ISBN 978-82-326-4456-8 (trykt utg.)
ISBN 978-82-326-4457-5 (elektr. utg.)
ISSN 1503-8181

Doktoravhandlinger ved NTNU, 2020:51

Ingun Næve

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Doktoravhandling

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Norges teknisk-naturvitenskapelige universitet
Avhandling for graden
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Trykket av NTNU Grafisk senter

Contents

Acknowledgements

This thesis was performed at the Norwegian University of Science and Technology (NTNU) and was funded by the Norwegian research council through the Industrial PhD program (grant number 241398/O30) and AquaGen.

First, I would like to thank my supervisor, Professor Elin Kjørsvik (NTNU) for sharing all her knowledge and for always asking the questions that kept me moving in the right direction. To my co-supervisor Maren Mommens (AquaGen), thank you for all the good discussions and distractions.

Thank you, Professor Augustine Arukwe (NTNU), for your guidance and encouragement during the writing of my papers. I would also like to thank Tora Bardal and Randi Røsbak (NTNU) for their help and training during my lab work.

I would like to thank my employer, AquaGen for giving me the opportunity to do an industrial PhD, and the staff in AquaGen who facilitated and assisted at all samplings. A special thanks to my colleague Trina Galloway for the support and input during the final stages of writing this thesis.

My family and friends, thank you for your support and cheering. Kristoffer, thank you for all the love, support and understanding, it would not have been possible without you. And darling Alfred, thank you for reminding me of the important things in life, such as blueberries and vacuum cleaners.

Abstract

In production of Atlantic salmon (*Salmo salar*) broodfish, handlings for lice and disease management, gradings and checks for progression of sexual maturation, are inevitable. These handlings cause stress responses and can challenge fish welfare and reproductive outcome. Tools that can contribute to a reduction in the number of handlings that are required for successful broodfish production and make handlings gentler, are needed. The purpose of this thesis was to establish ultrasound technology as a non-invasive tool in Atlantic salmon broodfish production and reproduction.

This thesis presents a novel method for sex identification of Atlantic salmon parr (from 50 g body weight) using ultrasound technology (Paper I). We found that a high frequency ultrasound probe gave 95 and 97 % correct identification of males and females, respectively, and that parr had a good tolerance of the procedure. Sex identification can be performed concomitant to mandatory vaccination during the freshwater phase, to avoid adding extra handlings of fish. Thus, it is no longer necessary to handle broodfish candidates for sex identification during the seawater phase, which reduces handling stress and risk of escapes. In papers II and III we established a non-invasive method for estimation of gonado-somatic index in Atlantic salmon females and males, using ultrasound technology (US-GSI). The female US-GSI model is well-suited for monitoring of sexual maturation, while the male US-GSI model can be a tool for maturation monitoring when observations of testis appearance (echogenicity) in ultrasound images are added. This method eliminates the need for sacrifice of broodfish or relying on GSI from deceased fish for maturation monitoring. Using the US-GSI method established in Paper II in broodfish where sexual maturation has been advanced by light and temperature control, we found that females expected to ovulate early and late in the stripping season could be identified in July (Paper IV). In the weeks leading up to ovulation we observed changes in oocyte echogenicity and found that this could be used to predict ovulation time. Using US-GSI in July to separate females that are expected to ovulate early and late, females that are expected to ovulate early can be checked for ovulation while not disturbing females that are expected to ovulate late. This procedure for broodfish

management can potentially reduce number of stressful handlings of females during a period when they are sensitive to the influence of stress on reproduction.

The ultrasound methods developed here can be used to establish a monitoring system that reduce handling of Atlantic salmon in seawater and in freshwater close to stripping. Such a system could also ease production planning and simplify broodfish handling logistics. These results also inspire further work to develop and refine the methods for maturation monitoring in Atlantic salmon using ultrasound.

List of papers

Paper I

I. Næve, M. Mommens, E. Kjørsvik, Sex identification of Atlantic salmon (*Salmo salar)* juveniles using ultrasound technology, submitted Aquaculture 28.06.19

Paper II

I. Næve, M. Mommens, A. Arukwe, E. Kjørsvik, Ultrasound as a non-invasive tool for monitoring reproductive physiology in female Atlantic salmon (*Salmo salar*), Physiological reports, 6 (9), 2018, e13640

Paper III

I. Næve, M. Mommens, A. Arukwe, J. Virtanen, M.E. Hoque, E. Kjørsvik, Ultrasound as a non-invasive tool for monitoring reproductive physiology in male Atlantic salmon (*Salmo salar*), Physiological reports, 7 (13), 2019, e14167

Paper IV

I. Næve, M. Mommens, A. Arukwe, E. Kjørsvik, Predicting ovulation and optimal stripping time in Atlantic salmon (*Salmo salar*) using non-invasive ultrasound technology to reduce handling stress, Manuscript

Introduction

Animal welfare is of increasing concern among the public and among producers of farmed animals such as cattle (*Bos taurus*), poultry, pigs (*Sus scrofa domesticus;* Kupsala *et al.*, 2015; Miranda-de la Lama *et al.*, 2017; Evans and Miele, 2019), and Atlantic salmon (Olesen *et al.*, 2010; Grimsrud *et al.*, 2013; Stien *et al.*, 2013). Common challenges to welfare in Atlantic salmon aquaculture include exposure to diseases and parasites, such as sea lice (*Lepeophtheirus salmonis*), stressful lice and disease treatment and handling operations, varying temperatures and oxygen saturation, and sexual maturation (Stien *et al.*, 2013; Noble *et al.*, 2018). The global Atlantic salmon production was 2.4 million tonnes in 2017, at an estimated value of 16.7 billion USD, and approximately half of this was produced in Norway (FAO, 2019). Through their breeding programs, broodfish producers deliver the starting material, genetically improved eggs with increased growth and disease resistance, to the aquaculture industry. There is an increasing demand for year-round supply of Atlantic salmon eggs due to smolt production in land-based recirculation aquaculture system (RAS) facilities, and broodfish producers manipulate the natural maturation pattern of Atlantic salmon to secure year-round deliveries. As broodfish are kept an additional year in seawater compared to fish for harvest, exposure to parasites and diseases with associated handling is prolonged. Further, securing sexual maturation at the desired time involves manipulation of environmental factors such as light and temperature, and in some cases extensive handling for sex identification and selection of broodfish candidates, monitoring of maturation progress and removing of gametes from the fish. Some of the methods used for monitoring sexual maturation require several handlings or sacrifice of fish, or they are invasive and stressful. Alternative non-invasive methods such as ultrasound may reduce the number of handlings and need to sacrifice fish, while still providing the necessary information at the right time, thus contributing to reduced stress and increased fish welfare in Atlantic salmon broodfish production.

Animal welfare

Definitions of animal welfare vary; from the functional perspective, where good biological function resulting in good growth and performance is viewed as good welfare,

to the nature-based definition which requires that animals should be able to perform species-specific behaviours in their natural environment to have good welfare, and to the feelings-based welfare definition which states that animals should be free from negative emotions and should be allowed to experience positive emotions (Fraser, 1999; Noble *et al.*, 2018). The five freedoms, originally suggested by the Brambell Committee for the British Farm Animal Welfare Council (FAWC) regarding welfare of terrestrial farm animals (FAWC, 2012), are often used to describe central factors in animal welfare, and states that animals should have

- Freedom from hunger and thirst
- Freedom from discomfort
- Freedom from pain, injury and disease
- Freedom to express normal behaviour
- Freedom from fear and distress

The five freedoms are reflected in European legislation (Council Directive 95/58/EC concerning the protection of animals kept for farming purposes, 1998), and the Norwegian law on animal welfare, where it is stated that animals, including fish, have an intrinsic value irrespective of their value to humans, and should be treated well and protected from harm and danger (Ministry of Agriculture and Food, 2009). In addition to the biological perspective, animal welfare is of increasing interest and concern in society, and expectations from the public must be taken into consideration when working with animal welfare, as pointed out by Ohl and van der Staay (2012).

A discussion regarding fish welfare is inevitably also about whether fish are self-aware and capable of feeling pain, which has been fiercely debated (Rose *et al.*, 2014; Key, 2016; Merker, 2016; Wadiwel, 2016; Sneddon *et al.*, 2018). Fish such as rainbow trout (*Oncorhynchus mykiss*) have the A-delta and C nerve fibres that conveys signal of noxious, tissue-damaging stimuli to the brain in higher vertebrates, although in different proportions (Sneddon, 2002; Dubin and Patapoutian, 2010). Further, using several noxious stimuli, deviant behaviours interpreted as reaction to pain, such as increased opercular movements, rocking behaviour, and long-term effects such as delayed resumption of feeding has been observed (Sneddon, 2003b; Sneddon *et al.*, 2003).

Morphine can ameliorate pain-related behavioural responses to noxious stimuli in rainbow trout (Sneddon, 2003a), and reduce post-test behavioural effects in goldfish (*Carassius auratus*; Nordgreen *et al.*, 2009). Further, there are homologies between the lateral and medial pallia in the teleost brain and the hippocampus and amygdala of mammals, which are the brain regions involved in spatial learning and emotional memory, respectively (Broglio *et al.*, 2005). However, the neocortex and associated brain structures that are central for translating a noxious stimulus to a conscious feeling of pain in humans, does not have a counterpart in fish, and this has been the main argument of Rose (2002) and Key (2016) for dismissing that fish are capable of feeling pain. The increasing amount of evidence that fish feel pain suggests that we should use the benefit of the doubt when handling fish. In an ethical farm animal production with increasing demand for transparency, this also comes down to whether one is willing to take the risk on whether fish feel pain or not. Despite the ongoing debate, authorities such as the European Food Safety Authority (EFSA) has concluded that fish can feel pain and fear, and might have some level of sentience (EFSA, 2009). They further give equal status to mammals, birds and fish, and recommend establishment of species-specific welfare indicators for fish to properly monitor and maintain their welfare (EFSA, 2009).

Welfare indicators and systems for evaluating welfare has mainly been studied or established for typical aquaculture species such as Atlantic salmon (Adams *et al.*, 2007; Oppedal *et al.*, 2007), sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*; Bagni *et al.*, 2007; Di Marco *et al.*, 2017) and Nile tilapia (*Oreochromis niloticus*; Boscolo *et al.*, 2011; Luo *et al.*, 2014). Recently, extensive reviews identified welfare needs of farmed Atlantic salmon and established operational welfare indicators for an easy evaluation of welfare in fish farms (Stien *et al.*, 2013; Noble *et al.*, 2018). These welfare indicators include factors at the level of the individual fish such as appetite, body condition, smoltification state, sexual maturity and sea lice infestation, and indicators at the cage or environment levels including temperature, light, salinity, water current, stocking density, and disturbance due to handlings (Stien *et al.*, 2013; Noble *et al.*, 2018). A method for evaluating welfare under practical farming conditions, the salmon welfare index model (SWIM), established by Stien *et al.* (2013), scores welfare on a scale from 0 to 1 (worst to best) during commercial grow-out. Several operational welfare indicators

are assessed and influence the SWIM score according to how severely they impact welfare. The knock-out levels of some welfare indicators such as very high water temperatures, high lice infestation levels, and emaciation are regarded as so detrimental that they automatically give a SWIM score of 0, irrespective of the score of all other welfare indicators (Stien *et al.*, 2013). During broodfish production, welfare indicators such as light regimes, temperature, salinity, sexual maturation status and handling stress are especially relevant due to practices undertaken to control sexual maturation.

The characteristic physiological response of an animal to noxious agents, stressors, named the general adaption syndrome by Selye (1936), facilitates adaptation to changing conditions. Within a short time of exposure to a stressor, catecholamines (adrenaline and nor-adrenaline) are released from the chromaffin cells in the kidney, making the fish capable of "fight-or-flight" by increasing respiration, O_2 transport capacity of red blood cells and blood glucose levels (Wendelaar Bonga, 1997). Through the hypothalamus-pituitary-interrenal (HPI) axis, corticotropin releasing hormone (CRH) released by the hypothalamus stimulates pituitary secretion of adrenocorticotrophic hormone (ACTH) which travels through the blood and stimulates release of the corticosteroid cortisol from the interrenal tissue within a mean time of 12.5 minutes (Wendelaar Bonga, 1997; Table 1 in Pankhurst, 2011). The main functions of cortisol during stress coping, are regulation of hydromineral balance and energy metabolism, enabling the animal to adapt to changing conditions (Wendelaar Bonga, 1997). Stress may also have deleterious effects on osmoregulation, growth, immune function and reproduction if the individual is not able to cope in a satisfactory way, or if the stressors are chronic (Wendelaar Bonga, 1997).

Both in production of Atlantic salmon for consumption and broodfish production, early sexual maturation in the seawater phase represent a welfare challenge as osmoregulatory capacity decreases in maturing individuals in preparation for spawning in freshwater, making further seawater residence challenging (Persson *et al.*, 1998). Further, wild Atlantic salmon perform sex-specific reproductive behaviours such as nest digging for females and aggressive behaviours such as display and fighting for access to spawnings for males (Fleming and Einum, 2010). If such behavioural needs are present

in captive broodfish, lack of opportunity to perform them because individuals are kept in a non-satisfactory environment might constitute a welfare issue.

In aquaculture, underwater lights are frequently used in net-pens to reduce risk of unwanted early maturation (Porter *et al.*, 1999; Leclercq *et al.*, 2011) and to advance sexual maturation in broodfish (Taranger *et al.*, 1998). As both placement of lamps and temperature gradients in net-pens can affect swimming patterns of Atlantic salmon, lights should be placed so that light control of maturation is achieved while also allowing fish to fulfil behavioural needs regarding swimming depths (Oppedal *et al.*, 2007). Further, sudden changes in light intensity may induce behavioural responses in Atlantic salmon corresponding to anti-predator behaviour, indicating that they are stressed (Mork and Gulbrandsen, 1994). Although some habituation take place (Folkedal *et al.*, 2010), it might be preferential to dim light intensities up and down during light manipulation to secure a low level of stress in fish.

Atlantic salmon are capable of acclimatising to temperatures between 0 and 22 °C, as long as the changes are gradual, and the holding water is sufficiently aerated (EFSA, 2008; Stien *et al.*, 2013). In Atlantic salmon broodfish production, temperature is used to influence sexual maturation after transfer from seawater to land-based freshwater facilities (Taranger *et al.*, 1998; Vikingstad *et al.*, 2016). Both sudden temperature increases and decreases can result in responses such as increased opercular beat rate, increased O_2 consumption and increased cortisol levels in salmonids (Bellgraph *et al.*, 2010; Folkedal *et al.*, 2010; Foss *et al.*, 2012; Overton *et al.*, 2019), thus temperature changes should be induced gradually to reduce possible stress responses. However, Foss *et al.* (2012) noted that in most direct transfers of fish between a wide range of temperatures, the effect of handling of fish was greater than the effect of the temperature challenge itself.

During seawater rearing in net-pens Atlantic salmon are exposed to diseases and parasites, the most common at the moment are salmon sea lice, the viral diseases pancreas disease (PD, caused by Salmonid alphavirus) and heart and skeletal muscle inflammation (HSMI, caused by *Piscine orthoreovirus*), and amoebic gill disease (AGD, caused by the amoeba *Paramoeba perurans*; Hjeltnes *et al.*, 2018). Depending on the severity of the

infestation or outbreak and regulations applying to the disease in question; surveillance, treatment or emergency harvesting may be required (Hjeltnes *et al.*, 2018). In general, disease and lice infestation increase the need for crowding and handling, and thereby unwanted handling stress (Foss *et al.*, 2012; Erikson *et al.*, 2016) which can deteriorate fish welfare and increase mortalities (Skjervold *et al.*, 2001; Overton *et al.*, 2018a), and be very costly to farmers (Liu and Bjelland, 2014). Handlings and sea lice increase risk of damage to the skin and mucus surface of the fish, and as skin and mucus are important in the immune system, this can increase the risk of infections (Ángeles Esteban, 2012; Xu *et al.*, 2013). The stress response can also modulate the immune system in fish, thus further increasing risk of infections (Fast *et al.*, 2008; Tort, 2011). As broodfish spend one extra year in seawater compared to fish farmed for consumption, they have longer exposure to disease and parasites, and might need more delousing and disease treatment, thus increasing the load of handling stress. Further, broodfish are handled more than fish farmed for consumption during the seawater phase, as sex identification, data collection and selection of broodfish candidates is necessary. After broodfish are transferred to freshwater for final maturation, weekly ovulation checks that involve crowding, netting and emersion, are necessary leading up to and during the stripping period (Springate *et al.*, 1984; Mommens *et al.*, 2015). Such repeated stressful handlings result in increased levels of cortisol in broodfish and can negatively affect reproductive outcome (Campbell *et al.*, 1992; Contreras-Sánchez *et al.*, 1998).

Since the numerous handlings broodfish are subjected to can affect both welfare and reproduction negatively, alternative non-invasive methods for monitoring maturation that can reduce the number of handlings, are needed. Non-invasive ultrasound technology is routinely used in human and farm animal medicine, and earlier studies have shown its potential for imaging of fish gonads (Martin *et al.*, 1983; Bryan *et al.*, 2007).

Non-invasive ultrasound technology

Ultrasound is a diagnostic tool in human medicine, such as pregnancy monitoring or examination of cardiac function. While audible sound is acoustic vibrations with a frequency of up to 20 000 cycles s^{-1} (Hz), waves with frequencies above 20 kHz are called

ultrasound (McDicken and Anderson, 2011). Medical ultrasound typically has frequencies in the range of 1 to 20 MHz, and these waves are created by piezoelectric crystals in the ultrasound transducer (Lieu, 2010). When placed under an electric current these crystals vibrate and emit waves that travel into tissues, and at tissue interfaces they are reflected to the transducer. Returning waves (echoes) initiate vibrations in the piezoelectric crystals, generating an electric voltage that is interpreted by the ultrasound machine in order to make an image of the scanned tissue (McDicken and Anderson, 2011). Based on the mean speed of ultrasound waves in soft tissues (1540 m s^{-1}) and the elapsed time from waves are emitted until an echo returns, the depth of reflecting tissues can be calculated and represented in live grey-scale ultrasound images (Lieu, 2010; McDicken and Anderson, 2011).

In human medicine, ultrasound has mainly two different uses; diagnostic and therapeutic. Diagnostic ultrasound usually employs frequencies from 1 to 20 MHz and is designed to generate ultrasound images with minimal interactions with tissues (Dalecki, 2004; McDicken and Anderson, 2011). In therapeutic ultrasound, such as high-intensity focused ultrasound, effects in tissues such as kidney stones or tumours are induced by focusing 1 to 10 MHz ultrasound waves thereby achieving very high intensities (ter Haar and Coussios, 2007). As the intention of therapeutic ultrasound is interaction with tissues, side-effects such as skin burns, damages to microvasculature and haemolysis, can occur (Dalecki, 2004; Izadifar *et al.*, 2017). However, when applying the "as low as reasonably achievable" (ALARA) principle and only using ultrasound when medically indicated, diagnostic ultrasound is regarded as safe for both operator and patient (Abramowicz *et al.*, 2008; ter Haar, 2011).

In farm animal production, ultrasound is used for estimation of backfat and marbling in cattle (Brethour, 2000) and in selective breeding for intramuscular fat in pigs (Schwab *et al.*, 2009). Several aspects of fish physiology and health have been studied using ultrasound, such as reproductive parameters in Coho salmon (*Oncorhynchus kisutch*; Martin *et al.*, 1983), detection of parasites in sockeye salmon (*Oncorhynchus nerka*) flesh (Boyce, 1985), heart anatomy in Atlantic salmon (Sande and Poppe, 1995; Poppe *et al.*, 1998), and cardiac function in zebrafish (*Danio rerio*; Nair *et al.*, 2016).

However, the bulk of studies on use of ultrasound in fish are related to reproduction. In many cultured and wild species, sex determination is either not possible due to lack of sexual dimorphism or external sexual morphology is not apparent until late in sexual maturation (Aksnes *et al.*, 1986; Newman *et al.*, 2008; Chebanov and Galich, 2013). Sexing by use of ultrasound has been studied in a species such as Atlantic cod (*Gadus morhua*; Karlsen and Holm, 1994), barfin flounder (*Verasper moseri*; Matsubara *et al.*, 1999), Atlantic halibut (*Hippoglossus hippoglossus*; Martin-Robichaud and Rommens, 2001), and hapuku (*Polyprion oxygeneios*; Kohn *et al.*, 2013), with higher accuracy in sexually maturing or large immature individuals than in smaller juveniles. Qualitative description of gonad appearance in ultrasound images at different stages of maturation includes parameters such as depth penetration in Atlantic halibut ovaries (Shields *et al.*, 1993) and brightness (echogenicity) of testes in ultrasound images of shovelnose sturgeon (*Scaphirhynchus platorynchus*; Wildhaber *et al.*, 2007). More advanced, quantitative ultrasound-based tools for monitoring or determining reproductive parameters such as gonado-somatic index (GSI; weight of gonads as percent of body weight), gonad volume or fecundity have also been established in species such as Neosho madtom (*Noturus placidus*; Bryan *et al.*, 2005), striped bass (*Morone saxatilis*; Jennings *et al.*, 2005), shovelnose sturgeon (Bryan *et al.*, 2007), white sucker (*Catostomus commersonii*; Macbeth *et al.*, 2011) and European eel (*Anguilla anguilla*; Bureau du Colombier *et al.*, 2015). None of these methods give instant results, as they often involve analysis of the ultrasound images after examination, thus requiring re-handling of fish for grading, or increased handling time to make extensive measurements while the fish are being examined.

Reproduction of wild, endangered and farmed sturgeon species has been studied intensively using ultrasound. Sturgeon caviar is a luxury product from eggs of wild or farmed sturgeons that lack sexual dimorphism, and they reach maturity at a high age (16 to 18 years for female beluga sturgeon, *Huso huso*; Chebanov and Galich, 2013). Early non-invasive sex identification might help optimise production and increase fish welfare by replacing invasive methods such as insertion of endoscope in the abdominal cavity (Hurvitz *et al.*, 2007; Chebanov and Galich, 2013; Munhofen *et al.*, 2013). In sturgeons, ultrasound has been used for sexing (Colombo *et al.*, 2004), sometimes with a description

of degree of maturity (Moghim *et al.*, 2002; Wildhaber *et al.*, 2005; Colombo *et al.*, 2007) or detailed descriptions of gonad appearance in ultrasound images (Wildhaber *et al.*, 2007). Traditional methods for sex determination and maturation monitoring, such as histological analyses of gonad tissues and plasma sex hormone analysis have also been used for comparison to observations in ultrasound images (Petochi *et al.*, 2011; Munhofen *et al.*, 2013). More advanced methods have involved several measurements such as gonad length and gonad and oocyte diameter from cross-section ultrasound images, followed by estimation of reproductive parameters such as volume of gonads, fecundity and GSI, with varying degree of accuracy (Bryan *et al.*, 2007; Albers *et al.*, 2013).

Martin *et al.* (1983) were the first to use ultrasound for sex determination of a salmonid, the Coho salmon, and demonstrated that a 5 MHz ultrasound probe could be used for sexing of maturing fish, but were not successful in determining sex of juveniles using a 15 MHz probe. Sex determination of adult Atlantic salmon and rainbow trout was achieved by Reimers *et al.* (1987), who also established an automatized system for sorting fish according to sex, although no efficiency of this system was reported. Mattson (1991) used ultrasound-based gonad diameter measurements above 10 and 25 mm for males and females, respectively, as a criterion to determine whether fish were maturing. Further he hypothesised that such measurements could be used for GSI estimation by breeders. Later studies have used ultrasound for identification of pre- and post-spawning anadromous rainbow trout (Evans *et al.*, 2004), for staging of sexual maturation in sex-reversed rainbow trout females (Hliwa *et al.*, 2014), and identification of maturing individuals and broodstock management of sockeye salmon (Frost *et al.*, 2014).

Reproductive parameters such as GSI and fecundity can thus be estimated using non-invasive ultrasound in many fish species, and there is a potential for using ultrasound to monitor sexual maturation in Atlantic salmon. Many of the ultrasound-based methods used in previous studies are too time-consuming or involve too much stressful handling to be applicable in intensive Atlantic salmon broodfish production. Further, the great technological development of ultrasound equipment since the first studies applying ultrasound in salmonids, might give new possibilities for use of these technologies in broodfish monitoring. Today a thorough knowledge of the Atlantic salmon life cycle and

factors influencing it, enable successful breeding and broodfish production. Studies examining how and when during the life cycle of Atlantic salmon ultrasound can be a tool for maturation monitoring could contribute to optimised broodfish production and a reduced number of handlings.

Wild and captive life cycle and reproduction of Atlantic salmon

Life cycle of wild Atlantic salmon

Wild Atlantic salmon can be found along both coasts of the Atlantic Ocean from New England in the United States to Ungava Bay in Canada on the west coast, through Greenland and Iceland and to the eastern coast from the Barents and Kara seas in the north to the Bay of Biscay and Northern Portugal in the south (MacCrimmon and Gots, 1979; Thorstad *et al.*, 2010).

Atlantic salmon has one of the most diverse life histories among fish (Figure 1; Klemetsen *et al.*, 2003; Thorstad *et al.*, 2010). As an anadromous species, Atlantic salmon migrates between freshwater spawning and nursing grounds and seawater feeding grounds (Thorstad *et al.*, 2010). There is a large plasticity in life histories, and not all individuals undertake long anadromous migrations, but simply stay in estuaries for one or more years before re-entering the river for spawning (Robitaille *et al.*, 1986). Due to postglacial land uplift, there are also non-anadromous, land-locked populations of Atlantic salmon where both males and females complete their whole life cycle in freshwater (Berg, 1985; Hauge *et al.*, 2016). Atlantic salmon are iteroparous, i.e. after spawning they can return to seawater for one or more years before they migrate to the river and spawn again (Ducharme, 1969). This contrasts Pacific salmon species such as sockeye salmon who are semelparous and die after completing sexual maturation and spawning (Groot, 1996).

