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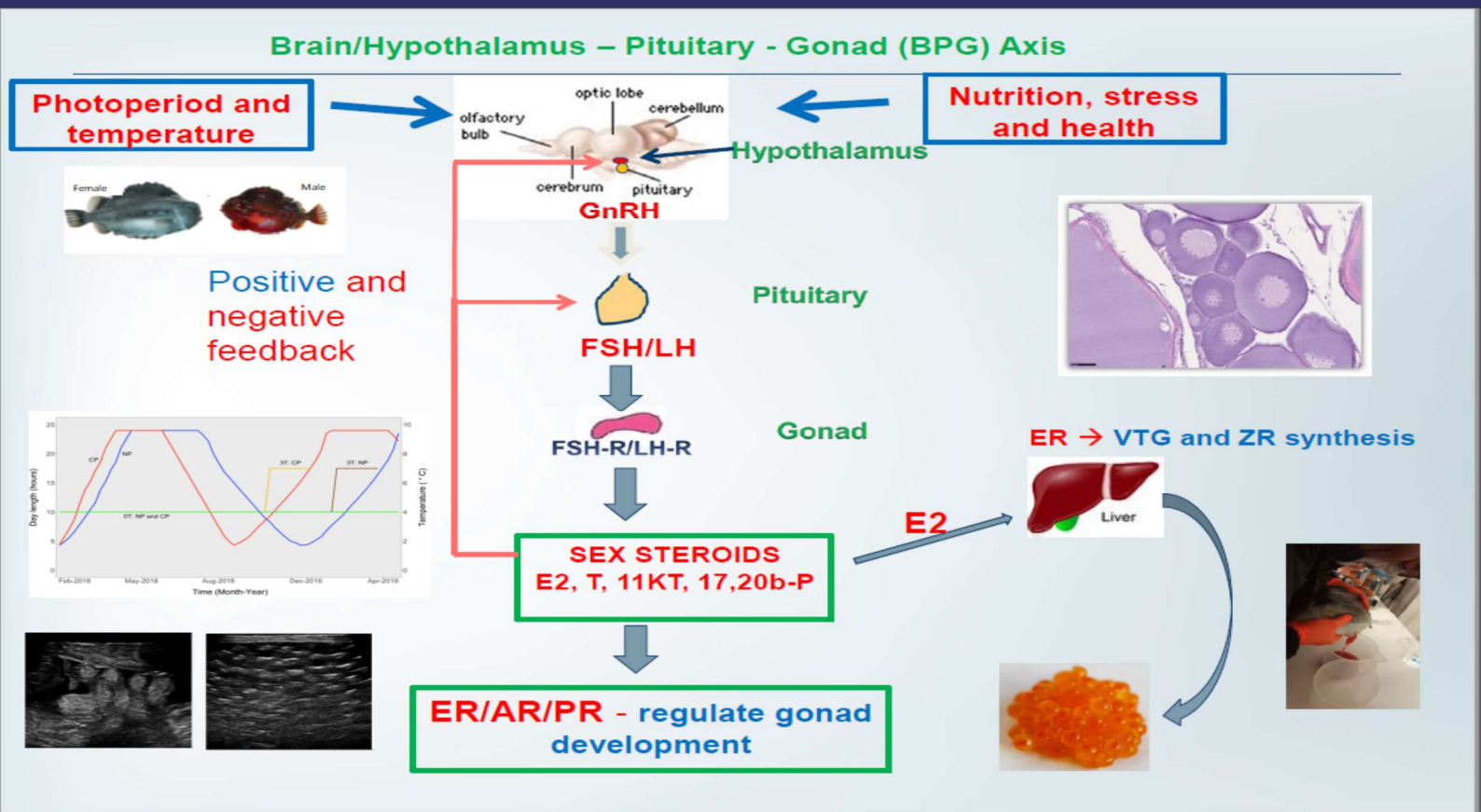
Norwegian Institute of Food, Fisheries and Aquaculture Research

Reproductive biology of lumpfish (*Cyclopterus lumpus*): a key to successful selective breeding.

