamamoto et al., 2011). Male charr, by contrast, exhibited relatively low threshold levels of body size and energy reserves to commence testicular development (Adams and Huntingford, 1997; Rice, 1999). Food deprivation failed to arrest testicular development in the present study. It appears that the timeframe for spermatogenesis is more flexible and the process is less sensitive to food deprivation. For instance, no food for six weeks at various time intervals between September and April

only inhibited testicular development temporarily; testis continued to grow after feeding resumed and the age 2 maturity rate in the fall was unaffected (Rice, 1999). Overall, the gender-specific sensitivity in response to food deprivation reflects the sexual dimorphism in energetic investment in reproduction with more energy required to produce eggs than sperm (Adams and Huntingford, 1997).

We are exploring the mechanism by which a long photoperiod in winter prevents sexual maturation among Arctic charr by histological analysis of the oocytes. Preliminary results suggest the 'short-to-long' photoperiod cue in fall is stimulatory to gonadal development, and the subsequent 'long-to-short' cue causes atresia among developing oocytes. In the present study, the long-to-short cue on February 1 was also associated with the significant decrease in mean condition factor and body weight among fish remaining immature age 2 from March onwards, confirming previous work on charr (Duston et al., 2003). Whether the decrease in somatic growth is a cause or an effect of maturation being 'switched off' is unclear. Among female rainbow trout, a 'long-to-short' switch in photoperiod that reduced the maturity rate was associated with a decrease in plasma IGF1 (Taylor et al., 2008). We speculate tracking both plasma IGF1 and oocyte development during the time interval which sexual maturation is switched off by the long-to-short photoperiod cue may reveal the linkage between somatic growth and the decision to commence sexual maturation.

Animal welfare concerns regarding rearing livestock under continuous light are valid for poultry (Schwean-Lardner et al., 2012) but not for Arctic charr we argue, since LL did not result in any negative effect on fish behavior, growth and survival. A positive influence of LL on welfare of farmed Atlantic salmon is recognized due to its effect on the

maturity rate (European Food Safety Authority, 2008). Sexual maturation is a major animal welfare issue among Atlantic salmon in sea-cages, associated with increased aggressive behavior, reduced appetite and immunity, and osmoregulatory stress due to the loss of salinity tolerance (Iversen et al., 2016). In Arctic charr, sexual maturation often induces *Saprolegenia* fungal infection causing significant mortalities (Nilsson, 1992; Liu and Duston, pers. observ.).

Among wild anadromous Arctic charr, somatic growth and energy accumulation are restricted to a short summer (1-3 months) of intensive feeding in coastal waters, whereas overwintering in freshwater is characterized by anorexia and energy depletion (Dempson and Green, 1985; Jørgensen et al., 1997). Clearly, this species exhibits an outstanding adaptation to a long-term nutrient poor environment. Food deprivation for 3 months at 5 °C in the lab had no effect on homeostasis and stress levels, indicated by plasma glucose, cortisol levels, liver glycogen and metabolic capacity (Jørgensen et al., 2013). At 10 °C, Fraser River charr not fed for 4.5 months also maintained homeostasis through the utilization of stored lipid, while whole body protein remained unchanged (Liu and Duston, 2016). Negligible weight loss and healthy status in response to winter food deprivation among several studies support the view that prolonged food deprivation is not a welfare issue (Frantzen et al., 2004; Frøiland et al., 2012; Striberny et al., 2015; Cassidy et al., 2018). The criticism that food deprivation is an unacceptable practice for farmed fish demands careful consideration (O'Halloran, 2017; Rollin, 2018). Among Arctic charr in the present and a previous study at 10 °C under the specific experimental conditions in our lab, there was no evidence the fish suffered (Liu and Duston, 2016). But, we must emphasize the risk is temperature and species-specific. For instance, rainbow trout (280 g)

subjected to food deprivation for 4 months at 5-9 °C resulted in a significant weight loss of 22% and decrease of plasma glucose and liver glycogen, although the stress hormone cortisol levels were not affected (Pottinger et al., 2003).

In conclusion, a long photoperiod overwinter from October to February can reliably suppress early sexual maturation among age 2 Arctic charr. The magnitude in the reduction of maturity rate caused by photoperiod manipulation compared with food deprivation indicates that the physiological 'decision' to commence maturation is more photoperiod-dependent. However, food deprivation offers an additional tool to secure the success of photoperiod manipulation in the case where LL shows unpredictable efficacy. Further studies are needed to elucidate the neuroendocrine control of photoperiod and nutritional factors in regulating age at first maturity in Arctic charr.

Table 5.1. Incidence of sexual maturation among two-year-old Arctic charr in October 2016. Between October 10, 2015 and February 1, 2016 (15 weeks), each of the 12 groups was subjected to one of three photoperiod regimes: simulated natural daylength (LDN), 24 h light (LL) or 18 h light:6 h dark (LD 18:6) and four feeding regimes: daily feeding, food deprivation either in the Fall (7 weeks, October 10-November 30), Winter (8 weeks, December 1-January 31) or Fall + Winter (15 weeks, October 10-January 31). From February 1 onwards all groups were fed daily and reared under LDN. Significant differences ($P<0.05$) in maturity rates within each sex between groups and between sexes within groups are indicated by uppercase and capital letters, respectively.

Group	Photoperiod	Feed regime				Maturity rate in October 2016				
	Oct. 10 – Feb. 1	Oct.	Nov.	Dec.	Jan.	Overall %	%♀	Nmat/N	%♂	Nmat/N
1	LDN	Daily Feeding				43	72^{a} A	18/25	27^{ab} B	12/45
2	LDN					46	52^{ab}	15/29	42^{a}	15/36
3	LDN					25	32^{b}	12/38	15^{bc}	4/27
4	LDN	No food				20	11^{c}	3/28	27^{ab}	11/41
5	LL					1	0^{c}	0/34	3^{cd}	1/33
6	LL	7 weeks				3	0^{c}	0/29	5^{cd}	2/37
7	LL			8 weeks		6	8^{c}	3/37	3^{cd}	1/32
8	LL	15 weeks				0	0^{c}	0/34	0^{d}	0/29
9	LD 18:6					6	6^{c}	2/35	6^{cd}	2/34
10	LD 18:6					3	7^{c}	2/29	0^{d}	0/38
11	LD 18:6					0	0^{c}	0/39	0^{d}	0/31
12	LD 18:6					6	0^{c} B	0/36	13^{bc} A	4/30

N_{mat}: number of mature fish; N: total number of fish. Black bar: not fed; Grey bar: fed.

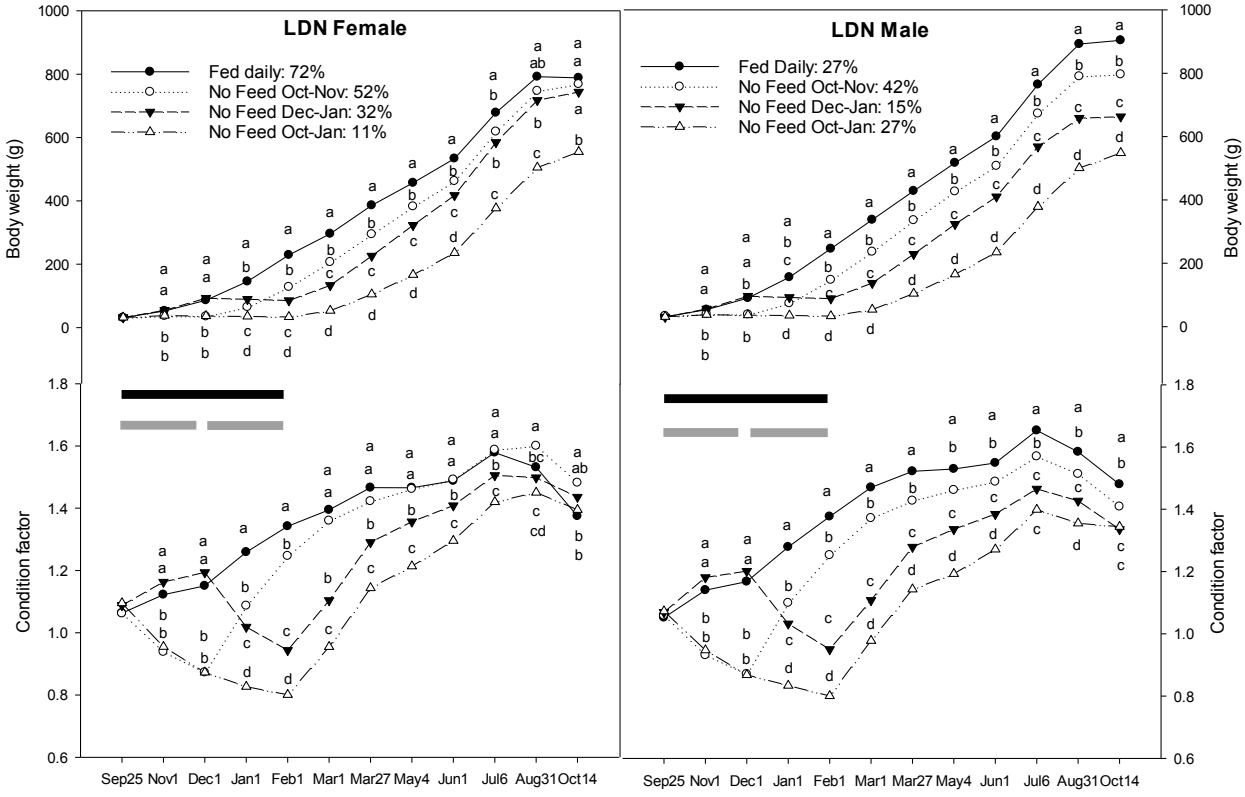

Figure 5.1. Mean body weight (BW, g; Top) and condition factor (CF=100*BW/Fork length^3; Bottom) of female (Left panel) and male (Right panel) Arctic charr under a simulated natural daylength cycle (LDN) and four feeding regimes: daily feeding, food deprivation either in Fall (October 10-November 30; grey bar), Winter (December 1-January 31; grey bar) or Fall + Winter (October 10-January 31; black bar). Maturing and immature fish are pooled since somatic growth was independent of maturation within each treatment. Maturity rate (%) is shown in the top-left of each panel. Means sharing the same letter at each date are not significantly different (P>0.05).

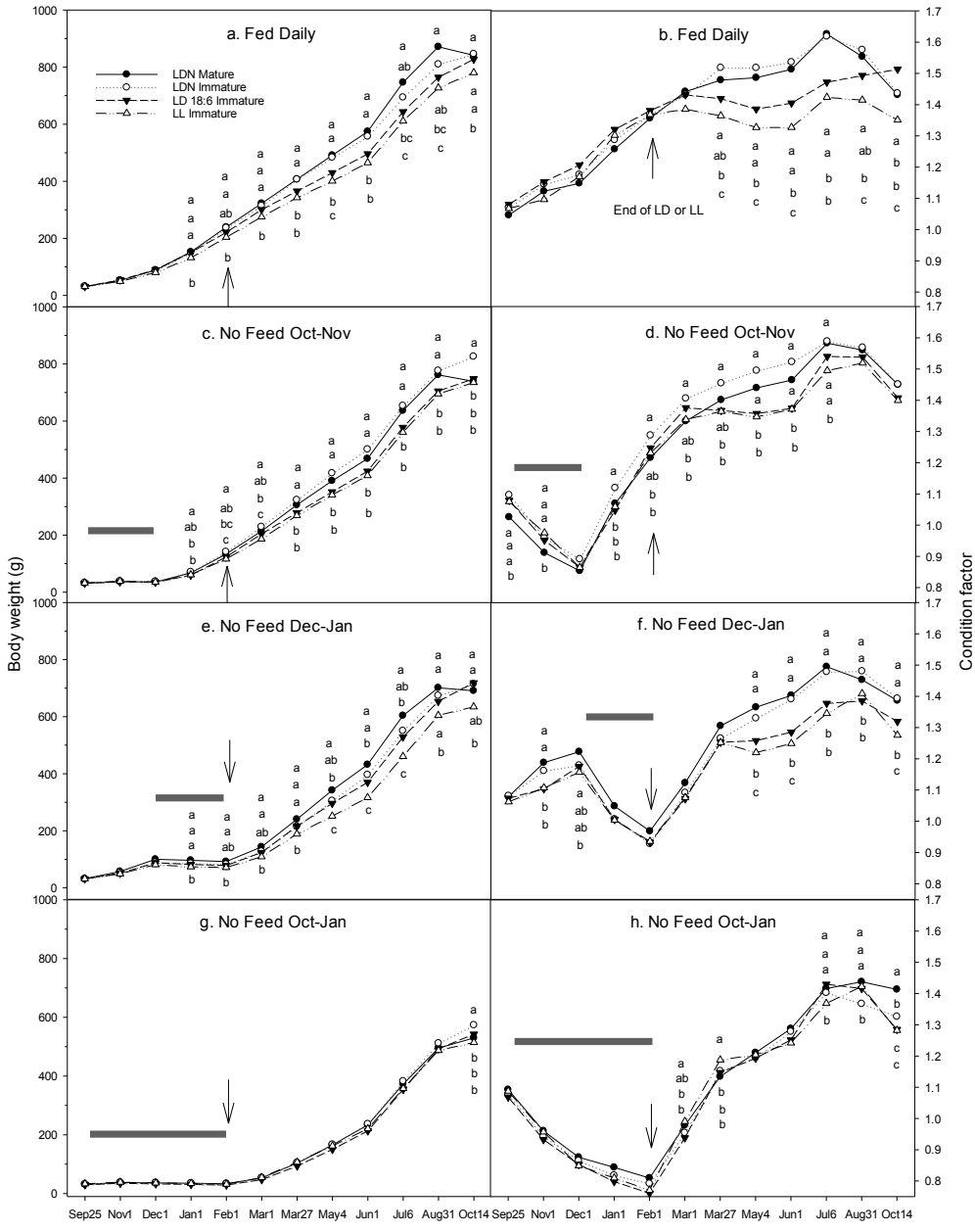

Figure 5.2. Mean body weight (BW, g; Left) and condition factor (CF=100*BW/fork length^3; Right) of Arctic charr reared at 9.5 °C under three photoperiods between Oct. 10 and Feb. 1: simulated natural daylength (LDN), 24 h light (LL) or 18 h light:6 h dark (LD 18:6). The comparison was made within each of four feeding regimes: daily feeding, food deprivation in Fall (October 10-November 30), Winter (December 1-January 31) or Fall + Winter (October 10-January 31). Number of mature fish was only sufficient in the LDN groups for analysis. Arrows indicate the timing of returning photoperiod from LL or LD 18:6 to LDN. Grey bar indicates periods of food deprivation. Within each feeding regime, means sharing the same letter at each date are not significantly different (P>0.05).

Chapter 6: Light Intensity and Suppression of Nocturnal Plasma Melatonin in Arctic Charr (*Salvelinus alpinus*)

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6.1 Abstract

The problem of early sexual maturation among farmed Arctic charr and other salmonids can be effectively reduced by 24 h light overwinter, provided it is bright enough to over-ride interference from the natural daylength cycle. To determine the threshold light intensity to suppress the nocturnal elevation of plasma melatonin, three groups of individually tagged fish (n=26-28/group ca. 1040 g) were reared on 12 h light: 12 h dark (LD 12:12) and subjected to nighttime light intensities of either 50-65, 0.1-0.3 or 0 (control) lux for five months (November to April). Daytime light intensity was 720-750 lux. Diel plasma melatonin profiles in both November and April were similar; mean daytime levels ranged from 20 to 100 pg/ml, and nighttime levels were inversely proportional to light intensity. In the control group at 0 lux, plasma melatonin increased about four-fold after lights-off, ranging between 320 and 430 pg/ml. Nighttime light intensity of 0.1-0.3 lux halved plasma melatonin levels to 140-220 pg/ml, and 50-65 lux further reduced the levels to one quarter of the control group, 68-108 pg/ml. Among the lit groups, daytime plasma melatonin levels were about 20-30 pg/ml, significantly lower than the nocturnal levels suggesting the diel hormonal rhythm was not completely abolished. Fish grew steadily from about 1100 g to 1600 g between November and April, independent of light intensity (P=0.67). Overall, the study demonstrated the sensitivity of pineal melatonin hormone to different light intensities in Arctic charr.

6.2 Introduction

In Atlantic Canada, unwanted early sexual maturation has greatly hindered the commercial farming of Arctic charr (Fraser River strain, Labrador stock). Our goal is to devise lighting protocols to minimize this problem (Liu and Duston, 2016, 2018). Photoperiod manipulation, specifically, continuous light overwinter, is used extensively to prevent early sexual maturation among farmed Atlantic salmon (*Salmo salar*; Iversen et al., 2016). Failure to reduce maturation can occur, particularly in large sea cages, when the intensity of the electric lighting is too low to override the ambient daylength cycle (Hansen et al., 2017). Measurement of the suppression of plasma melatonin is a useful tool to estimate the appropriate lighting requirements (Porter et al., 1999; Skulstad et al., 2013). Here, the objective was to determine the light intensity needed to suppress the nighttime elevation of plasma melatonin in Arctic charr.

Across the vertebrates, the pineal gland transduces light information into a hormonal melatonin signal reflecting the prevailing photoperiod, with circulating titers high during the night and low during the day (Falcón et al., 2011). Arctic charr, the northernmost freshwater fish, is no exception, the oscillation of plasma melatonin conforms to the extreme seasonal change of daylength in the circumpolar regions (Strand et al., 2008). Wild Arctic charr under thick ice in a sub-Arctic lake were able to track daylength despite very small changes in irradiance; melatonin was suppressed by intensities above a threshold of $1\text{-}10^{-2}$ to $1\text{-}10^{-3}$ W/m^2 (Strand et al., 2008; about 0.25-2.5 lux based on Thimijan and Heins, 1983). The Fraser River Arctic charr also overwinter under thick ice in Labrador (Dempson and Green, 1985; Latitude 56 °N). Here, two nighttime light

intensities were compared: 50-65 and 0.1-0.3 lux, at the extreme of what is hypothesized to suppress nocturnal melatonin secretion, against a 0 lux 'black-out' control group.

6.3 **Materials and methods**

6.3.1 Rearing conditions and photoperiod treatments

Arctic charr (Fraser River stock, Labrador strain) were supplied by a pedigreed breeding program at Shippagan, New Brunswick (Valorēs). In our lab, fish were reared under simulated light cycle (Latitude 45 °N) in a single tank (1200 L.) supplied with well water (10 °C; Oxygen saturation > 80%; total alkalinity 100 mg/L; total hardness 188 mg/L). In late October, two-year old immature fish (n=83, ca. 1040g), each identified with a passive integrated transponder (PIT) tag, were randomly stocked into three identical lightproof fiberglass tanks (26-28 fish/tank; 1.15 m diameter, 0.3 m water depth, 310 L volume, light blue interior) of a six-tank system. Each tank received a flow-through supply (8-10 L/min) of 10°C well water. Fish were hand fed to satiation once daily a commercial salmonid diet (protein 44-45%, fat 24-26%, fibre 1.3%, Corey Feed Mills Ltd., New Brunswick).