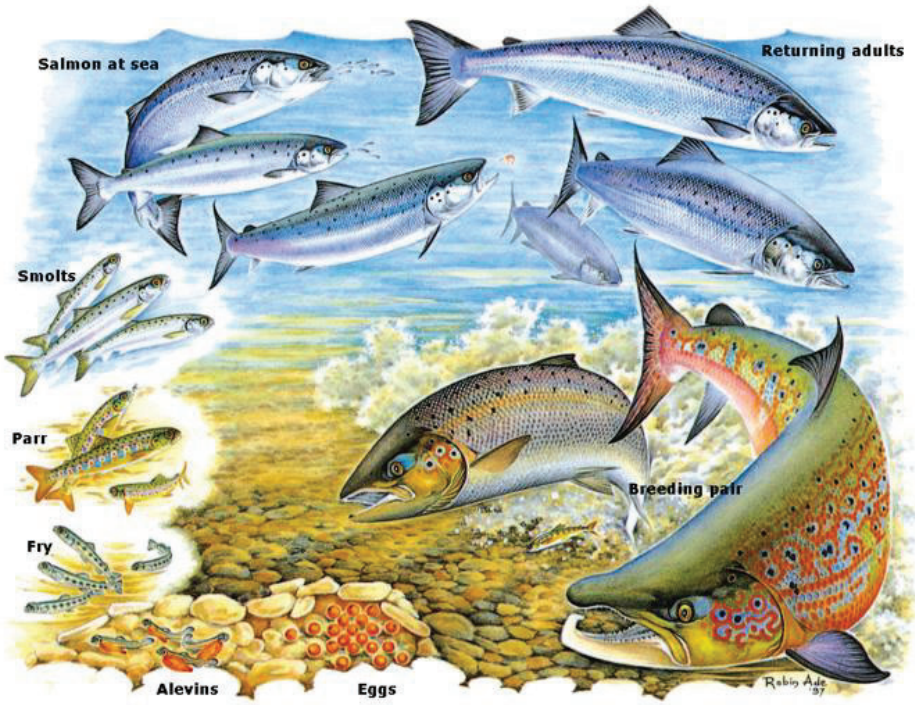


Figure 1. Atlantic salmon life history. Eggs spawned and fertilised at river spawning grounds in autumn hatch in spring, and alevins rely on their yolk sac while living in the gravel until they swim up to first feed. Parr spend a various number of years in the river before they either become sexually mature (males) or smoltify and migrate to seawater. Salmon stay for one or several years in seawater and can undertake long feeding migrations, before returning to spawn in their natal river. Breeders take advantage of this great plasticity in the Atlantic salmon life history when they use light and temperature signals to stimulate or inhibit sexual maturation at given times during the production cycle. Image reproduced with permission from owner and copyright holder, The Atlantic Salmon Trust.

During autumn, females dig nests called redds at river spawning grounds, into which eggs are spawned (Heggberget *et al.*, 1986; Fleming and Einum, 2010). Eggs are then fertilised by milt from one or several males, and covered by gravel for incubation (Fleming *et al.*, 1996). After a temperature dependent incubation time (Crisp, 1981; Heggberget and Wallace, 1984), eggs hatch in spring and alevins live in the gravel until the yolk sac has been depleted and fry emerge to first feed (Klemetsen *et al.*, 2003). Juveniles live in the river for one to several years, before they either adapt to seawater life through smoltification and migrate to seawater during spring, or become sexually

mature as parr (Metcalfe *et al.*, 1988; Jonsson *et al.*, 1998). These precocious males "sneak" in to fertilise eggs in spawnings with anadromous males and females (Myers and Hutchings, 1987; L'Abée-Lund, 1989), and after spawning they might smolt and migrate to seawater (Jonsson *et al.*, 1998). Anadromous individuals undertake a migration to seawater feeding grounds before returning to their natal river to reproduce (Hasler, 1966; Hansen *et al.*, 1993) after one (grilse, mainly males) or several seawater winters (Nævdal *et al.*, 1978; Jonsson *et al.*, 1997; Hutchings and Jones, 1998). In Pacific salmon such as Coho, "jacking", fish maturing after only a few months in seawater, is a common strategy (Gross, 1991), while this phenotype only occurs in farmed, not wild Atlantic salmon (Fjelldal *et al.*, 2011; Imsland *et al.*, 2014).

In fish species, such as salmonids, that live in a climate with pronounced seasonal variation, reproduction is timed according to environmental factors to secure emergence of fry under optimal conditions (Heggberget, 1988). It has been known for decades that changes in day-length influences the timing of sexual maturation in salmonids (Hoover, 1937; Hoover and Hubbard, 1937; McCormick and Naiman, 1984; Taranger *et al.*, 1998; Davies *et al.*, 1999). Fish register changes in daylength through production and release of the hormone melatonin from the light-sensitive pineal gland, found under a semi-transparent part of the scull called the pineal window (Nordtug and Berg, 1990). Melatonin is secreted from the pineal gland in darkness, and production is effectively inhibited when the gland is hit by light (Falcon *et al.*, 2001). Most fish species such as Nile tilapia and African catfish (*Clarias gariepinus*) have endogenous melatonin rhythms that are entrained by light (Martinez-Chavez *et al.*, 2008). The salmonid pineal however, responds directly to changes in light conditions (Alvariño *et al.*, 1993; Randall *et al.*, 1995), which make these species very responsive to light manipulation in captivity (Hoover, 1937; Taranger *et al.*, 1998; 1999). The precise mechanisms of photoperiod and melatonin influence on timing of reproduction are still under study (Maitra and Hasan, 2016), but evidence suggests that the kisspeptin and G-protein coupled receptor GPR54 system is involved (Chi *et al.*, 2017).

Temperature is an important factor affecting salmonid reproduction. Elevated seawater temperature in combination with high energy feed increase occurrence of jacks

(Jonsson *et al.*, 2013), and temperature during first seawater winter influences number of fish maturing as grilse in wild (Scarnecchia, 1983; Scarnecchia *et al.*, 1989) and farmed Atlantic salmon populations (Thorpe *et al.*, 1990). Wild Atlantic salmon spawn at decreasing river temperatures in the fall or winter (Heggberget, 1988). Furthermore, it has been shown that a marked drop in temperature can advance ovulation and increase egg survival and milt quality, while elevated water temperature during final maturation inhibits ovulation and reduces egg survival (Taranger and Hansen, 1993; King and Pankhurst, 2004; Vikingstad *et al.*, 2016). Negative effects of elevated temperature during final maturation include atretic and overripe oocytes within the ovary and inhibited milt production and reduced spermatocrit (Vikingstad *et al.*, 2016).

During sexual maturation and upstream migration and spawning, Atlantic salmon can deplete as much as 50 to 70 % of their energy reserves (Jonsson *et al.*, 1991; 1997), thus sufficient energy resources are vital for reproductive success. In females, much of this energy is invested into the lipid rich eggs, while in males less energy is invested in gametes and more in reproductive behaviour and development of secondary sexual characteristics (Fleming and Einum, 2010). Both in maturing parr (Rowe and Thorpe, 1990; Rowe *et al.*, 1991) and fish maturing in seawater (Thorpe *et al.*, 1990; Kadri *et al.*, 1996; Jonsson *et al.*, 2013) energy reserves in the spring before maturation influences decision to proceed with sexual maturation and spawning the following autumn. Sexual maturation also seems to influence growth, as a pubertal growth spurt is observed in maturing individuals compared to non-maturing individuals. Hunt *et al.* (1982) reported that maturing males were significantly heavier during spring than non-maturing males, but that non-maturing individuals caught up over the summer. The maturing individuals then showed a lower growth than non-maturing individuals, thus when it progressed too far, maturation had a deteriorating effect on growth (Hunt *et al.*, 1982).

Genetic factors interplay with environmental and intrinsic factors to determine age at sexual maturation of Atlantic salmon. Gjerde (1984) found that parental age at first sexual maturity influenced the age at maturity in offspring. A genetic marker associated with the vestigial-like family member 3 (vgll3) gene on chromosome 25, which is related to adiposity and puberty onset in humans, is involved in determining age at sexual

maturation in both wild and farmed Atlantic salmon (Ayllon *et al.*, 2015; Barson *et al.*, 2015). The alleles for early and late maturation had opposite dominance patterns between the sexes, the early allele was dominant in males, while the late allele was dominant in females (Barson *et al.*, 2015). However, recent findings suggests that captive females of the *mowi* strain mature after two seawater winters irrespective of early or late maturation genotype, and that the dominance patterns described for wild fish might not apply to captive males, which might indicate influence of other factors such as increased growth rate and light and temperature in captivity (Ayllon *et al.*, 2019).

The influence of salinity on salmonid reproduction has been less investigated than other environmental factors. However, Melo et al. (2014) showed that in male post-smolts, salinity in combination with continuous light (LL) treatment was more effective in stimulating sexual maturation than salinity combined with short photoperiod (LD 12:12) or freshwater combined with either LL or LD. Also, in female grilse, Magwood *et al*., (1999, cited in Bromage *et al.*, 2001) found that transfer to freshwater three to four months before regular broodfish transfer advanced ovulation and resulted in a more synchronised spawning period. Farmers rearing fish in RAS facilities also report higher occurrence of unwanted early sexual maturation at lower salinities, indicating that salinity affects maturation in Atlantic salmon (Table 2 in (Good and Davidson, 2016).

The great plasticity in Atlantic salmon life histories and the influence of light and temperature in initiation and completion of sexual maturation is utilised by broodfish producers. By adjusting timing of light and temperature stimuli, initiation of sexual maturation can be adjusted according to demand for fertilised eggs in the aquaculture industry. This also contributes to a higher utilisation of production capacity in Atlantic salmon broodfish production.

Life cycle of captive Atlantic salmon broodfish

Sea-based production

To imitate the declining river temperature that stimulate spawning in wild Atlantic salmon, a temperature decline, often called the temperature drop, is given approximately four weeks before planned stripping to initiate ovulation and spermiation and production

of expressible milt in captive broodfish production (Taranger and Hansen, 1993; Taranger *et al.*, 2015; Vikingstad *et al.*, 2016). As Atlantic salmon does not spawn in captivity, eggs and milt are collected by a gentle massage of the belly, so-called stripping. Salmonid eggs can be held in the abdominal cavity for approximately one week before there is a risk of quality deterioration and morphological changes due to post-ovulatory ageing (POA), also known as over-ripening (rainbow trout, Craik and Harvey, 1984; Springate *et al.*, 1984; Aegerter and Jalabert, 2004; Atlantic salmon, Mommens *et al.*, 2015). Thus, breeders perform weekly ovulation checks by manually palpating the belly of all females expected to ovulate soon (Billard and Jensen, 1996) and remove ovulated eggs within one week of detected ovulation. As the stripping window can be more than one month (Taranger and Hansen, 1993; Vikingstad *et al.*, 2016), number of handlings can become substantial for the females ovulating last. In some cases, a temperature drop may not be possible for technical reasons, or it may be desirable to advance and synchronise maturation, which can be done using a gonadotropin-releasing hormone analogue (GnRHa). In female salmonids, GnRHa has mainly yielded advanced ovulation with few negative effects on egg quality and progeny survival (Breton *et al.*, 1990; Taranger *et al.*, 1992; Slater *et al.*, 1995; Arabacı *et al.*, 2004). However, some studies report reduced egg fertilisation rates (Afonso *et al.*, 1999) or increased prevalence of malformations (Bonnet *et al.*, 2007) following GnRHa injection. This might in part be due to the timing of GnRHa injection, and it has been suggested that injection close to natural ovulation time could yield better fertilisation and survival rates (Taranger *et al.*, 1992; Afonso *et al.*, 1999). In males, use of hormonal stimuli accelerated spermiation and increased milt volume, without decreasing sperm counts or motility in species such as Atlantic salmon (Weil and Crim, 1983), sea bass (Sorbera *et al.*, 1996), yellowtail flounder (*Pleuronectes ferruginea*; Clearwater and Crim, 1998) and Atlantic cod (Garber *et al.*, 2009).

Fertilised Atlantic salmon eggs that are kept at 8 °C will hatch after approximately 62 days (500 degree-days; (Crisp, 1981). The number of days from fertilisation until hatch can be changed by adjusting the egg incubation temperature, with high temperature yielding shorter incubation time than low temperatures (Crisp, 1981; Heggberget and Wallace, 1984; Wallace and Heggberget, 1988). Increasing incubation temperature above a threshold of 8 °C increases occurrence of deformities, and is therefore not recommended

(Ørnsrud *et al.*, 2004). Fertilised eggs are delivered to customers just prior to hatching, or are hatched by the broodfish producer, initiating a new cycle. The hatched alevins rely on their yolk sac for approximately 34 days at 8 °C (approximately 270 degree-days), until they are ready for exogenous feeding (Jensen *et al.*, 1989). At this point, fry are transferred into larger tanks where first feeding on pelleted food takes place (Lemm and Hendrix, 1981; Stradmeyer *et al.*, 1988), and the preferred temperature during this period is 12-15 °C, which gives the best growth of parr (Elliott and Hurley, 1997; Noble *et al.*, 2018). During the freshwater phase, all parr must be vaccinated against the bacterial diseases furunculosis, vibriosis and cold water vibriosis (caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Aliivibrio salmonicida*, respectively; Ministry of Trade, Industry and Fisheries, 2008). As Norwegian aquaculture regulation (Ministry of Trade, Industry and Fisheries, 2008) states that fish can be graded and reared according to size unless this negatively affect fish health, fish are graded during freshwater production to separate them into groups with equal mean weight. A light regime consisting of a period of continuous light from first feeding followed by minimum four weeks of short photoperiod (winter signal) can induce smoltification (Berge *et al.*, 1995) in the same year as first feeding, resulting in seawater transfer of S0 smolts in August or September. Alternatively, smoltification takes place under natural or continuous light during spring the year following first feeding with transfer to seawater in May, as S1 smolts (Stefansson *et al.*, 1991). As in wild Atlantic salmon, some males will mature as parr rather than go through smoltification in preparation for seawater transfer (Stefansson *et al.*, 1991). Fjelldal *et al.* (2018) found that progressing maturation in freshwater production might impair osmoregulatory capacity, which can lead to reduced growth, difficulties in osmoregulating, reduced welfare and ultimately death if fish are transferred to seawater (Duston, 1994; Aunsmo *et al.*, 2008).

During the seawater phase, Atlantic salmon are kept in net-pens and grow to harvest size (approximately 4.5 to 6 kg) in 14 to 22 months. A difference in growth has been observed between males and females (Gjerde and Gjedrem, 1984; Acharya, 2011), where males reach a higher body weight at harvest than females. The high growth rates achieved in aquaculture increases risk of unwanted early sexual maturation (McCormick and Naiman, 1984; Skilbrei, 1989; Duston and Saunders, 1999). In production of Atlantic

salmon for consumption, sexual maturation before harvest is undesirable as this energetically demanding process reduces important quality parameters of the flesh such as skin and filet colour, flesh texture and fat and protein content (Aksnes *et al.*, 1986), which can lead to down-grading at the harvest plant (Ministry of Trade, Industry and Fisheries, 2013). The risk of unwanted sexual maturation shortly after seawater transfer, jacking, increases when large smolt that have been reared at high temperature and under continuous light during the freshwater phase, are transferred to seawater (Fjelldal *et al.*, 2011; Imsland *et al.*, 2014). Although jacking is predominately observed in males, incidents of females maturing shortly after seawater transfer ("jills") have been reported in Tasmanian salmon farming (Thorpe, 2004). Grilsing, maturation after one seawater winter, is most common in males, but can also be found in wild and farmed females (Youngson *et al.*, 1988; Hutchings and Jones, 1998; Ayllon *et al.*, 2019). To reduce prevalence of grilsing, continuous artificial light is used in the net-pens from winter to summer solstice during the first winter in seawater (Porter *et al.*, 1999; Leclercq *et al.*, 2011).

In broodfish production the best candidates to become broodfish are selected manually at harvest size and kept an additional year in seawater. A female Atlantic salmon produces approximately 1500 to 3000 eggs per kg of body weight giving a fecundity of 15 000 to 30 000 eggs for a 10 kg female (Klemetsen *et al.*, 2003), while male sperm producing capacity is in the range of 1.6×10^{10} spermatozoa per mL ejaculate, and mean 12 mL ejaculate per male (Kazakov, 1981). Thus, a sex ratio of one male per ten females is often regarded as sufficient in a commercial broodfish production setting. To optimise egg output from a limited production facility area there is a need for sex identification before transfer of broodfish to freshwater. Experienced personnel can identify sex of maturing broodfish manually as freshwater transfer approaches (Aksnes *et al.*, 1986; Youngson *et al.*, 1988), or ultrasound can be used to identify sex of maturing fish (Reimers *et al.*, 1987; Mattson, 1991).

After this selection and during the last year in seawater, broodfish are fed special broodfish diet, high on energy and marine oils to satisfy the nutritional requirements of broodfish during sexual maturation (Watanabe *et al.*, 1984; Eskelinen, 1989). It is

desirable to influence timing of sexual maturation which can be done by applying additional continuous light during the second winter in seawater. Continuous light from March to summer solstice and an 8 h light 16 h dark photoperiod (8:16 LD) after transfer to freshwater, advances maturation by approximately one month, while it can be delayed by one month by using continuous light from midsummer (Taranger *et al.*, 1998). Hansen *et al.* (2015) found that strict control of light and temperature during autumn and winter was needed for a six-month off-season ovulation and spermiation in Atlantic salmon. Thus, further advancement or delay of sexual maturation to produce out-of-season eggs is challenging in traditional seawater-based broodfish production as temperature control is difficult to achieve. This can, however, be achieved in land-based broodfish production.

Transfer of broodfish to freshwater takes place during spring or summer four to seven months before planned stripping time. As wild Atlantic salmon are known to not feed during the freshwater spawning migration, broodfish are not fed after transfer to freshwater (Kadri *et al.*, 1995). During the freshwater phase, temperature control and artificial light regimes are possible. An elevated temperature (up to 18 °C) combined with short day photoperiod (8:16 LD) is used to produce advanced broodfish groups that are ready for stripping in September (Taranger *et al.*, 1998; King *et al.*, 2003), while keeping broodfish in continuous light is used to delay maturation when stripping fish late in winter is desirable (Taranger *et al.*, 1998). During the freshwater phase, GSI can be used to monitor sexual maturation, and at stripping GSI is approximately 15 to 25 in females and 5 to 10 in males (Aksnes *et al.*, 1986; Jonsson *et al.*, 1991; King and Pankhurst, 2003; Melo *et al.*, 2014). Unfortunately, GSI measurements require sacrifice of valuable broodfish, or measurements in deceased fish which might not be representative of the population. Other quantitative methods for maturation monitoring such as sex hormone analysis (Nagahama and Adachi, 1985; Prat *et al.*, 1996) are costly and time-consuming, and involves tagging and re-handling of fish for grading based on results. Insertion of an endoscope into the abdominal cavity (Ortenburger *et al.*, 1996; Swenson *et al.*, 2007) or direct visual inspection of gonads through an incision (Melillo Filho *et al.*, 2016) gives instant results, but comes with increased risk of internal bleedings and infections.

Land-based production in RAS facilities in relation to maturation control

In recent years, RAS facilities have become more common in Atlantic salmon smolt production (Bergheim *et al.*, 2009; Dalsgaard *et al.*, 2013). Such facilities enable good control of production parameters, and large smolt can be produced and transferred to seawater at any time of year provided that fish are in a fit state for transfer at the prevailing environmental conditions (Ministry of Trade, Industry and Fisheries, 2008). It should however be kept in mind that the risk of jacking increases when large smolt reared at high temperature and continuous light in freshwater are transferred to seawater (Fjelldal *et al.*, 2011; Imsland *et al.*, 2014). The use of RAS facilities increases demand on broodfish producers for continuous delivery of fertilised eggs. The described advance or delay in timing of reproduction obtained by use of light and temperature control in sea-based broodfish production can be further extended by regulating egg incubation temperature until hatching. A high incubation temperature shortens the time until hatching, while a low incubation temperature increases time until hatching (incubation at 0.1 °C results in approximately 260 days from fertilisation until hatching; Wallace and Heggberget, 1988). Thus, using a combination of light and temperature to control broodfish maturation, and temperature control of egg development, it is possible to secure egg delivery from November throughout July. Production of broodfish in RAS facilities can be a solution for closing the gap in the delivery window of fertilised Atlantic salmon eggs. Rearing broodfish in land-based RAS facilities, light and temperature control can simulate opposite seasons compared to what fish would experience in the wild, thus making year-round stripping and egg delivery possible.

The biomass that can be produced in RAS facilities is limited compared to sea-based broodfish production, therefore establishing a desirable sex ratio when stocking facilities with juveniles is essential to optimise production. Further, non-invasive tools that enable breeders to monitor the response of fish to environmental stimuli intended to initiate sexual maturation, are needed. In both sea- and land-based broodfish production, light and temperature cues affect the reproductive biology of fish. Knowledge of reproductive physiology is therefore important when developing non-invasive methods for monitoring sexual maturation.

Fish reproductive physiology

Reproduction in fish is controlled by the brain-pituitary-gonad (BPG) axis (Figure 2). Neurons from the hypothalamus in the brain innervate the anterior lobe of the pituitary (hypophysis), and release gonadotropin-releasing hormone (GnRH) into the pituitary (Sherwood *et al.*, 1983). This stimulates release of the gonadotrophic hormones (GTH) follicle-stimulating hormone (FSH, previously known as GTH I) and luteinizing hormone (LH, previously known as GTH II; Swanson *et al.*, 1989; Breton *et al.*, 1998; Ando *et al.*, 2004) from distinct populations of hormone producing cells (Nozaki *et al.*, 1990; Shimizu *et al.*, 2003). In general, FSH is secreted during early phases of sexual maturation, when gonads grow and oocytes and spermatozoa are produced, while a surge in LH is seen at final maturation (Prat *et al.*, 1996; Breton *et al.*, 1998; Santos *et al.*, 2001). FSH and LH secreted from the pituitary travel in the blood to reach their target tissues, namely testes in males and ovaries in females (Andersson *et al.*, 2009), where sex hormones such as oestradiol-17β (E2), testosterone (T), 11-ketotestosterone (11-KT) and the maturation inducing hormone (MIH), 17α,20β-dihydroxy-4-pregnen-3-one, are produced.

Many factors other than hormones influence and regulate sexual maturation. The kisspeptin/GPR54 system has proven important for initiation and regulation of sexual maturation in both mammals (de Roux *et al.*, 2003; Seminara *et al.*, 2003) and fish (Felip *et al.*, 2009; Beck *et al.*, 2012; Chi *et al.*, 2017). Dopamine (Vacher *et al.*, 2000; 2002), neuropeptide Y (Kah *et al.*, 1989; Breton *et al.*, 1991; Peng *et al.*, 1993) and γ-aminobutyric acid (Mañanos *et al.*, 1999) all influence the production of FSH and/ or LH at various stages of maturation. Gonadotropin inhibiting hormone (GnIH) inhibits *in vivo* FSH and LH release in goldfish (Qi *et al.*, 2013), while it stimulates *in vitro* release of FSH and LH in precocious male sockeye salmon (Amano *et al.*, 2006). The sex steroids E2 and T regulate sexual maturation through negative and positive feedback mechanisms depending on maturational stage and sex (Dickey and Swanson, 1998; Saligaut *et al.*, 1998; Ando *et al.*, 2004).

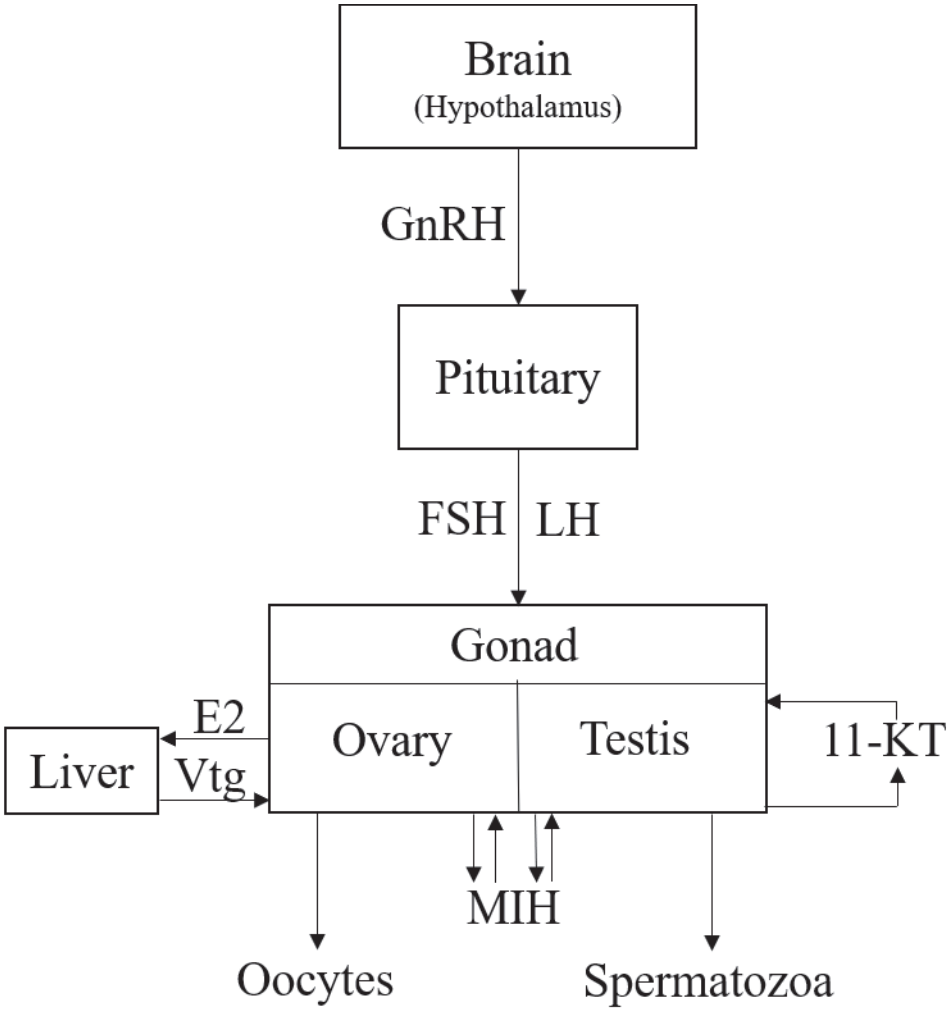


Figure 2. Schematic overview of the brain-pituitary-gonad (BPG) axis and sex hormones in fish. The hypothalamus secretes gonadotropin releasing hormone (GnRH), which stimulates pituitary production of follicle stimulating hormone (FSH) and luteinising hormone (LH). In ovaries FSH stimulates oestradiol 17β (E2) production, which again stimulates production of vitellogenin (Vtg) in the liver, that is incorporated into growing oocytes. In the testes FSH stimulates production of the main androgen, 11-ketotestosterone (11-KT) which stimulates spermatogenesis. In both ovaries and testes, luteinising hormone (LH) stimulates secretion of maturation inducing hormone (MIH) at final maturation.