Academic Final report



Velmurugu Puvanendran (Project Leader), Erik Burgerhout, Øyvind Hansen (Nofima); Helge Tveiten (Nofima/UiT); Elin Kjørsvik, Frank Mlingi (NTNU); Jonna Tomkiewicz (DTU, Denmark); Maren Mommens, Rikard Hageskal (AquaGen); Nina Iversen (Namdal Rensefisk).

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Contact information

Telephone: +47 77 62 90 00
post@nofima.no
www.nofima.no
NO 989 278 835 VAT

Main office in Tromsø
Muninbakken 9–13
Box 6122
NO-9291 Tromsø

Stavanger
Måltidets hus
Richard Johnsensgate 4
Box 8034
NO-4068 Stavanger

Sunndalsøra
Sjølsengvegen 22
NO-6600 Sunndalsøra

Ås
Osloveien 1
Box 210
NO-1433 ÅS

Bergen
Kjerreidviken 16
Box 1425 Oasen
NO-5844 Bergen

Report

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Title:
Reproductive biology of lumpfish (*Cyclopterus lumpus*): a key to successful selective breeding.

Tittel:
Reproduksjonsbiologi av rognkjeks (Cyclopterus lumpus): en nøkkel til vellykket avlsprogram.

Author(s)/project manager:
Velmurugu Puvanendran (Project Leader), Erik Burgerhout, Øyvin Hansen (Nofima); Helge Tveiten (Nofima/UiT); Elin Kjørsvik, Frank Mlingi (NTNU); Jonna Tomkiewicz (DTU, Denmark); Maren Mommens, Rikard Hageskal (AquaGen); Nina Iversen (Namdal Rensefisk).

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Summary/Recommendation:
A detailed description of lumpfish reproductive biology has been described in terms of gonadal development using histological analysis and physiology using sex steroid development. This knowledge of basic reproductive biology of lumpfish has adopted by our industry partners in their breeding activities in their facilities. Our results demonstrated that spawning of lumpfish can be shifted using photomanipulation to spawn different times of the year and spawning can also be synchronized by increasing the temperature prior to ovulation. This means that lumpfish broodstock can be photo-manipulated to spawn different times of the year. Compressing the natural photoperiod resulted in temporal shifts in levels of sex steroids and advanced gonad development, leading to earlier spawning. This knowledge has been currently implemented by our industry partners AquaGen and Namdal Rensefisk. Further, a non-invasive method for gender identification of lumpfish at smaller size (less than 50 g) using ultrasound technology has been developed in Cyclobreed project and the accuracy of this method is more than 90%. This is a huge benefit and important tool for the breeding companies, in this case our industrial partner AquaGen, in maintaining a proper sex ratio (increased females) in their breeding program. Currently AquaGen has implemented this tool in their breeding program for lumpfish.

Sammendrag på norsk:
En detaljert beskrivelse av reproduksjonsbiologien til rognkjeks er presentert. Den beskriver gonadeutviklingen basert på histologiske analyser og analyser av kjønnshormoner. Denne kunnskapen om basal reproduksjonsbiologi til rognkjeks blir benyttet av våre industripartnere i oppdrett av rognkjeks i deres anlegg. Resultatene våre har vist at fotomanipulering kan endre gytetidspunkt til rognkjeks. Gyting kan også synkroniseres ved å øke temperaturen før ovuleringen. Dette betyr at man, ved hjelp av fotomanipulering, kan få rognkjeks til å gyte til forskjellige tider på året. Komprimering av den naturlige fotoperioden medførte tilsvarende komprimering av utviklingen av kjønnshormoner og fremskyndet gonadeutvikling og tidligere gyting. Denne kunnskapen er nå tatt i bruk av våre industripartnere AquaGen og Namdal Rensefisk. I tillegg er det i Cyclobreed-prosjektet utviklet en non-invasive metode for kjønnsbestemmelse av små rognkjeks (under 50 g) ved hjelp av ultralyd. Denne metoden har en presisjon på over 90%. Dette er et viktig verktøy som er til stor nytte for avlsselskaper, i dette tilfellet vår industripartner AquaGen, for å oppnå en gunstig kjønnsfordeling (forhøyet andel hunnfisk) i deres avlsprogram. AquaGen har nå tatt i bruk denne metoden i sitt avlsprogram for rognkjeks.