On November 7, the photoperiod was increased from 10 h light and 14 h dark (LD 10:14; simulated natural daylength) to LD 12:12 to accommodate the sampling regime. The principal light source in each tank was an overhead fluorescent fixture (T5/865/ECO, General Electric, China; 6 tubes each 54 W; full spectrum) covered with shade cloth, 0.7 m above the water surface. Light intensity at the water surface during the day was between 720 to 750 lux (9.75-10.25 μmoles/sec/m^2). Photoperiod was regulated by a Sunmatch controller with no twilight (Aquabiotech, Quebec). The experimental factor was nighttime

light intensity with three nominal levels: 50-65 (0.68-0.88 μmoles/sec/m^2), 0.1-0.3 (0.0014-0.0041 μmoles/sec/m^2) and 0 lux (control). Nighttime illumination in each tank was provided by two spiral fluorescent bulbs (20 W, Spiralux Vita-lite #1171LN), each mounted separately in a metal can with a 15 cm diameter aperture facing downwards. Light intensity was adjusted by neutral density filters (Lee Filters, Andover, UK). Light intensity was measured at 13 positions at mid-water level using a spherical underwater light quantum sensor (Li-Cor, Model LI193SA; Light wavelength range 400-700 nm). Quantum units (micromoles/sec/m^2) were converted to lux based on Thimijan and Heins (1983) to allow comparison with previous studies, and provide more convenient units for farmers. Fish were acclimated to LD 12:12 and the respective nighttime illumination for three weeks prior to sampling on November 28-29.

6.3.2 Blood sampling and plasma melatonin analysis

The photo-phase of the LD 12:12 regime was from 08:00 to 20:00 h. Non-lethal blood samples were collected at five time points at four-hour intervals: 18:00, 22:00, 02:00, 06:00 and 10:00 h. Sampling at night was facilitated by a dim red beam headlight. At each sampling point, a total of five fish from each tank were randomly selected one at a time and anesthetized (MS-222, Syndel, 0.1 mg/L buffered with sodium bicarbonate). Body weight was measured (to 1 g) and PIT-tag recorded, then a blood sample (about 1 ml) was drawn from the Cuverian sinus close to the heart using a 19 gauge needle and 3 ml syringe, both rinsed with sodium heparin (180 IU/ml; Sigma). To avoid repeated sampling, each bled fish was allocated to a second set of three 'recovery' tanks with the same lighting conditions. After completion of sampling at each time point, the blood was centrifuged at 3000× at 4 °C and the plasma stored at –80 °C until analysis. To determine the repeatability

of the melatonin profiles, the sampling was repeated five months later on April 16-17. Specific growth rate (% day^{-1}) was calculated as $SGR = (e^q - 1) * 100$, where $q = (\ln(W_2) - \ln(W_1))/t_2 - t_1$. W_2 and W_1 are the mean body weight at time t_2, April 16 and t_1, Nov. 28. All procedures were approved by the local Animal Care and Use Committee (File No. 2014-026).

Plasma melatonin was measured using an enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmBH, Germany). The ELISA kit is designed for melatonin measurement in human serum or plasma, and is a competitive assay where melatonin in the sample competes with biotinylated melatonin for binding to a fixed number of antibody sites. The assay was validated for use with Arctic charr plasma by: i) testing a 2× dilution series of a high melatonin sample in phosphate–buffered saline over the range (5.8-186 pg/ml); and, ii) testing a subsample of a pool of low melatonin samples spiked with purified melatonin (Sigma-Aldrich, USA) to a nominal concentration of 200 pg/ml and then serially diluted 2× with the same pool to a level of 6.25 pg/ml. The curves were compared with the human plasma standard curve (3-300 pg/ml) and demonstrated parallelism, dilutional linearity, and a lack of sample matrix effects specific to charr plasma (Fig. 2).

Prior to running the assay, all samples and standards were passed through C18 Reversed-Phase Sep-Pak extraction columns (Waters Corp., USA) and the bound melatonin eluted with methanol. The methanol extract was dried using a Vacufuge™ (Eppendorf, Germany), and the residue resuspended in 0.15 ml water with vortexing. Nocturnal samples were diluted in assay buffer two-fold and were repeated if levels exceeded the highest standard. The ELISA protocol was followed, as per manufacturer's

instructions. Reference controls provided by the kit were within acceptable ranges. All standard and sample extracts were assayed in duplicate.

6.3.3 Statistics

Effect of light intensity (3 levels) and time (5 levels) on plasma melatonin was analyzed using two-way ANOVA followed by Tukey's *post hoc* test. Melatonin data was square root transformed to satisfy the normality and homogeneity of variance requirements for ANOVA. Data from the trials in November and April were pooled since there was no significant difference between plasma melatonin titers at the two sampling dates indicated by paired comparison. Effect of light intensity on mean body weight and specific growth rate (% day^{-1}) was analyzed using ANCOVA using initial weight as covariate. Statistical analyses were performed using Minitab 18.1 (Minitab Inc. PA. USA) and SAS 9.4 (SAS Institute, NC. USA). A probability level of $P<0.05$ was claimed significant.

All procedures were approved by the local Animal Care and Use Committee (File No. 2014-026).

6.4 Results

In the control group, mean plasma melatonin increased over four-fold from 98 pg/ml at 18:00 h (daylight) to 430 pg/ml at 22:00 h, two hours after 'lights off' ($P<0.05$; Fig. 3). During the 12 h night period at 0 lux, the mean plasma melatonin declined, but the change was not significant. Following lights-on at 08:00 h, mean plasma melatonin at 10:00 h was 99 pg/ml, a significant decrease from nighttime levels ($P<0.05$; Fig. 3). Both nighttime illumination treatments significantly reduced melatonin levels by over 50% compared to the 0 lux controls (Fig. 3). Nighttime light intensity of 0.1-0.3 lux halved the

mean plasma melatonin levels to 144-223 pg/ml, and 50-65 lux further reduced the levels to about 68-108 pg/ml. The daytime melatonin levels (18:00 and 10:00 h) of the two groups receiving nighttime illumination were also significantly lower than the control group ($P<0.05$), about 20-30 pg/ml, despite the identical lighting conditions during the day (Fig. 3). For the 0.1-0.3 lux group, the nocturnal plasma melatonin levels, although lower across the time course, increased 7 to 8.6-fold following lights-off, relative to the first daylight sample. In contrast, the 50-65 lux group, with similar low daytime melatonin as the 0.1-0.3 lux group, demonstrated only a maximum 3.4-fold increase in mean nighttime levels of melatonin relative to the first daylight sample. This group exhibited an overall flatter profile in melatonin; all nighttime points are statistically different from the darkened control group.

Mean body weight increased from about 1120-1180 g in November to 1610-1690 g in April, independent of night-time light intensity ($P=0.67$; Table 1). Specific growth rate was similar across the treatment groups, about 0.26-0.28 % day^{-1} ($P=0.63$; Table 1).

6.5 Discussion

The light intensity necessary to suppress a melatonin response in Arctic charr is very low. Illumination above 50 lux at the mid-water level is recommended to ensure the effectiveness of photoperiod manipulation and deserves to be further tested in a commercial setting. Nighttime illumination suppressed the plasma melatonin in Arctic charr dependent on light intensity, confirming previous studies on salmonids (Porter et al., 2001; Taylor et al., 2006; Vera et al., 2010).

The significant suppression of plasma melatonin among Fraser River charr by 0.1-0.3 lux at the mid-water level is similar to the situation of a wild charr under thick ice (Strand et al., 2008). Among brook trout (*Salvelinus fontinalis*), light exposure to 0.2 lux for 1 h at midnight failed to alter both pineal and plasma melatonin, whereas 2 to 20 lux was inhibitory (Zachmann et al., 1992). Acute exposure to light, however, may not reflect the true species light sensitivity since the minimum light intensity to suppress melatonin decreases with the increased duration of exposure (Aoki et al., 1998). Atlantic salmon were less sensitive to light at night than Arctic charr, their nocturnal melatonin was reduced only 50% by high intensity of 400 lux (at surface) in tanks and 340 lux (5 m depth) in a sea-cage (Porter et al., 1999, 2001). These differences in the sensitivity to light may represent species-specific adaptations to the photic conditions of their habitat. Despite the significant suppression of plasma melatonin in response to nighttime lighting, it is unknown whether fish perceive the illumination as moonlight (night) or day. Among both lit groups, daytime melatonin levels were significantly lower compared with the unlit group and their nocturnal phase, suggesting its entrainment of day/night. Whether the diel hormonal patterns can be completely abolished under higher nighttime light intensity should be tested in future studies. A confounding factor is that daylight intensity affects the definition of night or the threshold to suppress nighttime melatonin (Vera et al., 2010; Skulstad et al., 2013). High light intensity during the day appeared to reduce the sensitivity of the salmon pineal organ in response to night illumination (3000-12,000 lux in Porter et al., 1999, 2001), compared with only 200 lux for brook trout in Zachmann et al. (1992) and 750 lux for charr in the present study. This has important implications for setting the effective light treatment in commercial outdoor facility. Increasing the nighttime lighting

or using shade netting would be useful to reduce the interference of ambient light (Cowan et al., 2011).

The hypothesis that melatonin serves as a time-keeping agent to modulate seasonal reproduction in fish has no supportive evidence. The use of continuous light to reduce the maturity rate in Atlantic salmon and European sea bass (*Dicentrarchus labrax*) was associated with a suppression of plasma melatonin, but a causal link was lacking (Porter et al., 1999; Bayarri et al., 2010). Melatonin implantation among Arctic charr, rainbow trout and Atlantic salmon had no effect on seasonal feeding, growth patterns and/or sexual maturation, unlike mammals (Porter et al., 1998; Aarseth et al., 2010; Maiolo et al., 2015). An intra-pineal circadian clock underpinning the pineal melatonin synthesis and release was observed among most studied teleosts such as osmerids, pike and perciforms, but not salmonids (Falcón et al., 1989; Iigo et al., 2007; Strand et al., 2008). Among European sea bass, exogenous melatonin injection inhibited the expression of the gonadotropin-releasing hormone receptors in the brain, but its implantation for four months did not affect the profiles of gonadotropins and steroids during maturation (Servili et al., 2013; Alvarado et al., 2015). In addition, eyes and deep brain photoreceptors also contribute to the light perceptive and cognitive process among non-mammalian species, and the latter has been implicated to control seasonal reproduction in birds (Nakane et al., 2010).

The somatic growth of Arctic charr in the present study was independent of nighttime illumination and altered melatonin titers, confirming previous studies using continuous light (LL) (Bottengård and Jørgensen, 2008; Liu and Duston, 2018). Among Atlantic salmon parr and smolts, reared under LL, their somatic growth rate was independent of light intensity (range: 21-715 lux; Stefansson et al., 1993; Handeland et al., 2013). But in

a sea-cage, somatic growth from April to June was positively correlated with the nighttime light intensity ranging from 27 to 340 lux at 5 m depth under LL from January to June (Oppedal et al., 1997). It was suggested that the stimulatory effect on somatic growth was possibly due to the increase in daylength, rather than light intensity, associated with the alteration of a seasonal pattern of muscle formation (Boeuf and Le Bail, 1999; Johnston et al., 2003).

Overall, the results provide a baseline reference for studying the sensitivity of pineal melatonin secretion in response to nighttime lighting in Arctic charr and establishing the lighting protocol under captive conditions. Future functional studies are required to reveal the light transduction pathways in the brain and understand how fish integrate light information with seasonal reproduction.

Table 6.1. Mean body weight (BW; g) and specific growth rate (SGR; % day^{-1}) among age 2+ Arctic charr among three nighttime light intensities: 0 (control), 0.1-0.3 and 50-65 lux. Photoperiod was 12 h light and 12 h dark (20:00-08:00 h). Daytime light intensity was maintained at 720-750 lux in all three treatments. Values shown are means ± S.E. (n=23/group).

BW (g)	Control	0.1-0.3 lux	50-65 lux
November 26	1126 ± 30.5	1175 ± 42.0	1141 ± 34.8
April 16	1613 ± 42.7	1688 ± 60.7	1610 ± 57.2
$SGR_{Nov.-Apr.}$	0.28 ± 0.015	0.28 ± 0.011	0.26 ± 0.016

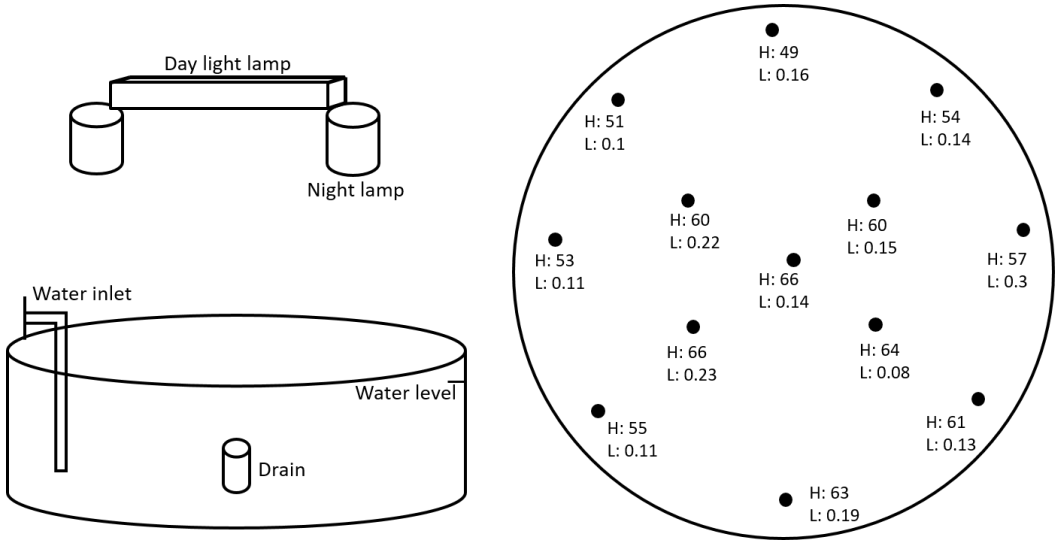


Figure 6.1. Left: Tank and light sources layout. The fiberglass tank was 1.15 m diameter, water depth 30 cm, and the interior was light blue. The distance between night lamp and the tank floor was 79 cm. Right: Plain section of the tank showing nighttime lux readings of high (H) and low (L) light intensity treatments measured at the mid-water level at 13 positions. The control treatment was in complete darkness at night (0 lux).

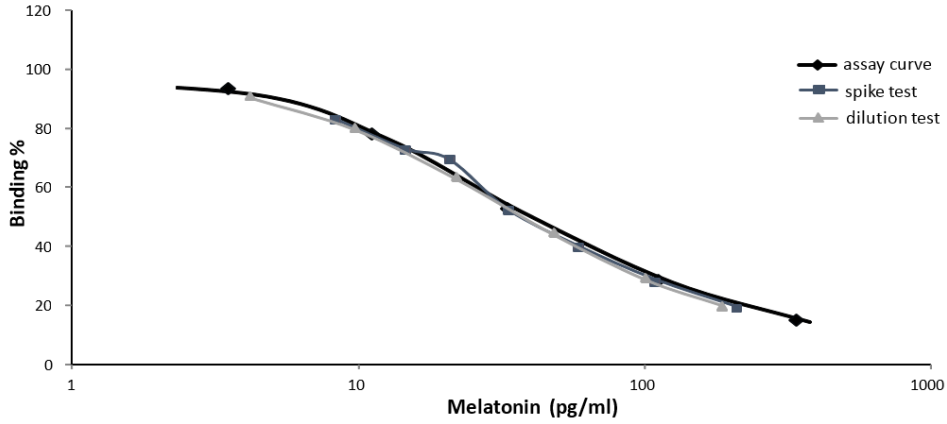


Figure 6.2. Validation of the commercial melatonin ELISA assay for Arctic charr plasma by the dilution tests with high endogenous melatonin and spiked plasma in comparison with the human plasma standards supplied with the kit.

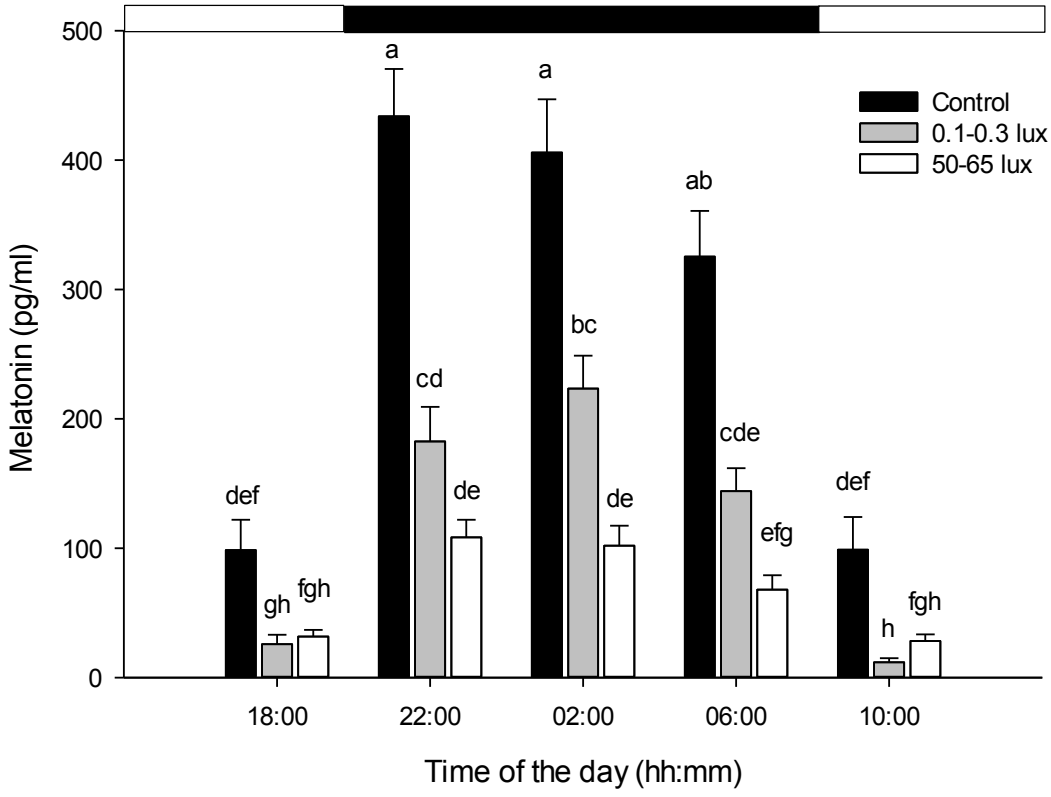


Figure 6.3. Plasma melatonin levels of Arctic charr under three nighttime light intensities: 0 (control), 0.1-0.3 and 50-65 lux, sampled in November and April (data pooled). Photoperiod was 12 h of light and 12 h of dark (20:00-08:00 h). Daytime light intensity was 720-750 lux in all three treatments. Means (S.E.; n=10) sharing the same letter are not significantly different (P>0.05).

Chapter 7: Gonadal Germ Cell Development in Response to Photoperiod Manipulation in Arctic Charr

Status: Manuscript in preparation.