Females

The paired ovaries are the site of oogenesis, the development and growth of oocytes, that eventually are ovulated and spawned or stripped from a female and fertilised by milt from the male (Lubzens *et al.*, 2010). The oocytes are enveloped in a single granulosa cell layer surrounded by the outer theca layer which consists of fibroblasts, collagen fibres, capillaries and special theca cells (Nagahama *et al.*, 1982). The theca cells produce oestrogen precursors (mainly T) that are converted into E2 by the granulosa cells (Nagahama *et al.*, 1982). Further, close to ovulation, the theca cells produce 17α-hydroxyprogesterone, which is in turn converted to MIH by granulosa cells (Young *et al.*, 1986).

Primordial germ cells are present from embryogenesis and differentiate into oocytes that after initiation of the first meiotic division are surrounded by granulosa and theca cells in the primary oocyte (Lubzens *et al.*, 2010). From this point, growth of the oocyte is separated into primary and secondary growth phases, and vitellogenesis. During primary growth the oocyte goes through the chromatin-nucleolus and perinucleolus stages (Figure 3A), which are independent of GTH stimuli from the pituitary (Tyler and Sumpter, 1996; Lubzens *et al.*, 2010). At the transition into the secondary growth phase cortical alveoli appear, and oil droplets (Figure 3A) are incorporated into the oocyte (Tyler and Sumpter, 1996; Taranger *et al.*, 1999). Although mainly regarded as a male androgen,11-KT has been related to pre-vitellogenic growth of oocytes in shortfin eel (*Anguilla australis*; Lokman *et al.*, 2007) and Atlantic cod (Kortner *et al.*, 2009), and in Coho salmon, Fitzpatrick *et al.* (1986) found higher 11-KT levels in ovulated females than those at pre-ovulatory stages, indicating functions of 11-KT in females as well.

FSH stimulates recruitment of oocytes into vitellogenesis (Tyler *et al.*, 1997) and production of the sex steroid E2 (Van Der Kraak and Donaldson, 1986; Swanson *et al.*, 1989; Planas *et al.*, 2000), depending on the stage of maturation. In the liver, production of zona radiata proteins, a major constituent of the eggshell, is initiated before vitellogenesis (Celius and Walther, 1998). During vitellogenesis E2 stimulates production of the yolk precursor vitellogenin (Vtg) in the liver (Maitre *et al.*, 1986), which is incorporated into the oocyte through endocytosis in clathrin coated pits (Selman and

Wallace, 1982; Tyler *et al.*, 1991; Mizuta *et al.*, 2017), leading to an increase in oocyte size (Tyler *et al.*, 1990). In salmonids vitellogenesis is a prolonged process, and accumulation of vitellogenin (Figure 3 B-D) can be seen ten to eight months before expected ovulation (Sumpter *et al.*, 1984; Tyler *et al.*, 1990; Andersson *et al.*, 2009).

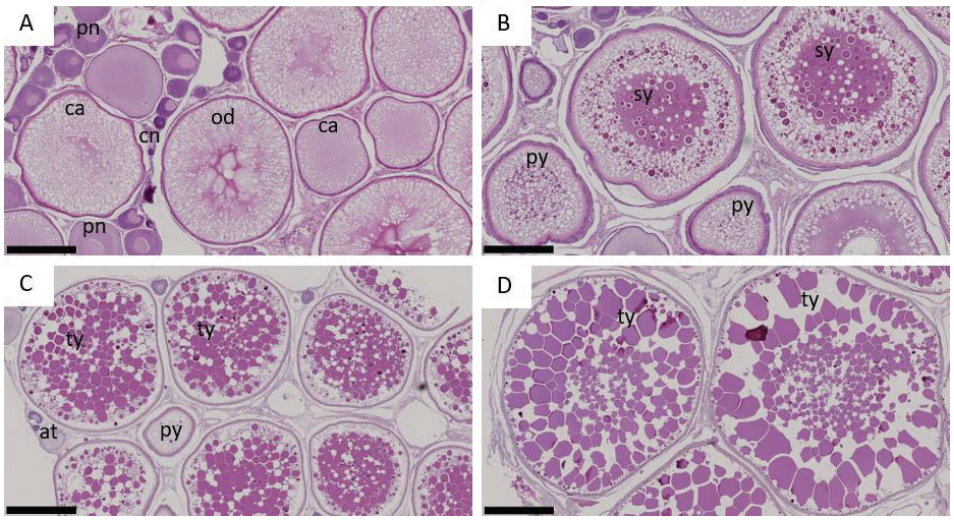


Figure 3. Histological sections from Atlantic salmon ovaries during oogenesis. Growth of oocytes during sexual maturation can be separated into primary and secondary growth phases (A), and vitellogenesis (B-D). at: atretic oocyte, ca: cortical alveoli stage, cn: chromatin nucleolus stage, od: oil droplet stage, pn: perinucleolus stage, py: primary yolk oocyte, sy: secondary yolk oocyte, ty: tertiary yolk oocyte. Scale bar is 500 μm in A and B, and 1 mm in C and D.

When vitellogenesis has been completed, a shift in enzymatic activity lead to LH stimulated production of MIH (Van Der Kraak and Donaldson, 1986; Planas *et al.*, 2000; Nakamura *et al.*, 2005), germinal vesicle breakdown, completion of the first meiotic division and final oocyte maturation (Nagahama *et al.*, 1983; Nagahama, 1994; Tyler and Sumpter, 1996). Hydration, the uptake of water into the oocytes, is central in fish that spawn pelagic eggs in seawater (Craik and Harvey, 1987), such as mummichog (*Fundulus heteroclitus*; Wallace and Selman, 1985), plaice (*Pleuronectes platessa*), and lemon sole (*Microstomus kitt*; Thorsen and Fyhn, 1996), and has been observed to a lesser degree in rainbow trout eggs (Bobe *et al.*, 2006; Milla *et al.*, 2006). At ovulation, the oocytes are

released from the follicle into the ovarian or abdominal cavity (Jalabert and Szöllösi, 1975; Jalabert *et al.*, 1978).

The seasonal variations in plasma levels of the reproductive hormones in salmonids reflect their functions. Plasma levels of FSH in female iteroparous salmonids generally have two distinct peaks, one during vitellogenic growth of the gonads and one at ovulation (Prat *et al.*, 1996; Gomez *et al.*, 1999; Santos *et al.*, 2001). The increase in FSH at or after ovulation might be related to the initiation of a new ovarian cycle (Prat *et al.*, 1996; Tyler *et al.*, 1997; Breton *et al.*, 1998; Santos *et al.*, 2001). This has been corroborated by histological studies of rainbow trout ovaries showing oocytes in primary growth phase present at ovulation (Grier *et al.*, 2007; Penney and Moffitt, 2014). In semelparous Coho salmon who die after spawning, no increase in FSH levels at ovulation has been observed (Swanson *et al.*, 2003). Plasma levels of E2 and T show relatively similar profiles during female reproduction, and reach maximum levels at the end of vitellogenesis, but prior to ovulation (Stuart-Kregor *et al.*, 1981; Fitzpatrick *et al.*, 1986; Goetz *et al.*, 1987; Norberg *et al.*, 1989; Estay *et al.*, 2003). LH and MIH remain undetectable or low throughout vitellogenesis, and show distinct peaks at ovulation (Fitzpatrick *et al.*, 1986; Swanson *et al.*, 1989; Prat *et al.*, 1996; Estay *et al.*, 2003).

Males

The paired testes are the site of production of the male germ cells, spermatozoa, from spermatogonial germ cells, and consist of two main compartments, the intertubular compartment with steroidogenic Leydig cells which are the main site for androgen production, vessels, macrophages and neural and connective tissue, and the tubular compartment containing Sertoli cells and germ cells (Nagahama *et al.*, 1982; Schulz *et al.*, 2010). Fish spermatogenesis is cystic, meaning Sertoli cells surround and support a clone of synchronously developing germ cells within each cyst (Vilela *et al.*, 2003; Schulz *et al.*, 2005). This contrasts mammalian non-cystic spermatogenesis, where Sertoli cells support several different developmental stages of germ cells at once (Schulz *et al.*, 2010).

In the first, proliferative phase of spermatogenesis, Sertoli cells encloses a single diploid spermatogonium A which then develops into spermatogonia B (Figure 4A) and proliferates by mitosis with incomplete cytokinesis, leaving them connected by

cytoplasmic bridges (Dziewulska and Domagała, 2003; Leal *et al.*, 2009). Spermatogonia B differentiate into primary spermatocytes (Figure B), which during the meiotic phase go through the first and second meiotic divisions, becoming secondary spermatocytes and haploid spermatids (Figure 4C), respectively (Dziewulska and Domagała, 2003; Leal *et al.*, 2009; Schulz *et al.*, 2010). Spermatogenesis is under endocrine control, and 11-KT is the major androgen in fish (Idler *et al.*, 1971; Miura *et al.*, 1991). In Japanese eel (*Anguilla japonica*) FSH stimulates 11-KT secretion from Leydig cells, resulting in full spermatogenesis from spermatogonia A to spermatozoa (Miura *et al.*, 1991; Ohta *et al.*, 2007).

Through the spermiogenic phase, the spermatids differentiate into spermatozoa (Figure 4D), and at spermiation they are released into the lobule by breaking the association between Sertoli cells (Dziewulska and Domagała, 2003). The spermatozoa acquire the ability to move as they migrate through the sperm duct (Morisawa and Morisawa, 1986), and together with the fluids produced during the migration, becomes the expressible milt that can be stripped from the male (Schulz and Miura, 2002). As in females, a shift in steroid production from 11-KT to MIH is seen as males enter the final phases of maturation (Sakai *et al.*, 1989). During the final stages of spermatogenesis, when spermatid and spermatozoa are present in testes, both FSH and LH are able to stimulate secretion of MIH, but as spermiation and milt production approaches, testes MIH production is more sensitive to LH than FSH (Planas and Swanson, 1995).

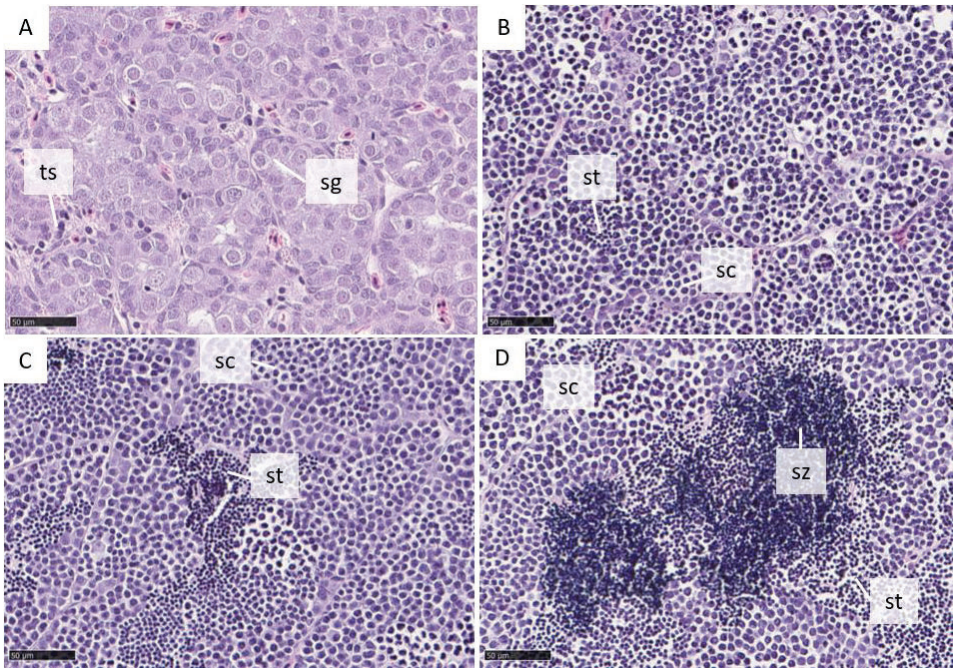


Figure 4. Histological sections from Atlantic salmon testes during spermatogenesis. During the proliferative phase spermatogonia (A) proliferates by mitosis with incomplete cytokinesis before differentiating into primary spermatocytes. During the mitotic phase secondary spermatocytes and spermatids (B and C) are formed. In the spermiogenic phase spermatozoa are formed (D). sc, spermatocyte; sg, spermatogonia; st, spermatid; sz, spermatozoa; ts, testicular somatic cells. Scale bars are 50 μm.

Seasonal variations in levels of reproductive hormones are seen in male salmonids as well. Plasma levels of FSH increase during testis growth and at spermiation (Prat *et al.*, 1996). 11-KT levels might show a small peak during spring, are elevated during testis growth and decrease before milt can be collected (Stuart-Kregor *et al.*, 1981; Hunt *et al.*, 1982; Fitzpatrick *et al.*, 1986). Plasma T levels follow the profile observed for 11-KT, although at considerably lower levels (Hunt *et al.*, 1982; Fitzpatrick *et al.*, 1986). LH and MIH remain undetectable or low throughout spermatogenesis, and show a distinct peak at spermiation or maximum milt production (Stuart-Kregor *et al.*, 1981; Scott and Sumpter, 1989; Swanson *et al.*, 1989; Prat *et al.*, 1996). Sakai *et al.* (1989) therefore concluded that MIH is involved in spermiation in male fish. As MIH is elevated during

milt production in rainbow trout, it has been suggested that it is involved in control of composition of seminal plasma (Baynes and Scott, 1985).

As sexual maturation is initiated months before stripping, many of the handlings broodfish are subjected to occur concomitant with, and might affect, the reproductive process as stress and elevated cortisol levels have been shown to influence fish reproduction.

The effects of stress on reproduction and broodfish welfare

As discussed in the chapter regarding animal welfare, handlings for lice and disease management, size gradings and examinations to check the status of sexual maturation, is necessary in production and can lead to stressed broodfish and reduced welfare. Other potential stressors such as light and temperature manipulations and sexual maturation in seawater might also negatively affect welfare.

Studies examining the effects of stress on reproduction has mainly used two different methods; various disturbances and cortisol implants. Typical disturbances used are comparable to handlings and situations that captive broodfish are exposed to, such as chasing, netting, crowding, confinement, reduction in water levels, emersion and noise, and result in elevated cortisol levels in broodfish, which is also transferred to eggs (Campbell *et al.*, 1992; 1994; Stratholt *et al.*, 1997; Contreras-Sánchez *et al.*, 1998). Cortisol implants and osmotic pumps are also frequently used to elevate broodfish cortisol to levels seen in stressed fish (Carragher *et al.*, 1989; Kleppe *et al.*, 2013; Eriksen *et al.*, 2015). Direct incubation of ovarian follicles and unfertilised and fertilised eggs in cortisol has also been used to isolate cortisol effects on progeny (Barkataki *et al.*, 2011; Li *et al.*, 2012).

The effects of increased cortisol levels on various reproductive parameters vary somewhat between studies. In Atlantic cod, increased cortisol levels during the last three weeks before spawning were associated with decrease in T levels, but no change was observed in E2 or Vtg level (Kleppe *et al.*, 2013). In brown trout (*Salmo trutta*) reduced levels of sex steroids was observed in both males (T) and female (T, E2 and Vtg) after 18 days with a cortisol implant (Carragher *et al.*, 1989), and T and 11-KT was reduced after

one month of chronic confinement in males (Pickering *et al.*, 1987). To examine the mechanism behind the cortisol inhibition of steroid synthesis, Barkataki *et al.* (2011) incubated rainbow trout ovarian follicles in cortisol, and hypothesised that the inhibition takes place early in the E2 and T synthesis pathway, in the theca cells.

Depending on when during reproduction the stressor is applied, several effects on spawning time has been observed. Repeated acute stressful events in the form of tank drainage and three minutes of air exposure during the last nine months before expected ovulation in rainbow trout resulted in a significant delay of ovulation time in stressed compared to control females (Campbell *et al.*, 1992). When applying various stressors comparable to those seen in aquaculture situations such as crowding, netting and making noise during final maturation (45 days) or vitellogenesis and final maturation (90 days) in rainbow trout, ovulation time was advanced by two weeks (Contreras-Sánchez *et al.*, 1998). Similar, although non-significant ($p = 0.054$) results has also been observed for Coho salmon (Stratholt *et al.*, 1997). In Atlantic cod, stressed broodfish performed significantly fewer courtship behaviours and showed a tendency for a more prolonged spawning period after capture and confinement stress for 10 weeks during the spawning period than non-stressed individuals (Morgan *et al.*, 1999).

The effects of stressors or cortisol implants in broodfish on gamete and progeny quality and quantity also vary. Acute stress from transportation and handling at the stripping day gave premature activation of spermatozoa in white bass (*Morone chysops*; Allyn *et al.*, 2001), and nine months of repeated acute stress in rainbow trout males resulted in reduced sperm densities (Campbell *et al.*, 1992), which might reduce capacity of spermatozoa to fertilise eggs. Increased cortisol has also been associated with reduced fecundity, egg size and gonad weight in some studies on salmonids (Carragher *et al.*, 1989; Campbell *et al.*, 1992; 1994), while others found no effect of cortisol on these parameters in Atlantic cod (Morgan *et al.*, 1999; Kleppe *et al.*, 2013). Stress and elevated cortisol also affect the final outcome of reproduction, seen as reduced fertilisation rates (Eriksen *et al.*, 2015), reduced survival to eyed stage, hatch and/ or first feeding (Campbell *et al.*, 1992; Contreras-Sánchez *et al.*, 1998; Eriksen *et al.*, 2006; Medeiros *et*

al., 2016) and increased occurrence of malformations of progeny (Morgan *et al.*, 1999; Eriksen *et al.*, 2006; 2015).

In rainbow trout, genetic differences in magnitude of the cortisol response to stressful stimuli has been identified, making it possible to establish low- and high-responding lines (Pottinger and Carrick, 1999; Fevolden *et al.*, 2002; Øverli *et al.*, 2002). Given the effects of cortisol on reproduction, use of low-responding lines might contribute to increased quality of the reproductive output and increased welfare in intensive aquaculture settings. Today, anaesthetics are commonly used to facilitate handling and reduce stress in commercial aquaculture settings. The anaesthetics used should not only render fish immobilised and handleable, but also prevent a rise in blood cortisol levels (Olsen *et al.*, 1995; Iversen *et al.*, 2003).

Stress responses are in themselves not detrimental, but instead help the animal adjust to changing conditions, and it is only when the animal is not able to adapt or the stressor becomes chronic that stress might be detrimental and pose a welfare challenge. Although each stressful event alone does not cause a decline in welfare, the accumulated load of handlings, temperature and light changes, the energetically costly reproductive process and reduced immune function, might however negatively affect welfare of broodfish. As especially vitellogenesis is a prolonged process in Atlantic salmon, handlings during the last year before stripping are concomitant with this process, and a reduction in number of handlings during this period could improve reproductive outcome and broodfish welfare. Sex identification of broodfish candidates is usually performed during this period, and if this could be performed much earlier during production, handlings in seawater during vitellogenesis could be avoided. Further, all females are handled weekly to check for ovulation as expected stripping approaches and during the stripping season. This results in many handlings especially of females that ovulate late in the stripping season and reducing this number could be beneficial for reducing broodfish stress and increasing welfare and reproductive outcome. Non-invasive ultrasound has shown potential in previous studies on fish reproduction and use at selected times during maturation might give valuable information to breeders. This require systematic studies to identify times during the life cycle of captive broodfish when ultrasound can be a basis

for well-informed decisions regarding broodfish maturation and to identify its potential in reducing handling of broodfish.

Study aims

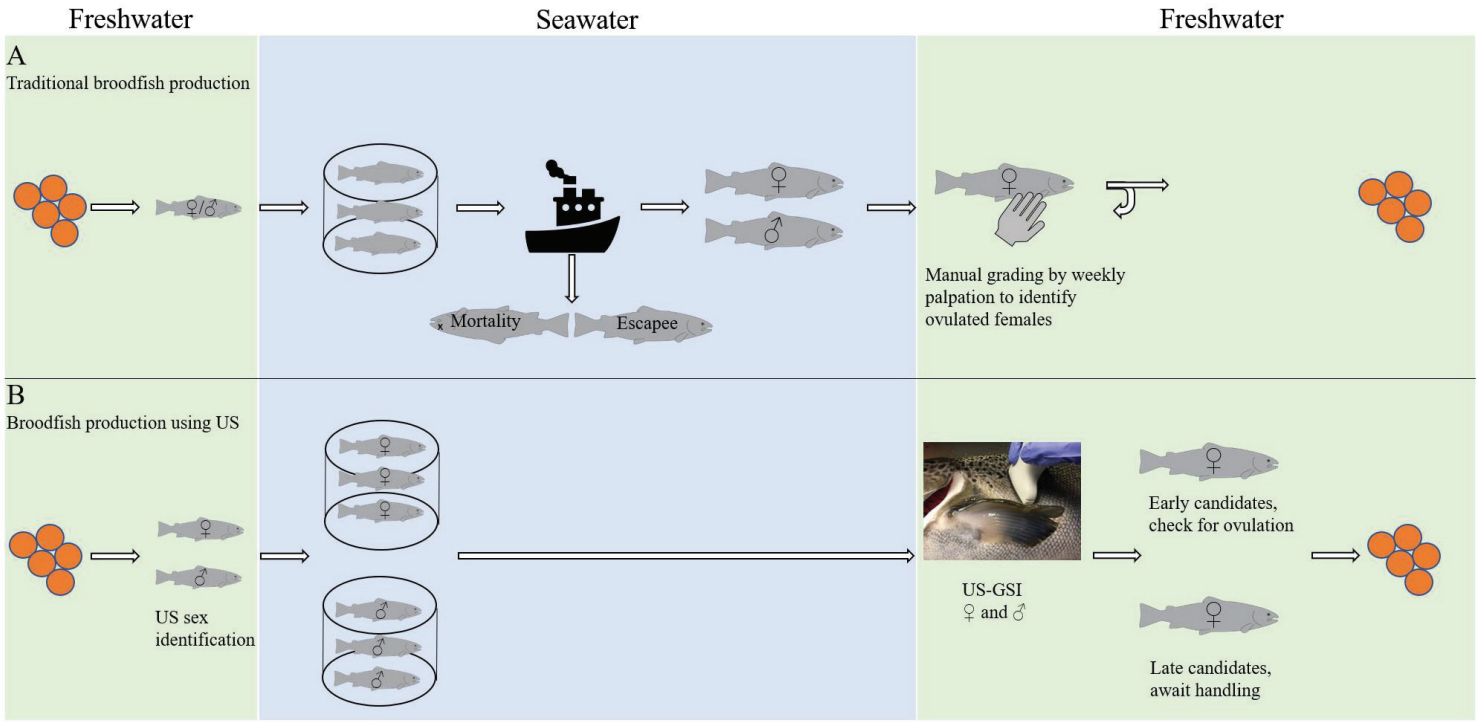
The main objectives of this thesis were to establish non-invasive ultrasound-based sex identification of parr in order to reduce the need for handling in seawater, and to develop ultrasound-based methods for monitoring sexual maturation that can help reduce the number of handlings of Atlantic salmon broodfish. Four studies with the following specific goals were conducted.

- Paper I: The goals of this study were to determine a) whether ultrasound technology can be used to separate the sexes of juvenile Atlantic salmon before seawater transfer, b) if low or high frequency ultrasound is better suited for sex identification, c) at which fish size this can be done most accurately, and d) how the fish tolerate the ultrasound examination
- Paper II and Paper III: The goals of these studies were to a) establish a non-invasive method for monitoring sexual maturation of Atlantic salmon females (Paper II) and males (Paper III), and b) examine if ultrasound could replace more invasive methods for maturation monitoring
- Paper IV: The goals of this study were to examine a) if ultrasound can be used to identify final maturation and ovulation in female Atlantic salmon, b) if and at which point after freshwater transfer, ultrasound can be used effectively to predict ovulation, and c) to establish an ultrasound-based method for prediction of ovulation that reduces handlings and secures optimal timing of stripping and egg quality.

Main results and discussion

In these studies, several methods were developed that can be used for non-invasive management and monitoring during Atlantic salmon broodfish production, thus easing broodfish management and reducing the number of handlings compared to regular broodfish production (Figure 5). At the parr stage, sex identification using ultrasound has been achieved. During sexual maturation ultrasound-based GSI (US-GSI) measurements have been established for both sexes. The use of US-GSI and observations of oocytes in ultrasound images can be implemented as tools for managing females to reduce the number of handlings during final maturation.

Figure 5. Atlantic salmon broodfish production. In traditional Atlantic salmon broodfish production (A, upper panel), smolts are transferred to seawater and reared in mixed sex net-pens. Due to limited space in land-based broodstock facilites for final maturation, sex identification of broodfish candidates takes place during seawater rearing. This process is stressful to fish, and increases the risk of escapes and mortalities. Broodfish candidated are then transferred to the land-based freshwater facilities for final maturation and stripping. As the expected stripping season approaches, all females are checked weekly for ovulation to reduce risk of post-ovulatory ageing of eggs. In ultrasound-based broodfish production (B, lower panel), the sexes can be separated during freshwater rearing, before smoltification takes place. By rearing fish separately during the seawater phase, additional handling for sex identification, with associated risks of escapes and mortality, are uneccessary. After transfer to freshwater, one ultrasound-based gonado-somatic index (US-GSI) estimation can be performed in July to separate females that can be expected to ovulate early and late in the stripping season. Females expected to ovulate early should undergo weekly ovulation checks from three weeks after the temperature drop, while females expected to ovulate late can be left undisturbed until five weeks after the temperature drop. Using changes in oocyte echogenicity observed in ultrasound images, handlings of females that are not expected to ovulate soon, can be avoided. All handlings described in this figure come in addition to handlings for size gradings, and sea lice and disease management.


Freshwater
Seawater
Freshwater
A
Traditional broodfish production
Mortality
Escapee
Manual grading by weekly palpation to identify ovulated females
B
Broodfish production using US
US sex identification
US-GSI ♀ and ♂
Early candidates, check for ovulation
Late candidates, await handling

Sex identification of juvenile Atlantic salmon in freshwater (Paper I)

Sex identification of Atlantic salmon juveniles before transfer to seawater was established using both low and high frequency ultrasound probes. Sex identification was performed before onset of smoltification (weight ranging 50 to 219 g), and parr showed good tolerance to the ultrasound grading procedure. Using high frequency ultrasound, it is possible to effectively grade fish before transfer to seawater. This reduces the need for stressful handling to identify sex during the seawater production of broodfish and can be used to optimise sex ratio in land-based broodfish production in RAS facilities.