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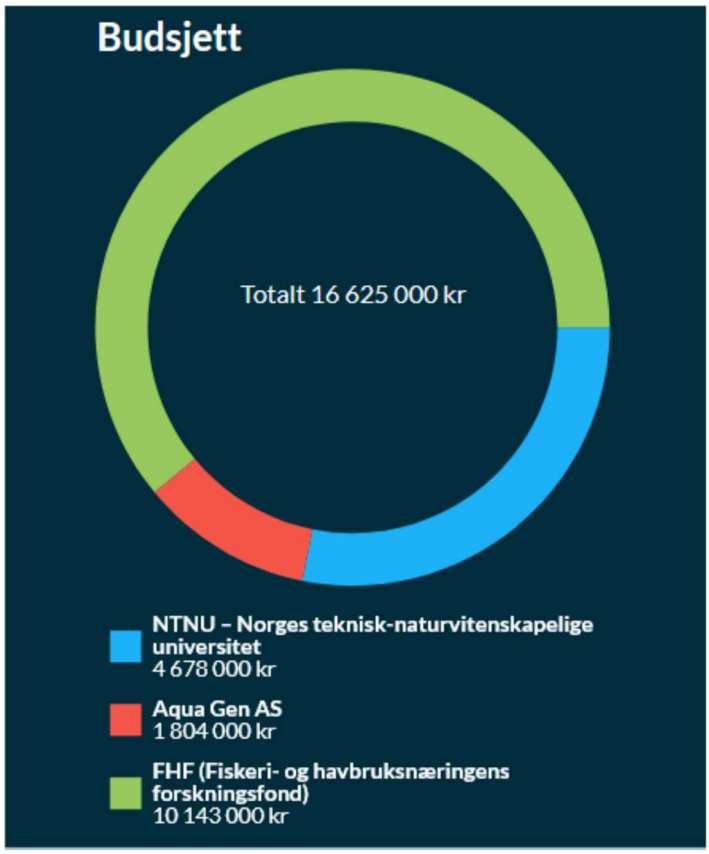
Preface

This report provides an overview of results from the project “Reproductive biology of lumpfish (*Cyclopterus lumpus*): a key to successful selective breeding”. Short name: Cyclobreed. Originally, the project period was set from 01 September 2017 to 30 September 2021 and Nofima as the responsible research institution. The project has been carried out with funding from the Fiskeri- og havbruksnæringens forskningsfinansiering (FHF; Project number: 901418). The main partners of the project were Nofima, NTNU, AquagGen, Namdal Rensefish. The total budget of the project was NOK 16,625,000 with major contribution from FHF (NOK 10,143,000), NTNU for PhD student and supervisory support (NOK 4,678,000) and AquaGen (NOK 1,804,000). The reference group was comprised of Henriette Glosvik (MOWI), Claudia Wittwer (Arctic Cleanerfish) and Marco Schaer (Salmar settefisk). Due to the covid pandemic, laboratories in Norway and Denmark were closed and sample analyses were delayed which has resulted a delay in completion of the project. As a results, the project period was extended by nine months to 30 June 2022 with the approval of FHF. This final report describes activities and results from the entire project period 2017-2022.