7.1 Abstract

To better define the mechanism by which photoperiod manipulation suppresses sexual maturation, gonadal development was quantified by histological analysis among yearling PIT-tagged Arctic charr (ca. 60 g body weight) reared at 10 °C and fed daily under either 1) LDN: simulated natural daylength (control); 2) CLL: constant 24h light (LL) since 1st feeding and throughout the trial; 3) LNO: LL for 11 months from November 1, 2016 to October 2017, or 4) LNF: LL for 3 months from November 1, 2016 to February 1, 2017, followed by LDN until the end. In October 2017 at age 2, the incidence of maturation (sexes pooled) was greatly reduced to 19% by the LNF regime compared with 63% under LDN and 28% in CLL and 40% in LNO groups. Histological analysis was a useful indicator of gametogenesis, whereas GSI failed to detect the early changes of gonadal development. Development of both oocytes and spermatocytes supported the hypothesis the physiological 'decision' period for commencing maturation started in October/November and lasted to April or May, when individuals committed to gametogenesis. The abrupt increase from LDN to LL in November stimulated gonadal development among some individuals, but also inhibited the initiation of sexual maturation among the others. The abrupt reduction from LL to LDN on February 1 further stimulated the completion of sexual maturation among fish that had already started, but also disrupted gametogenesis among some others, associated with a decrease in GSI and an increase in oocyte atresia, hence, the dichotomous response. Predicting which individuals would mature based on initial body size and fat content was impossible. Once maturation had

commenced, significant differences in mean somatic growth and fat content were evident between maturing and immature fish of both sexes.

7.2 Introduction

Among salmonids, the entrainment of seasonal reproduction by an out-of-phase photoperiod cycle is understood (reviewed by Bromage et al., 2001). An unresolved question is the mechanism by which maturation is delayed or arrested by a long photoperiod in winter. Among Atlantic salmon (*Salmo salar*), after the winter solstice, an abrupt switch from natural daylength (LDN) to 24 h light (LL) could be either stimulatory or inhibitory to gonadal development among individuals, termed a 'dichotomous' response (Schulz et al., 2006; Andersson et al., 2013). Both studies concluded that the onset of LL causally arrested the gonadal development that had already started under LDN. The direction of the response was related to body size among females, with larger fish being stimulated, while smaller fish remained immature, when they likely would have matured under LDN (Andersson et al., 2013). The data supported the hypothesis the reduction of incidence of maturation in response to LL or long photoperiod is associated with a shift of a decision period in the fall, which only allows bigger or fatter fish to mature (Duston and Bromage, 1988; Thorpe et al., 1998). Further support was derived from male Atlantic salmon post-smolts exposed to LL; sexual maturation was induced only among larger individuals at elevated temperature which stimulated somatic growth (Imsland et al., 2014; Fjelldal et al., 2018). Fraser River Arctic charr reared in captivity and fed to satiation, by contrast, yielded no support for the 'body size hypothesis' in this thesis. Mean body size and condition factor was a poor predictor of whether an individual would mature or not (Duston et al., 2003; Liu and Duston, 2016; 2018). Taking a new approach, the current

study included lethal sampling through the proposed decision period to allow gonadal development to be quantified by histology, in addition to non-lethal sampling of body size and estimation of fat content using a non-invasive meter (Johansson et al., 2016).

In Arctic charr, the most effective photoperiod regime to suppress maturity at age 2 was LL from November to February which reduced the maturity rate to less than 20%, compared to 70% among controls reared under simulated natural daylength (LDN; Liu and Duston, 2018). To investigate the nature of the dichotomous response by which an LL zeitgeber can either stimulate or arrest maturation, the objective here was to quantify gametogenesis following two signals: the switch from LDN to LL in November then back to LDN in February. Photoperiod history is an essential component of a successful photoperiod regime to prevent sexual maturation, specifically the LDN from first feeding in spring ensures the onset of LL signal in the fall occurs at the correct physiological time (Randall and Bromage, 1998). By contrast, constant LL exposure has been applied throughout the production cycle of both *Salmo salar* and *Oncorhynchus mykiss* in land-based culture which resulted in unwanted maturation and associated downgrade of yield and fillet (Weber et al., 2015; Good et al., 2016). The importance of photoperiod history was examined here by comparing charr reared under constant LL from 1st feeding until the end of trial at age 2, with groups reared under LDN then receiving the direction of change of daylength between LL and LDN.

7.3 Materials and methods

7.3.1. Rearing conditions and photoperiod treatments

Diploid Arctic charr (Fraser river stock, Labrador strain) of mixed sex from 10-15 families were supplied by a pedigreed breeding program at Valorēs in Shippagan, New Brunswick. They were reared under 24 h light (LL) after hatch (February 2016) until shipment to the Aquaculture Centre, Truro on July 5, 2016. Fish were divided into two groups, one group (n=1200) received simulated natural daylength with no dawn or dusk (LDN, Latitude 45°N), the other group (n=400) was maintained under LL until the onset of the trial in November 2016, and for 12 months thereafter. The LL group was illuminated by an LDN photoperiod regime from ceiling lights during daytime. At night, the group was supplemented with a spiral fluorescent bulb lit (20 W, Spiralux Vita-lite # 1171LN) that was permanently switched on, providing down-welling light at an intensity of 60-85 lux at the water surface. The two groups were reared in separate rooms in identical 1200 L tanks supplied with well water (9.5-10 °C; oxygen >80%, total alkalinity of 100 mg/L; total hardness of 188 g/L) and hand-fed once daily a commercial salmon diet to satiation (protein 43%, fat 14%, fibre 3%; EWOS Canada Ltd., British Columbia). In September, each of the 1600 fish were identified with a Passive Integrated Transponder (PIT) tag inserted into the body cavity.

On October 31 2016, both groups of fish were distributed among eight 500 L lightproof tanks in a recirculating system (total volume 5000 L). Fish (n=400) that were reared under LL from first feeding were randomly divided between two tanks, with 80 fish in the non-lethal sampling group and the remaining 320 fish in the lethal sampling group. Similarly, the fish reared under LDN fish (n=1200) were randomly distributed into six tanks with 80 fish stocked in each of three tanks for monthly non-lethal sampling. The other three tanks were each stocked 310-330 fish for monthly lethal sampling. On

November 1, four photoperiod treatments commenced in each set of four tanks: 1) LDN (control); 2) constant LL since July 2016; 3) LL for 11 months from November 1 2016 to October 19, 2017; 4) LL for 3 months from November 1 2016 to February 1 2017 (Fig. 7.1). The simulated natural photoperiod (Latitude 45°N) with no twilight was regulated using a computer system (Delta Controls) with Orcaview 3.30 software. Temperature was constant at 10°C. Each tank was lit by an incandescent bulb (40 W) providing about 70-80 lux at the water surface. Initial average body weight in November was 60 g. The fish were hand-fed a salmonid feed (protein 43%, fat 14%, fibre 3%; EWOS Canada Ltd., British Columbia) once per day to satiation.

7.3.2. Data sampling and analysis

In the non-lethal sampling group, at monthly intervals each fish was anaesthetized (MS222, 0.1 g/L) to record fork length (FL; to 0.1 cm), body weight (BW; to 1 g) and PIT-tag number. Condition factor (CF) was calculated as $CF = 100\ (BW/FL^3)$. Whole body lipid content of each fish was estimated using a microwave transmittance fat meter (non-destructive) that was calibrated for Arctic charr (Fatmeter, Model FM 692, Distell, Scotland). One measurement was taken between the dorsal fin and lateral line and a second measurement between the lateral line and pelvic fin, following the manufacturer's directions. Briefly, the handheld unit houses a microwave oscillator that emits a low-powered wave (frequency, 2 GHz ±20 MHz; power, 2 mW) that interacts with water in the somatic tissues at a given location. Due to the strong inverse relationship between the water and lipid content in fish tissues, the meter converts water concentration to estimate lipid content. The Pearson correlation coefficient was 0.73 ($P< 0.01$) between lipid content measured by the Distell fat meter and whole carcass analysis of 18 Arctic charr using a

high temperature solvent extraction system (ANKOM XT15, USA; AOAC, 2011). About one year later, October 19, 2017, all fish were euthanized and dissected to determine sex, maturity status and gonadosomatic index calculated as GSI% = 100*(Gonad weight/BW).

Lethal sampling was conducted on a random sample of 30 fish from each of the four photoperiod treatments monthly from November 2016 to July 2017. Initial sampling in November 2016 was conducted only among fish in the LDN and constant LL treatments since in the other two treatments only 8 days had elapsed since the photoperiod was increased from LDN to LL. Each fish was euthanized (MS222) then measured for BW (to 1 g), FL (to 0.1 cm), and whole body lipid content (%) using the fat meter. The fish was then dissected, both gonad strands were weighed (to 0.1 g), then preserved in 10% buffered formalin solution (Fisher Scientific) for subsequent histological analysis. CF and GSI were calculated. After the final lethal sampling event in July, between 90 to 100 fish remained in each treatment. These fish were reared to the end of the trial on October 19, 2017 to provide additional data on the final incidence of maturation. Females were classified as 'immature' if the gonads comprised only of primary oocytes (perinucleolus stage) or GSI < 0.5%, and if male gonads were at the spermatogonium stage, with GSI < 0.1%. Fish were classified as sexually mature if gametes were freely expressed, and classified as maturing if females had commenced vitellogenesis with germ cells dominated by yolk stage oocytes or males had at least spermatocytes visible.

7.3.3. Histological analysis

Gonads were processed for histology at the Nova Scotia Provincial Pathology lab, Truro. The fixed gonads were dehydrated through a graded series of ethanol baths, embedded in paraffin wax, then sectioned (5 μm thick) and stained with

haematoxylin/eosin. Gonads from between 7 and 16 females and 6 to 14 males were analyzed per treatment on November 9, December 12, January 18, February 23 and April 12, to cover the fall-winter physiological 'decision' period.

A digital image was taken at three locations selected at random on each paired ovary cross-section using a Leica microscope under 40-fold magnification (DM750; Leica Microsystems, Switzerland). The number of oocytes per field ranged from about 20 to over 300 depending on the oocyte size. The proportion of oocytes within each of seven developmental stages was quantified using ImageJ software (https://imagej.nih.gov/ij/). The seven-stage scale devised for female rainbow trout (Bromage and Cumaranatunga, 1988) was adopted, following previous studies (*Salvelinus alpinus*, Frantzen et al., 1997; *Salmo salar*, Taranger et al., 1999). Stage 1: primary growth, perinucleolus stage; nucleoli located near or along the nuclear membrane (Fig. 7.2 S1). Stage 2: secondary growth, cortical alveolus; cortical alveoli (vesicles) appear in the periphery and later throughout cytoplasm (Fig. 7.2 S2). Stage 3: lipid drop; lipid droplets appear in the perinuclear and later in the periphery region, the zona radiata is formed (Fig. 7.2 S3). Stage 4: vitellogenesis, peripheral yolk granule; incorporation of yolk derived from vitellogenin, appearance of small granules in the peripheral regions of cytoplasm (Fig. 7.2 S4 early and late). Stage 5: yolk granule migration; yolk globules aggregate further and fill the whole central area (Fig. 7.2 S5 early and late). Stage 6: germinal vesicle breakdown, yolk filled structure (Fig. 7.2 S6). Stage 7: ovulation; oocytes are ovulated into the body cavity. For each fish, the diameter of the twelve most advanced oocytes (4 oocytes per image) were measured using the ImageJ software. Atretic oocytes were characterized by shrinkage, irregular shape, rupture of the nucleus, cell vacuolization, fragmentation of the zona

radiata, and degeneration of the yolk (Fig. 7.2). The percentage of atretic cells relative to the total number of follicles was calculated. Data from each of the three images from each female were pooled and means calculated.

Male gonads were examined under 400-fold magnification and stage of development estimated by the appearance and relative quantity of six cell types. The stages of spermatogenesis were characterized according to Rice (1999). Stage 1: primary (type A) spermatogonium; large and distinct cytoplasm with a central round nucleus, less basophilic than secondary spermatogonia (Fig. 7.3 S1). Stage 2: secondary (type B) spermatogonium; reduced size and cytoplasmic and nuclear content (Fig. 7.3 S2). Stage 3: primary spermatocyte; the nucleus is strongly basophilic due to aggregation of the chromatin (Fig. 7.3 S3). Stage 4: secondary spermatocyte; reduced germ cell size and denser nucleus (Fig. 7.3 S4). Stage 5: spermatid, a further reduction in size and the distinctly round shape of the darkly staining nuclei (Fig. 7.3 S5). Stage 6: Spermatozoa, dark concentrations of sperm heads, cytoplasmic elimination (Fig. 7.3 S6). Stage 7: spermiation, spermatozoa lying free in the lumens of the lobules, seminal fluid readily discharged when pressure is applied to the abdomen of fish. Notably, some males displayed ovotestis, with oocytes scattered within testis tissue (Fig. 7.3 Oa, Ob).

7.3.4 Statistics

Incidence of maturity was analyzed using the CATMOD procedure with the generalized logits response function (SAS 9.4, 2013). When the treatment effect was significant, the proportions were further compared using the contrast statement of the CATMOD procedure. GSI and oocyte size were analyzed using the T-test for the November initial sampling as there were only two treatment groups analyzed. For the other

results, one-way ANOVA was used to compare the four treatment groups at each sampling point. Tukey tests were applied for multiple means comparison when significant differences were revealed in the ANOVA. The development of ovarian and testicular stages was analyzed using the Fisher exact probability test due to the small sample size. Among non-lethal sampled fish, the effect of photoperiod treatment on body size, condition factor and lipid content was analyzed using repeated measures (MIXED procedure; SAS 9.4, 2013) as a four-factor factorial: photoperiod (4 levels), maturity (mature or immature), sex (male or female) and date (repeated factor). The most appropriate covariance structure for the MIXED procedure was autoregressive order 1 or compound symmetry structure determined by Akaike's Information Criterion and Schwarz's Bayesian Criterion (Littell et al., 2006). Statistical significance was determined when $P < 0.05$.

All procedures were approved by the local Animal Care and Use Committee (File No. 2016-119).

7.4 Results

7.4.1 Incidence of sexual maturation

The incidence of sexual maturation at age 2 was significantly affected by the interaction of photoperiod*sex ($P<0.01$). Under a simulated natural daylength cycle (LDN control), the overall maturity rate was 63%; females maturing at a significantly higher rate than males, 77 vs. 48% ($P<0.05$; Fig. 7.4). 24h light exposure from the first feeding stage onwards (CLL) significantly reduced the incidence of maturity in both sexes compared to controls, females again maturing at a significantly higher rate than males (overall 28%, ♀ 40% vs. ♂ 15%; Fig. 7.4). The LNO photoperiod regime, 11 months of LL starting

November 1, reduced the overall maturity rate to 40%, with the effect of sex not significant (P=0.37). The most effective photoperiod regime for reducing the maturity rate was LDN up to November 1, then LL for three months to February 1 followed by LDN; the overall maturity rate was 19% with no significant difference between sexes (P=0.46; Fig. 7.4).

7.4.2 Female gonadosomatic index and ovarian development

The change in gonadosomatic index (GSI%), oocyte diameter and stage of development during the proposed decision period between November and April are presented in both Figure 7.5 and Table 7.1. Under simulated natural daylength (LDN) through to late February all females were sexually immature judging from their mean GSI, which remained around 0.16%. Among the three LL treatments, the mean GSI between November 9 and December 12 was not significantly different from the LDN controls (Fig. 7.5, top panel). The diameter of the leading cluster of oocytes and their stages, by comparison to GSI, proved a more sensitive indicator of gonadal development. On November 9, mean oocyte diameter of the LDN group was significantly larger than the CLL sample (388 vs. 330 μm; P=0.01; Fig. 7.5, bottom panel). Oocyte development was marginally, but not significantly, more advanced among charr reared under LDN compared to CLL, 75 vs. 62% at stage 2 (cortical alveolous; 6 out of 8 fish, vs. 8/13; P=0.65), and fewer at stage 1 (perinucleolus 25 vs. 38%; Table 7.1). By mid-December, however, all four groups exhibited a similar mean oocyte diameter (P=0.39), which had increased to about 420 μm, and oocyte development at stage 2 among 71 to 75% of fish (P>0.05). The transition from primary to secondary oocyte growth was clearly evident from November through December, and by mid-January there were significant differences between photoperiod treatments (Fig. 7.5, bottom panel; Table 7.1). By mid-January, it

was clear that both LL treatments that started in November (LNO, LNF) stimulated oocyte development; the mean GSI and oocyte diameter were higher than both the control and CLL treatment groups (Fig. 7.5, both panels). Moreover, oocyte development had advanced to stage 3 (Lipid drop) among 70-81% of females reared under LL starting November 1, compared to only 46% at stage 3 in the LDN controls (Table 7.1). The slowest oocyte development in mid-January was among fish on LL since 1st feeding (CLL), only 22% were at stage 3 (Table 7.1).

Between February 23 and April 12 ovarian development exhibited three important changes. First of all, under LDN, the gonadal growth among females was not significant through to mid-April, mean GSI was around 0.23%. But mean oocyte size increased from about 540 μm on Feb. 23 to 650 μm on April 12, with the oocytes of most individuals at stage 3 (69%) and 2 (23%) in later. One female had advanced to stage 4 (8%; 1/13) with an oocyte diameter of 1070 μm, marked the initiation of vitellogenesis, (Fig. 7.5 bottom panel; Table 7.1).

Secondly, a dichotomous response to LL that started in November was evident. In the LNO group, mean GSI exhibited a four-fold increase between late-February and mid-April (0.23 vs. 0.85%), but between individuals there was a clear divergence in oocyte diameter, either <800 or >1000 μm (Fig. 7.5 bottom panel, April 12). Among the individuals with oocytes <800 μm, the gonadal growth was arrested at the oil drop stage with a mean GSI of 0.2%. A stimulatory effect of LL was evident in 5 of 14 fish, their mean GSI was 2.2% associated with the accumulation of vitellogenin, evident by stage 4 and stage 5 oocytes (stage 4: peripheral yolk granule; stage 5: yolk granule migration; Table 7.1).

The third important change between February and April was evident in the LNF group, the abrupt decrease in photoperiod from LL to LDN on February 1 inhibited ovarian development through April, mean GSI remained ~0.25% and oocyte diameter was <800 μm (Fig. 7.5, both panels). The regressive effect on ovarian development of the switch from LL to LDN on February 1 was confirmed by histological analysis. On Feb. 23, one female that was stimulated by the LDN to LL cue on November 1 exhibited 23% atresia among stage 4 vitellogenic oocytes, indicated by the disintegration of the zona radiata and engulfment of the yolk (Fig. 7.2, early atresia). On April 12, one female exhibited advanced atresia, >90% of oocytes at the vitellogenic stage were atretic, with a highly convoluted basal membrane and the yolk was almost completely reabsorbed (Fig. 7.2, late atresia). On May 11, a further two females from the LNF treatment exhibited mild atresia (ca. 15% of total oocytes) with the vitellogenic oocytes exhibiting an irregular shape and limited incorporation of yolk granules. Among the other three treatment groups that did not experience a decrease in photoperiod on February 1, by contrast, advanced oocyte atresia was not evident (Table 7.1).