Testing ultrasound probes with low (6 to 18 MHz centre frequency, scanning performed at 10 MHz) and high (10 to 22 MHz centre frequency, scanning performed at 22 MHz) frequency, sex identification of parr before transfer to seawater was possible. Sex was determined by localising one or both ovaries in the ultrasound image to identify a female (Figure 6A), and if no ovary was observed, the individual could be either a male or a female where the ovaries were not discernible, and was classified as a male (Figure 6B). With the low frequency probe correct sex identification varied and mean correct identification was 87 and 78 % for males and females, respectively. A considerable increase in percent correct identification, and reduction in variation, was achieved using a high frequency ultrasound probe, with 95 and 97 % correct identification of males and females, respectively. Sex identification using a high frequency probe could be performed with high accuracy within seconds, which makes sex identification of groups of Atlantic salmon intended for broodfish production before seawater transfer possible (Figure 5B). In contrast, other methods claiming to achieve rapid sex identification of Atlantic salmon by use of PCR, identifying the male-specific sexually dimorphic on the Y chromosome (*sdY*) gene, still require tagging and repeated handling of fish (Eysturskarð *et al.*, 2017).

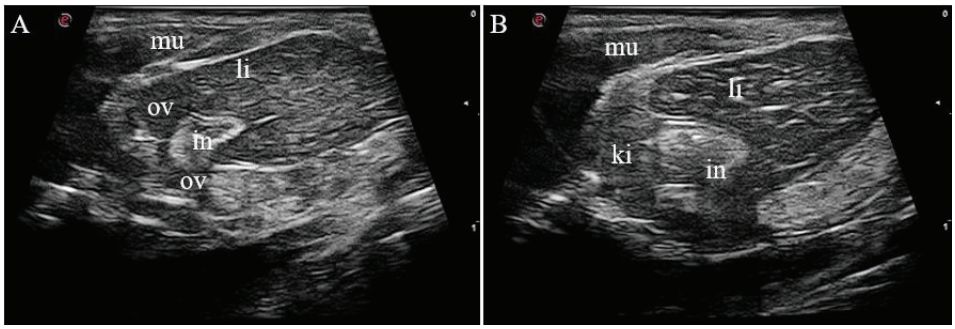


Figure 6. Sex identification of juvenile Atlantic salmon parr using high frequency ultrasound. In females (A), one or both ovaries could be identified between the intestine and the abdominal wall in the ultrasound image. If no ovaries could be detected, the individual was classified as a male (B). in, intestine; ki, kidney; li, liver; mu, muscle; ov, ovary. Depth is denoted to the right in each ultrasound image.

During smoltification, fish are more vulnerable to handling stress and descaling than parr (Carey and McCormick, 1998; Zydlewski *et al.*, 2010). Thus, handling fish close to or at smoltification increases the risk of handling stress which can result in reduced osmoregulatory capacity. Fish transferred to seawater without the sufficient osmoregulatory capacity have reduced growth, suffer and eventually die (Duston, 1994; Aunsmo *et al.*, 2008). Therefore, sex identification should be performed well in advance of smoltification, i.e. at lower mean weight, to ensure optimal fish health and welfare. Using the high frequency ultrasound probe, sex identification was high in all weight classes, but especially in the lowest weight class (50 – 59.9 g). We also saw an increasing trend in GSI with decreasing body weight class, which might be a reason for easier sex determination in lower weight classes. Thus, performing sex identification using ultrasound well in advance of smoltification can minimise smoltification-related challenges to fish health and welfare after handling and secure high accuracy in sex determination. During freshwater rearing, fish are handled for mandatory vaccinations, and can be graded according to size if the handling does not challenge fish health (Ministry of Trade, Industry and Fisheries, 2008). As any handling might be stressful, adding extra handlings is undesirable, and it would be reasonable to perform sex identification concurrent with vaccination. Fish can then go through smoltification

undisturbed and males and females can be placed in separate net-pens in seawater for on-growth.

By separating the sexes during the freshwater phase, one has eliminated the need to crowd and handle the much larger fish for sex identification in seawater. Given the number of handlings that are necessary for sea lice treatment, disease management, and data collection and selection of broodfish candidates, any reduction in handlings performed during the seawater phase might be beneficial for the welfare of broodfish (Erikson *et al.*, 2016; Overton *et al.*, 2018a; Overton *et al.*, 2018b). Further, if selection of candidates for breeding is performed only in males, females could be left undisturbed which might benefit growth of gonads and give a better outcome of reproduction (Campbell *et al.*, 1992). In addition, 59 % of escapes reported in Norwegian aquaculture in 2018 were due to "operational causes" (Directorate of Fisheries, 2019), meaning they happened during operations involving the net-pen or handling of fish. This is especially critical in broodfish production, as escaped maturing individuals can migrate to nearby rivers and spawn with wild Atlantic salmon (Glover *et al.*, 2013; Heino *et al.*, 2015).

Few studies report on sexing of juvenile fish, although in juvenile Atlantic halibut (four years old, two kg body weight), Martin-Robichaud and Rommens (2001) achieved 100 % correct sex identification, and in juvenile beluga sturgeon (three years old, three to six kg body weight) 97 % correct sex identification was reported (Masoudifard *et al.*, 2011). A relatively high frequency (15 MHz) was used in the first attempt at ultrasound-based sex identification of a juvenile salmonid, Coho salmon, but sex identification of juveniles was not possible at that time (Martin *et al.*, 1983). The frequency used by Martin *et al.* (1983) is between the low and high frequencies that gave mean correct identification between 78 and 97 % in our study. There has been a great technological development of ultrasound equipment since 1983, and it is therefore likely that a corresponding 15 MHz probe would be sufficient for sex identification using today's technology. In a recent assessment of precocious parr maturation in wild Atlantic salmon, Nevoux *et al.* (2019) used a low frequency (5 MHz) probe, and reported that sex identification was only possible in mature males where testes filled the abdominal cavity. This contrasts our study, where identification of ovaries using a 22 MHz probe made sex determination possible. In general, high frequency ultrasound gives good detail resolution but decreased

depth penetration, while low frequency ultrasound has high depth penetration, but lacks resolution (Chan and Perlas, 2011). Thus, the composition and size of the structure or tissue of interest should be considered when choosing frequency for ultrasound examination, and high frequency ultrasound is more suited for identifying the very small gonads of Atlantic salmon parr.

Ultrasound-based GSI in Atlantic salmon males and females (Papers II and III)

New methods for non-invasive estimation of GSI using ultrasound in female and male Atlantic salmon was established. By measuring left gonad length using ultrasound, US-GSI can be estimated within seconds, thus replacing the need for sacrifice of fish to calculate GSI (Figure 7).

The US-GSI models for female and male Atlantic salmon were based on ultrasound measured length of the gonads of advanced broodfish during the last year before final maturation (Figure 7). To standardise, all ultrasound measurements were made on the left gonad. Measured gonad length was used to estimate gonad weight and with measured body weight, GSI can be calculated (indirect method; Figure 7A and B). Gonad length can also be used to estimate GSI directly, that is without using body weight (direct method; Figure 7C and D). Both linear and exponential models were made for direct and indirect US-GSI estimation, but the linear models are recommended for maturation monitoring of broodfish after transfer to freshwater (Figure 7E and F; refer to Figure 1 in Paper II and Paper III for details). In most females, the left ovary was longer than the right ovary, but some females where the right ovary was longer were observed. Using left ovary length to calculate US-GSI in these females would underestimate GSI. In these cases, it is recommended to use the length of the longest ovary to calculate US-GSI. Using the direct US-GSI method, it takes a few seconds to perform the single measurement needed, and instant results are provided, given the necessary equation has been plotted into an excel sheet beforehand. The quick and non-invasive US-GSI method presented here can be used for maturation monitoring after transfer to freshwater (Figure 5). If the results presented here are implemented, it is no longer necessary to sacrifice fish or rely on GSI calculated from deceased fish to estimate GSI and monitor sexual maturation.

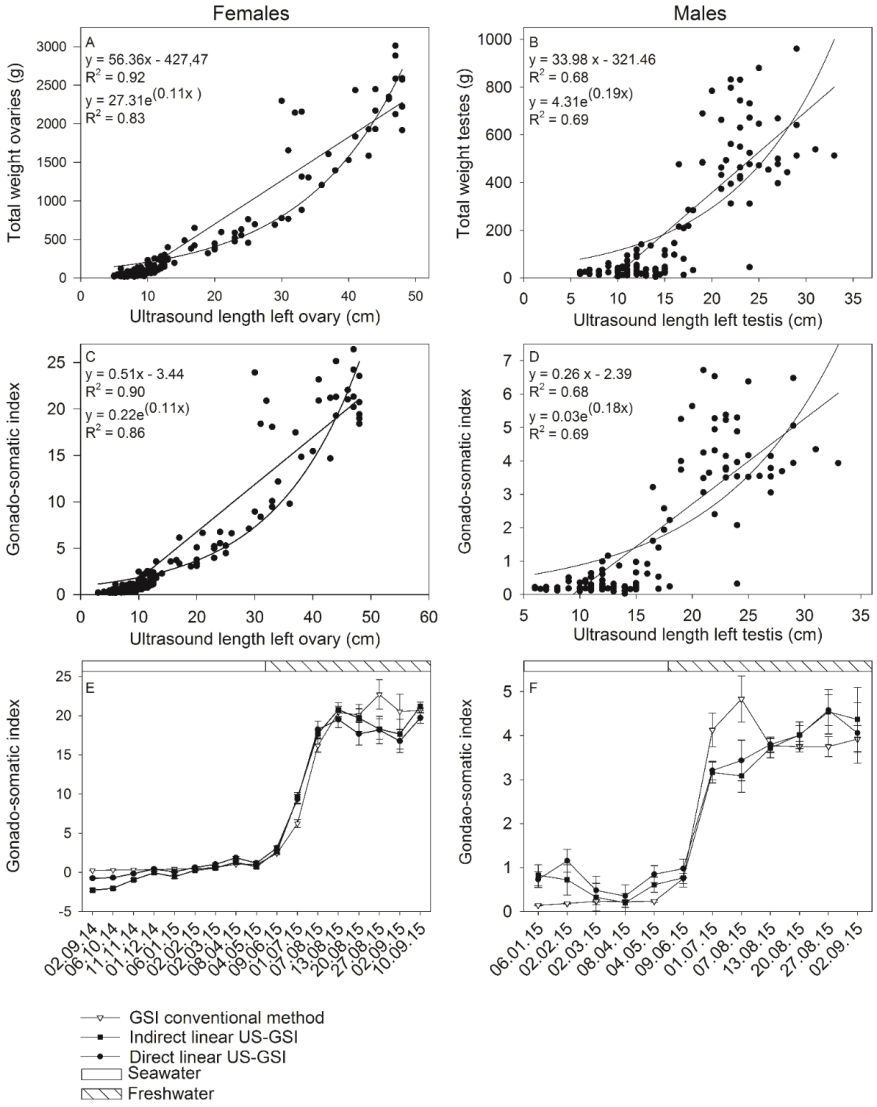


Figure 7. Establishing ultrasound-based gonado-somatic index (US-GSI) in female (left side) and male (right side) Atlantic salmon. Length of the left ovary (A) and testis (B) can be used to estimate total ovary and testes weight, respectively. Estimated gonad weight and measured body weight can be used to calculate indirect US-GSI. Length of left ovary (C) and testis (D) can be used to calculate direct US-GSI. Linear models (E, F) estimated US-GSI better than exponential models and are recommended for use. Data in E and F are mean ± SEM. Vertical bars at the top in E and F represent rearing conditions.

Anatomical differences between the gonads of males and females made ultrasound examinations more challenging in males, as has been observed in Neosho madtom (Bryan *et al.*, 2005), shovelnose sturgeon (Wildhaber *et al.*, 2007) and European eel (Bureau du Colombier *et al.*, 2015). In Atlantic salmon females, the left ovary was a clearly defined, oval structure that grew in thickness and in a posterior direction as maturation progressed and was easily measured using ultrasound. In contrast, the left testis spanned the abdominal cavity throughout the sampling period, and it could be difficult to discern from other tissues at early samplings, and to define the thickened part for measuring as maturation progressed. Thus, precision of gonad length measurements by ultrasound compared to measurements with a ruler, differed between the sexes (R^2 = 0.98 and 0.77 in females and males, respectively; refer to Figure 1 in Paper II and Paper III for details). These results are, however, more accurate than those obtained by Mattson (1991), who compared area measurements of gonads in ultrasound images with measurements made with a ruler, and reported R^2 = 0.86 and 0.57 for females and males, respectively. The accuracy of the male US-GSI model could probably be improved with practice and experience, as considerably more time has been spent measuring females than males during this work.

As discussed, the US-GSI model for males is less accurate than for females and was not able to reproduce an increase and decrease in GSI observed during the last weeks before stripping (Figure 7F). This pattern of GSI observed before stripping could be related to loss of cellular material (Stanley, 1969; Schulz *et al.*, 2010) and germ cells (Vilela *et al.*, 2003; Schulz *et al.*, 2005) during final maturation of spermatids and spermatozoa. However, the US-GSI method can be used to monitor gonad development of males after freshwater transfer when adding other observations from ultrasound images. During the final months of maturation, we observed a change in testis brightness, echogenicity, in ultrasound images (Figure 8) that corresponded with progress in spermatogenesis observed in histological sections. The left testis appeared black in ultrasound images during the seawater phase (Figure 8A) and became progressively more grey as maturation progressed after freshwater transfer (Figure 8B and C), and spermatocytes and spermatids were observed in histological sections of left testis. Such changes in echogenicity of testes has also been described in rainbow trout (Evans *et al.*,

2004) and shovelnose sturgeon (Wildhaber *et al.*, 2007), while the opposite was observed in starry sturgeon (*Acipenser stellatus*; Moghim *et al.*, 2002). In males with testes dominated by spermatozoa, the left testis appeared bright grey or white in ultrasound images (Figure 8D). Using US-GSI in combination with observations of echogenicity during the final months of maturation, ultrasound can be used to determine maturation stage of Atlantic salmon males during the final months of maturation more accurately. Thus, there seems to be potential for further experiments into final maturation, spermiation and milt production in Atlantic salmon males using ultrasound.

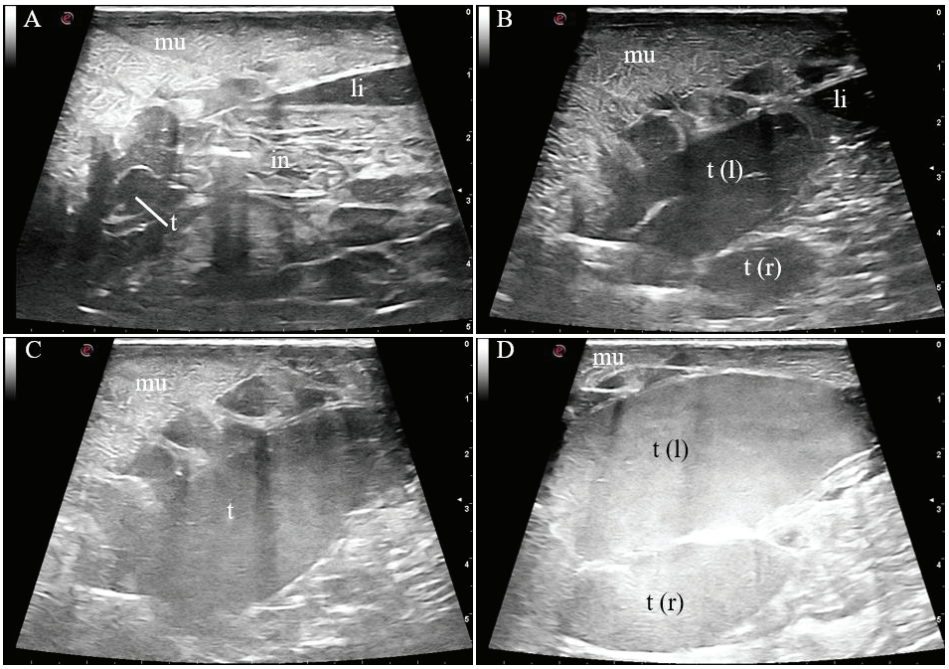


Figure 8. Changes in echogenicity of Atlantic salmon testes during sexual maturation. In January (A) testes were small and could be difficult to discern in ultrasound images. In June, after freshwater transfer, increased size was observed (B). As maturation progressed in July testes became grey in ultrasound images (D) and spermatids were observed in histological sections. All males with bright white testes (D) had spermatozoa present in histological section. in, intestine; li, liver; mu, muscle; t, testis; t (l), left testis; t (r), right testis.

Methods for GSI estimation using ultrasound in species such as Neosho madtom (Bryan *et al.*, 2005), sturgeons (Bryan *et al.*, 2007; Albers *et al.*, 2013), northern pike (*Esox lucius*; Macbeth *et al.*, 2011), white sucker (Macbeth *et al.*, 2011) and European

eel (Bureau du Colombier *et al.*, 2015) were all based on measurements in cross section ultrasound images. Using area measurements from cross section images and ultrasound-based gonad length, Bureau du Colombier *et al.* (2015) achieved very accurate models for estimation of gonad mass ($R^2 = 0.97$). The US-GSI models presented here may have been improved using area measurements as done by others. A model for estimation of gonad volume in shovelnose sturgeon, using gonad length and three cross sectional ultrasound images obtained equal level of precision for males and females ($R^2 = 0.88$; Bryan *et al.*, 2007), thus extra measurements might add extra value especially for monitoring male maturation. However, these measurements are time-consuming and quite cumbersome with the equipment we used, making this approach less applicable for large-scale monitoring of whole broodfish groups. If instant results from the US-GSI examination are needed for grading of fish during broodfish production, these extra, time-consuming measurements would increase emersion time for individual fish while calculations were made, or additional handling would be necessary if fish were put back in the water and had to be re-handled for grading when results were ready. This increased handling stress could have negative impact on fish welfare and reproduction (Campbell *et al.*, 1992). Should one simply be interested in an overview of the variation in a population, but not in grading fish based on the result, a brief examination saving an image for later calculation is sufficient.

Extra measurements such as cross sectional area of ovaries and oocytes from ultrasound images and measured oocyte density has enabled others to make models that predict fecundity (Bryan *et al.*, 2005; Whiteman *et al.*, 2005; Bryan *et al.*, 2007; Macbeth *et al.*, 2011; Albers *et al.*, 2013). Some of these measurements involve capturing and analysing up to 20 ultrasound images, where image capturing alone was reported to take up to 8 minutes, followed by later image analysis (Macbeth *et al.*, 2011). Thus, these methods are more suitable for registering and monitoring, than grading of fish, which must progress rapidly. Fecundity prediction could be of interest to Atlantic salmon broodfish producers and those that rear Atlantic salmon and rainbow trout for caviar production, given shorter examination time and more instant results. We used a linear array probe (3 to 13 MHz centre frequency, scanning performed at 3 to 5 MHz) for maturation monitoring. This probe has a flat, 5 cm wide footprint, and emits ultrasound

waves straight down into the tissue. As gonads grew in diameter during maturation it was challenging to fit the whole cross-section of the gonad within the footprint of the probe (Figure 9). Curvilinear probes that have a convex shape allow a wider field of view with increasing depth (Whittingham, 2007). This option was tested, but as the flank of the fish is also convex the contact surface between fish and probe was reduced, and the images obtained did not have additional width. We therefore suggest that when it is desirable to image the whole ovary, such as for ovary diameter or cross section area calculations for fecundity models, one should try scanning with a linear array probe with a larger footprint than the one used here.

We experienced some difficulties sectioning oocytes for histological analysis from females close to final maturation (samplings in August and September). Estay *et al.* (2003) fixed brown trout oocytes in Gilson's fixative to achieve better fixation of large oocytes, while Idler *et al.* (1981) reported sectioning at up to 12 µm of yolk filled oocytes. In paper II we hypothesised that 11-KT peak at final maturation might be involved in final maturation in Atlantic salmon female. This peak might also have been the initiation of the next ovarian cycle, as 11-KT is involved in growth of pre-vitellogenic oocytes in other species (Lokman *et al.*, 2007; Kortner *et al.*, 2009). Histological sections from this period could perhaps have helped clarify this.

Monitoring of final maturation in Atlantic salmon females (Paper IV)

Using the US-GSI model established in Paper II two months before expected ovulation, females in the advanced broodfish segment could be separated into groups expected to ovulate early and late in the stripping season. In the final weeks before ovulation, changes in appearance of eggs in ultrasound images could be used to estimate time until ovulation. This enables breeders to reduce the number of handlings of broodfish during the weeks leading up to and through the stripping season, thus reducing handling stress of broodfish.

We found that females that ovulated early within the advanced broodfish segment had higher US-GSI up to two months before ovulation than females ovulating later. Using this, female broodfish can be separated into two cohorts expected to ovulate early and late (Figure 5). During the period when ovulation is expected, females are checked by a weekly palpation of the belly to avoid POA of eggs (Springate *et al.*, 1984; Aegerter and

Jalabert, 2004; Mommens *et al.*, 2015). Females expected to ovulate early would need weekly checks for ovulation from an earlier time point, while those expected to ovulate later could be spared the handlings during maturation checks identifying early ovulated females. Hence, one handling up to two months before ovulation could spare females ovulating late several stressful handlings. As handlings and other stressors close to ovulation can reduce fertilisation rates (Eriksen *et al.*, 2015) and reduce survival and increase prevalence of deformities in progeny (Campbell *et al.*, 1994; Morgan *et al.*, 1999; Eriksen *et al.*, 2006), any reduction in the number of handlings during final maturation could increase welfare and output from reproduction. In broodfish groups where reproductive biology is being pushed for production of off-season eggs, a close follow-up might be necessary to make sure fish respond to the environmental cues as expected. Depending on results from US-GSI examinations, the breeder could adjust light and temperature conditions to ensure optimal maturation of fish. For more standard groups, such as advanced broodfish, a close follow-up might not be necessary, nor feasible, as it adds unnecessary handlings without necessarily providing extra information. The reduced workload on staff could also increase precision and quality of ovulation checks, ensuring more females are stripped at an optimal time.

Two weeks after temperature drop, we observed a change in egg echogenicity in the females that ovulated first, using ultrasound (Figure 9). Oocytes appeared grey in ultrasound images throughout vitellogenesis (Figure 9A to C), before a progressively larger black spot was observed spreading from the centre of the oocyte (Figure 9D and E). We hypothesised that this was hydration of Atlantic salmon oocytes observed using ultrasound, as hydration has been shown for salmonids although to a smaller degree than in saltwater species (Bobe *et al.*, 2006; Milla *et al.*, 2006). It took approximately two weeks from the first observation of changed egg echogenicity (Figure 9 D and E), until the female had ovulated and was ready for stripping. Other studies in Coho (Martin *et al.*, 1983) and sockeye (Frost *et al.*, 2014) salmon also report similar hydration, but not ovulation, of eggs in ultrasound images, but none quantified this in a way that was applicable for broodfish management.

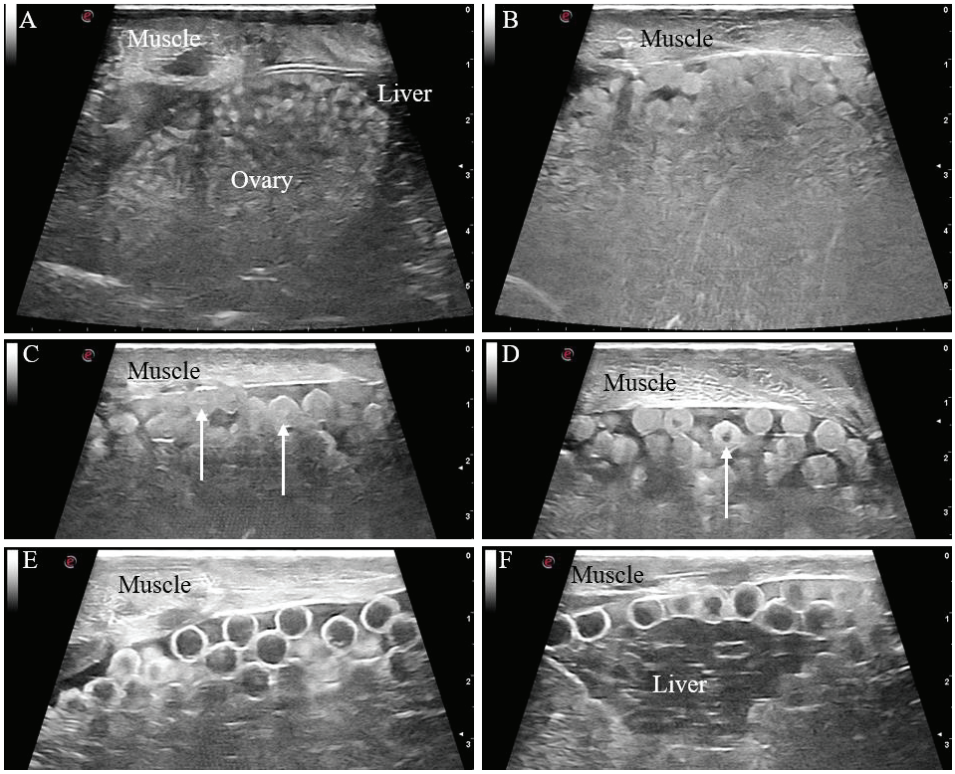


Figure 9. Changes in oocyte echogenicity during sexual maturation of Atlantic salmon females. During vitellogenesis ovaries grew in length and diameter, until the whole cross-section of the ovary did not fit within the footprint of the probe (A and B). As maturation progressed, a white shadow (C) followed by an increasingly larger black spot (D and E) was observed spreading from the centre of the oocyte. After ovulation, eggs "rolled" away under the light pressure of the probe, revealing the liver in the ultrasound image (F).

Combining the observed changes in oocyte echogenicity with the prospect of separating females into groups according to US-GSI up to two months before ovulation, it is possible to reduce handlings even further. Using ultrasound when fish are checked for ovulation, one can grade females that have not ovulated yet according to presence or absence of the black spot in oocytes. Females where the black spot is observed should be checked regularly, as they might be expected to ovulate within one to two weeks, while those where oocytes are uniformly grey could be left undisturbed for two additional weeks. This could help further reduce unnecessary handling of females that mature late

within the broodfish group. We also found that ultrasound can be used to verify that all eggs are ovulated from the ovary, as ovulated eggs "rolled" away from the light pressure of the ultrasound probe when placed at the clavicle, and revealed the liver, which had been obstructed from sight by the ovary during maturation (Figure 9F). The management of females in the final months before ovulation and stripping that has been suggested here does, however, require production facilities that are flexible and allows separation of females according to results from ultrasound examinations (Figure 5).