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1 Summary

Currently, lumpfish are used in salmon sea cages to control the lice, but off-season production of lumpfish juveniles is not possible due to lack of knowledge of reproductive biology and physiology of farmed lumpfish. Manipulations of photoperiod and temperature in temperate farmed fishes affect sexual maturation and spawning. In the CycloBreed project, we studied to describe the effects of photoperiod and temperature on gonadal development and sex steroid profiles. We have also aimed to develop a non-invasive sex determination method of lumpfish using ultrasound.

In our study, different photoperiod did not affect the growth of lumpfish. Our study showed that gender identification can be successfully done using ultrasound and, in most cases, above 90% correctly. Ovary development was higher in the short photoperiod groups. The low levels female sex steroids in the continuous photoperiods agree with low testosterone and estradiol 17-β levels related with exposure of haddock to continuous photoperiod. Our results indicated that lumpfish ovary maturation was affected by temperature. Our study showed that lumpfish spawned sparsely under continuous photoperiod, but distinctly under compressed photoperiods. For the first time, our study has also elaborately described gonad development in farmed lumpfish histologically. It was further demonstrated that, photoperiod and temperature manipulations can be applied to control sexual maturation and spawning of broodstock, for better broodstock management and ensured year-round supply of right-sized lumpfish juveniles. Our results confirmed that a combination of short photoperiod and higher temperature could provide better management tool in controlling the gonadal maturation and synchronized spawning in lumpfish. This detailed histological profiling of gonadal development along with sex steroid profile during gonadal development will be useful for the breeding companies to manipulate the photoperiod and temperature in their operations. Use of ultrasound in our study showed that this technique can successfully be used to identify the gender of lumpfish from juveniles (less than 50 g) to adults which is very important tool in maintaining a proper sex ratio in breeding programs and our industry partners in the Cyclobreed, AquaGen and Namdal Rensefisk, are currently using this technique in identifying the gender of the lumpfish at an early stage.

1.1 Summary in Norwegian

Rognkjeks benyttes i dag i lakseoppdrett for å kontrollere lakselus, men produksjon av rognkjeks utenom ordinær sesong har ikke vært mulig på grunn av mangelfull kunnskap om reproduksjonsbiologien og fysiologien til oppdrettet rognkjeks. Manipulering av fotoperiode og temperatur påvirker kjønnsmodning og gyting hos fisk fra tempererte områder. I Cyclobreed-prosjektet har vi beskrevet effekten av fotoperiode og temperatur på utviklingen av gonader og kjønnshormoner hos rognkjeks. Vi har også hatt som mål å utvikle en non-invasive metode for kjønnsbestemmelse av rognkjeks ved hjelp av ultralyd.

I dette prosjektet ble det ikke funnet at forskjellige fotoperioder påvirket vekst hos rognkjeks. Studien viser at det er mulig å benytte ultralyd til kjønnsbestemmelse, som oftest med mer enn 90 % korrekt resultat. Gonadeuvikling var høyere i grupper med kort fotoperiode. Lave nivåer av hunnlige kjønnshormoner ved kontinuerlig lys samsvarer med lave testosteron og østradiol 17-β nivåer hos hyse holdt under kontinuerlig lys. Resultatene våre indikerer at ovariemodning hos rognkjeks påvirkes av temperatur. Studiet vårt viste sparsommelig (spredt?) gyting ved kontinuerlig lys, men til eksakte tider ved komprimerte fotoperioder. Studiet vårt har også, for første gang, grundig beskrevet gonadeutvikling hos oppdrettet rognkjeks histologisk. Det ble videre demonstrert at fotoperiode- og temperaturmanipulering kan benyttes til å kontrollere kjønnsutvikling og gyting hos stamfisk. Det gir bedre stamfiskkontroll og sikrer mulighet for årstidsuavhengig levering av rognkjeksyngel av rett størrelse. Våre resultater bekrefter at en kombinasjon av kort fotoperiode og høyere temperatur er et bedre styringsverktøy for å kontrollere gonadeutvikling synkronisering av gyting hos rognkjeks. Denne detaljerte histologiske kartleggingen av gonadeutvikling, sammen med kjønnshormonprofiler under