Figure 7.6 presents a broader perspective of ovary growth through to completion of maturation in October. Under the control photoperiod, LDN, an increase in GSI was first evident between June and July (Fig. 7.6A). On June 15, only 29% (4/14) of females were judged to be maturing, GSI averaged 1.5%. By late-July, 86% (12/14) were maturing, their mean GSI was 2.5%, compared to < 0.5% among immature fish. By October under LDN, 79% of females were mature with a mean GSI of 10% (Fig. 7.6A). Under the LNO regime, by contrast, the 3-months of LL from November 1 to February 1 that was stimulatory to some females resulted in a mean GSI of females increasing from 2.2 to 6.3%

between April and July compared to LDN controls mean GSI of 0.6-2.5% (Fig. 7.6A vs. C). By October, the mean GSI of mature females in both the LNO and LDN groups was similar, around 10%, but the incidence of maturity in the LNO group was only 54% (30/56), a consequence of the inhibitory effect of the LNO regime. The strongest dichotomous response resulted from LL for three months from Nov. 1 to Feb. 1 (LNF group). Among females in this group, between February and July, 82% (55/67) were immature, with a GSI of <0.5%. The 18% of females sampled between February and July that were judged to be maturing (GSI ranging between 0.5 and 1.2%) was a similar incidence to the overall final maturity rate in October of 21% (9/43; Fig. 7.6D). Notably, one female sampled in mid-April had a GSI of 8.9% and was close to ovulation, an isolated example.

Charr reared under LL since 1st feeding (CLL) exhibited a chronology of GSI change similar to the LDN controls, but the incidence of maturing females was significantly lower. Between April and July under CLL, 17% (9/54) were maturing compared to 34% (19/54) in the LDN controls (Fig. 7.6A vs. B). Under CLL, two fish had very high GSI values, 5.9% (May 11) and 8.0% (July 24), hence the large error bars (Fig. 7.6B), indicating the constant light was stimulatory to some individuals, whereas seven other maturing females had GSI values between 0.5 and 0.9%. The mean GSI of the mature females increased to 8.5% in October (Fig. 7.6B).

7.4.3 Male gonadosomatic index and testicular development

Male Arctic charr exhibited a dichotomous response to photoperiod signals similar to females, but more rapidly, with some stimulated fish producing milt between February and July, several months ahead of controls. Under LDN, between November and April, testicular development progressed gradually from stage 1 (primary spermatogonium) to 2

(secondary spermatogonium; Table 7.2), with the mean GSI remaining unchanged, <0.05%. On April 12, one of the ten males sampled had initiated spermatogenesis, evident by stage 3 primary spermatocytes (Table 7.2). In both mid-May and mid-June, only 6% of males (1/16) were identified as maturing, GSI: 0.1-0.5%. By late-July, by contrast, 69% (11/16) of males were maturing, their GSI increased 11-fold from June reaching about 3.5% (Fig. 7.7A). In October, 48% of males (19 of 40) in the LDN group were mature with a mean GSI of 2.4%.

The stimulatory nature of the switch from LDN to LL on November 1 among the LNO and LNF treatment groups was clearly evident about 6-weeks later. In mid-December, spermatogenesis was initiated among 31% (stage 3, 4/13; LNO) and 45% (stage 3, 5/11; LNF) of the males, at least four months earlier than the LDN controls (Table 7.2). GSI data, by contrast, was less sensitive, indicating only two of the 14 males were stimulated and maturing in each of the LNO and LNF groups (GSI: 0.1-0.4%; Fig. 7.7C, D). By January 18, males stimulated by the LL signal starting November 1 were at stage 4 (2^{nd} spermatocyte) or 5 (spermatid), indicating they had entered the late stages of spermatogenesis (LNO: 5/12, GSI~1.7%; LNF: 2/16, GSI~0.6%' Fig. 7.7C, D; Table 7.2). The LL-LDN signal on February 1 (LNF) played two roles, stimulating the completion of gametogenesis and reducing the incidence of maturation. The stimulatory effect of the LL-LDN signal was evident by 21% (15/70) of the maturing males between April and July which all had produced milt. Histologically, the LL-LDN signal accelerated the transformation of germ cells from spermatocytes/spermatids to spermatozoa and the testis consisted of mainly spermatozoa and a few primary spermatogonia spread at the peripheral area of a cyst. By comparison, the inhibitory component of the dichotomous signal of the

switch from LL to LDN on February 1 was evident by the lower incidence of maturing/mature males sampled between April and July and in October, was 21% and 23%, respectively, significantly lower than the control group (LDN: 48%, 19/40).

Maintaining LL throughout the summer resulted in the advancement in the timing of gametogenesis among 40% of males (24 of 60), their GSI increased from 2.7 to 3.7% between April and July (Fig. 7.7C). Milt was not expressed by these fish indicating functional maturity was not reached. On April 12, histological anlaysis indicated that spermatozoa were enclosed by spermatocytes and spermatogonia within each cyst and not free in the lumen.

Among males reared under LL since 1st feeding (CLL), most exhibited a chronology of gonadal development similar to the LDN controls, matching the females in the CLL treatment. Between November 9 and April 12 the fish exhibited little gonadal activity with a mean GSI of 0.03-0.05% and testicular development at stage 1&2. Testes were stimulated by the constant LL exposure among two individuals. On January 18, germ cells of one male (1/10) were at stage 6 spermatozoa with a GSI of 5.8%, and on February 23, one of the 16 males had advanced to stage 5 spermatids with a GSI of 2.5% (Fig. 7.7B). A low incidence of maturing males was indicated between April and July, 15% (10/64) based on GSI. Among fish that were maturing, the mean GSI increased from 1.8% in mid-June and 5.8% in mid-July. By October, the mean GSI was 2.5% and the maturity rate was 20% (10/48; Fig. 7.7B).

7.4.4 Intersexuality

Intersexuality in gonad morphology was observed for a total of 25 male Arctic charr sampled between November 2016 and April 2017, with oocytes scattered within a testis (Fig. 7.2, Oa, Ob). The oocytes were all at the early perinucleolous stage. In the control LDN group, intersex gonads were observed in only three fish, accounting for 3.6% of the sampled males (3/82). Among the photoperiod-treated groups, the incidence of intersex fish was greater, 11% in CLL (8/71), 14.5% in LNO (8/55) and 10% in LNF (6/58) treatment groups. At the end of the trial in October, only one hermaphrodite was mature/maturing in the LNO group (n=100); the left gonad comprised of enlarged oocytes attached to the anterior end and of whitish testis attached to the posterior end, whereas the right gonad consisted of testis only.

7.4.5 Somatic growth and lipid content relative to sexual maturation

Among the PIT-tagged non-lethally sampled fish, body weight (BW) data was pooled from males and females since the highest order of interaction effect was Date*Photoperiod*Maturity ($P<0.01$), indicating the effect of sex was insignificant. Between November and May the mean body size of immature and incipient mature fish was not significantly different in three of the four photoperiod treatments (LDN, CLL, LNO; Fig. 7.8). In the LNF group, by comparison, a divergence in mean body weight was evident by mid-March indicating the switch from LL to LDN on February 1 advanced the timing of sexual maturation. By May 1 this difference was significant, the maturing fish having a smaller body size (Fig. 7.8D). A similar divergence in body weight became evident in the LNO, CLL and LDN on June 22, August 10 and September 27 respectively (Fig. 7.8). This sequence was also a measure of the stimulatory effect of each photoperiod

regime. Mean body weight of maturing fish at the end of the trial was 623, 600 and 500 g in the CLL, LNO and LNF treatment groups, respectively, 20-40% smaller than immature fish, which exceeded 800 g. Mean CF was similar in immature and incipient mature fish in all four treatment groups throughout the trial (Fig. 7.8). The only difference detected was at the end of the trial under LDN control, mature fish were significantly thinner than the immature fish (Fig. 7.8A).

Whole-body lipid estimates provided further evidence the LNF treatment was the most stimulatory of the four photoperiod regimes in advancing the timing of completion of gonadal development (Fig. 7.9). The LNF treatment was also the most effective at inhibiting sexual maturation, but whole body lipid estimates were a poor predictor of the outcome of the physiological decision to either mature or not. Variation in mean lipid content was mainly due to an effect, rather than cause, of sexual maturation, resulting in the redirection of energy reserves from somatic to gonadal growth in a four-way interaction (Date*Photoperiod*Maturity*Sex; $P<0.01$). Among all four treatment groups, immature charr exhibited similar mean lipid profile during the trial, increasing from about 8% in November to 14% from May onwards. In the control group (LDN), the mean lipid content was similar between immature and maturing fish between November and June independent of sex, thereafter it decreased significantly to about 11% and 9% among maturing females and males respectively ($P<0.05$; Fig. 7.9A). Among charr reared under 24h light from 1st feeding (CLL) the lipid profiles were similar to the LDN group during the initial 6 months, increasing to about 12-14% in May (Fig. 7.9B). The subsequent decrease in lipid among maturing fish in the CLL group was detectable from May 1 onwards, about 7 weeks earlier than the LDN controls, again the males losing significantly

more lipid than females (Fig. 7.9A, B). Among maturing males in both LNO and LNF treatments, the decline in lipid was evident from late-January onwards, several months ahead of the LDN controls, further evidence of the stimulatory effect of the switch from LDN to LL on November 1 on the timing of completion of maturation, among individuals physiologically capable of responding. The switch on February 1 from LL to LDN (LNF group) was an additional stimulatory cue, as evidenced by the rapid decline in male lipid levels reaching a low in late-June, compared to late-September among males in the other three groups. Among maturing females in groups LNF and LNO, the timing of the decline in lipid was several months later than males, and the magnitude of the decrease significantly less than maturing males (Fig. 7.9C, D).

7.5 Discussion

The importance of photoperiod in modulating germ cell development during the fall/winter period that is critical for the physiological decision to mature was demonstrated by the histological analysis of gonads. GSI, by contrast, was less sensitive to characterize gametogenesis. A dichotomous response that gonadal development was stimulated among some individuals but inhibited among others was identified in response to 24h light exposure, confirming early work on Atlantic salmon (Schulz et al., 2006; Anderson et al., 2013). What is more important is the abrupt reduction of photoperiod from LL to LDN also played two roles, either inhibiting gametogenesis or stimulating its completion. The changes of somatic growth and lipid content were independent of the decision of whether to mature or not, but reflecting the stimulatory effect of photoperiod manipulation in a sex-dependent manner.

The dichotomous nature of a LL signal on the gonad means that the development of germ cells was stimulated among some fish, but arrested among others. The mechanism that the LL signal inhibits germ cell development was dependent on the direction of change of photoperiod and stage of gonad. The first switch in photoperiod from LDN to LL inhibited the initiation of sexual maturation, since the germ cells among some individuals did not surpass the previtellogenic and spermatogonial stages. This confirmed Schulz et al., (2006) that a LL signal started in February arrested maturation during the early testicular development and fish remained immature thereafter, compared with the maturing fish under LDN. In the same experiment, but among females, LL arrested vitellogenesis by inhibiting the recruitment of oocytes from oil drop to vitellogenic stages and prevented vitellogenesis (Andersson et al., 2013). LL inhibited the activation of the brain-pituitary-gonadal axis, evident by reduced pituitary follicle-stimulating hormone and sex steroids (Andersson et al., 2013). This differs with the present study among Arctic charr. The arrestment of vitellogenesis and spermatogenesis was mainly detected after the reduction of daylength from LL to LDN in February, disrupting a maturation process that had already started. In a recent study on post-smolt *Salmo salar*, male maturation was initiated by LL and 12°C for six-weeks (started early December), evident by androgen 11-ketotesterone, but arrested subsequently after returning the photoperiod to simulated natural daylength (7-19 h light; Fraser et al., 2019). The differences between studies might be related to the variation of gonadal development and the timing when LL was applied or switched off. In Arctic charr, the combination of both switches in photoperiod that contributed to the overall suppression of sexual maturation was reaffirmed by the incidence of maturation from both the present and previous studies (Duston et al., 2003; Liu and Duston, 2018). Overall, the LDN-LL switch in November inhibited the initiation

of sexual maturation, whereas the LL-LDN switch arrested the gametogenesis that had already commenced.

Oocyte atresia is commonly referred as a mechanism for fine-tuning fecundity and maintaining ovarian homeostasis (Bromage and Cumaranatunga, 1988; Lubzens et al., 2010). The severe atresia observed here indicates that it may also serve as a mechanism for an individual to omit from the reproductive cycle and to ensure the production of quality offspring at the proper time of year. It can be hypothesized that a threshold of germ cell development or sufficient length of vitellogenesis is required for the completion of gametogenesis, especially under an advanced photoperiod. Alternatively, an individual fish would abort sexual maturation to conserve the energy and wait for the next cycle. The indirect evidence comes from salmonid studies that the degree of advancement in the timing of ovulation/spermiation was coupled with the reduction of proportion of mature fish (Duston and Bromage, 1988; Liu and Duston, 2018). In other species, female striped bass (*Morone saxatilis*), for example, condensing photothermal cycle to six months resulted in 50% females (4 out of 8) failing to complete maturation associated with reduced steroid hormones, vitellogenin levels and severe atretic oocytes (Blythe, et al., 1994).

The stimulatory aspect of the directional change in daylength can be interpreted by the photoperiod entrainment of seasonal gonadal maturation. The early arrival of an increasing daylength followed by a decreasing one acts as two synchronizers forcing the adjustment of a putative internal 'clock' that was running behind the time, which orchestrates the germ cells to compensate in development (Randall et al., 1998; reviewed by Bromage et al., 2001 and Migaud et al., 2010). In the present study, male Arctic charr responded to the advanced photoperiod more rapidly compared with females. The duration

for completing spermatogenesis was as short as four months, whereas vitellogenesis required 6 months at least. The process of exogenous vitellogenesis is more complex and requires the uptake of yolk secreted by a liver (Borg et al., 2004). The sex-dependent physiological changes in response to an advanced photoperiod was also demonstrated by the changes in lipid content and body weight, with males declining significantly earlier than females.

Clear evidence was presented of the existence of two critical decision periods that were defined by the seasonal change of daylength. The onset of sexual maturation was marked by the proliferation of spermatogonia in testes and development of oocytes from perinucleolous stage to secondary growth under a decreasing daylength in the fall. The second critical period was indicated by the start of vitellogenesis or spermatogenesis from April onwards under an increasing photoperiod. In Arctic charr, the previtellogenic or spermatognial development which occuring during this period was sensitive to food deprivation and photoperiod manipulation, which added support for the 'decision period' hypothesis (Liu and Duston, 2019; Current study). The duration of the critical periods, however, seems quite long, as evidenced by the variability of germ cell development between individuals. The first histological sign of gametogenesis under LDN was detected in late-April in both sexes, but onset of vitellogensis or spermatogenesis can spread over several months between April and June (Frantzen et al., 1997; Rice, 1999).

The unchanged GSI during the fall/winter period in the present study was, however, contrasted with the progression of germ cell growth obtained from histology, indicating the former cannot accurately reflect the early gonadal development. GSI did not increase significantly until June/July, coincided with the timing of seasonal migration/feeding

pattern in the wild (Dempson and Green, 1985). It is possible that a strategy might be employed by this fish to avoid over-expenditure of reserved energy during the early reproductive cycle when food resource is scarce. Also, the ontogenetic variation among individuals is likely the consequence of adaptation to the extreme fluctuation of environments, which provides an opportunity to further explore the interaction of genotype and environment or epigenetics on maturation (Sloat et al., 2014).

Studies on Atlantic salmon parr or post-smolt maturation have provided evidence that 24h light exposure combined with large body size, or enhanced growth due to high temperature, triggers sexual maturation among males (Imsland et al., 2014; Melo et al., 2014; Fjelldal, et al., 2011, 2018). That individuals with larger body size or higher growth rate can better respond to the stimulation of photoperiod is not disagreed. Whether the inferior growth or size is causally related to the inhibition of a LL signal remains questionable. Among Arctic charr exposed to either LL or LDN, maturing and immature fish shared similar trajectories of growth, condition factor and fat content during early stages of gonadal development were as repeatedly reported (Liu and Duston, 2016; 2019). These findings argue against the life-history models proposed for Arctic charr that the physiological basis for sexual maturation is to attain the threshold levels of fish size, growth rate and/or lipid reserves during the critical decision period (Rikardsen et al., 2004). I speculate that the 'threshold' hypothesis does not apply to the captive conditions of daily feeding and high temperature since surplus energy was supplied by the manufactured diet with high nutrient value and lipid content. Alternatively, the hypothesis stands when fish were deprived from food overwinter (Liu and Duston, 2016, 2019). This discrepancy was reconciled by the two-step gated mechanism that the control of sexual maturation can be

influenced at two levels by growth-independent (step 1: photoperiod) and growth-dependent factors (step 2: growth, food availability; Liu and Duston, 2016).

Salmonids exhibit gonochoristic sexual development whereby the undifferentiated gonad develops directly and only into an ovary or a testis (Devlin and Nagahama, 2002). Hermaphroditism is aberrant but has been observed among salmonid families (Kinnison, et al., 2000). Previously, a wild Arctic charr found in the Scottish Loch possessed both a mature testis and ovary (Fraser, 1997). To my knowledge, this is the first histological evidence for hermaphroditism among juvenile Arctic charr under captivity, where perinucleolus oocytes were dispersed among spermatogonia. A mature intersexual gonad was observed in one fish at age 2 indicating that a charr hermaphrodite can reach maturity. The incidence of intersexuality was relatively high, about 1.6% of the population, compared to 0.1-0.3% found among the wild salmonids (Kinnison et al., 2000). Whether the increased occurrence of intersexual gonads is associated with photoperiod manipulation remains open to further investigation. It is known that photoperiod affects the sex steroid feedback system and hormones, which might lead to disorder in gonadal development (Chiasson and Benfey, 2007; Shao et al., 2013).

To sum up, the current study unified the theory by which the prevention of unwanted sexual maturation among salmonids is dependent on both an abrupt increase and decrease of photoperiod. Identification of the critical decision periods and their linkage with the development of germ cells provided the foundation to better understand the relationship between photoperiod and the brain-pituitary-gonadal axis.

Table 7.1. Arctic charr ovarian development under four photoperiod regimes expressed as a percentage of individual fish with oocytes in each of six stages 1: perinucleolous; 2: cortical alveolous; 3: lipid drop; 4: peripheral yolk granule; 5: yolk granule migration; 6: germinal vesicle breakdown. The mean percentage of atretic oocytes (At) is also presented. Comparisons made only among numbers in bold within each month across all four treatments. Within each month, means sharing the same letters are not significantly different at the 5% level. 'ns' indicates there was no significant difference within a month ($P > 0.05$).