During this work, fish have been taken out of the water for ultrasound examination, partly because the ultrasound probes used were not designed for immersion to more than five cm of water. Waterproof ultrasound probes are however available and scanning in water should be tested to further reduce the stress of emersion (Campbell *et al.*, 1992). Given the effects of handlings and stress on reproduction and fish welfare, US-GSI examination of fish should only be performed when necessary, and not in excess. All experiments related to maturation monitoring presented here (Papers II – IV) were performed on advanced broodfish. It is therefore strongly recommended that for other broodfish groups, i.e. normal, late, off-season, one should perform a one-time extensive and thorough registration to have a good basis for comparison. Further, these data should be used to identify the optimal timing for examination of broodfish with ultrasound for each group so that the number of handlings can be reduced while securing enough data to make decisions that gives the best possible maturation and timing of stripping. For advanced broodfish production, given a flexible production facility, we recommend one US-GSI check in July and then weekly ovulation checks using both palpation and ultrasound tools to identify optimal stripping time, from approximately three and five weeks after the temperature drop in the groups expected to ovulate early and late, respectively.

Conclusions and further studies

- Paper I: Ultrasound is well suited for sex identification in juvenile Atlantic salmon. We recommend use of a high frequency ultrasound probe for sex identifications as it gives higher accuracy than low frequency ultrasound. Fish showed good tolerance to this procedure. With automatization this technology might be of interest to regular farmers as well as to broodfish producers.
- Paper II: Ultrasound technology is well suited to substitute traditional invasive methods during sexual maturation monitoring in Atlantic salmon females. Ultrasound-based ovary length measurements correlated strongly with GSI and sex hormone levels. The methods offer a possibility to reduce stress and improve animal welfare in broodstock management.
- Paper III: Ultrasound is suitable for maturation monitoring in male Atlantic salmon. The US-GSI models provide valuable information about progress in maturation and correlated to levels of sex hormones and spermatogenic development. In addition, it is specific enough for grading purposes when supplemented with observation of echogenicity. Using ultrasound technology, more invasive methods are not needed, and therefore the use of ultrasound will reduce stress and improve welfare during maturation monitoring.
- Paper IV: Using ultrasound, two time points after freshwater transfer were identified, when US-GSI can be used to identify females that will ovulate early or late within a broodfish group; in July or around the temperature drop. Changes in oocyte echogenicity that were related to sex hormone levels were also identified, and can be used to predict ovulation time, reduce unnecessary handling and thereby increasing fish welfare, and ensuring optimal stripping time of each female. Based on these findings we recommend one US-GSI check in July, and weekly checks from three and five weeks after temperature drop in females expected to ovulate early and late, respectively.

Ongoing activities:

- Studies aiming to identify an optimal monitoring system to reduce handling and secure good reproduction using ultrasound are being performed in delayed and off-season Atlantic salmon broodfish groups.
- Knowledge from these studies is at present being transferred to other aquaculture species. In lumpfish (*Cyclopterus lumpus*), sex identification of juveniles and monitoring of sexual maturation is being studied. In rainbow trout, ultrasound is being used in studies to establish protocols for advanced and delayed maturation groups, and studies for juvenile sex identification are planned.
- Given the differences in growth patterns between males and females, sex separated rearing might be of interest to the whole aquaculture industry, but as sex identification of parr is a labour-intensive process, automatization is needed. Such automatization requires substantial innovation and investments, and therefore a project has been initiated to examine if the benefits of sex separated rearing in large-scale Atlantic salmon production for harvest, are great enough to perform such costly innovation work.

Further studies:

- Results of GnRHa injections to advance and synchronise ovulation in females have varied, while results in males have been mainly positive. We have shown changes in echogenicity of both testes and ovaries close to final maturation and suggest that further studies should investigate if ultrasound can be used to optimise the timing of GnRHa injection, especially in females.
- Post-ovulatory ageing of eggs is associated with changes in egg morphology and reduced fertility. Further studies should be undertaken to examine if, and at which point after ovulation, ultrasound can be used to identify such changes, and if this can contribute to increased egg quality.
- Fecundity estimation has been established in several fish species. A quick and non-invasive method for fecundity estimation could be of interest to Atlantic salmon broodfish producers. Studies should be undertaken to establish if, and at

which point during sexual maturation, such estimates can be obtained in Atlantic salmon females.

- The main focus in these studies has been on females. Further studies could be initiated to optimise the US-GSI model for males, and further examine how the observed changes in testes echogenicity can be used to secure optimal male maturation. When a one male to ten females sex ratio is used, it is especially important to get maximum “value” from each selected male.

List of abbreviations

11-KT, 11-ketotestosterone; **ACTH**, adrenocorticotropic hormone; **AGD,** amoebic gill disease; **BPG**, brain-pituitary-gonad axis; **CRH,** corticotropin releasing hormone; **EFSA**, European Food Safety Authority; **E2**, oestradiol-17β; **FAO**, Food and Agriculture Organisation of the United Nations; **FAWC**, Farm Animal Welfare Council; **FSH**, follicle stimulating hormone; **GnIH**, gonadotropin inhibiting hormone; **GnRH**, gonadotropin-releasing hormone; **GnRHa**, gonadotropin-releasing hormone analogue; **GSI**, gonado-somatic index; **GTH**, gonadotrophic hormone; **HSMI**, heart and skeletal muscle inflammation; **HPI**, hypothalamus-pituitary-interrenal axis; **LH**, luteinizing hormone; **MIH**, maturation inducing hormone; **PD**, pancreas disease; **POA**, post-ovulatory ageing; **RAS**, recirculation aquaculture systems; **sdY**, sexually dimorphic on the Y chromosome; **SWIM**, salmon welfare index model; **T**, testosterone; **US-GSI**, ultrasound-based gonado-somatic index; **vgll3**, vestigial-like family member 3; **Vtg**, vitellogenin

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Paper I

This paper is awaiting publication and is not included in NTNU Open

Paper II

Physiological Reports

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Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Ultrasound as a noninvasive tool for monitoring reproductive physiology in female Atlantic salmon (*Salmo salar*)

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Keywords
Animal welfare, endocrinology, histology, reproduction, ultrasound.

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Funding Information
This work was funded by the Norwegian Research Council (241398/O30) and AquaGen AS Norway.

Received: 29 September 2017; Revised: 29 January 2018; Accepted: 31 January 2018

doi: 10.14814/phy2.13640

Physiol Rep, 6 (9), 2018, e13640, https://doi.org/10.14814/phy2.13640

Abstract

Aiming to explore ultrasound technology as a noninvasive method for maturation monitoring, we compared ultrasound observations and measurements in female Atlantic salmon (*Salmo salar*) during the last year before ovulation with standard, invasive methods such as gonadosomatic index (GSI), gonad histology and sex hormone analysis. Ultrasound measurements of ovaries correlated strongly ($R > 0.9$, $P < 0.01$) with ovary weight and GSI, and could be used as a noninvasive tool for GSI estimation. Using ultrasound, we were able to identify females with advanced oocyte development and elevated sex hormone and GSI levels earlier than previously observed. Histological studies confirmed these observations showing oocyte yolk accumulation 10 months before ovulation and 8 months before significant increase in sex hormones. Levels of the sex hormone 11-keto testosterone (11-KT) indicated a new role of this hormone at final maturation in salmon females. We propose the use of ultrasound as an alternative method to traditionally used invasive methods during sexual maturation monitoring in wild and farmed Atlantic salmon broodstock populations. Eliminating sacrifice of valuable broodfish, and reducing handling stress, would improve animal welfare in present-day broodstock management.

Introduction

Salmonid species are of great economic and nutritional value, and as a consequence their reproduction has been thoroughly studied over decades (Ducharme 1969; Bun Ng and Idler 1978). Triggered by shortening day length and decreasing river temperature, wild Atlantic salmon (*Salmo salar*) spawn in autumn (Heggberget 1988; Webb and McLay 1996). Photoperiod and temperature control enables breeders in commercial Atlantic salmon farming to advance and delay spawning according to market demands for fertilized eggs (Pankhurst and King 2010; Taranger et al. 2010; Vikingstad et al. 2016).

The gonadosomatic index (GSI), gonad weight as percent of body weight, is a measure for maturational status used in fish. Atlantic salmon females typically ovulate and spawn at GSI > 20. In studies covering different aspects of salmonid reproduction, GSI is typically reported in combination with one or more other methods of maturation index, such as sex hormone analysis (Nagahama and Adachi 1985; Prat et al. 1996; Taranger et al. 1998; King and Pankhurst 2003), histology of gonad tissues (Sumpter et al. 1984; Taranger et al. 1999; Estay et al. 2003; Grier et al. 2007), endoscopy (Ortenburger et al. 1996; Swenson et al. 2007) and expression of genes related to sexual maturation in relevant tissues (Campbell et al. 2006; Luckenbach et al. 2008).

GSI estimation, gonad histology, and gene expression studies require fish sacrifice, and while endoscopy and blood sample analysis are less invasive, they might still pose a risk to the health and survival of examined fish (Ortenburger et al. 1996; Swenson et al. 2007) and induce handling related stress (Fast et al. 2008; Eriksen et al. 2015). In addition, most of these methods are limited to

provide maturation information on individual fish only once. To monitor maturation in a broodstock population over time, several individuals must be sacrificed, which might not be feasible when working with wild, endangered species or valuable, farmed broodfish. At present, maturation monitoring in wild Atlantic salmon populations relies on GSI measurements requiring sacrifice. In farmed Atlantic salmon, GSI is often estimated from deceased fish, where low fitness may result in a nonrepresentative GSI estimate and maturation status.

Ultrasound technology, a noninvasive technique using echoes from high frequency sound waves to visualize internal organs, has been tested in wild and domesticated fish species, such as European eel (*Anguilla anguilla*; Bureau du Colombier et al. 2015), shovelnose sturgeon (*Scaphirhynchus platorynchus*; Colombo et al. 2004; Bryan et al. 2007; Colombo et al. 2007), sockeye salmon (*Oncorhynchus nerka*; Frost et al. 2014) and striped bass (*Morone saxatilis*; Blythe et al. 1994; Jennings et al. 2005). Ultrasound has been explored as a noninvasive tool for sexing (Colombo et al. 2004; Wildhaber et al. 2005; Guitreau et al. 2012; Frost et al. 2014; Hliwa et al. 2014; Du et al. 2017), basic maturation monitoring (Martin-Robichaud and Rommens 2001; Moghim et al. 2002; Colombo et al. 2007; Wildhaber et al. 2007; Petochi et al. 2011) and development of advanced methods for estimation of gonad volume, egg size, fecundity and maturity status (Blythe et al. 1994; Bryan et al. 2005, 2007; Jennings et al. 2005; Whiteman et al. 2005; Newman et al. 2008).

Although previous studies indicated the usefulness of ultrasound for maturation monitoring in Atlantic salmon (Mattson 1991), its use has been limited to sex differentiation (Reimers et al. 1987; Mattson 1991) and health inspections (Poppe et al. 1998). We therefore compared ultrasound measurements with traditional invasive methods for maturation monitoring (i.e., GSI, sex hormone analysis and histology). Our goals were to establish (1) a noninvasive method for sexual maturation monitoring in Atlantic salmon using ultrasound technology (2) if ultrasound technology can be an adequate alternative to other invasive methods.

Materials and Methods

Ethical approval

All animals were reared and transported according to Norwegian aquaculture legislation. Euthanasia was performed according to Annex IV in European directive 2010/63/EU. In accordance with Norwegian and European legislation related to animal research, formal approval of the experimental protocol by the Norwegian Animal Research Authority (NARA) is not required because the experimental conditions are practices undertaken for the purpose of recognized animal husbandry. The authors have understood the ethical principles the journal operates under, and confirm that this work complies with the checklist provided by Grundy (2015).

Fish husbandry

Atlantic salmon fry of the AquaGen strain were start-fed from February 2012 and transferred to net-pens at a commercial AquaGen sea site in the Hemne fjord (63 °N, 9 °E) in Norway as 1-year old smolts, in May 2013. During seawater phase, fish were fed according to appetite with Ewos Opal 120 until 1 year before ovulation, when they were fed with Ewos Opal Breed until freshwater transfer. Photoperiod treatment was performed according to commercial broodstock production protocols (Fig. S1). In May 2015, the broodfish were transferred to indoors circular freshwater tanks (8 m diameter, 1.5 m depth, 60 m^3), and given an autumn light and temperature signal (Fig. S1), until temperature was dropped to induce final maturation and ovulation. Fish were not fed after freshwater transfer as maturing wild Atlantic salmon are not known to feed during spawning migration in rivers (Kadri et al. 1995). Water temperature (Fig. S1) and oxygen levels were recorded daily at 3 and 6 m depth in net-pens during the seawater phase (58–116% and 75–148%, respectively), and in freshwater tanks (97–114%).

Experimental design and sampling

Starting in September 2014, 20 females were examined once a month until the temperature drop in August 2015, and then five females were examined weekly until stripping in September 2015. Mean female weight increased from 5.75 ± 0.23 kg in September 2014 to a maximum of 12.56 ± 0.41 kg in April 2015 (see Table S1 for further details). After freshwater transfer, females were not fed, which led to a small nonsignificant decrease in weight (see Table S1). Before sex could be determined by secondary sexual characteristics, ultrasound was used for sexing (see below). Fish were sacrificed with an overdose of tricaine methanesulphonate (200 mg/L, Pharmaq, Norway), followed by spinal transection. Blood was drawn from the caudal vein using heparinized vacuum tubes (Terumo, Japan), and after centrifugation at 2.4 × 1000 *g* for 10 min in 4°C (Micro Star 17R, VWR, USA), plasma was collected and kept on ice for 1–5 h until it could be frozen at −80°C. Body weight was registered to the nearest 20 g using a digital scale (SFE 60K20IMP, Kern & Sohn GmbH, Germany) and fork length to the nearest cm, using a measuring tape. Ultrasound length of left ovary was measured by placing a ruler at the base of the

pectoral fin and the ultrasound transducer by the clavicle to locate the anterior tip of the ovary. The transducer was moved in a posterior direction along the ruler until the posterior tip of the ovary was observed in the ultrasound image. Ovary length was measured as distance traveled along the ruler. Cross-sectional ultrasound images (3–5 depending on ovary length) were captured and compared to histological observations in ovaries. The left ovary was gently removed, and length measured to the nearest mm to verify ultrasound length. Weight of left and both (from December 2014) ovaries was registered to the nearest 0.1 g using a digital scale (MFD, A&D, Japan and Scanvaegt DS-673SS, Denmark). Cross-sectional tissue slices, about 0.5–1 cm thick, from the anterior half of the gonad were collected and fixed in 4% formaldehyde solution in phosphate buffer (pH 6.9, Merck Millipore, Germany) for histological analysis.

Ultrasound specification

A MyLab Alpha ultrasound unit (Esaote, Italy) with a linear array 3–13 MHz probe was used for ultrasound examinations. Ultrasound scanning of fish was performed at 5–7 MHz frequency, with focus point at 25–40 mm, and signal amplification (gain), of approximately 80–90%.

Sex hormone analysis

Plasma concentrations of sex hormones –estradiol (E2), testosterone (T), 11-ketotestosterone (11-KT), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and maturation inducing hormone (MIH) were analyzed, using enzyme-linked immunosorbent assay (ELISA) kits from Cayman chemical (Ann Arbor, MI, USA). Frozen plasma samples were thawed on ice and sex hormones were extracted with organic solvent. Briefly, plasma samples were thoroughly mixed with diethyl ether (1:4 plasma:solvent) by vortexing, and the two phases were left to separate. The aqueous phase was frozen in liquid nitrogen, and the sex hormone containing ether phase decanted into a new glass tube before repeating the process. The organic phase was allowed to evaporate overnight and dry extracts were resuspended in 500 μL ELISA buffer, and stored at −80°C until analysis.

Samples were analyzed in duplicate according to the manufacturers protocol, with incubation times of 60 min for MIH, FSH and LH, 70 min for T and E2, and 90 min for 11-KT. Absorbance readings were performed at 410 nm (11-KT, E2, MIH and T) and 450 nm (FSH and LH), using a Cytation 5 Imaging plate reader (BioTek Instruments, USA). Standard curves were prepared using Microsoft Excel (Microsoft, USA) by linear regression fit of logit transformed data (E2, T, 11-KT, MIH) or by 4-parameter logistic fit, using optical density and standard concentration (FSH, LH, web-based software: elisaanalysis.com), both as instructed by the manufacturer.

Histology

Tissue samples for histology were fixed and stored in formaldehyde at 4°C until dehydration in a tissue processor (TP1020, Leica Biosystems Nussloch GmbH, Germany), and then embedded in paraffin. Tissue was sectioned at 4 μm section thickness using a microtome (2055/RM2255, Leica Biosystems Nussloch GmbH, Germany) and stained with standard hematoxylin and eosin (H&E) staining. Microscopy slides were scanned, using a digital slide scanner (NanoZoomer, Hamamatsu photonics, Japan) with up to 40× magnification, and images were exported from the scanner software (NDP, Hamamatsu photonic, Japan) as jpg-files. Samples from the August and September 2015 samplings were not sectioned as high amounts of lipid rich yolk made this very difficult.

Oocyte development stage was determined according to Taranger et al. (1999) and Andersson et al. (2009; Table S2). Area fraction, area of oocytes in one stage as a fraction of the total tissue area in a section, was determined using a grid plugin for ImageJ (National Institutes of Health, USA). A grid of 231 crosses (Hamilton and Megown In Press, USDA Forest Service Remote Sensing Applications, https://www.fs.fed.us/eng/rsac/invasivespecies/documents/0087-RPT3.pdf) each covering an area of 0.4 mm^2 was overlaid an image at 1.25× magnification for September 2014 to May 2015 samples, while for June and July 2015 samples, magnification was reduced to 0.7–0.8, while number of crosses in the grid was kept at 231, each now covering 1.1 or 1.2 mm^2. A cross with center in the tissue qualified as a hit, and was quantified by a cell counter plugin for ImageJ. Oocytes with traits corresponding to more than one developmental stage, were classified according to the most advanced trait. Number of hits within one oocyte stage was multiplied by area covered by each cross, and divided by the total area of the section to calculate area fraction. "Other tissues" accounted for 15–25% of the area throughout the whole sampling period. As the goal was to quantify the area of different oocyte stages in the ovary, it was removed before further calculations were done. Chromatin nucleolus stage oocytes were so small and few that they did not give area fractions, and were excluded from the area fractions figure.

To relate sex steroid levels to oocyte development in addition to sampling time, females were rearranged in four different categories according to their dominating

area fraction. Females with area fraction dominated by chromatin nucleolus, perinucleolus, cortical alveoli, and oil droplet stage oocytes were defined as previtellogenic. Females that had started vitellogenesis were divided into three groups; primary, secondary, and tertiary yolk oocyte females (Table S2). Individuals where ovaries contained oocytes in several stages were classified according to the oocyte stage with the highest area fraction.

Data analysis and statistics

Preparation of data was done, using Microsoft Excel (Microsoft, USA). Figures were made, using Sigmaplot 13 (Systat Software, USA). GSI represents the weight of the gonads as percent of the body weight, and was calculated as: GSI = (gonad weight/body weight)*100. The condition factor, *K*, was calculated as $K = 100*\text{weight}/\text{length}^3$.At ovulation, ovarian tissue is degraded, and eggs are released to the abdominal cavity. For fish that had ovulated and were ready to spawn, GSI was defined as zero, as the ovaries could not be dissected and weighed. In September to November 2014, left ovary weight but not total ovary weight was registered. The linear relationship ($R = 0.99$, $P < 0.001$, eq. 1) between left ovary weight (x) and total ovary weight (y) from December 2014 to March 2015 was used to estimate total ovary weight used to calculate GSI for these samplings.

$$y = 1.74x + 1.46 \quad (1)$$

Maturation variation, measured as GSI, was low until March 2015, and the number of samples used for sex hormone analysis and histology was reduced to $n = 6$ per month between September 2014 to February 2015 and 10 per month from March to June 2015, while from July to September 2015, all 20 females were analyzed (Table S1). Statistical analyses were done, using SPSS Statistics 21 (IBM Analytics, USA). All data were tested for normality using Shapiro–Wilk test with significance level of $P < 0.05$ representing nonnormality. Sex hormone (E_2, T, 11-KT, LH and MIH) and GSI data were tested for monthly and weekly significant differences using a general linear model (GLM) followed by a least squared difference (LSD) post hoc test to identify significantly different sampling groups. Significance level was set at $P < 0.05$. Data for FSH were not normally distributed, and ANOVA on ranks followed by Dunn's procedure was chosen for this dataset. Significance level was set at $P < 0.05$. All correlations involving GSI measurements and sex hormones were performed, using Pearson product-moment correlation, with $P < 0.05$. Linear and exponential curves and equations were fitted using regression curve estimation in SPSS Statistics, with $P < 0.05$.

Results

During the seawater phase, mean GSI showed a non-significant, gradual increase from 0.23 ± 0.01 to 1.02 ± 0.07 (Fig. 1 in Figs. S2 and S3). After freshwater transfer, mean GSI increased exponentially, and reached a peak of 22.71 ± 1.88 at the end of August 2015, just before ovulation. As final maturation progressed and eggs were released from the ovaries, GSI fell to 0.

There was a strong correlation between left ovary length and total ovary weight ($R = 0.94$, $P < 0.01$, Fig. 1A), and left ovary length measured by ultrasound correlated well with real left ovary length ($R = 0.99$, $P < 0.01$, Fig. 1B). Further, there were strong correlations between left ovary length measured by ultrasound, and both total ovary weight (Fig. 1C) and GSI (Fig. 1D). Linear and exponential equations were fitted, and these can be used to estimate GSI directly from ultrasound length of left ovary, or indirectly by estimating total ovary weight from ultrasound length of left ovary and then calculating GSI, using fish body weight. Measured GSI of females was plotted with GSI estimated using linear and exponential equations given in Figure 1C and D to examine models further (Fig. 1E and F). Both linear models underestimated GSI during the seawater phase and at final maturation. The exponential models were close to measured GSI during the seawater phase when ovary length was small, but overestimated GSI largely at later stages when ovary length increased.

Left ovary cross-section ultrasound images from each female were examined in relation to histological observations in said females to look for easily discernible structures in the ultrasound images. From September 2014 to January 2015, the ovarian tissue was a uniform mass in the ultrasound image and only a growth in ovary area could be observed (Fig. 2A–E).11 In September 2014, oocytes were predominantly previtellogenic, (Figs. 2A–B and 3) while in January 2015, vitellogenesis had started and ~40% of the observed oocytes were in primary and secondary yolk stage (Figs. 2C–F and 3). In February 2015, one female with tertiary yolk oocytes was observed histologically, and this was the first female where oocytes were discernible in ultrasound images (Fig. 2G–H and 3). In the following months, the occurrence of discernible oocytes observed in ultrasound images increased and were all confirmed to be females with tertiary yolk oocytes. By May 2015, mean area fraction of tertiary yolk oocytes had increased from 3 to 64 % while primary yolk oocytes were reduced to 6%. After freshwater transfer, this development continued, and ended at a total dominance of 95% tertiary yolk oocytes in July 2015 (Fig. 3).

Linear models for ultrasound-based GSI measurements correlated stronger with sex hormone levels (Fig. 2 in

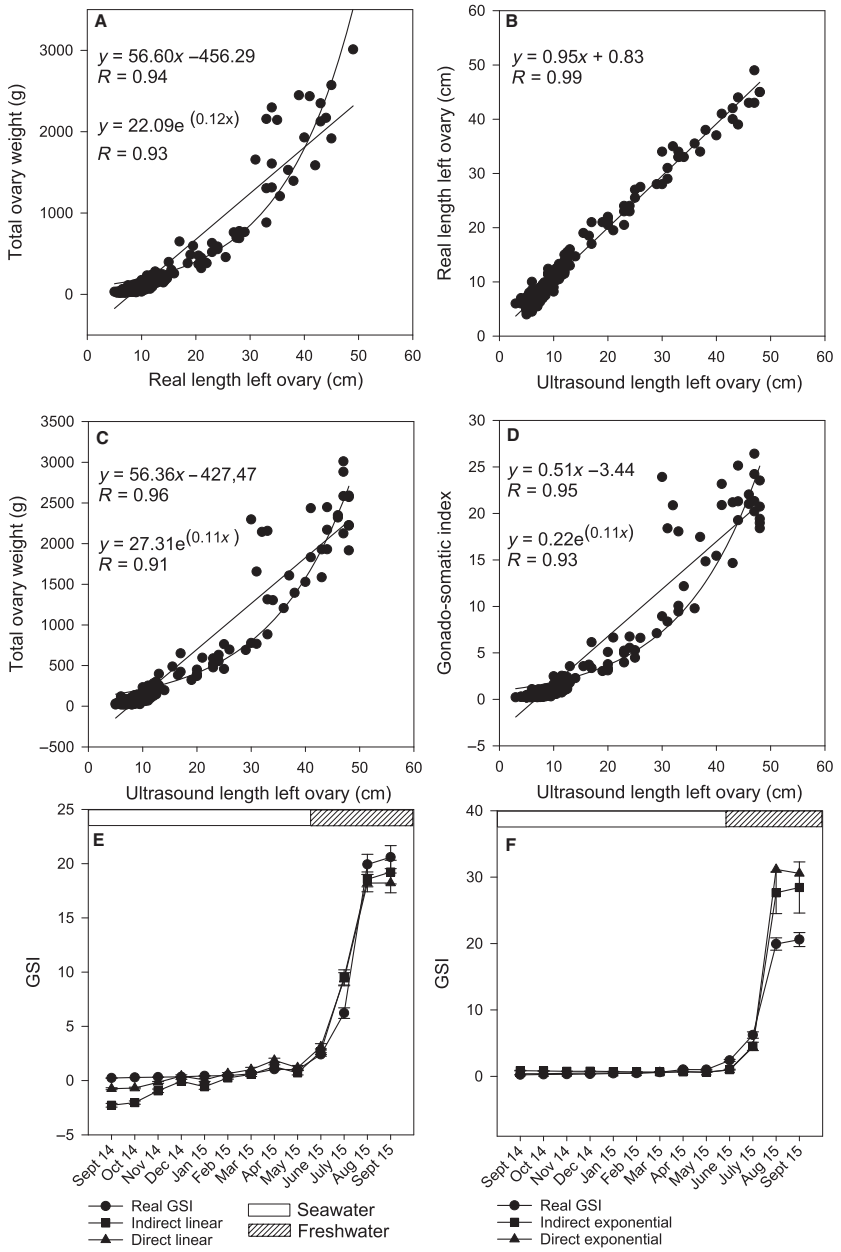

Figure 1. Estimating GSI in Atlantic salmon females from ultrasound measurements. Relationship between real left ovary length and total ovary weight (A) and between ultrasound length and real length of left ovary (B). Linear and exponential models for estimating GSI indirectly by first estimating the total ovary weight and using body weight to calculate GSI (C), or directly from ultrasound length of left ovary (D). Comparison of GSI estimated by linear (E) and exponential (F) method and real GSI of females sampled during the last year before ovulation. Horizontal bars at the top represents changes in environment conditions. Data in E and F are mean ± SEM.