gonadeutviklingen, kan avlsselskaper nyttiggjøre seg for å manipulere fotoperiode og temperatur i produksjonen. Vi har også vist at ultralyd kan benyttes til å kjønnsbestemme rognkjeks fra yngel (mindre enn 50 gram) til voksen. Det vil være et viktig verktøy for å oppnå en gunstig kjønnssammensetning i avlsprogram og for våre industripartnere i Cyclobreed, AquaGen og Namdal Rensefisk, benytter allerede denne teknikken til å bestemme kjønn hos rognkjeks på et tidlig stadium.

2 Introduction

Salmon lice – The major threat to the salmon industry: The ongoing battle to combat sea lice remains one of the major issues facing salmon the industry today as indicted by the worlds' largest salmon producer, the Marine Harvest AS (Intrafish, 2015). Whilst a number of preventative methods and post-infestation treatments are currently available, including both therapeutic and non-therapeutic approaches, the direct costs, associated with prevention and chemical treatments are significant, as are the indirect costs relating to negative public opinion due to environmental contamination and creation of lice strains resistant to currently available treatments (Imsland et al. 2014; Bjørn et al 2001; Sevatdal et al. 2005). This has increased the necessity to develop new and alternative technology to combat the salmon lice problem and aquaculture companies around the world are striving to find sustainable methods.

Current use of cleaner fish in salmon sea lice control: cleaner fish such as lumpfish (*Cyclopterus lumpus*) and different wrasse species (*Symphodus melops*, *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Labrus berggylta*), effectively feed lice off salmon. Between wrasse and lumpfish, wrasses being the preferred choice of cleaner fish because they effectively feed on the lice even as adults. Currently, mostly wild caught wrasse species (but also some farmed) and farmed lumpfish (ca. 12 million per year) are used in ca. 50% of the commercial salmon cages for lice control (Norwegian Directorate of Fisheries: Norwegian Aquaculture statistics. 2014). Wrasse fishery stops during winter/spring and opens in May in south Norway and, June/July for the rest of the country, which is far too late to meet the needs for year-round supply of cleaner fish (Helland et al. 2014). Moreover, further exploitation of wild wrasse is considered unsustainable. Thus, attempts are being made to develop culture techniques for wrasse species[7], but the number and quality of farmed fish produced is not yet sufficient to meet the current or future commercial needs. Whilst offering some potential in the multifaceted battle against sea lice control, wrasse use is not without its limitations; the fish are particularly sensitive to cold-water temperatures making them unsuitable for use in many farms, especially the salmon farms of northern Norway. Moreover, they enter a dormant state at low water temperature (Espeland et al. 2010). More recently, evidence has shown that lumpfish also feed on sea lice even at very low temperature (Imsland et al. 2014). A number of governments and aquaculture companies around the world are now researching this species as an additional and alternative cleaner fish to be exploited in the ongoing battle against sea lice. Lumpfish appear to be particularly attractive for lice control under Norwegian seawater conditions ((Imsland et al. 2014). Unlike wrasses, lumpfish is relatively easy to farm from start feeding; thus, use of lumpfish for biocontrol of salmon lice has increased significantly over the last three years in all regions from Rogaland to Finnmark County.

Need for developing captive broodfish: With increased demand for cleaner fish, several new companies have chosen to breed lumpfish. In Norway, about 16 licenses are now granted (compared to 5 in 2013) for lumpfish production with geographical locations ranging from the south to the north of Norway for an estimated total production of 5 million juveniles in 2014 and an increase to 12-14 million juveniles for 2015 (Norwegian Directorate of Fisheries: Norwegian Aquaculture statistics. 2014). For efficient salmon lice control in sea cages, farmed lumpfish must be supplied at the right smaller size, because lumpfish in the sea cages grow along with salmon and larger lumpfish tend to feed on pellets rather the salmon lice. Thus, year around supply of right size lumpfish is required which necessitates adequate broodstock spawning at predictable periods. To achieve this, knowledge on basic lumpfish reproductive physiology is needed.