	LDN: simulated natural daylength								CLL: 24h light (LL) from 1st feeding							
Month	1	2	3	4	5	6	At	n	1	2	3	4	5	6	At	n
Nov. 9	25	**75** ns					2.6	8	38	**62** ns					2.2	13
Dec. 12	9	**73** ns	18				3.2	11	14	**71** ns	14				3.7	7
Jan. 18	18	36	**46** ab				3.8	11	14	64	**22** b				3.5	14
Feb. 23	0	40	**60** ns				7.2	10	0	17	**75** ns	8			6.6	12
Apr. 12	0	23	**69** ns	8			7.9	13	0	9	**73** ns	18			6.8	11
	LNO: LL Nov. 1 onwards								LNF: LL Nov. 1 to Feb. 1 then LDN							
Nov. 9	N/A								N/A							
Dec. 12	0	**75** ns	25				5.4	12	17	**75** ns	8				3.8	12
Jan. 18	0	13	**81** a	6			10.2	16	10	20	**70** ab				5.5	10
Feb. 23	0	20	**60** ns	20			6.6	10	0	13	**80** ns	7			5.7	15
Apr. 12	0	7	**64** ns	0	29		7.8	14	0	17	**58** ns	17		8	9.2	12

Table 7.2. Testicular development among Arctic charr under four photoperiod regimes expressed as a proportion of individual fish with testis in each of six stages 1: primary (type A) spermatogonium; 2: secondary (type B) spermatogonium; 3: primary spermatocyte; 4: secondary spermatocyte; 5: spermatid; 6: spermatozoa**.** Comparisons made only among numbers in bold but no significant difference was found (P>0.05).

	LDN: Simulated natural daylength							CLL: 24h light (LL) from 1st feeding						
Month	1	2	3	4	5	6	n	1	2	3	4	5	6	n
Nov. 9	**57**	43					7	**50**	50					10
Dec. 12	38	**62**					8	29	**71**					14
Jan. 18	8	**92**					12	14	**71**				14	7
Feb. 23		**100**					14	17	**75**			8		12
Apr. 12	10	**80**	10				10		**100**					9
	LNO: LL Nov. 1 onwards							LNF: LL Nov. 1 to Feb. 1 then LDN						
Nov. 9			N/A							N/A				
Dec. 12	8	**62**	31				13	9	**46**	45				11
Jan. 18		**70**		10	20		10	8	**77**			15		13
Feb. 23	13	**87**					8	8	**76**			8	8	12
Apr. 12		**78**				22	9	8	**76**				16	12

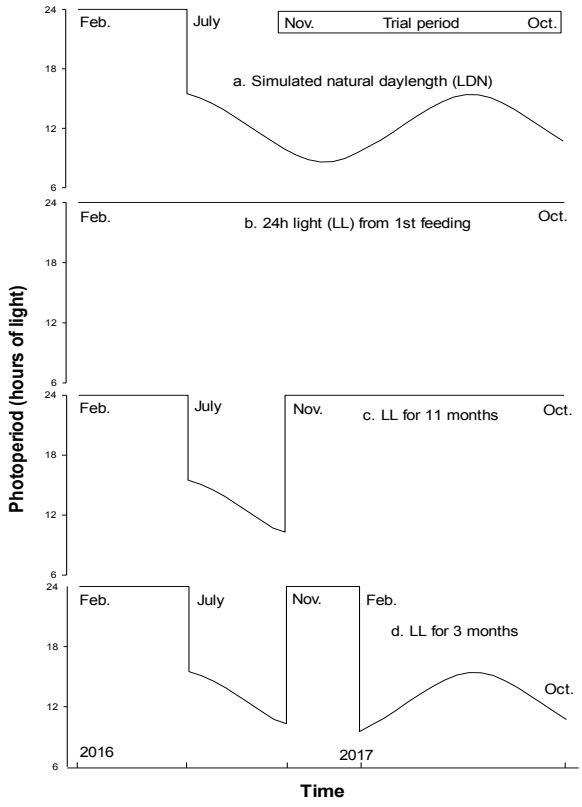


Figure 7.1. Photoperiod history of Arctic charr from 1st feeding through four experimental regimes from November 1, 2016 to October 19, 2017: a. simulated natural daylength cycle (LDN; Latitude 45°N control); b. constant 24h light (LL) throughout two years of life; c. LL for 11 months from November 1 2016 to October 19, 2017; d. LL for 3 months from November 1 2016 to February 1 2017, then back to LDN. Temperature was 10°C.

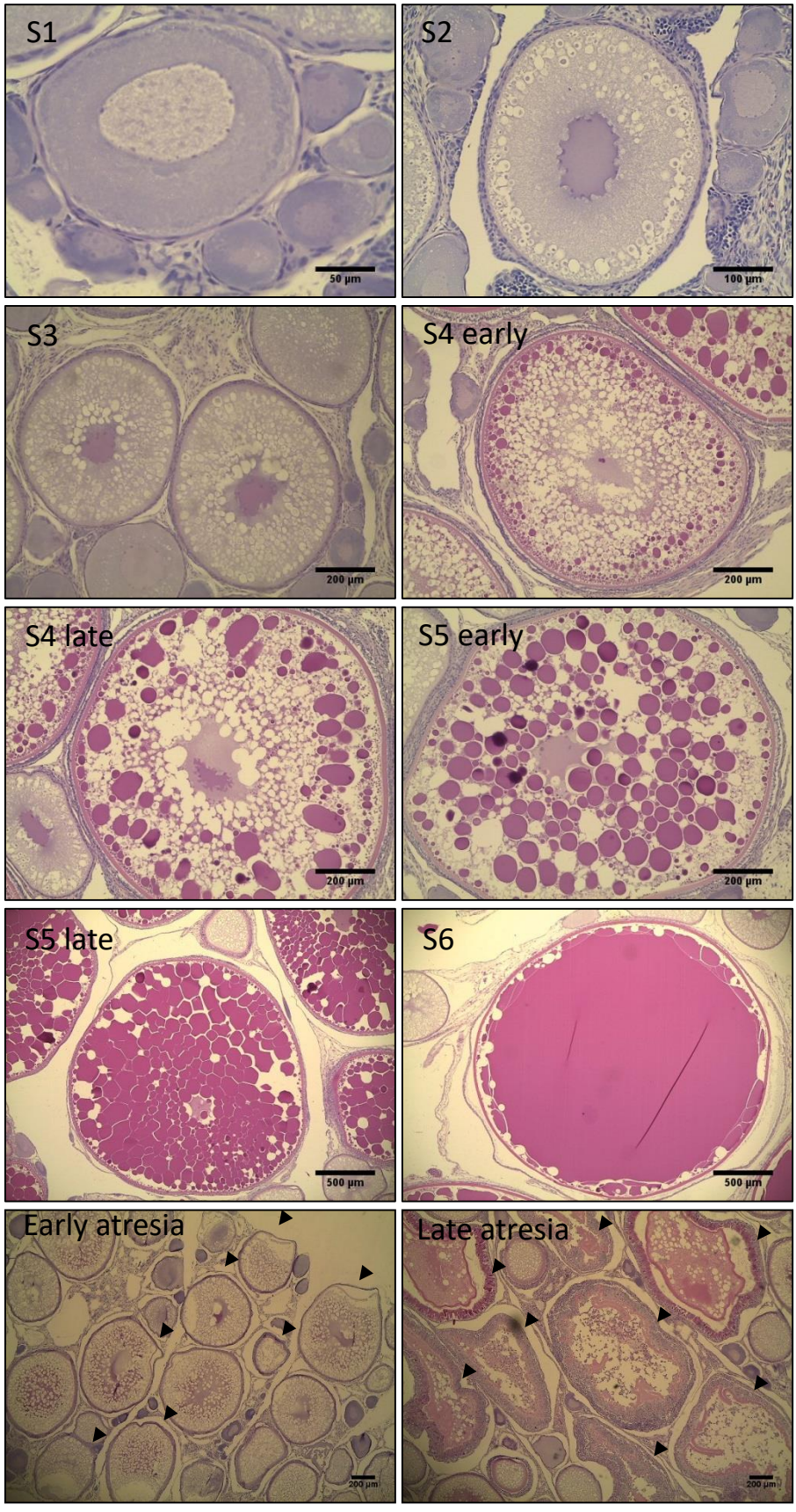

S1
50 µm
S2
100 µm
S3
200 µm
S4 early
200 µm
S4 late
200 µm
S5 early
200 µm
S5 late
500 µm
S6
500 µm
Early atresia
200 µm
Late atresia
200 µm

Figure 7.2. Female Arctic charr germ cell development. Stage 1: early perinucleolus and late perinucleolus stages (S1); Stage 2: cortical alveolus stage (S2); Stage 3: lipid drop stage (S3); Stage 4: early peripheral yolk granule stage (S4 early); Stage 4: late peripheral yolk granule stage (S4 late); Stage 5: early yolk granule migration stage (S5 early); Stage 5: late yolk granule migration stage (S5 late); Stage 6: germinal vesicle breakdown stage (S6); Early and late stage of atretic oocytes, indicated by black triangle. Scale bar of each image is located at the bottom right.

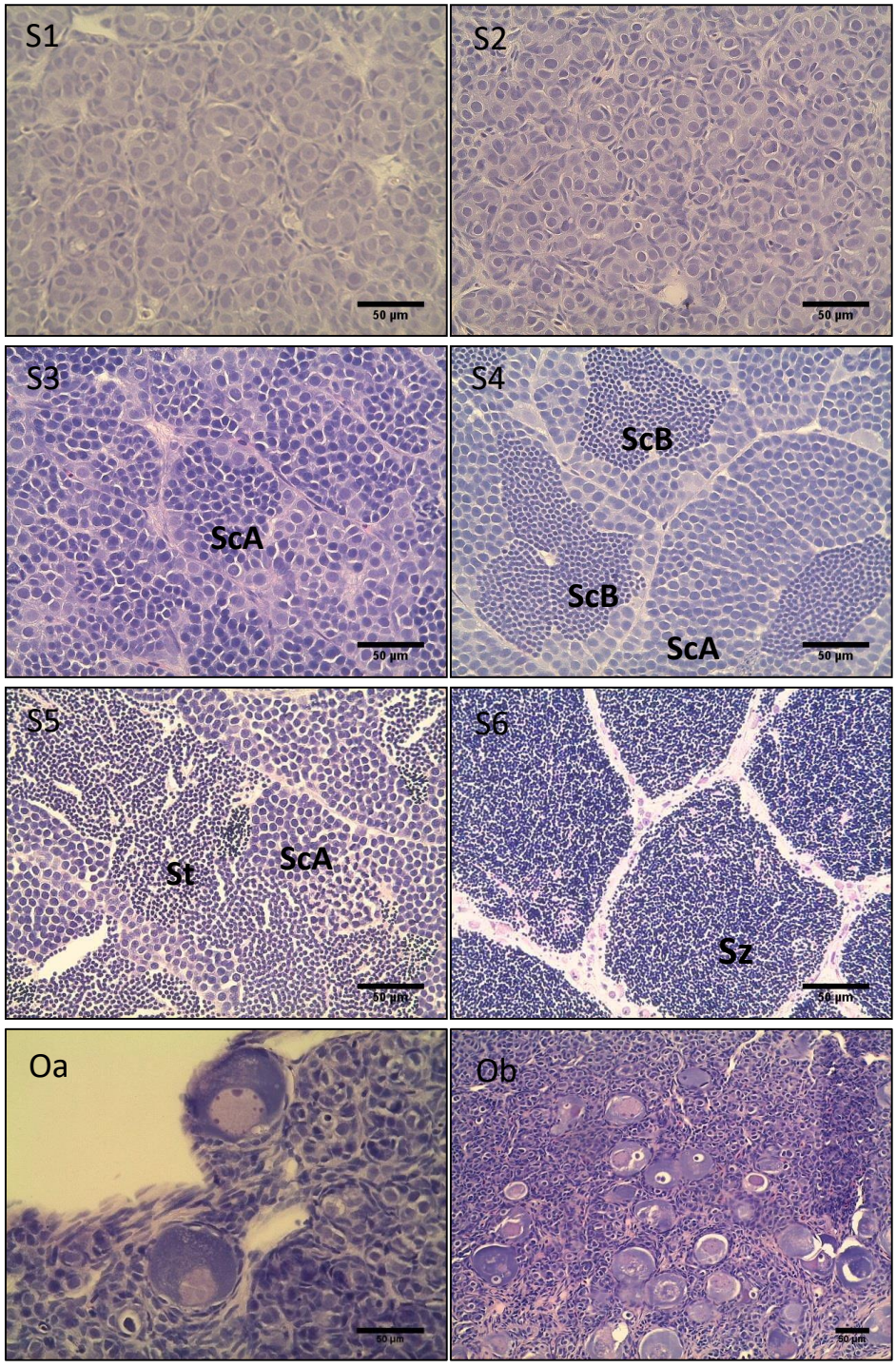


Figure 7.3. Male Arctic charr germ cell development. Stage 1: primary (type A) spermatogonium stage (SgA); Stage 2: secondary (type B) spermatogonium stage (SgB); Stage 3: primary spermatocyte (ScA); Stage 4: secondary spermatocyte (ScB); Stage 5: spermatid stage (St); Stage 6: spermatozoa stage (Sz); Intersex morphology: ovotestis a (Oa, 400×) and b (Ob, 200×): oocytes scattered within testis tissue. Scale bar = 50 µm.

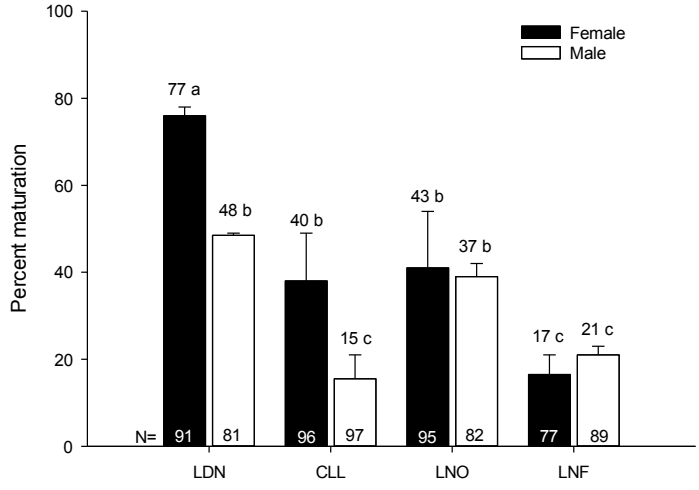


Figure 7.4. Incidence of sexual maturation (mean ± standard error) among female (black) and male (white) Arctic charr at 2 years of age in response to four photoperiod regimes: LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. N is the number of fish in each category. Bars sharing the same letter are not significantly different at the 5% level.

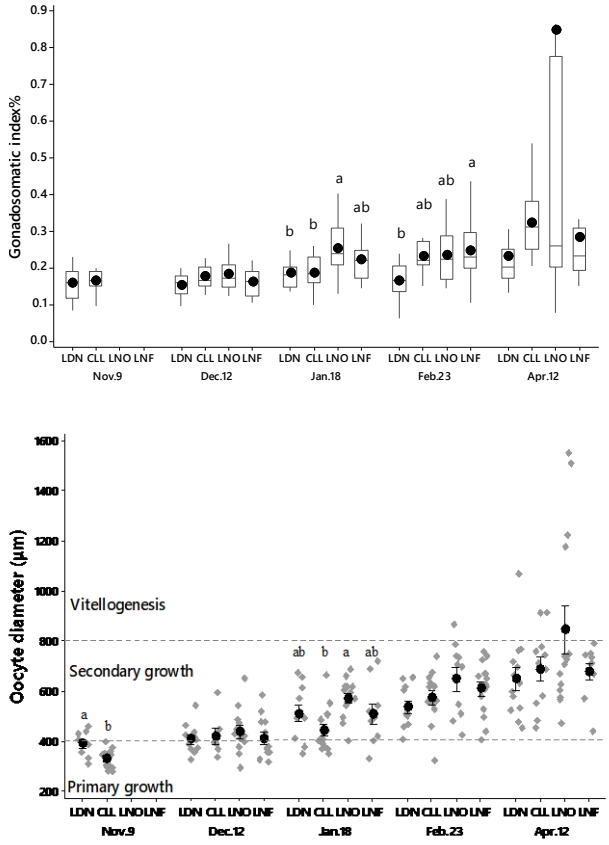


Figure 7.5. Female Arctic charr gonadosomatic index (%; top panel) and diameter (µm; bottom panel) of the leading cohort of oocytes from November 9, 2016 to April 12, 2017. Top: Boxes denote the 25, 50 and 75% quartiles, whiskers show the range. The black circle in each box is the mean. Bottom: Grey diamonds denote to the mean oocyte diameter of individual fish; black circles and error bars show the group mean and standard error. The four photoperiod regimes were LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light (LL) throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. In LNF, one female sampled in April was excluded in the figure with GSI of 8.9% and oocyte diameter of 2460 µm. Within each sampling date, means sharing the same letter are not significantly different at the 5% level.

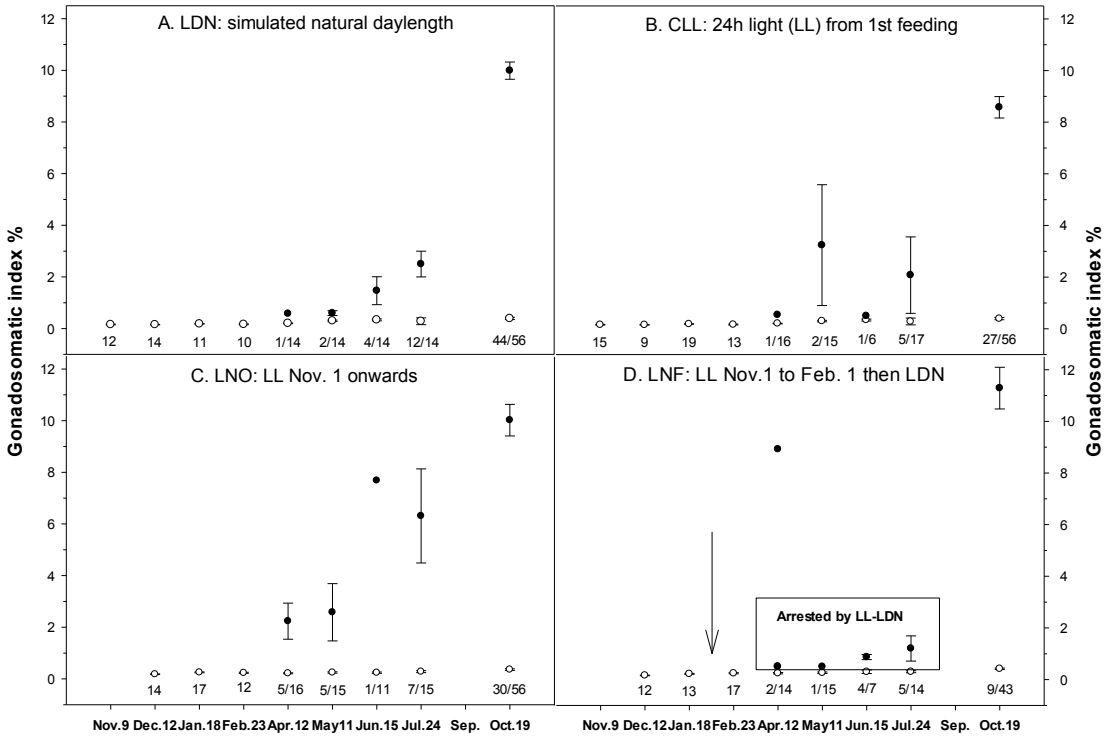


Figure 7.6. Female gonadosomatic index (GSI%; mean ± standard error) comparing maturing (black circle) and immature (open circle) Arctic charr based on the threshold of 0.5% from November 2016 to October 2017. Four photoperiod regimes were LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light (LL) throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. Arrow indicates the timing of an abrupt drop in photoperiod from LL to LDN. The number of maturing fish and sampling size are indicated along the x-axis.