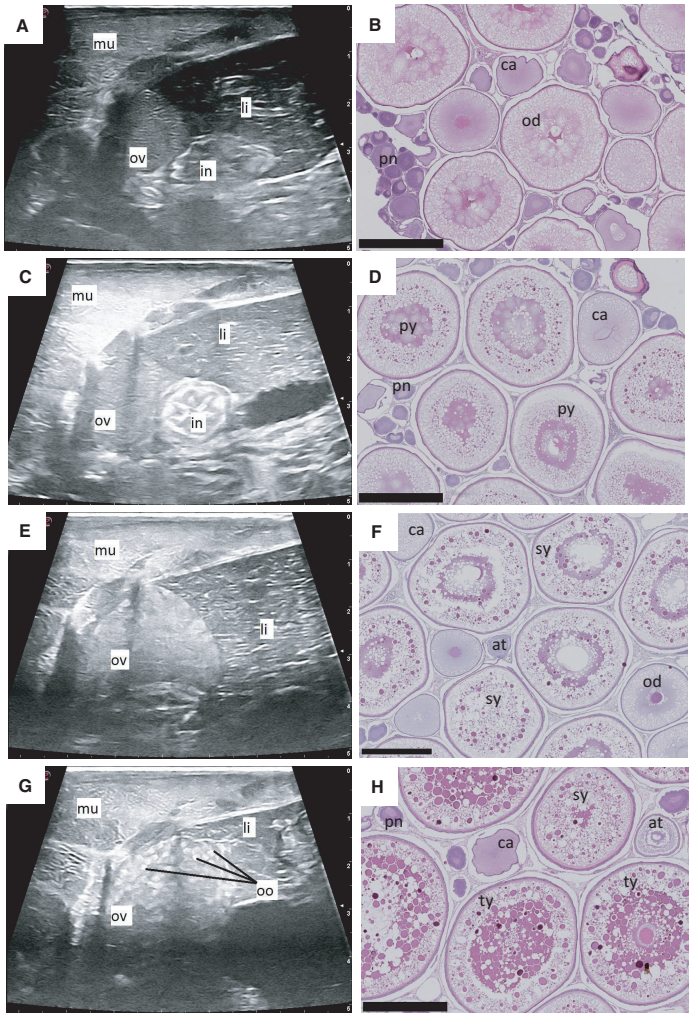

Figure 2. Vitellogenesis in female Atlantic salmon during the last year before ovulation, visualized using ultrasound and histology. Ultrasound (A) and histology (B) of previtellogenic female in September and December 2014, respectively. Ovaries in ultrasound images remain a uniform mass (C) as vitellogenesis has started (D) in some females in December 2014. A growth in ovary area (E) can be observed in ultrasound images as vitellogenesis progresses in February 2015 (F). As some females reach tertiary yolk oocyte stage of vitellogenesis in February 2015, oocytes becomes clearly visible in ultrasound images (G and H). at, atretic oocyte; ca, cortical alveoli stage; in, intenstine; li, liver; mu, muscle; od, oil droplet stage; oo, oocyte; ov, ovary, pn: perinucleolus stage, py: primary yolk oocyte, sy: secondary yolk oocyte, ty: tertiary yolk oocyte. Scalebar in ultrasound images (right side) is 5 cm. Scalebar in all histology slides is 1 mm.

Figs. S2 and S3) than exponential models, with the exception of MIH (Table 1). Highest correlations (R = 0.74–0.81) were found between ultrasound-based GSI and levels of 11-KT, independent of model type. When females were rearranged according to the dominating oocyte stage based on area fractions, the presence of vitellogenic oocytes concurred with a significant increase in sex hormone levels (Fig. 4). Mean plasma levels of E2 increased from those seen in previtellogenic females to those with secondary and tertiary yolk stage oocytes (Fig. 4A). T levels were also significantly higher in females with tertiary yolk oocytes than females with

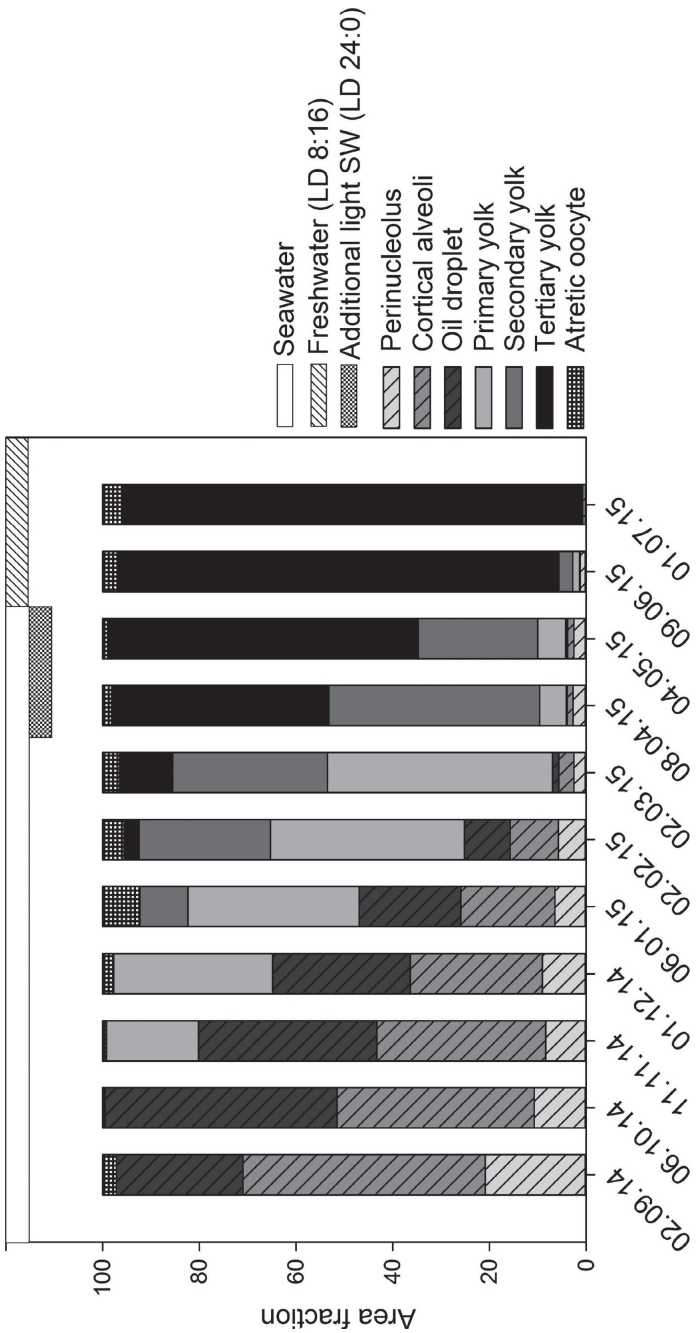

Figure 3. Gonad development of female Atlantic salmon during the last year before ovulation described as area fractions of different oocyte stages in histological sections. The area of oocyte development stages was calculated as percent of total area in sections from 12 to 2 months before ovulation. Horizontal bars at the top represent changes in environment conditions. Data are mean values.

Table 1. Correlations of sex hormone levels to ultrasound-based GSI estimates and real GSI of Atlantic salmon females during the last year before ovulation. Numbers are R, $P < 0.01$ for all correlations. 11-KT, 11-keto testosterone; E2, oestradiol; FSH, follicle-stimulating hormone; GSI, gonado-somatic index; LH, luteinizing hormone; MIH, maturation inducing hormone, T, testosterone.

	Linear indirect	Linear direct	Exponential indirect	Exponential direct	Real GSI
E2	0.63	0.62	0.48	0.46	0.77
T	0.71	0.72	0.62	0.62	0.88
11-KT	0.81	0.81	0.80	0.78	0.74
FSH	0.50	0.50	0.34	0.33	0.32
LH	0.77	0.76	0.60	0.58	0.57
MIH	0.48	0.46	0.51	0.48	0.27

previtellogenic, primary and secondary yolk oocytes (Fig. 4B). Plasma levels of LH were similar in females with previtellogenic, primary and secondary yolk oocytes, but doubled in tertiary yolk oocyte females (Fig. 4C). FSH levels were higher in secondary and tertiary yolk oocyte females than in previtellogenic and primary yolk oocyte females (Fig. 4D). Levels of 11-KT was low in females with previtellogenic, primary and secondary yolk oocytes, but were significantly higher in tertiary yolk oocyte females (Fig. 4E). The pattern described for the other hormones was not observed for MIH as plasma levels dropped from previtellogenic females to females with primary yolk oocytes and then increased in secondary and tertiary yolk oocytes (Fig. 4F). Ultrasound estimated GSI (direct exponential approach) was low in females with previtellogenic, primary, and secondary yolk oocytes (0.44, 0.52 and 0.57, respectively), while it was significantly higher (2.33, $P < 0.05$) in females with ovaries dominated by tertiary yolk oocytes (Fig. 4).

Discussion

GSI has been a standard method for evaluating progress in sexual maturation of salmonids. Here, a new, noninvasive method that uses ultrasound for estimating GSI in females from 1 year before ovulation has been presented. We found that ultrasound-based ovary length measurements can be used to monitor GSI in maturing females and it can give an accurate picture of maturational progress in combination with oocyte size observations by ultrasound.

The first application of ultrasound in a salmonid fish, was aimed at sex differentiation for broodstock management and estimating amount of sexual maturation in harvest fish (Martin et al. 1983; Reimers et al. 1987). Mattson (1991) expanded this concept by measuring ovary diameter using ultrasound, and suggested that gonad length and diameter could be used to estimate gonad weight. Models have been developed for estimating GSI in females, by combining area measurements in cross-sectional images of ovaries and external length measurements (European eel; Bureau du Colombier et al. 2015), or by converting calculated ovarian volume to weight through stage specific density factors (pallid sturgeon, *Scaphirhynchus albus*; Albers et al. 2013). These methods require detailed measurements and calculation after ultrasound examination for GSI estimation, and are thus little applicable for real-time GSI estimation when working with live fish. In this study, we found two models for real-time GSI estimation in live females, using ultrasound-based ovary length measurements that are performed in a few seconds. GSI can either be modeled directly from ultrasound measured left ovary length, or indirectly by combining modeled total ovary weight with female body weight measurements. Linear and exponential models for both direct and indirect GSI estimation were developed in this study. Direct and indirect linear models performed equally well after freshwater transfer. Both exponential models have good performance during seawater phase when ovaries are short, but are not recommended after freshwater transfer due to an increasing overestimation of GSI with increasing ovary lengths. Both approaches provide real-time GSI results that can be used instantly to grade females according to their maturational status, and examinations can be performed on the same female repeatedly. Ultrasound equipment has lately gone from large stationary scanners to portable units, therefore this technology has become more accessible for field studies and other applications. This noninvasive approach to maturation monitoring would improve animal welfare according to two of the three Rs in the guidelines for animal welfare. Eliminating the need to sacrifice fish or the use of other invasive methods, and the possibility to examine the same individual repeatedly, reduces the number of fish needed for examination and refines maturation monitoring (Russell and Burch 1959).

The observed extensive oocyte development during seawater phase did not correlate with performed monthly GSI and sex hormone measurements. Used independently to histological studies, these methods should therefore be

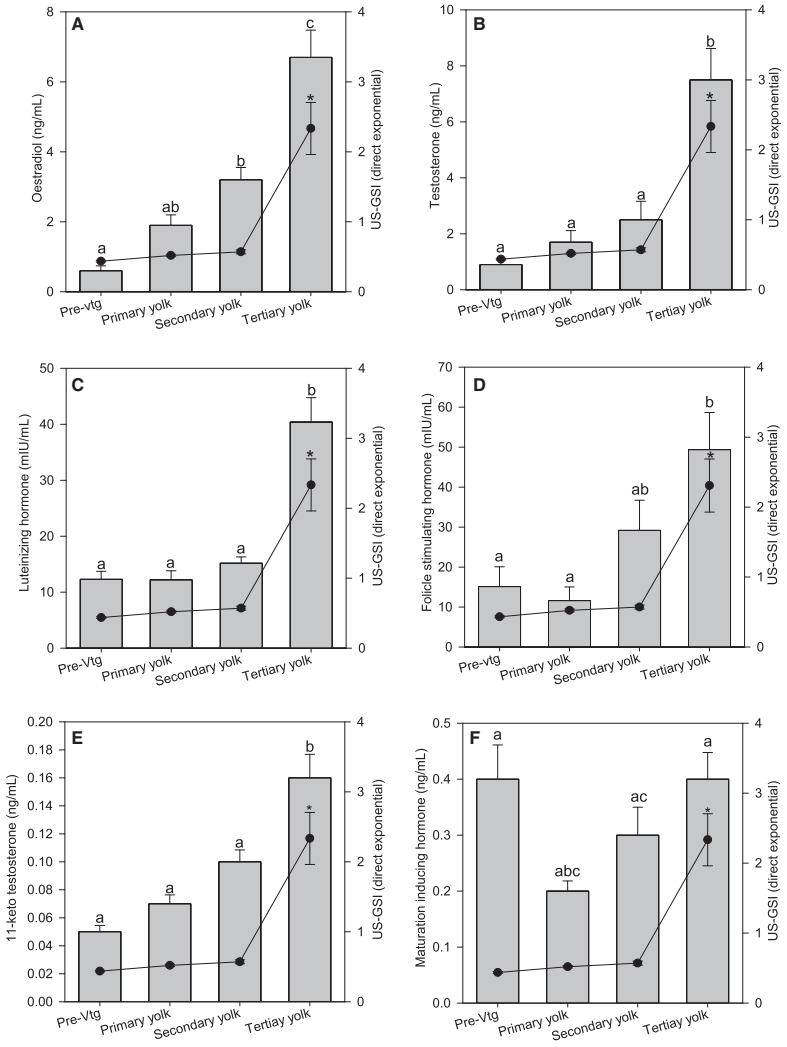

Figure 4. Sex hormone levels in female Atlantic salmon grouped according to dominating oocyte stage area fraction during maturation. Oestradiol (A), testosterone (B), luteinizing hormone (C), 11-keto testosterone (E) and maturation inducing hormone (F): pre-vtg; previtellogenic females (n = 21; September 2014–February 2015), primary yolk oocytes (n = 17; November 2014–March 2015), secondary yolk oocytes (n = 14; January–May 2015), tertiary yolk oocytes (n = 44; March–July 2015). Follicle stimulating hormone (D): Pre-vtg: previtellogenic females (n = 13; September 2014–January 2015), primary yolk oocytes (n = 12; December 2014–March 2015), secondary yolk oocytes (n = 14; January–May 2015), tertiary yolk oocytes (n = 43; March–July 2015). Bars are mean values and S.E.M. The line represents mean ultrasound estimated GSI and S.E.M. GSI was estimated from ovary length using direct exponential approach (Fig. 1D). Letters indicate significant differences in sex hormones, $P < 0.05$. Asterisks represents significant different US-GSI measures.

applied with care when monitoring Atlantic salmon female maturation in seawater.

Ultrasound could not clearly visualize changes in oocyte development in the last 12 to 8 months before ovulation, since the only observation was an increase in diameter of a uniformly structured ovary. From 7 months before ovulation and onwards, tertiary yolk oocytes were distinguishable in ultrasound images. When individual

females were rearranged according to oocyte development stage, strong correlations between ultrasound estimated GSI, plasma sex hormone levels and oocyte development was revealed. Females with more developed oocytes, that is, tertiary yolk oocytes, showed higher ultrasound estimated GSI and level of hormones involved in inducing vitellogenesis, such as FSH and E2, indicating that hormonal processes had been initiated earlier than what could be observed from the monthly hormone profiles. This is in accordance with findings of Taranger et al. (1999), where increased plasma E2 levels were observed in January in females that matured the following autumn. Thus, ultrasound examination could replace sacrifice for histology sampling and less invasive blood sampling in Atlantic salmon females.

All females in this study matured normally although some unexpected findings included the presence of vitellogenic oocytes in November 2014 (Fig. 3), 8 months before significant changes in GSI (Fig. 1 in Figs. S2 and S3), and sex hormones levels were observed (Fig. 2 in Figs. S2 and S3). This is somewhat earlier than expected compared to other studies in Atlantic salmon females, where secondary growth phase oocytes were observed equally early under similar photoperiod treatment, but not any vitellogenic oocytes (Taranger et al. 1998, 1999; Andersson et al. 2009). Recently, high expression of vitellogenin receptors in ovaries from females during the same period have been observed, supporting our observation (Andersen et al. 2017) The early development of vitellogenic oocytes found here appears to be more similar to observations in rainbow trout (*Oncorhynchus mykiss*; Sumpter et al. 1984).

Low levels of 11-KT were described for the first time in maturing Atlantic salmon females (Fig. 2 in Figs. S2 and S3). Studies in short-finned eel (*Anguilla australis*) and Atlantic cod (*Gadus morhua* L.) have indicated that 11-KT stimulates previtellogenic oocyte growth (Lokman et al. 2007; Kortner et al. 2009a,b), and it has also been shown to induce silvering-related changes in female short-finned eels (Rohr et al. 2001). Fitzpatrick et al. (1986) reported that female Coho salmon (*Oncorhynchus kisutch*) with more advanced maturation status, had higher plasma levels of 11-KT than less mature females, although nonsignificant. In our study, levels of 11-KT in females were very low (1–2 ng/mL) compared to other steroids (E2: ~40 ng/mL). However, there was a significant step-wise increase and decrease during the freshwater phase toward ovulation. A role of 11-KT in previtellogenic oocytes could not be confirmed in this study, as levels remained low during the short period before yolk accumulation started in November 2014. On the other hand, we observed increasing levels of 11-KT closer to ovulation, suggesting that 11-KT may be involved in regulation or stimulation of final maturation and ovulation in Atlantic salmon.

Ultrasound technology is well suited to substitute traditional invasive methods during sexual maturation monitoring in Atlantic salmon broodfish populations. Ultrasound-based ovary length measurements correlated strongly with GSI and sex hormone levels. In addition, oocyte size observations by ultrasound corresponded well to histologically identified stages of oocyte development. These methods offer a possibility to reduce stress and improve animal welfare in broodstock management of both wild and farmed Atlantic salmon populations.

Acknowledgments

The authors express thanks to staff at AquaGen Kyrksæterøra for assistance during all samplings; Tora Bardal and Randi Røsbak at Department of Biology, NTNU for valuable help and training in histology and sex hormone analysis; Nina Santi for comments on the manuscript.

Conflict of Interest

All authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Rearing conditions. Temperature (blue), and natural (black) and artificial (dashed line) light profiles during seawater and freshwater phases. Horizontal boxes represent seawater and freshwater phases.

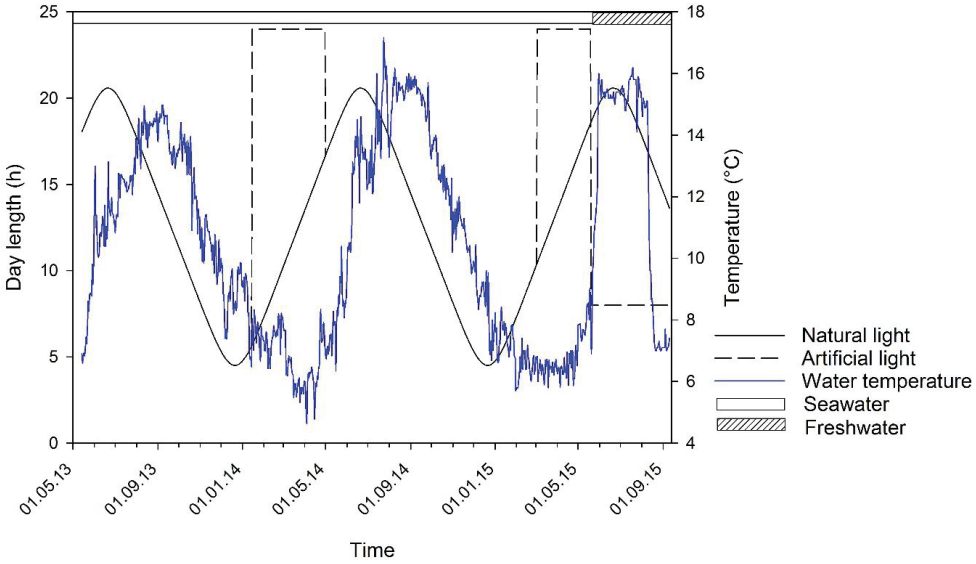
Figure S2. Gonado-somatic index (GSI) in female Atlantic salmon during the last year before ovulation. Samplings from September 2014 through July 2015 are monthly, while August and September 2015 samplings are weekly. Horizontal bars at the top represents changes in environment conditions. Data are mean ± SEM. Letters indicate significant differences, $P < 0.05$. Break in x axis indicates sampling frequency changing from monthly to weekly.

Figure S3. Profiles of plasma sex steroids in female Atlantic salmon during the last year before ovulation. Samplings are monthly from September 2014 through July 2015, and then weekly from there. (A) oestradiol, (B) testosterone, (C) luteinizing hormone, (D) follicle stimulating hormone, (E) 11-keto testosterone hormone, (F) maturation inducing hormone. Data are mean ± SEM. Letters indicate significant differences, $P < 0.05$. Horizontal bars at the top represents changes in environment conditions. Break in x axis indicates sampling frequency changing from monthly to weekly.

Table S1. Mean fish weight (±SEM) and *K* factor (±SEM) for all samplings. Number of samples analyzed for sex hormone concentration and histology for each sampling. 11-KT, 11-keto testosterone; E2, oestradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; MIH, maturation inducing hormone, T, testosterone.

Table S2. Histological classification of oocytes in sections from Atlantic salmon ovaries (Taranger et al. 1999; Andersson et al. 2009).

Supplements paper II



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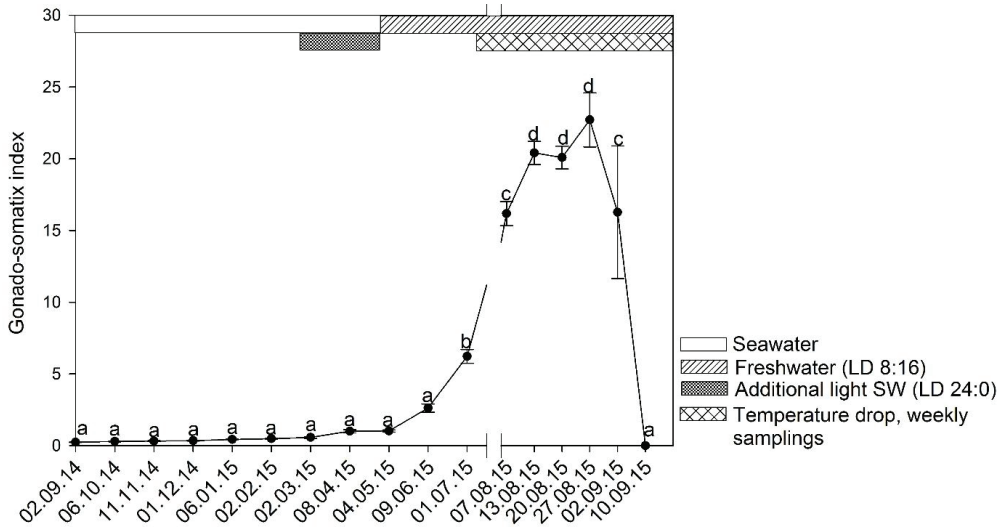

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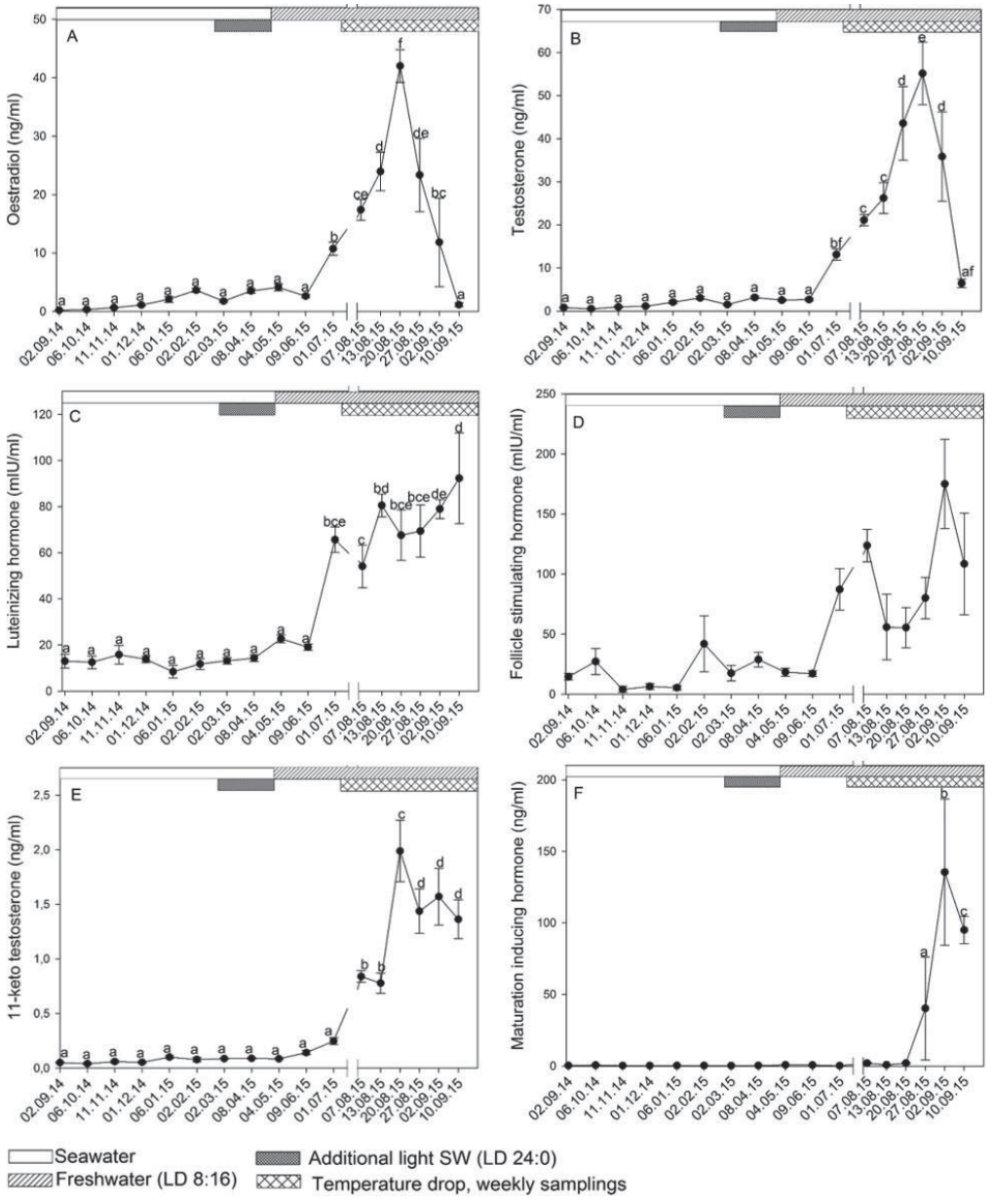

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Sampling date (n)[1]	Fish weight (kg) (mean ± S.E.M)[2]	K factor (mean ± S.E.M)	11-KT, E2, LH, MIH, T (n)	FSH (n)	Histology (n)
02.09.14 (20)	5.75 ± 0.23	1.20 ± 0.02	6	3	6
06.10.14 (20)	6.08 ± 0.16	1.16 ± 0.02	6	5	6
11.11.14 (20)	7.22 ± 0.20	1.28 ± 0.02	6	2	6
01.12.14 (20)	8.12 ± 0.29	1.31 ± 0.01	6	5	6
06.01.15 (20)	8.36 ± 0.40	1.26 ± 0.03	6	5	6
02.02.15 (20)	9.90 ± 0.38	1.42 ± 0.04	6	4	6
02.03.15 (19)	10.93 ± 0.45	1.44 ± 0.04	10	9	10
08.04.15 (15)	12.56 ± 0.41	1.50 ± 0.01	10	10	10
04.05.15 (20)	11.60 ± 0.31	1.39 ± 0.08	10	10	10
09.06.15 (20)	11.09 ± 0.35	1.30 ± 0.02	10	10	10
01.07.15 (20)	10.55 ± 0.36	1.30 ± 0.03	20	19	20
07.08.15 (5)	11.43 ± 0.75	1.28 ± 0.03	5	5	
13.08.15 (5)	10.26 ± 0.41	1.22 ± 0.03	5	5	
20.08.15 (5)	9.88 ± 0.75	1.17 ± 0.05	5	5	
27.08.15 (5)	10.76 ± 0.43	1.16 ± 0.03	5	5	
02.09.15 (5)	10.48 ± 0.55	1.21 ± 0.05	5	5	
10.09.15 (5)	10.21 ± 0.54	1.18 ± 0.02	5	5	

[1]In March and April 2015, fewer females were sampled due to weather conditions.