Reproductive biology of lumpfish: Only sporadic information is available on the reproductive biology of wild lumpfish on reproductive season, nesting, parental care and behaviour (Goulet et al. 1986). Captive commercial production of lumpfish has been attempted since 2013 but no information on reproductive biology and physiology of captive broodfish of lumpfish is available. The lumpfish breeding

season seems to range from March to August (Mitamura et al. 2007) but peaking in April and August (per. obs.). Lumpfish are heterosexual and males are usually smaller than females (Goulet et al. 1986). During breeding season, males usually become bright reddish/orange colour while females are bluish green colour. It is reported that wild lumpfish mature in 4-6 years (Goulet et al. 1986) but intensively bred broodfish mature in 2 years (per. obs.). It is not known if the lumpfish are batch or multiple spawners, but some personal observations and grey literature indicates that they could be batch and multiple spawners (Kennedy et al. 2015).

Photoperiod and temperature manipulation on reproductive physiology: Seasonal changes in photoperiod and temperature influence the gonadal maturation of many animals including fish (Bromage et al. 2001). Photoperiod is regarded as the key environmental factor for manipulating puberty and gonadal development in fish species living at moderate to high latitudes, securing spawning in the appropriate season with the most favourable conditions for the offspring (Taranger et al. 2010). For many aquaculture fish species in temperate regions photoperiod manipulation alone appears to be sufficient for successful shifting of spawning time but water temperature and nutritional factors could play a modulating role (Bromage et al. 2001). Unfavourable water temperature has been shown to affect the spawning rhythm, fertility, and egg quality in temperate and cold-water finfish species (Tveiten 2008; Tveiten & Johnsen 1999; Brown et al. 2006). Further, in high latitude fishes, photoperiod has been suggested to affect the early gonadal development (vitellogenesis) while temperature and nutrition, as modulators, determine the final oocyte maturation and ovulation (Pankhurst & Porter 2003). Higher water temperatures during cytoplasmic growth and pre-vitellogenesis have been shown to affect the gonadal development, especially the vitellogenesis in Atlantic cod (Tveiten 2008) and common Atlantic wolfish *Anarhichas lupus* (Tveiten & Johnsen 1999). Ovulation has also been shown to be affected by the higher water temperature in Coho salmon *Oncorhynchus kisutch* (Flett et al. 1996), rainbow trout *O. mykiss* (Pankhurst et al. 1996). Further, increasing spring temperature trigger and synchronize the spawning in most temperate and cold-water finfish species (Pankhurst & Porter 2003).

Endocrine control of reproduction: The brain–pituitary–gonad (BPG) axis regulates the reproductive function in vertebrates, including fish. External and internal signals are primarily integrated in the brain by the hypothalamic neurons producing the gonadotropin-releasing hormones (GnRHs). The GnRHs stimulate the synthesis and secretion of gonadotropins (GTHs) in the pituitary, which are critical modulators of gametogenesis and gonadal maturation, through the mediation of gonadal steroids and growth factors (Schulz & Miura 2002; Yaron et al. 2003).