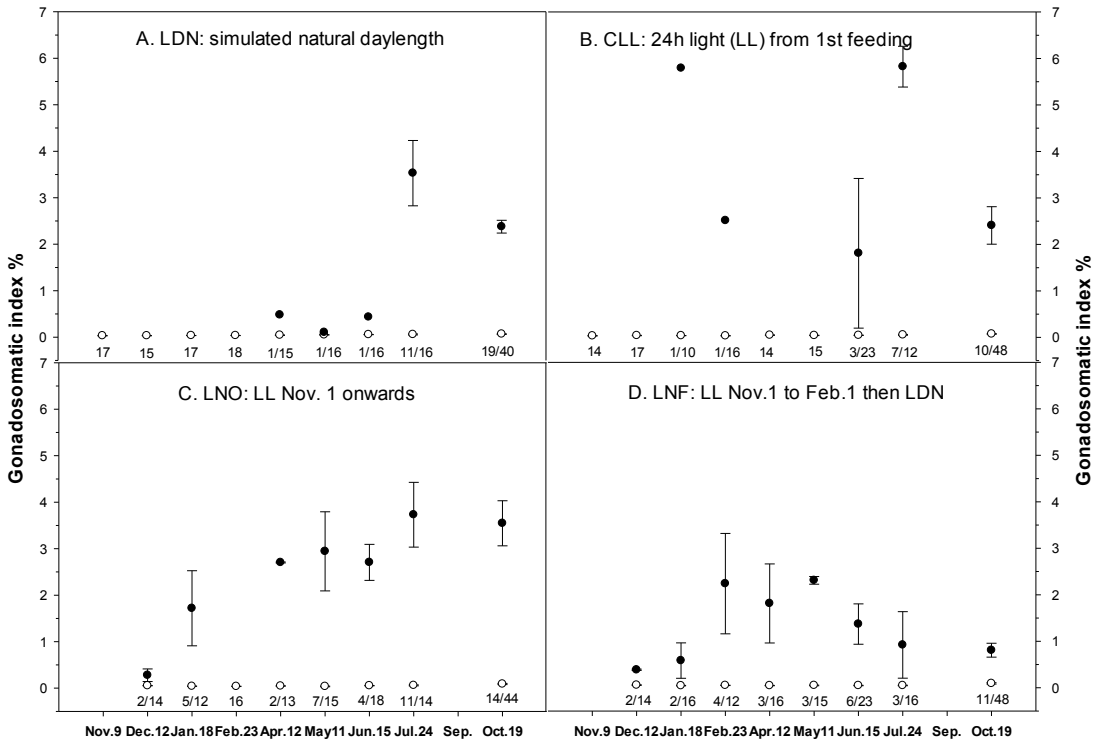


Figure 7.7. Male gonadosomatic index (GSI%; mean ± standard error) comparing maturing (black circle) and immature (open circle) Arctic charr based on the threshold of 0.1% from November 2016 to October 2017. Four photoperiod regimes were LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light (LL) throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. The number of maturing fish and sampling size are indicated along the x-axis.

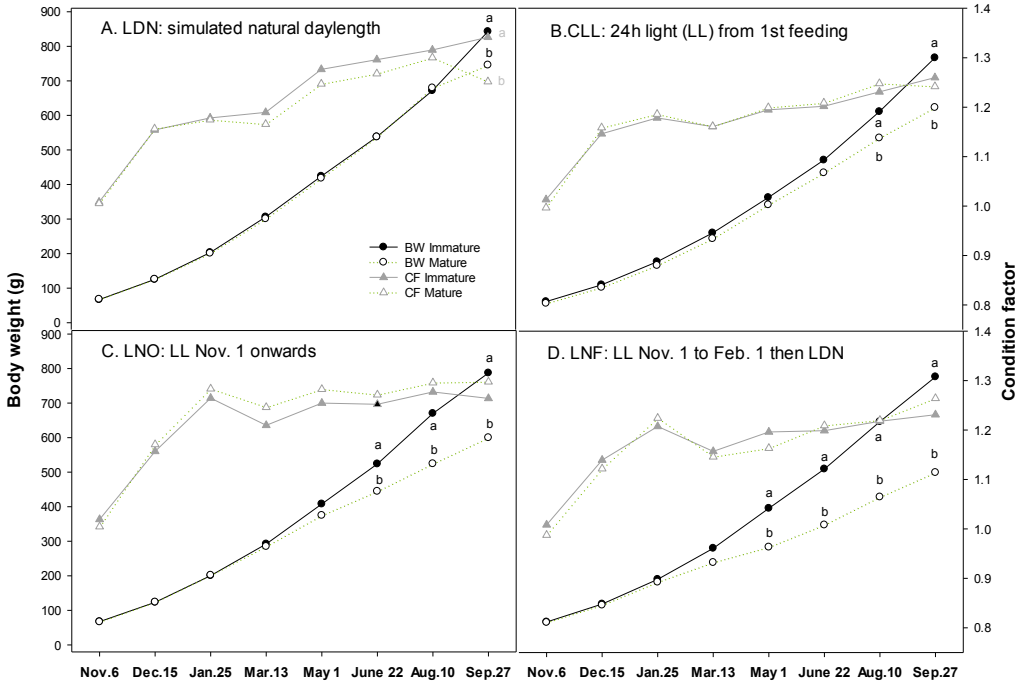


Figure 7.8. Mean body weight (BW; black color) and condition factor (CF=100*BW/Fork length^3; grey color) comparing maturing and immature Arctic charr from November 2016 to September 2017. Sexes are pooled. Four photoperiod regimes were LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light (LL) throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. Within each month and treatment, means sharing the same letter are not significantly different at the 5% level.

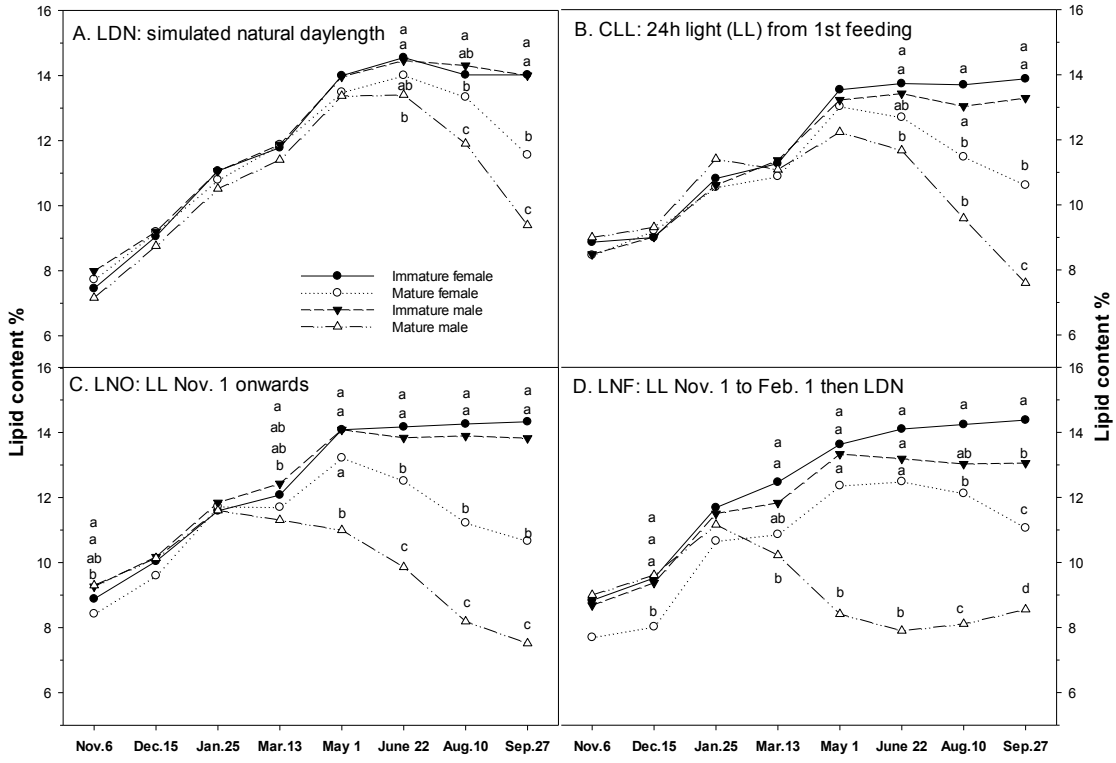


Figure 7.9. Mean whole-body lipid content (%) of Arctic charr non-lethally estimated by the Distell fatmeter. Four photoperiod regimes were LDN: simulated natural daylength (Lat: 45°N); CLL: constant 24h light (LL) throughout two years of life; LNO: LL for 11 months from November 1, 2016 to October 19, 2017; LNF: LL for 3 months from November 1, 2016 to February 1, 2017. Within each month and treatment, means sharing the same letter are not significantly different at the 5% level.

Chapter 8: Testing the Endogenous Circannual Rhythm Hypothesis

8.1 Abstract

The hypothesis an endogenous circannual rhythm governs seasonal reproduction among salmonids is widely accepted but has little supportive evidence. To investigate if Arctic charr possess such an internal 'clock', individually identified adults that had matured for the first time in November 2015 (age 2) were reared from January 2016 in light-proof tanks supplied with flow-through freshwater at constant 10 °C under either constant short photoperiod, 8h light and 16h dark (LD 8:16; 18 ♀, 10 ♂), or continuous light (LL; 14 ♀, 7 ♂). Food was offered daily, but some fish failed to eat, lost weight and did not re-mature. Among the nine females under LD 8:16 that did mature, the timing of ovulation was desynchronized between October 2016 and February 2017, suggestive of a 'free-running' rhythm. Under LL, 11 females matured and became heavily gravid by May 2016 but failed to ovulate. Ovulation was induced in two females by transferring them from LL to LD 8:16, suggesting the completion of maturation requires a short photoperiod cue, seemingly rejecting the endogenous rhythm hypothesis. Among individual males, the period of spermiation under LD 8:16 lasted for 2-4 months and under LL up to 7 months, but a long-term rhythm was not evident. The trial terminated prematurely after 17 months for welfare concerns due to the weight loss among 60% of the fish, possibly due to a dominance hierarchy.

8.2 Introduction

Globally, most habitats exhibit seasonal changes in environmental conditions to which plants and animals entrain their life cycles. In some animals, an endogenous

calendar has been identified which entrains to the annual cycle of daylength to anticipate the time of year and adjust their physiological state. William Rowan (1925) was the first to document this concept through his studies on a bird, greater yellowlegs (*Tringa melanoleuca*), which breeds in Alberta in spring and migrates to South America in autumn, a round trip of up to 26,000 km. Hatch date is very consistent May 26-29. Rowan not only demonstrated that change in daylength provided the cue to regulate reproduction, he hypothesized there was an internal timer or physiological rhythm that kept the birds 'on time' despite the complex changes in photoperiod they experienced during their annual migration (Rowan, 1925). Since then, endogenous circannual rhythms have been reported in reproduction, molt, migration or hibernation among mammals and birds reared under constant environmental conditions (Farner, 1985; Gwinner, 2003). Seasonal changes in daylength, by contrast, only act on the endogenous response system to fine-tune the timing of behavior that is specific to the local ecology (Gómez-Brunet et al., 2008; Helm et al., 2009; 2013). Among teleosts, a circannual rhythm was first implicated in the seasonal growth of brown trout (*Salmo trutta*; Brown, 1946), and later to reproductive cycles among three-spined stickleback (*Gasterosteus aculeatus*; Baggerman, 1957) and catfish (*Heteropneustes fossilis*; Sundararaj et al., 1982). Among other salmonids, female rainbow trout (*Oncorhynchus mykiss*) exhibited the best evidence of an endogenous driven circannual rhythm of sexual maturation (Duston and Bromage, 1991; Randall et al., 1998).

Gwinner (1986) proposed three criteria must be satisfied for a seasonally occurring rhythm to be considered endogenously driven, analogous to the characteristics of circadian rhythms. Firstly, under a constant environment, a circannual rhythm should persist for at least two cycles with a periodicity that is close to, but different from one year. This is

because the self-sustaining 'clock' is progressively drifting away from the calendar year in the absence of seasonal cues and thus the rhythm can be termed as 'free-running'. For instance, exposure of an African stonechat (*Saxicola torquata axillaris*) to a constant equatorial photoperiod and temperature showed that the testicular and molt cycles occur successively for 12 years with an average period of rhythmicity about 9 months (Gwinner, 2003). Secondly, the rhythm can be entrained by an environmental *Zeitgeber*. Photoperiod is the most reliable predictor of seasons away from the equator and is used by many organisms to time seasonal events. Thirdly, the free-running rhythm is temperature compensated. It means that circannual periodicity is unaffected by the differences of experimental temperature, unlike the temperature coefficient Q_{10} property where the rate of physiological processes increases with the increase of temperature. This third property remains controversial because temperature often modifies the 'circannual' hibernation rhythm of ground squirrels (*Spermophilus lateralis*) in a phase-dependent manner (Mrosovsky, 1990; Freeman and Zucker, 2000). The biggest deterrent to studying the circannual rhythms is the long-time frame. Entrainment studies, by contrast, can be completed with a year or so, hence the photoperiod control of the reproductive cycle in salmonids has been widely studied and included Arctic charr (Frantzen et al., 2004; Liu and Duston, 2018; Reviewed by Bromage et al., 2001; Taranger et al., 2010). Interpretation of the data from such studies generally acknowledges the response is underpinned by an endogenous circannual 'clock' without any critical analysis. To address this unsatisfactory state, the current study tested whether the rhythm of sexual maturation in Arctic charr can be self-sustaining when reared under a constant photoperiod.

Among teleosts, evidence of an endogenous circannual rhythm of sexual maturation has been reported for male three-spined stickleback (Baggerman, 1957; Bornestaf and Borg, 2000), female rainbow trout (Duston and Bromage, 1991), catfish (Sundararaj et al., 1982), and sea bass (*Dicentrarchus labrax*; Prat et al., 1999). None of the evidence satisfies Gwinner's three criteria. Among male sticklebacks, nest building was the response variable for tracking a free-running rhythm of reproduction. At 20 °C, under LD 16:8 they (n=14) exhibited up to two breeding cycles at an interval of about 150 days, but under LD 8:16 they failed to mature (Baggerman, 1957). Bornestaf and Borg (2000) indicated the breeding cycles were irregular under constant LD 12:12 or 16:8 at 17 °C, showing patterns of annual, biannual and even continuous nest-building behavior, leading the investigators to question the circannual rhythm hypothesis for seasonal breeding in sticklebacks. Selecting sticklebacks as the experimental animal for circannual studies is perhaps surprising since the life-span is usually less than two years. Among female rainbow trout, the circannual reproductive rhythm was clearly self-sustained under constant LD 6:18 and 9 °C for up to 51 months, but there was large variation in the timing of ovulation between individuals from Nov. to May, suggesting the 'clock' was highly inaccurate (Duston and Bromage, 1991). The periodicity of the rhythm, however, was only about 6 months under LL or LD 18:6, similar with the male stickleback (Baggerman, 1957; Duston and Bromage, 1986). Bi-annual spawning among rainbow trout was related to the observations that some strains spawn twice a year naturally (Colihueque et al., 2015). But why a 'clock' should run at two or more different speeds in response to constant photoperiod remains obscure. By contrast, catfish exhibited a rhythm of ovarian development that self-sustained for two cycles under constant both LL and darkness, but the rhythm was only demonstrated at a population level since individuals were euthanized

at each sampling point to obtain the ovaries (Sundararaj et al., 1982). Similarly, in sea bass two reproductive cycles were reported at the population level under both LD 9:15 and LD 15:9, with the periodicity about 12 months. Moreover, the study was confounded by the annual temperature cycle ranging from 12 to 28 °C, which can be a strong entrainment cue for bass (Prat et al., 1999). Together, these four studies emphasize the rather weak evidence for a circannual reproductive rhythm in teleosts, and thus provide a sufficient rationale for further experimental work.

8.3 Materials and methods

Arctic charr (Fraser River population, Labrador strain) were produced by a pedigreed breeding program at Valorēs (Shippagan, New Brunswick). Fish were raised under LL from first feeding in February 2014 to 10 g body weight in June, followed by LD 18:6 until August when the fish were trucked from Shippagan to Truro, NS. Each fish was identified in August 2014 by a passive integrated transponder tag (PIT-tag) inserted into the body cavity. Afterward, a week of conditioning took place in a 1200 L tank supplied with flow-through 10 °C well water and simulated natural daylength (45°N, LDN). On August 9, 2014, fish were distributed into eight 500 L light-proof tanks (90 fish/tank) in a recirculation system (total volume 5000 L) for a photoperiod manipulation trial (Aug. 2014-Oct. 2015; Chapter 3). Temperature was constant 10 °C. Fish grew rapidly, mean body weight increased from ca. 24 g in August, 2014 to about 1000 g in October, 2015.

At the end of the trial in October 2015, a proportion of age 2 mature/maturing fish were selected from two photoperiod treatment groups: a) LDN: n=28, 18 ♀,10 ♂; b) constant 24h light (LL): n=21, 14 ♀, 7 ♂. All fish were healthy and gross anomalies were

absent. A female was defined as sexually mature if it expressed eggs when gentle hand pressure was applied to the abdomen, or defined as maturing if secondary sexual characteristics were prominent, including a protruding genital pore, soft and enlarged abdomen and darkened skin color. Sexually mature males expressed milt, and maturing males typically exhibited a hooked jaw, thin abdomen and bright orange coloration. The fish were reared in the 500 L tanks until January 6, 2016, then transferred into two 1500 L light-proof fiberglass tanks each with a flow-through supply of well water (Diameter: 1.8 m; Water depth: 0.65 m; constant 10 °C; oxygen saturation > 80%, total alkalinity of 100 mg/L; total hardness of 188 mg/L). The fish reared under LDN in 2015 were subjected to the experimental regime of constant short photoperiod, LD 8:16. By contrast, the fish reared under constant LL in 2015 continued with LL during the trial. The photoperiod history of each group of fish is illustrated in Figure 8.1. Notably, the LDN fish were all defined as mature and their eggs were stripped between October 2015 and February 2016. By contrast, no LL fish expressed gametes in January 2016, a consequence of their photoperiod history, but all were categorized as 'maturing'. Light intensity at the water surface was 70-80 lux provided by an incandescent bulb (40 W) positioned centrally at a height of about 1 m above the water surface. Fish were hand-fed once daily a commercial salmonid diet (protein 44-45%, fat 24-26%, fibre 1.3%, Corey Feed Mills Ltd., New Brunswick). The pellet size was 5.5 to 7.5 mm. Stocking density was 66 kg/m^3 (LD8:16) and 56 kg/m^3 (LL) in the respective treatment group in 500 L tanks, and following stocking into the 1500 L tanks in January 2016 was 25 kg/m^3 and 21 kg/m^3, respectively.