[2] There was significant increase in body weight between October and November 2014, and monthly from January to April 2015.

Table S2. Histological classification of oocytes in sections from Atlantic salmon ovaries (Taranger et al., 1999; Andersson et al., 2009).

Oocyte stage	Description	Phase
Chromatin nucleolus stage	Small oocyte with visible nucleolus and chromatin threads	Primary growth phase
Perinucleolar stage	Blue staining nucleoli visible around the nucleus	“
Cortical alveoli stage	Cortical alveoli around the periphery (and in center, depending on section) of oocyte	Secondary growth phase
Oil droplet stage	Oil droplets in the center of the oocyte	“
Primary yolk stage	Small amounts of yolk appear in the periphery of the oocyte	True vitellogenesis
Secondary yolk stage	Yolk globule fills more of the oocyte, the globule increases in size	“
Tertiary yolk stage	Whole oocyte filled with large yolk globules	“
Atretic oocyte	Breakdown of oocyte content and membranes, increasing folding of follicular layers	Other
Other tissues	Blood vessels, connective tissue, adipose tissue	

Paper III

Physiological Reports

Open Access

Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

Ultrasound as a noninvasive tool for monitoring reproductive physiology in male Atlantic salmon (*Salmo salar*)

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Keywords
Animal welfare, histology, male reproduction, sex hormones, ultrasound.

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Funding Information
This work was funded by the Norwegian Research Council (241398/O30) and AquaGen AS, Norway.

Received: 13 May 2019; Revised: 18 June 2019; Accepted: 19 June 2019

doi: 10.14814/phy2.14167

Physiol Rep, 7 (13), 2019, e14167, https://doi.org/10.14814/phy2.14167

Abstract

We examined the potential for ultrasound as a noninvasive tool for maturation monitoring in Atlantic salmon (*Salmo salar*) males. Ultrasound examination and measurements were compared to common practices for maturation monitoring such as gonadosomatic index (GSI), sex hormone analysis, and histological analysis of spermatogenesis. There were significant correlations ($R^2 = 0.68$, $P < 0.01$) between ultrasound-based measurements of the left testis and total testes weight and GSI, and ultrasound could be used for noninvasive GSI measurements. Echogenicity of ultrasound images corresponded to the histological stages observed, which added nuance to ultrasound-based GSI measurements during final weeks preceding stripping. We propose that ultrasound can be used as an alternative to more invasive methods for sexual maturation monitoring in wild and farmed Atlantic salmon males. Using ultrasound technology, we have established a quick and noninvasive method that could reduce the number of stressful handlings and unwanted sacrifice of broodfish required for maturation monitoring in Atlantic salmon males.

Introduction

The salmonid species are of great cultural and economic value, and knowledge and control of their reproduction is an important step both in management of wild, endangered populations and for commercial production. In the wild, Atlantic salmon (*Salmo salar*) return from seawater migration to their natal river for spawning (Hansen et al., 1993). During upstream river migration, decreasing photoperiod and water temperature induces final maturation of eggs and sperm and initiates spawning (Heggberget, 1988; Webb and McLay, 1996). In broodfish production, light and temperature control is used to advance and delay maturation in order to secure year-round production and supply of fertilised eggs for customer demands (Pankhurst and King, 2010; Taranger et al., 2010; Vikingstad et al., 2016). Gonadosomatic index (GSI) is an invasive method that is most commonly used to monitor sexual maturation in fish. It is based on gonad weight as a percentage of total body weight, and the method requires unwanted sacrifice of valuable broodfish. In farmed broodstock, it is therefore often performed on deceased fish, which might not be representative for the population.

The GSI method for monitoring male maturation (Schulz et al., 2006; Fjelldal et al., 2011) is used alone or in combination with other invasive and semiinvasive methods such as histological analysis (Dziewulska and Domagała, 2003, 2005), gene expression in relevant tissues (Melo et al., 2014) and single or repeated blood

sampling for plasma sex hormone analysis (Youngson et al., 1988; Sakai et al., 1989). Blood sampling has the advantage of being less invasive but involves time-consuming and costly analysis which requires tagging of individual fish and repeated handlings. Crowding and netting involved in handlings can induce stress response (Waring et al., 1992; Carey and McCormick, 1998) and have negative effects on fish health (Pickering and Pottinger, 1989; Fast et al., 2008) and reproductive outcome (Campbell et al., 1992; Allyn et al., 2001). Insertion of an endoscope in the abdominal cavity for sexing and maturation monitoring gives instant results, but comes with increased risk of infection and internal bleedings (Ortenburger et al., 1996; Swenson et al., 2007), which also is the case for cutting several centimeter-long incisions for visual inspection of gonads (Melillo Filho et al., 2016). In search of noninvasive methods for maturation monitoring, sex steroid assays from holding water (Ellis et al., 2013), scoring of external morphology (Youngson et al., 1988), and computed tomography (Müller et al., 2004) have been suggested. None of these methods appear to be precise or practical enough for maturation monitoring at present.

Ultrasound is a noninvasive method for imaging of internal organs and tissues based on reflection of emitted sound waves from organs back to the transducer (Kossoff, 2000), giving grey-scale images that have been used for sexing and male maturation monitoring in several species such as rainbow trout (*Oncorhynchus mykiss*; Evans et al. (2004)), European eel (*Anguilla anguilla*; du Colombier et al., 2015) and Chinese sturgeon (*Acipenser sinensis*; Du et al., 2017). These methods range in complexity from sexing of immature males based on exclusion (Newman et al., 2008), to descriptions of testes appearance in ultrasound images at different stages of maturity (Colombo et al., 2007; Wildhaber et al., 2007) and measurements such as external ultrasound-based testis length and area in cross section ultrasound images (Evans et al., 2004; Bryan et al., 2007). In male Atlantic salmon, ultrasound has been used for sexing by exclusion and there have been attempts at measurement of gonad diameter for determination of maturity status (Reimers et al., 1987; Mattson, 1991).

Inspired by our previous work regarding use of ultrasound for maturation monitoring in females (Næve et al., 2018), we compared ultrasound examinations of Atlantic salmon males with established methods for maturation monitoring such as plasma sex hormone and histological analyses. The goals of this study were to a) establish ultrasound as a noninvasive tool for maturation monitoring in Atlantic salmon males and b) examine if ultrasound can replace more invasive methods for maturation monitoring such as blood and tissue sampling.

Materials and Methods

Ethics statement

Fish included in this experiment were reared according to the Norwegian aquaculture legislation. Euthanasia was performed according to European directive 2010/63/EU, Annex IV. According to Norwegian and European legislation concerning animal research, experimental conditions and sedation in this study are practices undertaken for the purpose of recognized animal husbandry, and therefore formal approval of these experiments by the Norwegian Animal Research Authority (NARA) was not requested.

Fish husbandry

Atlantic salmon fry of the AquaGen strain were first fed in February 2012 and transferred to seawater as 1-year-old smolts in March 2013. During the seawater phase, fish were fed according to appetite (Ewos Opal 110/112 and Ewos Opal Breed 3500 from September 2014). Fish were reared under natural photoperiod, except for two periods of continuous light during the first and second seawater winters to prevent grilsing and to advance maturation, respectively (described in Næve et al. (2018)). In May 2015, fish were transferred to indoor circular freshwater tanks (8-m diameter, 1.5-m depth, 60 m^3) for final maturation and stripping. Autumn light and temperature signals were given from freshwater transfer. In August 2015, a temperature drop was given to induce final maturation. Water temperature (see also Næve et al. (2018)) and O_2 levels were registered daily at 3- and 6-m depth during the seawater phase (58 to 116 % and 75 to 148 %, respectively) and freshwater rearing (97 to 114 %).

Experimental design

Fish samplings started from September 2014, and 10 to 20 males (Table S1) were sampled monthly until the temperature drop (see fish husbandry), when five males were sampled weekly until running milt was observed in all sampled males (02.09.15). Before sex could be identified by external traits, ultrasound was used to identify males (see ultrasound specifications, below). Fish were sacrificed with an overdose of tricaine methanesulfonate (200 mg/L, Pharmaq, Norway) before the spinal cord was severed. Using heparinised vacuum tubes (Terumo, Japan), blood samples were drawn from the caudal vein and centrifuged at 2.4 x 1000 g for 10 min at 4 °C (Micro Star 17R, VWR, USA). Plasma was collected and stored on ice for 1 to 5 h prior to freezing at −80°C. Body weight was registered to the nearest 20 g using a digital scale (SFE 60K20IMP, Kern & Sohn GmbH, Germany) and length

to the nearest centimeter (cm) using a measurement tape. From January 2015, ultrasound length of left testis was measured by placing the ultrasound probe at the clavicle and a ruler at the basis of the pectoral fin and moving the probe in a posterior direction while tracing the left testis in the ultrasound image. Ultrasound-based left testis length was registered as distance traveled along the ruler by the ultrasound probe. Cross-section ultrasound images (one to four per individual) of left testis were captured from each individual. Testes were gently dissected out and length of left testis and testis weight (left and total) was registered to the nearest 0.1 g using a digital scale (MFD, A&D, Japan and Scanvaegt DS-673SS, Denmark). Cross-sectional tissue samples (0.5 to 1 cm thick) were cut from the anterior half of the testis and fixed in 4 % formaldehyde solution in phosphate buffer (pH 6.9, Merck Millipore, Germany).

Ultrasound specifications

Ultrasound examination was performed using a MyLab Alpha ultrasound machine (Esaote, Italy) with a linear array probe (3 to 13 MHz) placed at the clavicle. Examinations were performed at 3 to 5 MHz, with focus at 25 to 40 mm and gain (signal amplification) of 80 to 90 %.

Hormonal analysis

Plasma levels of 11-keto testosterone (11-KT), maturation inducing hormone (MIH) and testosterone (T) were measured using enzyme immuno-assay (EIA) kits (Cayman chemical, USA). Steroid sex hormones were extracted from frozen plasma samples using diethyl ether. Briefly, 500-µL plasma was thoroughly mixed with diethyl ether (1:4 plasma:solvent) by vortexing. The phases were separated by freezing in liquid nitrogen, and the organic phase was decanted. The process was repeated, and the combined organic phase was evaporated overnight at 25°C. The dry extracts containing steroid hormones were resuspended in 500 µL of EIA buffer and frozen at −80°C pending analysis. Samples were analysed in duplicate as described by kit manufacturer. Absorbances were read at 410 nm for all three hormones using a Cytation 5 Imaging plate reader (BioTek Instruments, USA). Plasma steroid levels were calculated from standard curves fitted with linear regression of logit transformed data as instructed by the manufacturer.

Histology

Tissue for histological analysis was fixed and stored in phosphate buffered formaldehyde at 4°C until dehydration by a tissue processor (TP1020, Leica Biosystems Nussloch GmbH, Germany). Testis slices were embedded in paraffin and sectioned at 4-µm thickness using a microtome (2055/RM2255, Leica biosystems, Nussloch GmbH, Germany), before hematoxylin and eosine (H&E) staining. Glass slides were scanned at 20x magnification with a digital slide scanner (NanoZoomer, Hamamatsu photonics, Japan) and images viewed and exported from scanner software (NDP, Hamamatsu photonics, Japan) as jpg.-files. Ten images were exported from each slide, each covering an area of approximately 0.71 mm^2. A track map in the scanner software was used to avoid overlapping images.

Spermatogenesis was evaluated in three randomly chosen images from individuals selected for further analysis. A point grid of 112 crosses covering 6500 µm was overlaid with each image using Grid plugin for ImageJ (National Institutes of Health, USA). Cells and tissues in testis sections were classified according to Dziewulska and Domagała (2003, 2005), Leal et al. (2009), Schulz et al. (2010) and Melo et al. (2014). Using a cell counter plugin for ImageJ, number of points (intersection of each cross) hitting each tissue type was counted. Area fraction for each tissue or cell type was calculated as number of points hitting each tissue type divided by total number of points.

Spermatogenic maturity index (SMI) was introduced by Tomkiewicz et al. (2011) as a method for evaluating the spermatogenic progress in histology slides of testis. SMI ranges from 0 (only testicular somatic cells present) to 1 (only spermatozoa present) and was calculated by weighting area fractions (F) of each tissue or cell type, according to the following equation:

$$SMI = 0.0F_{Ts} + 0.25F_{Sg} + 0.5F_{Sc} + 0.75F_{St} + 1.0F_{Sz}$$

Sc, spermatocytes; Sg, spermatogonia; St, spermatids; Sz, spermatozoa; Ts, testicular somatic cells.

Further classification of substages within each spermatogenic stage was not performed as it was not possible with the applied method.

Data analysis and statistics

Data were prepared using Microsoft Excel (Microsoft, USA). Figures were made using SigmaPlot 14 (Systat Software, USA). GSI, gonad weight as percent of body weight, was calculated as.

$$GSI = (\text{gonad weight}/\text{body weight}) \times 100$$

Condition factor, K, was calculated as.

$$K = 100000 \times \left(\text{weight}/\left(\text{length}^3\right)\right)$$

At September throughout November 2014 samplings, total testes weights were not registered. There was a linear

relationship between left and total testes weight at the rest of the samplings (Pearson's correlation, $R^2 = 0.992$, $P < 0.01$). The following equation was used to calculate total testes weight for September throughout November 2014 samplings, and estimated total testes weight was used for GSI calculation for these samplings.

$$\text{Total testes weight} = (1.80 \times \text{left testis weight}) + 1.20$$

Variation in maturation (observed as GSI) was low during the first months (September 2014 throughout February 2015) of this study. Therefore, the number of males analysed for sex hormones and testis development represented by histological sections was reduced for these samplings (Table S1). From September 2014 throughout February 2015 samplings, five and six individuals were selected randomly for histology and sex hormone analysis, respectively. From March until June 2015, the five males with the highest and lowest GSI were analysed for sex hormones and histology. Statistical analyses were performed using IBM SPSS Statistics 25 (IBM, USA). Data were tested for normality using Shapiro–Wilk test, with $P < 0.05$ indicating nonnormal data. GSI, SMI, and sex hormone data were nonnormal and included some outliers, they were tested for differences between groups using Kruskal–Wallis H-test followed by Dunn's test with Bonferroni correction for pairwise comparisons ($P < 0.05$ for SMI and GSI and $P < 0.01$ for 11-KT, MIH, and T). Linear and exponential ultrasound-based GSI models were fitted using regression curve estimation, with $P < 0.05$. As these data were nonnormal, included outliers and did not have symmetrical distributions, the Sign test ($P < 0.05$) with Bonferroni correction was used to identify differences between the estimated GSI based on ultrasound measurement and the GSI measured by conventional method for each sampling. Correlations between SMI, sex hormone plasma levels, GSI, and ultrasound-based GSI models were performed using Pearson product moment correlation, $P < 0.05$.

Data were rearranged into four groups according to GSI (<1; 1 to 2.9; 3 to 4.9 and 5 to 7) rather than monthly samplings to study differences in levels of SMI and sex hormones between these groups. Data were not normally distributed (Shapiro–Wilk test, $P < 0.05$), and differences between GSI groups were tested using Kruskal–Wallis H-test followed by Dunn's test with Bonferroni correction for pairwise comparisons ($P < 0.05$ for SMI and MIH, $P < 0.01$ for 11-KT and T).

Results

There was a strong correlation between length of left testis and total testis weight (Figure 1A, Pearson's correlation; $R^2 = 0.61$, $P < 0.01$), and between length of left testis (Figure S1) and ultrasound measured length of left testis (Figure 1B, $R^2 = 0.79$, $P < 0.01$). Ultrasound measured left testis length correlated strongly with total testis weight (Figure 1C, $R^2 = 0.68$, $P < 0.01$) and GSI (Figure 1D, $R^2 = 0.68$, $P < 0.01$) and linear and exponential models for estimating total gonad weight and GSI using ultrasound-based length of left testis were fitted. Applying these models, GSI was estimated indirectly using total testis weight calculated from ultrasound measurement and registered body weight, or directly from ultrasound measurement of left testis (Figure 1C and 1, respectively). Both linear models followed each other closely, and overestimated GSI (nonsignificant) during the seawater phase, while they first under- and then overestimated GSI during the freshwater phase (Figure 1E). The exponential models followed GSI calculated by conventional method closely during the seawater phase. Indirect exponential model significantly under-estimated GSI in June and July, and then greatly overestimated (nonsignificant) GSI prior to stripping (Figure 1F). Direct exponential model significantly underestimated GSI for the July sampling, but estimated GSI more accurately in the final weeks before stripping.

Left testis could not be discerned from internal organs and adipose tissue in ultrasound images during September throughout November 2014 samplings. During these months, sexing was done by exclusion as ovaries were visible in ultrasound images. From December 2014, left testis was observed in some individuals as a black, oval, or circular structure near the intestine and kidney. From January 2015, it was possible to measure ultrasound-based left testis length in some individuals (Figure 2A). During seawater phase, little growth in left testis size was observed in ultrasound images. In histological sections from this period (Figure 2B), area fraction of spermatogonia and testicular somatic cells increased (11 to 37%) and decreased (89 to 63%), respectively (Figure 3A). At the first sampling after freshwater transfer (June 2015), left testis was black in ultrasound images, with increased size (Figure 2C) and spermatocytes were observed in histological sections from some individuals (Figures 2D, 3A). In the July 2015 sampling, testes in several males had outgrown the footprint of the ultrasound probe, and we were not able to capture the whole left testis in the cross-section ultrasound image. At the July sampling, only one male had testis that appeared black in the ultrasound image, and left testis of most males appeared grey (Figure 2E). In these males, spermatocytes and spermatids could be observed in histological sections (66 and 20%, respectively, Figure 3A), while area fractions of spermatozoa were very low (1 to 3%, Figures 2F, 3A). In July, two individuals had testes that were bright grey, almost white

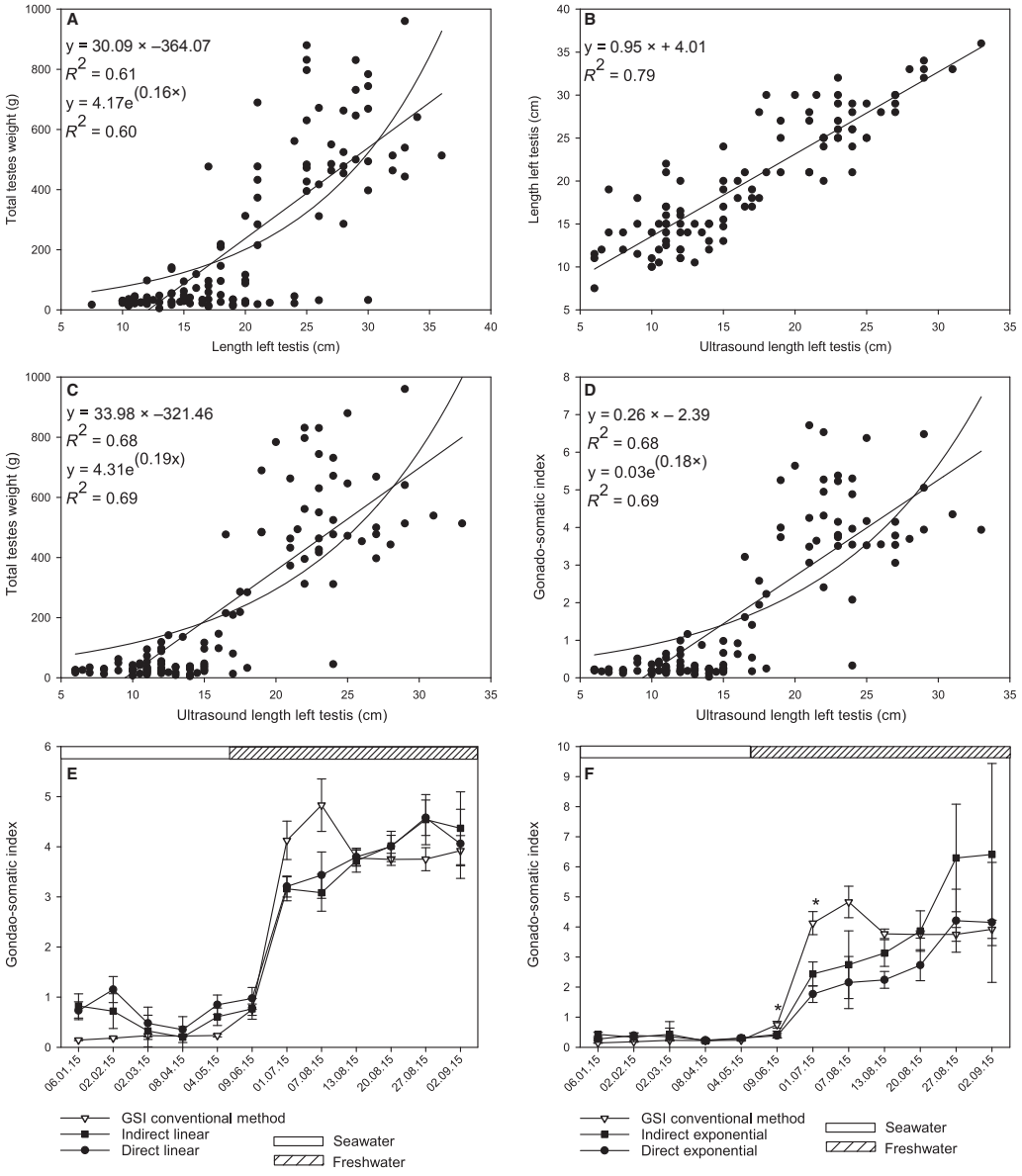

Figure 1. Ultrasound-based estimation of gonadosomatic index in Atlantic salmon males during the last year before stripping. Correlation (Pearson's correlation) between testis length and weight (A) and ultrasound length of testis (B). Correlations between ultrasound length of testis and total testis weight (C) and gonadosomatic index (GSI; D) were used to make direct (calculating GSI directly from ultrasound measurement) and indirect (calculating GSI by estimating ovary weight first) linear and exponential models for GSI estimation. GSI estimated using direct and indirect linear (E) and exponential models (F). Horizontal bars at the top (E and F) represent rearing conditions and data in E and F are mean ± SEM. Asterix in F represent significant differences between indirect exponential model and GSI in June, and between both exponential models and GSI in July (Sign test with Bonferroni correction, $P < 0.05$).

in ultrasound images (Figure 2G), and these individuals had spermatozoa that accounted for ≥10% of testicular area fraction (Figures 2H, 3A). During August and September 2015 an increasing number of males with bright grey or white testis were observed. During the same period, the area fraction of spermatozoa increased to complete dominance (87 %) at stripping (Figure 3A).