Gender identification and maturation monitoring: Many fish display dimorphism in growth and external morphological characteristics as part of their secondary sexual characteristics (Darwin 1871). The lack of non-invasive techniques to identify gender and track maturity stages are common management handicaps in captive broodstock management. In most sexually reproducing species, the gender ratio tends to be close to 1:1. However, due to the high sperm density in males, in commercial broodfish production a ratio of 1 male to 10 females is considered most cost-efficient. Genetic gender markers, such as sdY in salmonids, or sex steroids level analysis protocols in sturgeons are being used for gender determination but require invasive sampling (Yano et al. 2013; Falahatkar & Poursaeid 2014). Gonadal maturity has traditionally been monitored using gonadal anatomy or weight in sacrificed broodfish, or biopsy and endoscopy in living broodfish (Hurvitz et al. 2007). Maturity status varies between individuals of a broodstock population and accurate methods for maturation monitoring can help broodstock managers to decide when to induce final oocyte maturation and ovulation. Recently, ultrasound (US) technology has been applied as a non-invasive and fast method for gender determination and maturation monitoring in several farmed fish species (Novelo & Tiersch 2012). Due to differences in gonadal and maturational echogenicity, US-based staging systems are being developed for gender and maturity determination prior to the development of external morphological

characteristics. Lumpfish display a sexual dimorphism in growth, but no external morphological sex characteristics until they approach maturation (Bagge 1964; Davenport 1985).

Egg quality: Control of gamete quality in lumpfish is important, as availability of high-quality eggs and sperm from broodstock fish is necessary to close the lifecycle for species in breeding programs. The only relevant (biologically) method today to estimate gamete quality is to observe the fertilisation success, and subsequently the embryonic development and survival and possible deformities. Yolk composition, maternal hormones, and post-ovulatory ageing of eggs seem to have a significant effect on egg viability and in CycloBreed project, we focused on the egg lipid profile and its energetic status.

Lipid profile of lumpfish eggs: Lipids, especially the essential polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series, are known to affect fecundity, egg quality, hatching success, and deformities in marine fish (Salze et al. 2005; Izquierdo et al. 2001; Pickova et al. 1997; Fuiman & Perez 2015). The relative egg content of these lipids and the fatty acid profiles may be altered due to maternal differences in condition factor or environmental conditions such as light and temperature and season. The energetic status of the ovulated egg reflects the energy available to drive the processes of activation, fertilisation, and early cell cleavage. We have found that the content of adenylated phosphates declines quite rapidly during post-ovulatory ageing in cod eggs, and it was a more sensitive parameter of egg viability than blastomere morphology or fertilisation success in cod (Kjørsvik et al. unpublished). However, the same pattern was not found in rainbow trout eggs (Aegerter & Jalabert 2004), so species-specific differences seem likely.

The importance of maternal genes: We still know very little about the molecular signature of a “high quality” fish egg (brooks et al. 1997), despite of its crucial importance in aquaculture. There is now an increased attention to the role of maternal mRNAs in early development, especially in mammals and several model species. Maternal mRNAs accumulate in the oocytes during oogenesis, and the maternal factors direct the fertilisation and early embryonic development until activation of the embryonic transcription. This “maternal-embryo transition” happens around the mid/late-blastula stage in teleost fish (Kane & Kimmel 1993; Iwamatsu 2004). During this stage, there will be a gradual degradation of maternal mRNAs, and a transcriptional activation of the zygotic genes (Tadros & Lipshitz 2009). In a few studies, maternal mRNAs have been identified as potential molecular markers for egg quality in fish, by use of microarrays (Bobe & Labbe 2009). In rainbow trout, maternal mRNA showed differential abundance in eggs of varying quality (Aegerter et al. 2005; Bonnet et al. 2007). In the Atlantic halibut, a few genes related to early cell division and export of proteins and RNAs from the nucleus were correlated to the rate of symmetric blastomeres or to hatching success (Mommens et al. 2014). External factors such as broodstock environment during oogenesis (temperature and light) can trigger changes in the abundance of maternal mRNAs in the unfertilized eggs, but our knowledge of the maternal mRNAs in fish oocytes is limited (Traverso et al. 2012). An increasing number of genes are found to be important for fish egg and larval quality, demonstrating the complexity of cellular and physiological processes in fertilisation and early embryonic development (Aegerter et al. 2005; Yeong Kwon et al. 2001; Greenwood & Gautier 2005). Gene expression related to mitochondrial energy metabolism and to endocrine and intra-cellular regulation seem to be of specific interest (Goldenthal & Marín-García 2004). RNA-sequencing is a promising tool for differentiating between the maternal gene pool in high- and low-quality eggs. We plan to compare egg quality with the embryonic transcriptome obtained by applying RNA sequencing on unfertilized eggs and embryos (Aanes et al. 2011), a method, which provides a considerably more precise measurement of transcript abundance than microarrays, due to the high number of sequences reads.