The number of females chosen was greater than males because the timing of ovulation is a discrete indicator of the periodicity of the maturation rhythm, whereas males

can release sperm for several months making it difficult to define the periodicity. At monthly intervals between November 2015 and May 2017, all fish were anesthetized (MS222, 0.1g/L) and measured for body weight (BW; 1 g) and fork length (FL; 0.1 cm). Sexual maturity was assessed by applying gentle pressure to the abdomen. Timing of ovulation for each female was defined as the date some eggs could be stripped even if the majority of eggs had not been ovulated, and were stripped out a month later. Secondary sexual characteristics for both sexes were recorded. By July 2016, 12 out of 13 females in the LL treatment were abnormally heavily gravid, but had not ovulated. One female was euthanized on July 7 from the LL treatment to allow examination of the ovaries. On the same date, two females were transferred from the LL tank to the LD 8:16 tank to determine whether a short day was required for ovulation.

The trial was terminated in May 2017 since 29 out of 49 fish (60%) were not feeding and were emaciated. Somatic growth and the reproductive cycle of individual fish is presented. Within each treatment, a two sample t-test was used to compare the initial body size of fish that subsequently either gained or lost body weight during the trial. A probability level of $P<0.05$ was claimed significant. All procedures were approved by the local Animal Care and Use Committee (File No. 2015-082).

8.4 Results

After reaching first maturity at age 2 in November 2015, both sexes reared under LD 8:16 exhibited a divergence in body weight, some fish gained body weight, but over 50% lost weight because they failed to accept food (Fig. 8.2 and 8.3). No signs of aggression were observed, either behavioral or wounds. Among females that subsequently diverged in body size, their initial body weight was similar (Gained: 1108 g vs. Lost 1179

g). Among the seven females that gained weight, n=4 initially lost weight for about six months, then started eating and growing (Fig. 8.2 top). From October 2016 onwards, somatic growth either stopped or slowed, associated with the final stages of the second reproductive cycle and development of secondary sexual characteristics (Fig. 8.2; top). The timing of ovulation ranged from October 27, 2016 to February 20, 2017. Partial ovulation was evident among five individuals (n=5) resulting in eggs being stripped in two consecutive months. These fish showed rapid growth after the completion of maturation with mean body weight of 1975 g. The nine females under LD 8:16 that did not feed from November 2015 onwards continued to lose weight and exhibited an average of 27% loss of body weight during the 17 months to April 2017. By the end of the trial, they were less than half the size of fish that fed, only average at 793 g (Table 8.1). The incidence of maturity age 3 among females that lost weight was only 22% (2 out of 9; Fig. 8.2, bottom).

Males reared under LD 8:16, like females, exhibited a divergence in body weight, five gained and four lost (Fig. 8.3). Five fish (initial ca. 1336 g) lost weight for between 2 and 6 months, but then began feeding and gaining weight between March and July 2016. The final mean body weight was 1890 g ranging from 1390 g to 2650 g (Table 8.1). By contrast, four males lost weight progressively throughout the trial from 982 g in November 2015 to 775 g in April 2017 (Table 8.1; Fig. 8.3). All individuals expressed milt at age 2 between November and December 2015 prior to the trial start. At age 3, only four fish expressed milt between October 27, 2016 and April 6, 2017. The duration of spermiation was between 1-4 months, although the milt was very watery, indicative of poor quality. Secondary sexual characteristics among males were evident during two phases, from January to May and again between September and November 2016 (Table 8.2).

Under LL, five of eleven females gained weight during the trial, the other six lost weight (Table 8.1; Fig. 8.4, top). Among all eleven, only two fish ovulated during the trial, on February 26 and October 27, 2016, respectively. The other nine females clearly underwent sexual maturation prior to the trial onset judging from their secondary sexual characteristics including orange coloration and heavily enlarged and soft abdomen, but did not ovulate. On July 7, 2016, one euthanized female had a healthy looking ovary with orange oocytes (Fig. 8.5). The gonadosomatic index was 27% (Gonad weight=248 g, body weight= 923 g). Two females that were transferred from LL to LD 8:16 on July 7 2016 were checked on July 25, August 16 and September 23. Eggs were stripped out from both fish on September 23. One female ovulated completely on that day, the other one had eggs stripped out on both September 23 and October 27. Among the seven males, the individuals with the largest initial body weight (Jan. 2016) was the only fish that gained weight during the trial, reaching 2780 g at the end. The other six fish lost an average of 37% of their body weight from 1083 g in Nov. 2015 to a mean final weight of 681 g (Table 8.1; Fig. 8.4, bottom). Three males expressed milt continuously for 4-7 months and exhibited full secondary sexual characteristics throughout. The other four males did not express milt but did exhibit secondary sexual characteristics of a hook jaw, dark skin and orange coloration of abdomen throughout the trial (Table 8.2).

8.5 Discussion

The experiment was confounded by a divergence in feeding and growth that forced it to be terminated after 17 months. None of the three criteria for an endogenous circannual rhythm were satisfied. The failure of heavily gravid females to ovulate under LL, but subsequently ovulating following transfer to a short photoperiod, is evidence for

rejecting the circannual rhythm hypothesis. In previous studies, by contrast, female rainbow trout reared under LL, ovulated about every six months, supporting an endogenous rhythm hypothesis (Duston and Bromage, 1991). The contrasting response to constant 24h light between Arctic charr and rainbow trout indicates the dogma that all salmonids possess the same mechanism controlling sexual maturation must be reconsidered and perhaps abandoned. Contradictory and perplexing results are common to all published attempts to define a circannual rhythm of sexual maturation in teleosts, indicating the true mechanism remains elusive.

The results obtained from female Arctic charr reared under LL was contrary to the response of female rainbow trout, also reared under LL at 10 °C, which exhibited an advance of the first reproductive cycle by two months followed by recurring six-month cycles of ovulation and associated sex steroids (Duston and Bromage, 1986). The rainbow trout response supported the hypothesis of an endogenous mechanism controlling seasonal reproduction although the periodicity was outside the 7-15 months limit for a circannual rhythm, suggested by Gwinner (1986). Among two heavily gravid Fraser River Arctic charr, ovulation following their transfer from LL to LD 8:16 indicates a short or decreasing photoperiod is necessary for completion of the 1st cycle of ovarian development. Norwegian Hammerfest Arctic charr (n=13 females, age 3; matured previously age2) reared under LL from February to November completed ovulation over a protracted period of 15 weeks from July until October (Frantzen et al., 2004). Over 50% of ovulation occurred between September and October after the temperature drop from 9 to 4 °C during August-September, indicating that change of temperature was a confounding factor and may synchronize the timing of ovulation (Frantzen et al., 2004). Nevertheless, the

existence of an endogenous circannual oscillation controlling reproductive development was proposed since alteration of photoperiod clearly modified the timing of ovulation, in line with the entrainment criterion (Frantzen et al., 2004).

Arctic charr females under a constant LD 8:16 completed ovulation, suggesting this fish can express an endogenous rhythm of gonadal maturation provided the photoperiod conditions are permissive. Their response was similar to female rainbow trout reared under LD 6:18 that exhibited up to three self-sustained cycles of gonadal maturation and ovulation, with a periodicity between 11-15 months (Duston and Bromage, 1991). Why Arctic charr and rainbow trout respond differently under constant long photoperiod remains unclear. *Oncorhynchus mykiss* have been domesticated for over 100 years and they originated from the coastal riverine system in California (32-42°N) with relatively mild climate and ice-free winter conditions. The Arctic charr used here, by contrast, were retrieved from the Fraser River Labrador (56°N) in 1980s, a habitat that experiences about six-month of ice-covered winter conditions (Dempson and Green, 1985). The differences in selective pressure between two species may result in the variation of photoperiodic response. Indeed, comparing two populations of three-spined stickleback, either from Alaska (61°N) or Oregon (43°N), provided evidence of latitudinal variation in reproduction in response to the change of daylength (Yeates-Burghart et al., 2009). In a six-week trial, increasing the photoperiod from LD 12:12 to LD 16:8 stimulated the gonadal development in females and kidney growth in males among the Alaska population but failed to cause similar responses among the Oregon population (Yeates-Burghart et al., 2009). It is hypothesized that the reproductive cycle in Arctic charr is also controlled by the circannual clock but the daylength sets the limits to the expression of biological

rhythms. This supports the theory derived from birds that endogenous programs and exogenous '*Zeitgeber*' constitute a functional entity that provides as a whole for adaptive temporal programming (Gwinner, 1989). The hypothesis that Arctic charr, like many high latitude species, utilize circannual oscillators to cope with the fluctuation of seasonal environment requires further testing (Jørgensen et al., 2014; Hawley et al., 2017).

Environmental regulation of the final maturation process and ovulation is mediated by hormonal signals including the pituitary luteinizing hormone (LH) and follicular maturation-inducing steroid (MIS; 17, 20ß-dihydroxy-4-pregnen-3-one; Gillet et al., 2011). High temperature of 10 °C was indicated to be inhibitory to LH and MIH in Arctic charr and caused temporary delay of ovulation (Gillet et al., 2011). In the present study at 10°C, two females ovulated within two months after the transfer from LL to LD 8:16 suggesting the blockage of ovulation was temperature independent. The switch from LL to LD 8:16 would increase the nocturnal melatonin levels, a hormone that conforms to daylength with the levels only elevating during the nighttime. It can be hypothesized that the melatonin signal triggers the reproductive neuroendocrine cascades that are responsible for the completion of maturation and ovulation in Arctic charr. The attempts to connect melatonin with early development of sexual maturation in salmonids have all failed (Porter et al., 1998; Mailolo et al., 2015). The importance of melatonin in final maturation stage, however, has been recognized in Indian carp (*Catla catla*). *In vitro* study indicated melatonin accelerates oocyte maturation through its stimulatory effect on MIH (Maitra et al., 2005; Maitra et al., 2013). Nevertheless, the role of melatonin during final stages of oocyte development can be tested by LL exposure and melatonin implantation in Arctic charr.

Some male Arctic charr completed spermatogenesis and expressed milt under both photoperiod regimes, although the endogeneity was not evident given the nature of spermiation lasting for several months. The observed poor quality of milt in charr agreed with an earlier study on male rainbow trout that constant photoperiod exposure such as LD 8:16 or LD 16:8 impaired the process of spermatogenesis with lower number of spermatocytes at both 8 and 16 °C (Breton et al., 1977). Measurement of male steroid profiles is probably a better prediction of reproductive cycles compared with the timing of milt expression. Nevertheless, the persistence of secondary sexual characteristics under LL implied that fish retained at the maturation stages throughout the trial and did not finish the first reproductive cycle, similar with the female results.

The disparity of somatic growth in the present study was unexpected and seriously confounded the study. The reduction of stocking density from 60 kg/m^3 to 20 kg/m^3 prior to the trial may exacerbate the problem of social hierarchies with the larger fish dominating the tank and feeding. The behavior change and growth patterns in relation to the age of fish differed readily before and after age 1. The problem was never observed among underyearlying Fraser River charr reared at a stocking density of <10 kg/m^3 in 500 L tanks (Liu and Duston, 2019). Grading the same stock underyearlings did not seem to improve the growth of smaller individuals compared with the ungraded groups (Duston, unpubl. data). Growing juvenile charr (ca. 177 g) at densities of 30, 60, 90, 120 and 150 kg/m^3 found fish exhibited higher cortisol levels at 30 kg/m^3 compared with other densities suggesting the development of social hierarchies, but growth and fin health were unaffected (Sevier et al., 2019). The Hammerfest stock, by contrast, stocking density of less than 20 kg/m^3 suppressed the growth of age +0 charr associated with the increased

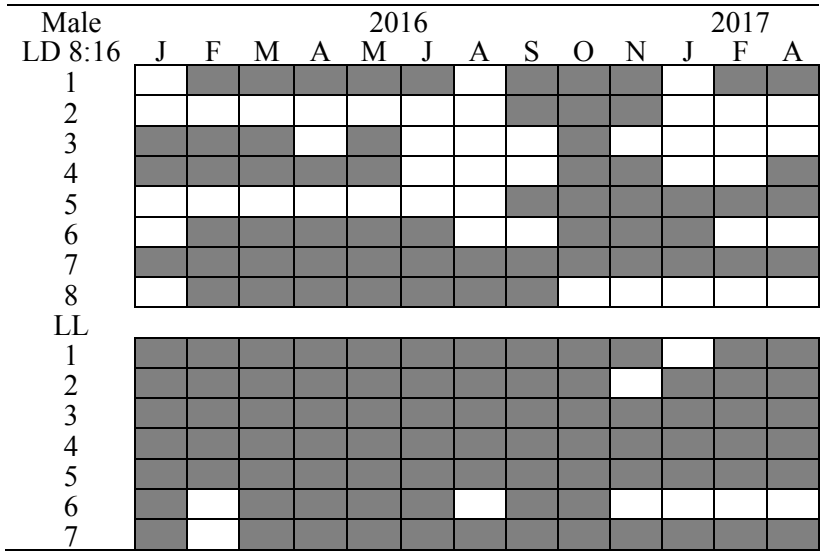
problems of social hierarchies and agonistic behavior compared with 60 and 120 kg/m^3 (Jørgensen et al., 1993). Increased water velocity or swimming speed can reduce the agonistic activity and break down the social hierarchy (Christiansen and Jobling, 1990). The growth pattern of Arctic charr is also suggested to be endogenously controlled due to the presence of annual cycles of food consumption and growth under constant LD 12:12 and 4°C, similar with the wild conspecifics (Sæther et al., 1996). But somatic growth is also strongly influenced by the reproductive status of the individual in relation to the changes of sex steroids (Tveiten et al., 1996). In the present study, poor growth and significant variation between individuals may have been due to the combined effects from sexual maturation, social hierarchy and photoperiod.

Overall, the reproductive rhythms in fish maybe more appropriate to be termed as 'long-term endogenous cycles' rather than 'circannual' until the criteria proposed by Gwinner (1986) are satisfied. Arctic charr, a polar-region species, may pose a stronger photoperiod-dependent timing mechanism compared to salmonids indigenous to lower latitudes, since the conditions that are conducive to growth and reproduction are limited to a very short period of time. This view, however, is against the long-running assumption that all salmonids share a common mechanism in controlling reproduction (Bromage et al. 2001). Comparative studies of the rhythmic reproduction between salmonid species under tightly controlled environmental conditions may reveal that variations among species resulted from selection pressures during evolution. Nonetheless, the endogenous controlled reproductive rhythms that interact with photoperiod or daylength deserve further investigation because they are the foundation of all theories that attempt to explain the control of seasonal breeding.

Table 8.1. Mean (S.E.) body weight (g) of Arctic charr that subsequently either gained or lost body weight between the start (December 2015) and end (April 2017). Photoperiod was under either constant 8h light:16h dark (LD 8:16) or 24h light (LL) at 10°C. Two sample t-test was performed within each sex and treatment, except the males from LL since only one male gained weight. $P<0.05$ was claimed significant.

	LD 8:16			LL		
	Gained	Lost	P value	Gained	Lost	P value
Female	n = 7	n = 9		n = 5	n = 6	
Dec. 2015	1108 (111)	1079 (66)	0.74	1098 (148)	1167 (159)	0.41
Apr. 2017	1975 (180)	793 (42)	<0.01	1635 (285)	1022 (194)	0.12
Male	n = 5	n =4		n = 1	n = 6	
Dec. 2015	1299 (70)	982 (36)	<0.01	2155	1083 (115)	-
Apr. 2017	1791 (229)	775 (95)	<0.01	2780	681 (80)	-

Table 8.2. Male Arctic charr secondary sexual characteristics presence (grey boxes) and absence (white boxes) under either constant 8h light:16h dark (LD 8:16; n=8 males) or 24h light (LL; n=7). The characteristics included hook jaw, orange coloration of abdomen, darkening skin color. The treatment started January 6, 2016. Temperature was constant 10°C.



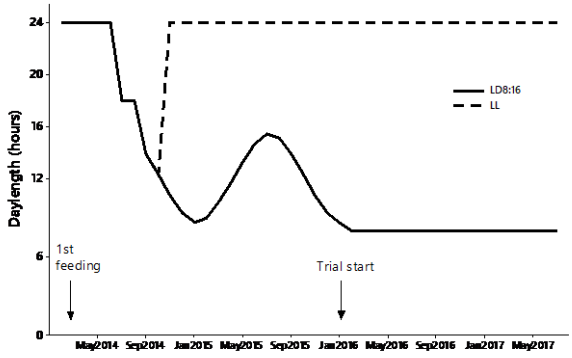


Figure 8.1. Photoperiod history of the two experimental groups of Arctic charr. Fish were raised under LL from 1st feeding in February to June, 2014, followed by LD 18:6 until August. The short photoperiod experimental fish were reared under simulated natural daylength (LDN; 45°N) from August 2014 to January 6, 2016, then LD 8:16 until the trial ended May 15, 2017. The second group were exposed to LDN for two months (August-September, 2014), then constant LL for 34 months from October 2014 until May 15, 2017 at constant 10 °C.

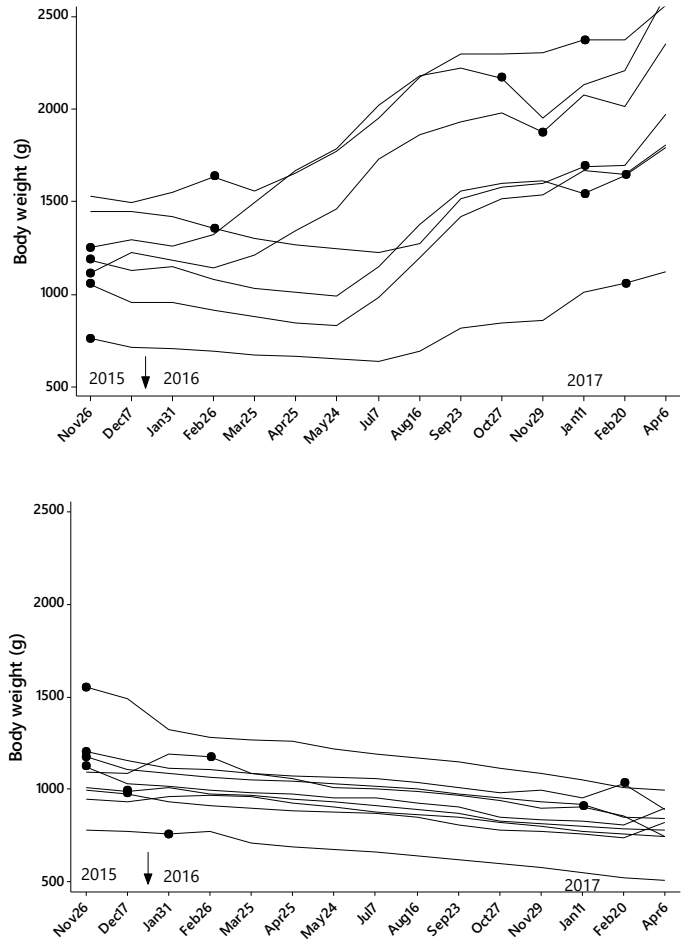


Figure 8.2. Short photoperiod, females. Body weight and timing of ovulation (Solid black circle) of individual female Arctic charr between November 26, 2015 and April 6, 2017. Top panel: fish that gained weight (n=7). Bottom panel: fish that lost weight (n=9). Photoperiod was constant 8h light: 16h dark for 17 months starting January 6 2016 (arrow), with simulated natural daylength (45°N) prior to that. Temperature was 10 °C. Note: the dates on the X-axis are presented categorically, not linearly.