During the seawater phase the mean GSI of Atlantic salmon males was stable at low levels (Figure 3B). At the first sampling after freshwater transfer in June 2015, GSI had increased slightly, followed by an exponential increase to a peak value of 5 in early August 2015 (Figure 3B). GSI declined to July 2015 levels and was stable at approximately 4 during the rest of the freshwater phase, including at final maturation. Mean SMI was low during seawater phase but showed a slight increasing trend during the final months in seawater (Figure 3A). After freshwater transfer SMI increased exponentially from 0.2 in June to 0.9 at stripping in September 2015. GSI was strongly correlated (Pearson's correlation) to SMI and plasma levels of T and 11-KT (Figure S2) while the correlation to MIH was moderate (Table 1). Both linear models for ultrasound-estimated GSI had strong correlation to SMI, T, and 11-KT and moderate correlation to MIH. Exponential ultrasound GSI models had strong correlation to SMI and moderate correlation to plasma levels of T, 11-KT, and MIH (Table 1).

Rearranging males in groups according to GSI showed similar trends in SMI and all three sex hormones measured. SMI (Figure 4A) and plasma 11-KT, T,and MIH (Figure 4B–D) levels were low in males with GSI less than 1 and increased to a peak in males with GSI ranging 3 to 4.9. Males with the largest gonads, namely those with GSI between 5 and 7, had lower levels of sex hormones and SMI than males with GSI between 3 and 4.9.

Discussion

Using ultrasound technology, we have established a quick and noninvasive method that could reduce number of stressful handlings and unwanted sacrifice of broodfish required for maturation monitoring in Atlantic salmon males.

The ultrasound-based GSI method requires one measurement of left testis length, and calculations can easily be performed in an excel sheet, giving instant results. Indirect and direct ultrasound-based GSI methods that were established here both have linear and exponential alternatives available. The indirect approach calculates GSI based on measured body weight and total testis weight estimated from ultrasound-based left testis length measurement, while the direct approach estimates GSI from ultrasound measurement of left testis length. Linear indirect and direct methods for ultrasound-based GSI are suitable for GSI estimation after freshwater transfer although it should be kept in mind that they deviate somewhat from GSI measured by conventional method. Direct and indirect exponential models underestimate GSI too much during early freshwater phase (June and July). Thus, these models are not recommended for maturation monitoring. For GSI estimation during seawater phase, exponential models are recommended, as these follow GSI calculated by conventional method closely in seawater. However, none of the models presented here were able to represent the peak and decline in GSI observed during the weeks preceding stripping.

In early reports on use of ultrasound in salmonids, simple sexing of maturing individuals was achieved (Martin et al., 1983; Reimers et al., 1987). Subsequently, attempts at maturation assessment using area measurements in cross-section ultrasound images were attempted. However, these methods involved some uncertainties (Mattson, 1991; Evans et al., 2004). In our experience, performing such measurements using ultrasound equipment can be cumbersome and time-consuming, prolonging handling time and stress for each fish. Using ultrasound-based GSI method reported here, results are obtained within seconds, reducing handling time and stress to the broodstock. To ease handling of large male broodfish and keep handling stress and subsequent risk of reduced fish health and welfare at a minimum (Fast et al., 2008; Tort, 2011; Conte, 2004), we strongly suggest that ultrasound-based GSI measurements should be performed using anesthesia (Iversen et al., 2003).

A corresponding method for GSI estimation was recently established for Atlantic salmon females (Næve et al., 2018). In the present study, R^2-values for ultrasound-based GSI model in males were lower than those reported in salmon females. In addition, the observed variations were higher than those observed in females, giving a less accurate GSI estimate in males. Ultrasound-based ovary length measurements are quite easy to perform because ovaries are clearly defined and increase in size in an anterior to posterior direction as maturation progresses. Atlantic salmon testes are elongated structures spanning the length of the abdominal cavity from early life stages (Laird et al., 1978). Therefore, testes do not grow in length during sexual maturation as in females, but rather in thickness, starting anteriorly and narrowing toward the anus. When measuring left testis length, the thickened part was registered, and it could be challenging to define the end of testis thickening, both when using ultrasound and measuring tape. With practice and experience, accuracy of ultrasound-based length measurement could probably be increased, giving more exact models

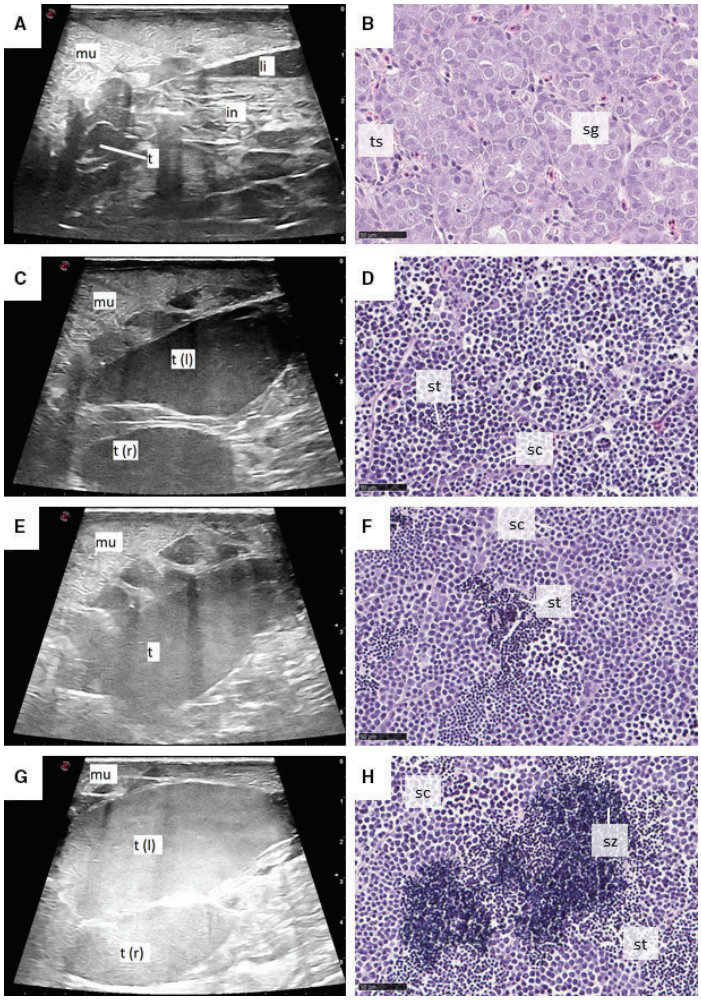

Figure 2. Spermatogenesis in Atlantic salmon males during the last year before stripping, represented by ultrasound images and histological sections. Ultrasound-based left testis length was measured from January 2015, when testes were small in ultrasound images (A) and testis histology was dominated by spermatocytes and testicular somatic cells. Growth in testis size accompanied by presence of spermatocytes and spermatids (C and D) was observed in June 2015. In July 2015, change in echogenicity gave grey shade of testis in ultrasound images (E) and spermatids were observed in some histological sections (F). From July, males where testes were bright grey, almost white in ultrasound images were observed (G), these males often had spermatozoa present in histological sections (H). Scalebar (right) in ultrasound images is 5 cm. Scalebar in histology images is 50 μL. in, intestine; li, liver; mu, muscle; sc, spermatocyte; sg, spermatogonia; st, spermatid; sz, spermatozoa; t, testis; t (l), left testis; t (r), right testis; ts, testicular somatic cells.

and ultrasound-based GSI measurements for male Atlantic salmon.

Echogenicity describes the amount of sound waves that are reflected to the ultrasound transducer from different tissues (Lieu, 2010). Low echogenicity gives a dark appearance of an organ or tissue in ultrasound images, while high echogenicity gives a bright grey or white appearance. During these samplings, we observed a change in testes echogenicity that was related to testis maturation. Not all males at the spermatozoa stage had

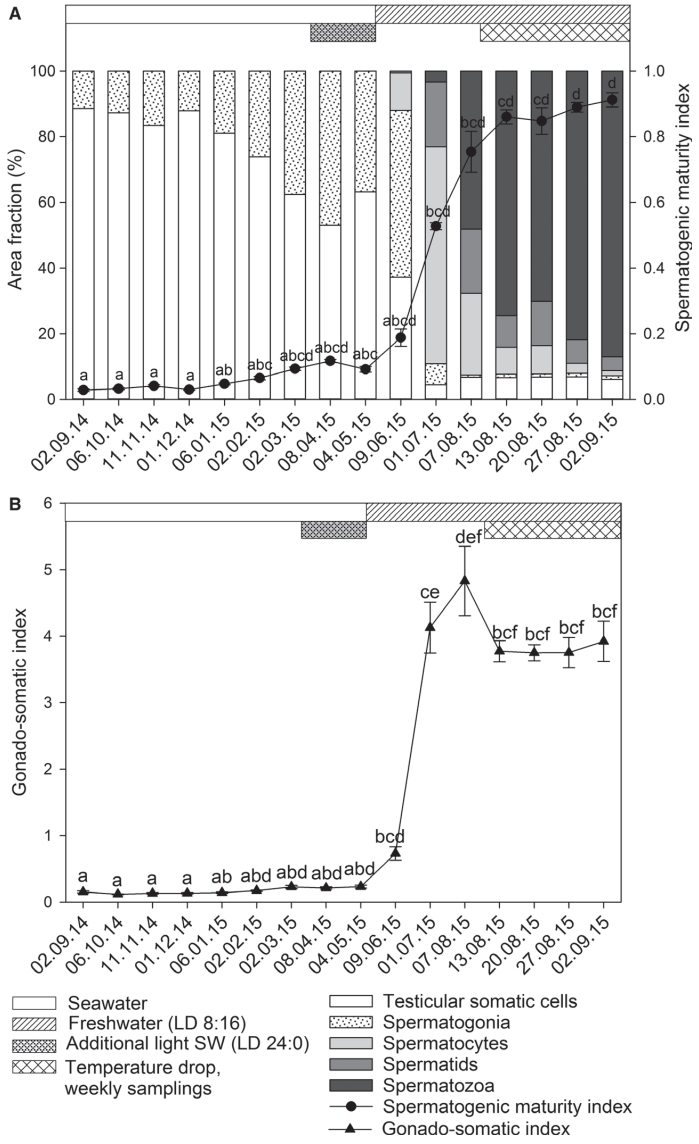

Figure 3. Area fractions of stages in spermatogenesis and spermatogenic maturity index (SMI) and gonadosomatic index (GSI) in Atlantic salmon males during the last year before stripping. SMI (A) and GSI (B) data are mean ± SEM. Bars in A represent fractions of cells and tissues in different stages of testis maturation. Letters indicate significant differences (Kruskal–Wallis H test) between samplings, $P < 0.05$ for SMI and GSI. Horizontal bars indicate rearing conditions. SW, seawater.

bright grey or white testes in ultrasound images, but all males that had bright grey or white testes had spermatozoa present in histological sections. These differences in echogenicity could be used as a noninvasive method for maturation monitoring after freshwater transfer in Atlantic salmon males. At present, this method should be regarded as a semi-objective approach for evaluating testes development using ultrasound. However, when

Table 1. Correlations between GSI, SMI, and plasma levels of the sex hormones T, 11-KT, and MIH in male Atlantic salmon during the last year before final maturation.

	GSI conventional method	Indirect linear US-GSI	Indirect exponential US-GSI	Direct linear US-GSI	Direct exponential US-GSI
SMI	0.86	0.85	0.65	0.84	0.64
T	0.68	0.62	0.41	0.62	0.41
11-KT	0.57	0.55	0.35	0.54	0.34
MIH	0.38	0.45	0.44	0.41	0.41

Numbers are R, $P < 0.01$ for all correlations (Pearson's correlation). 11-KT, 11-keto testosterone; GSI, gonadosomatic index; MIH, maturation inducing hormone; SMI, spermatogenic maturity index; T, testosterone; US-GSI, ultrasound-based gonadosomatic index. Full correlation matrix can be found in Table S2.

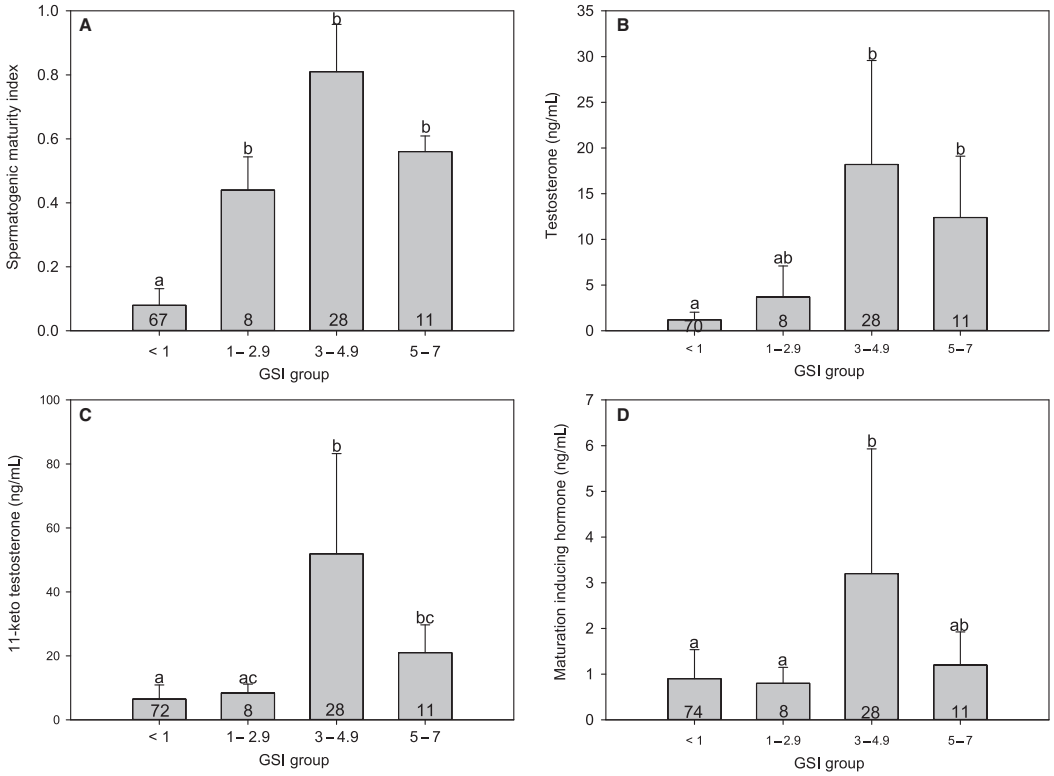

Figure 4. Levels of spermatogenic maturity index (SMI; A) and testosterone (T; B), 11-keto testosterone (11-KT; C) and maturation inducing hormone (MIH; D) in Atlantic salmon males grouped according to gonadosomatic index (GSI). Data are mean ± SD. Numbers at the base of each bar represents n. Letters indicate significant differences between GSI groups (Kruskal–Wallis H-test), $P < 0.05$ for MIH and SMI, and $P < 0.01$ for 11-KT and T. GSI group < 1 includes individuals from September 2014 throughout June 2015 samplings, GSI group 1 – 2.9 includes individuals from June and July 2015 sampling, GSI group 3 – 4.9 includes individuals from July, August and September 2015 samplings, and GSI group 5 to 7 includes individuals from July and 07.08.2015 samplings.

coupled with computer-assisted grey-scale analysis techniques, it may be possible to quantify these observed differences in grey-scale as has been done in human studies (Reimers et al., 1993; Pillen et al., 2007).

The high correlation observed between ultrasound-based GSI and SMI and plasma sex hormone levels indicates that ultrasound is a promising noninvasive tool for identifying individual progression during spermatogenesis

and high plasma androgen levels. Levels of sex hormones reported here were in accordance with previous findings in salmonid species (Fitzpatrick et al., 1986; Scott and Sumpter, 1989; Tveiten et al., 1998), although MIH concentrations were in the lower range of what has been previously reported (reviewed in Scott et al. (2010)). A decrease in GSI calculated by the conventional method was observed before final maturation, as has been observed in several other species such as brown trout (*Salmo trutta*; Billard, 1983) and rainbow trout (Scott and Sumpter, 1989; Prat et al., 1996). The decline in GSI during final maturation could be due to loss of cellular material during meiotic cleavage and maturation of spermatids into spermatozoa (Stanley, 1969; Schulz et al., 2010). Further, a germ cell loss of up to 30 % has been reported in Nile tilapia (*Oreochromis niloticus*) during spermiogenesis (Vilela et al., 2003; Schulz et al., 2005).

Given that GSI peaks and declines during the month before running milt was observed, using only GSI or ultrasound-based GSI during this period might not be sufficient to conclude on reproductive stage alone. For example, a male with GSI of around four could either be at the spermatocyte and spermatid stage with GSI still increasing and quite some time until final maturation (typically seen in July), or he could be at the spermatozoa stage, approaching spermiation and be ready for stripping within short time (typically end of August). In the absence of additional information, GSI or ultrasound-based GSI is not informative enough to certainly determine maturation status at this point. Adding observations of echogenicity gives more nuance. Using both ultrasound-based GSI value and observed echogenicity (i.e., black/ grey/ bright white testis in ultrasound image), ultrasound examination can provide a more accurate estimate of the progression of maturation in individual male Atlantic salmon.

In conclusion, this study has shown that ultrasound is suitable for maturation monitoring in male Atlantic salmon. The ultrasound-based GSI models provide valuable information about progress in maturation, levels of sex hormones and spermatogenic development. In addition, it is specific enough for grading purposes when supplemented with observation of echogenicity. The method is quick and easy to perform for trained personnel, and it requires only basic knowledge of fish anatomy of the operator. Using ultrasound technology, more invasive methods are not needed, therefore we believe the use of ultrasound will reduce stress and improve welfare during maturation monitoring of both farmed and wild fish, including endangered salmonids.

Acknowledgments

The authors thank the staff at AquaGen Kyrksæterøra for assistance during samplings, and Tora Bardal and Randi Røsbak from the Department of Biology, NTNU, for help and training during histology and sex hormone analysis.

Conflict of Interest

All authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.:

Figure S1. Atlantic salmon left testis lenth measurement. During sexual maturation testes thickness increase in an anterior to posterior direction. The clearly thickened part of testis was measured when measuring testis length.

Figure S2. Plasma levels of testosterone (A), 11-keto testosterone (B) and maturation inducing hormone (C) in Atlantic salmon males during the last year before stripping. Data are mean ± SEM. Horizontal bars at the top indicate rearing conditions. Letters indicate significant differences (Kruskal-Wallis H-test) between samplings, $P < 0.01$. SW, seawater.

Table S1. Body weight (mean ± S.E.M.) and K factor (mean ± S.E.M.) for Atlantic salmon males sampled during the last year before final maturation and stripping.

Table S2. Full correlation matrix between GSI, SMI and plasma levels of the sex hormones T, 11-KT and MIH in male Atlantic salmon during the last year before final maturation.

Supplements Paper III

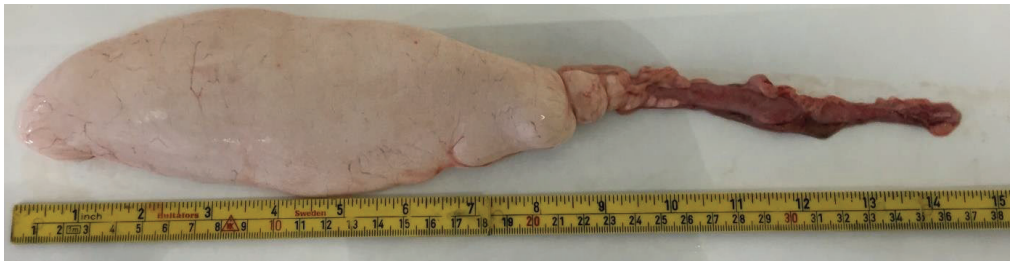

Figure S1. Atlantic salmon left testis lenth measurement. During sexual maturation testes thickness increase in an anterior to posterior direction. The clearly thickened part of testis was measured when measuring testis length.

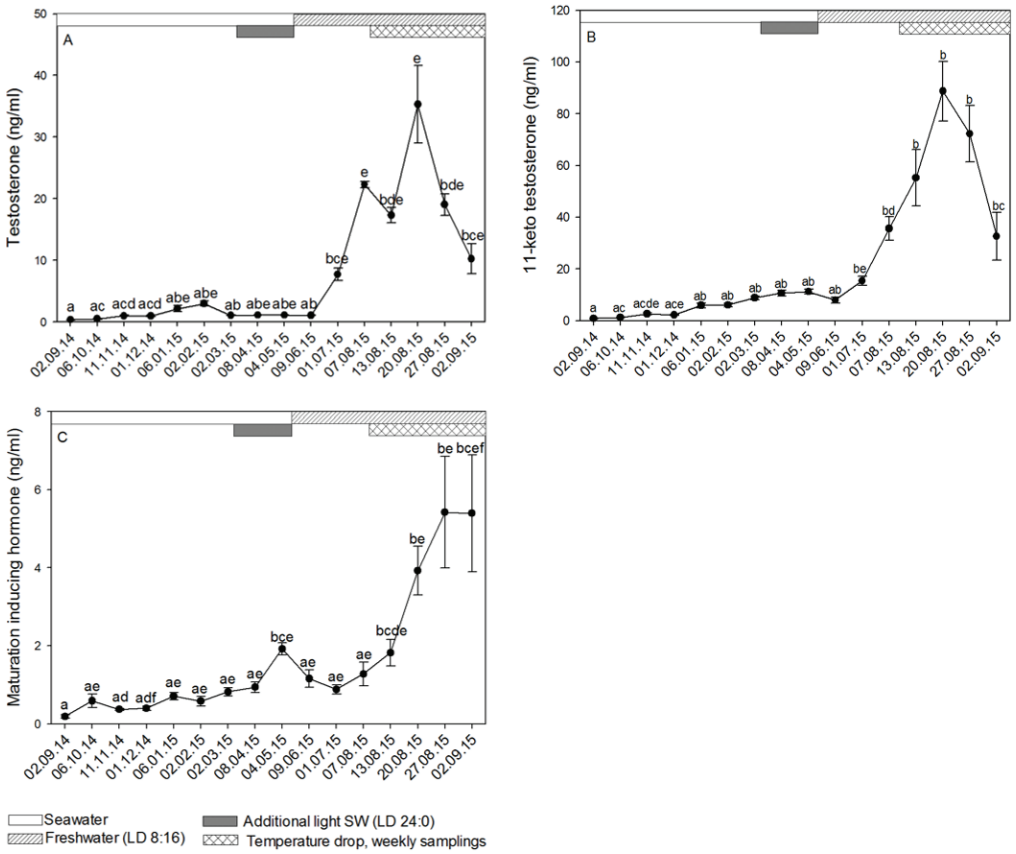

Figure S2. Plasma levels of testosterone (A), 11-keto testosterone (B) and maturation inducing hormone (C) in Atlantic salmon males during the last year before stripping. Data are mean ± SEM. Horizontal bars at the top indicate rearing conditions. Letters indicate significant differences (Kruskal-Wallis H-test) between samplings, $P < 0.01$. SW, seawater.

Table S1. Body weight (mean ± S.E.M.) and K factor (mean ± S.E.M.) for Atlantic salmon males sampled during the last year before final maturation and stripping. Number of individuals analysed for sex hormones (testosterone, 11-keto testosterone and maturation inducing hormone) and histology is also listed. 11-KT, 11-keto testosterone; MIH, maturation inducing hormone; T, testosterone

Date (n)	Body weight (kg)	K factor	Histology (n)	11-KT (n)	MIH (n)	T (n)
02.09.14 (16)	6.5 ± 0.2	1.18 ± 0.02	5	6	6	6
06.10.14 (19)	7.2 ± 0.3	1.12 ± 0.02	5	6	6	6
11.11.14 (10)	9.1 ± 0.5	1.23 ± 0.03	5	6	6	6
01.12.14 (12)	9.5 ± 0.5	1.27 ± 0.04	5	6	6	6
06.01.15 (11)	9.4 ± 0.5	1.18 ± 0.04	5	6	6	6
02.02.15 (14)	12.6 ± 0.7	1.40 ± 0.03	5	6	6	6
02.03.15 (14)	12.2 ± 0.9	1.30 ± 0.07	10	10	10	9
08.04.15 (12)	15.3 ± 0.5	1.45 ± 0.03	10	10	10	9
04.05.15 (13)	14.1 ± 0.7	1.31 ± 0.03	10	10	10	9
09.06.15 (20)	13.5 ± 0.4	1.20 ± 0.02	10	9	10	9
01.07.15 (20)	13.3 ± 0.5	1.14 ± 0.02	20	20	20	20
07.08.15 (5)	14.2 ± 0.6	1.08 ± 0.02	5	5	5	5
13.08.15 (5)	13.2 ± 0.5	1.09 ± 0.05	5	5	5	5
20.08.15 (5)	12.9 ± 0.9	1.08 ± 0.04	5	5	5	5
27.08.15 (5)	13.2 ± 0.6	1.06 ± 0.05	5	5	5	5
02.09.15 (5)	12.2 ± 1.1	1.02 ± 0.03	5	5	5	5

Table S2: Full correlation matrix between GSI, SMI and plasma levels of the sex hormones T, 11-KT and MIH in male Atlantic salmon during the last year before final maturation. Numbers are R, $p < 0.01$ for all correlations (Pearson's correlation). 11-KT, 11-keto testosterone; GSI, gonado-somatic index; MIH, maturation inducing hormone; SMI, spermatogenic maturity index; T, testosterone; US-GSI, ultrasound-based gonado-somatic index.

	Total gonad weight	GSI conventional method	Indirect linear GSI	Indirect exponential GSI	Direct linear GSI	Direct exponential GSI	T	11-KT	MIH	SMI
Total gonad weight		0.98	0.79	0.57	0.82	0.59	0.67	0.56	0.35	0.84
GSI conventional method	0.98		0.84	0.59	0.83	0.59	0.68	0.57	0.38	0.86
Indirect linear US-GSI	0.79	0.84		0.81	0.97	0.80	0.62	0.55	0.45	0.85
Indirect exponential US-GSI	0.57	0.59	0.81		0.80	0.99	0.41	0.35	0.44	0.65
Direct linear US-GSI	0.82	0.82	0.97	0.80		0.83	0.61	0.54	0.41	0.84
Direct exponential US-GSI	0.59	0.59	0.80	0.99	0.83		0.41	0.33	0.41	0.64
T	0.67	0.68	0.62	0.41	0.62	0.41		0.80	0.48	0.77
11-KT	0.56	0.57	0.55	0.35	0.54	0.33	0.80		0.61	0.77
MIH	0.35	0.38	0.45	0.44	0.41	0.41	0.48	0.61		0.59
SMI	0.84	0.86	0.85	0.65	0.84	0.64	0.77	0.77	0.59	

Paper IV

This paper is awaiting publication and is not included in NTNU Open