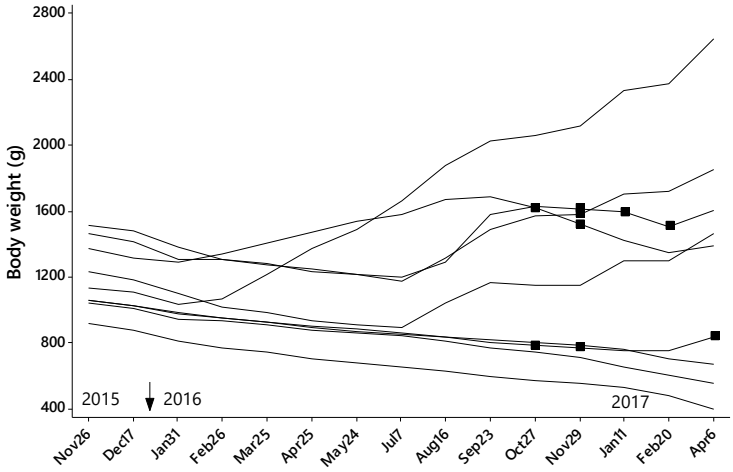


Figure 8.3. Short photoperiod, males. Body weight and timing of spermiation (square) of individual male Arctic charr (n=9) between November 26, 2015 and April 6, 2017. Constant 8h light: 16h dark for 17 months started January 6 2016 (arrow), with simulated natural daylength (45°N) prior to that. Temperature was constant 10°C. Note: the dates on the X-axis are presented categorically, not linearly.

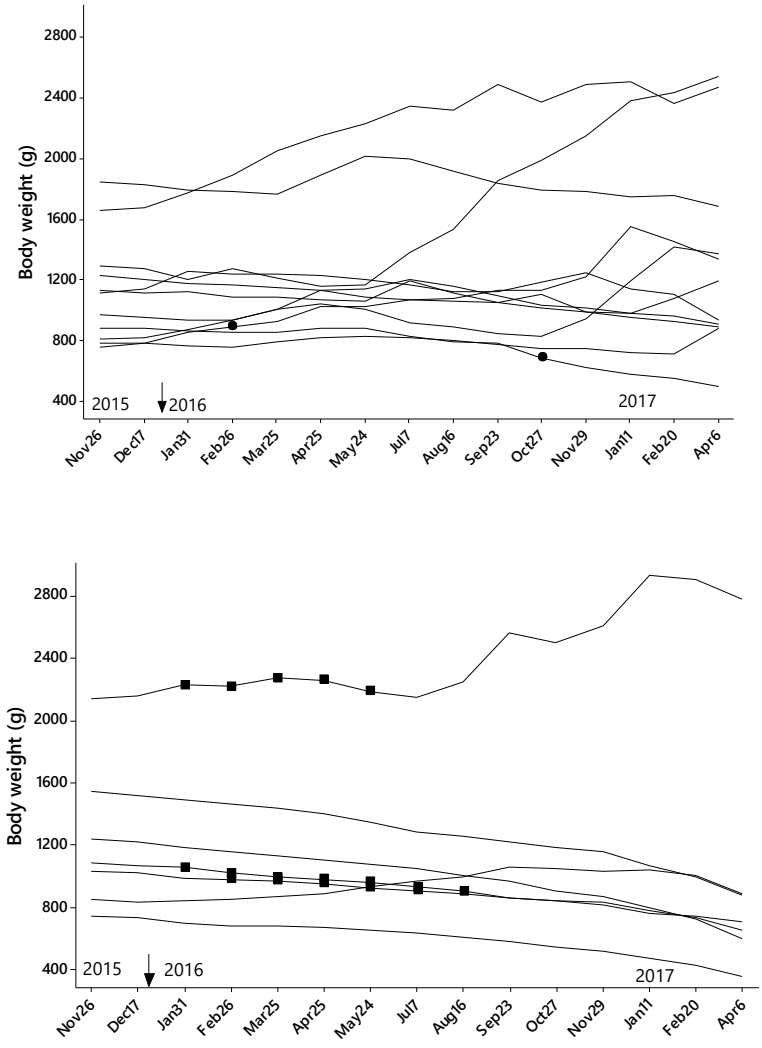


Figure 8.4. Long photoperiod. Body weight of individual female (top panel; n=11) and male (bottom panel; n=7) Arctic charr between November 26, 2015 and April 6, 2017. Timing of ovulation (circle) and spermiation (square). Photoperiod was constant 24h light exposure since October 1, 2015 for 34 months. The treatment started January 6, 2016. Temperature was constant 10°C. Note: the dates on the X-axis are presented categorically, not linearly.

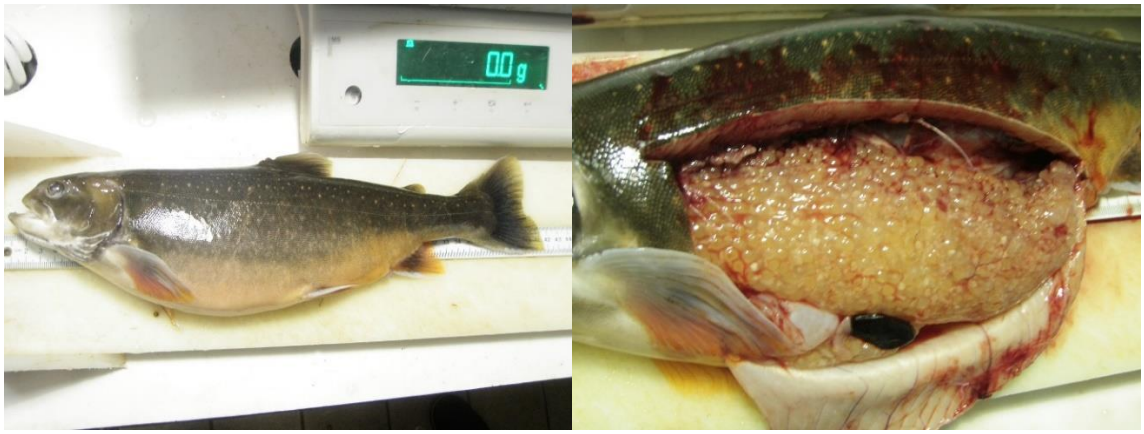


Figure 8.5. Long photoperiod. A heavily gravid female Arctic charr (left) that failed to ovulate was euthanized and dissected on July 7, 2016. The weight of ovaries (right) was 248 g and the gonadosomatic index was 27% (GSI=100*Body weight/Gonad weight). Photoperiod was constant 24h light exposure since October 1, 2015 for 34 months. The treatment started January 6, 2016. Temperature was constant 10°C.

Chapter 9: General Discussion and Conclusions

The new knowledge presented here can potentially reduce the problem of early maturity in the diploid Arctic charr grow-out industry and improve the overall productivity and sustainability. Through the manipulation of photoperiod, food deprivation and temperature, the aim of <20% maturity was achieved repeatedly, indicating the protocol is reliable which should be used to formulate industry guidelines. The physiological mechanisms by which sexual maturation is triggered or delayed by the environmental factors were elucidated.

The working hypothesis is that photoperiod manipulation acts via entrainment of an endogenous circannual 'clock' controlling the timing during the year of a 'critical decision period' during which the process of gonadal maturation commences. Histological analysis of Arctic charr gonads confirmed that the existence of two decision periods that are defined by the seasonal change of photoperiod. The decision to enter puberty is made in the fall, when daylength is decreasing. Around November and December, the initiation of gonadal development was evident by mitotic proliferation of spermatogonia and growth of oocytes from the primary to secondary stages. Between April and May, spermatocytes and vitellogenic oocytes were recruited under an increasing photoperiod, marking the commitment to sexual maturation. It has been hypothesized that the first critical period occurs when the fish makes an assessment of resources and the decision to either begin investment in gonad tissue or to postpone development (Thorpe et al., 1998; Sloat et al., 2014). The second critical period then occurs in spring when lipid stores or energy reserves remain sufficiently high overwinter, fish decide either sustain maturation or halt/delay maturation (Thorpe et al., 1998). Undoubtedly, body size and lipid reserves are

determinants that can dictate whether or not an Arctic charr commences maturation. Fall, Winter and Fall+Winter food deprivation treatments that suppressed somatic growth during the decision periods resulted in a clear step-wise reduction in the incidence of maturation among females (Liu and Duston, 2019). Among males, food deprivation failed to prevent testicular development, associated with the lower requirement for completing spermatogenesis compared to vitellogenesis. The efficacy of food deprivation on reducing the incidence of maturity was dependent on its duration and sex of the fish (Liu and Duston, 2019).

Photoperiod manipulation was more effective than food deprivation in suppressing the problem of sexual maturation. At constant 10 °C and daily feeding, the best photoperiod regime to suppress age 2 maturity is 24h light (LL) starting in October/November and ending it between February and April. An LL start date in the autumn was of major importance in reducing the maturity rate in Arctic charr. By contrast, long-term LL exposure or delaying LL onset date significantly impaired the efficacy (Fig. 9.1). Photoperiod history determines the reproductive response to changes in daylength. Periods of simulated natural daylength (LDN) before and after LL serve as entrainment cues for fish to recognize the time of year. By contrast, the absolute daylength was less important. Comparison of long photoperiod using LL and 18h light and 6h dark (LD 18:6) under the same timeframe from October to February, with LDN before and after that, resulted in similar efficacy in suppressing age 2 maturity (Liu and Duston, 2019). Overall, the direction of change in daylength was far more important than the absolute daylength in preventing early sexual maturation.

The ‘coupled’ response to LL of a reduction in the incidence of maturation in some charr with an advance in the timing of gamete release in others conforms with studies on rainbow trout (Duston and Bromage, 1988; Randall et al., 1998) and Atlantic salmon (Taranger et al., 1998; 1999). This consistent response supports the hypothesis the same endogenous timing mechanism controls both the physiological decision to mature and the rate of gametogenesis across the salmonids. The key to better understanding the underlying mechanism is the dichotomous response of gonadal development in response to a LL signal. This was initially identified among Atlantic salmon in sea-cages reared under LL from February until June which stimulated gonadal development among some individuals but arrested others (Schulz et al., 2006; Andersson et al., 2013). The LL signal inhibited the brain-pituitary-gonadal axis evident by reduced follicle-stimulating hormone, sex steroids and retarded germ cell development (Andersson et al., 2013). Among Arctic charr, the inhibitory effect of LL on germ cell development was dependent on the timing of LL onset/switch-off and stage of gonad. The abrupt increase in photoperiod from LDN to LL in November stimulated gonadal development among some individuals, but inhibited the initiation of sexual maturation among the others, whereas the drop of daylength from LL to LDN in February further suppressed the gonadal development among individuals that had already commenced the process. The second switch from LL to LDN also stimulated the completion of gametogenesis, mainly, among males. This finding was also supported by the incidence of maturation at age 2.

These abrupt changes from LDN to LL or LD 18:6 and then back to LDN advance a ‘decision period’ for maturation, resulting in some individuals being physiologically unready and remaining immature (Duston and Bromage, 1988; Taranger et al., 1999). The

cause of this unreadiness was neither small body size nor poor condition overwinter, suggesting other factors may be in play (Liu and Duston, 2018, 2019). Future experiments should test if the genetic/family effect associated variation between individuals masks the picture. By comparison, combined LL overwinter with suppressed somatic growth through food deprivation and/or lower temperature can further reduce the incidence of maturation to <10% among age 2 Arctic charr (Fig. 9.1; Liu and Duston, 2016). Temperature or food availability, via its effect on growth, was an enabling factor affecting the magnitude of the response to photoperiod signal. The additive effect supports the hypothesis that individuals failed to mature because their growth rate and energy reserves were below a genetically determined threshold at a specific time of year (Thorpe et al., 1998; Taranger et al., 2010). To reconcile the influence of somatic growth and photoperiod on physiological decision to mature in Arctic charr, a two-step gating model was proposed (Liu and Duston, 2016). In the first step, prevention of sexual maturation is solely affected by photoperiod, a growth independent factor, which defines a period of time during which individuals have an opportunity to commence gonadal maturation. The second step is only possible for those who have passed the first step and have a faster growth rate and larger body size (Liu and Duston, 2016).

Light information is transduced by the pineal gland through a hormonal signal melatonin, with high titers during the night and low during the day (Falcón et al., 2011). Measurement of plasma melatonin in Arctic charr indicated that the light intensity necessary to elicit a melatonin response is very low, about 0.1-0.3 lux at the mid-water level, comparable with a wild charr under thick ice (Strand et al., 2008; Liu et al., 2019). And illumination above 50 lux at the mid-water level is recommended to ensure the

effectiveness of photoperiod manipulation and deserves to be further tested in a commercial setting (Liu et al., 2019).

The hypothesis of an endogenous circannual rhythm governing seasonal reproduction in Arctic charr is not supported here, although counfounded by the variation in somatic growth and possibly social hierarchy associated with low stocking densities. A short photoperiod cue appeared to be essential for the completion of gametogenesis with ovulation, suggesting the expression of a circannual rhythm is photoperiod dependent. This is contrary to rainbow trout that exhibited a free-running rhythm in ovulation under either constant long or short photoperiod (Duston and Bromage, 1986, 1991). Better understanding of this subject is further constrained by limited attempts of testing the endogenous circannual rhythm hypothesis and perplexing results. The present research nevertheless raised the need to investigate the endogenous circannual rhythm or clock which underpins the photoperiod entrainment of seasonal reproduction.

The animal welfare concerns regarding the use of food deprivation and continuous light were not evident in the present research, but Arctic charr maybe a special case. Neither food deprivation nor LL resulted in any negative effect on fish behavior, growth and survival. Prolonged winter food deprivation that causes no distress among Arctic charr is also supported by other studies (Jørgensen et al., 2013; Cassidy et al., 2018). By contrast, sexual maturation is a major threat to animal welfare. Maturing Arctic charr are often infected by the *Saprolegnia* fungal disease causing significant mortalities. The health benefits of preventing sexual maturation and shortened duration of production period should be factored into any consideration of welfare issues when rearing salmonids.

With the advance of genomic tools, identification of genetic markers that are pertinent to the commercially important traits including age at maturity and growth rate is now widely adopted to analyze the within- and between-family component of genetic variation (Houston and Macqueen, 2018). The high-throughput sequencing techniques enable the studies of genetic mapping, genome-wide association studies, population genetics and single nucleotide polymorphism discovery, which would be beneficiary for the breeding program using the marker-assisted selection (Houston and Macqueen, 2018). For instance, studies on wild Atlantic salmon revealed that age at maturity is significantly affected by the vestigial-like family member 3 gene (*VGLL3;* an adiposity regulator); this single locus accounted for > 30% of individual variation in the age that *Salmo salar* undergo maturation in the wild (Ayllon et al., 2015; Barson et al., 2015). Recently, Arctic charr genome has been published (Christensen et al., 2018). This also opens new routes for the genomic comparisons between different groups of salmonids and analysis of their unique physiology and commercially important traits using the new genomic tools. It would be worthwhile to investigate if the *VGLL3* gene is also involved with the control of age at maturity in Arctic charr.

To conclude, this thesis has increased the understanding of environmental control of sexual maturation in Arctic charr, particularly the role of photoperiod. The potential of the use of photoperiod manipulation in the charr farming industry has been established.

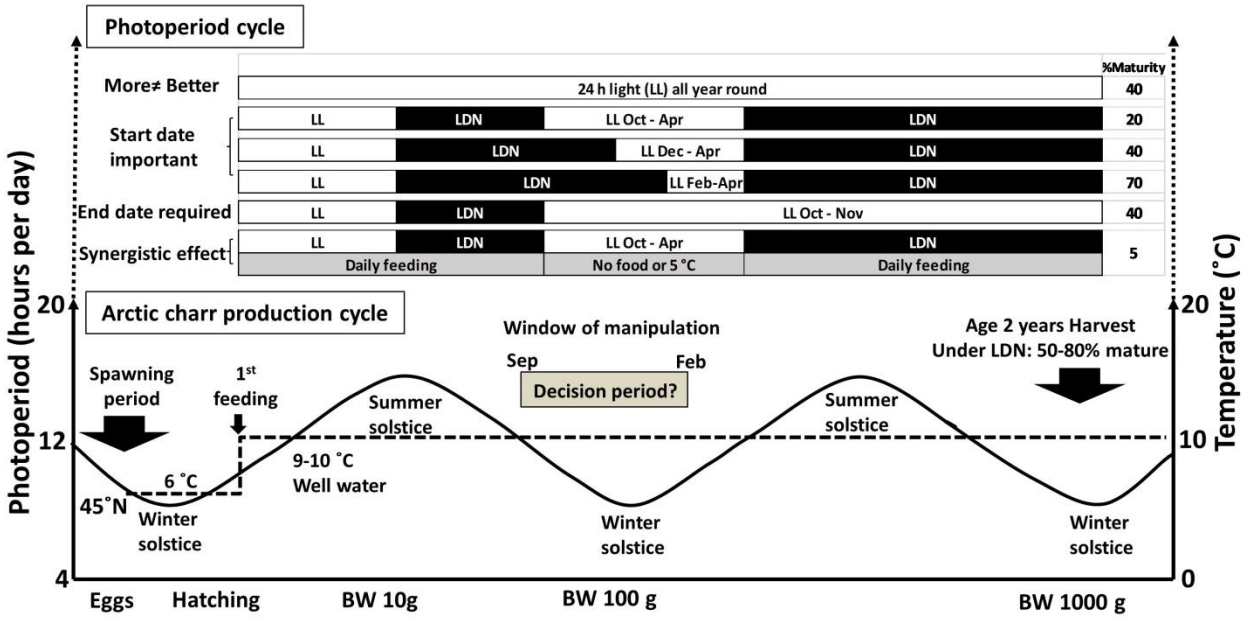


Figure 9.1. Top: Five photoperiod regimes comprising different periods of 24h light (LL; white bar) and simulated natural daylength (LDN; black bar). The sixth regime combined LL overwinter with food deprivation or 5 ˚C. Incidence of sexual maturation among age 2 Arctic charr was indicated on the right. Bottom: Arctic charr takes about two years to grow from egg to 1 kg body weight (BW). Temperature was maintained at 6 ˚C during egg incubation and increased to 9-10 ˚C (well water) from first feeding to the end of production (dotted line). The control fish received LDN (Latitude 45 ˚N; solid line; Adopted from Yossa et al., 2019; Produced by Qi Liu).

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