As described above, our project will enlighten environmental control (photoperiod and temperature) over lumpfish gonadal maturation mechanisms and will provide the important monitoring tools to start a selective breeding program. The techniques developed with the help of light and

temperature manipulation will enable to produce groups of juvenile lumpfish fry at different times of the year and this will allow salmon farmers to increase lice eat effectiveness of lumpfish fry in salmon cages. This will strengthen the international reputation of Norwegian salmon industry through reduction/elimination of the use of chemical therapeutics for controlling lice. Thus, our project results help the salmon industry both economically and socially through increasing profitability and reputation. This is within the internal strategic initiatives created by Nofima in 2013 to combat the salmon lice problem by using cleanerfish. Within these strategic initiatives of Nofima, preliminary experiment on showed that manipulation of photoperiod and temperature can affect the gonadal development. Further, AquaGen has also identified the importance of understanding the reproductive biology of lumpfish for their initiatives to start a selective breeding program. This initiative is well within the FHFs' action plans for development of medicine free salmon lice combat. Experiments within the Nofimas' strategic initiatives have showed lice eating ability of lumpfish can have a genetic base and Aquagen stresses out the importance of understanding the reproductive biology and physiology for a selective breeding program of lumpfish. All these together falls within another action plan of FHF of increasing the lice eating ability of cleanerfish, thus reducing the number of cleanerfish deployed in sea cages.

3 Objectives

Four main objectives were set in the project. Those were;

1. Acquire the knowledge on lumpfish reproductive biology and physiology under varying environmental conditions and the underlying mechanisms involved.

2. Developing techniques to produce batches of juvenile lumpfish at different times of the year using photoperiod and temperature manipulation.

3. Compare the short- and long-term manipulation of photoperiod and temperature on gonadal maturation, spawning and egg quality.

4. Develop a non-invasive early gender identification using ultrasound enabling the breeding companies to reduce the males in the tanks at an early stage.

Secondary objectives

1. Clarify the influence of photoperiod and temperature on lumpfish reproductive development and spawning time.

2. Investigate how environmental signals are mediated within the BPG axis.

3. Assess the effects of advanced/compressed cycles of photoperiod and temperature on gamete quality.

4. Provide knowledge about the mode of lumpfish ovary development – synchronous vs. asynchronous oocyte development (i.e. do they spawn one or several times during the same season).

5. Gain information about lumpfish iteroparity – i.e. can lumpfish spawn successive seasons and what is post spawning mortality rates.

6. Investigate if repeat spawning fish is better egg producers in terms of quality and quantity.

7. The ultimate objective is to apply all information collected for the project into a selective breeding program and increasing the efficiency of lice eating of lumpfish.

4. Project Organisation

Nofima: Nofima is Europe's largest institute for applied research within the fields of fisheries, aquaculture and food. The institute has around 420 employees with an annual turnover of about NOK 500 million (€ 62 mill.). Nofima carries out internationally recognized research and develops solutions that provide a competitive edge throughout the value chain. Nofima has established several excellent integrated research facilities including Center of Marine Aquaculture and Aquaculture Research Station, Tromsø. All the experiments (except for some parts of WP2) will be conducted at Nofimas' research facilities. Expertise and labs are also available for reproductive endocrinology analysis.

Norwegian University of Science and Technology (NTNU): Marine science and technology is a strategic focus area at NTNU. The marine aquaculture group at NTNU have high international status and experience (since 1986) in research on cultivation of marine fish larvae and planktonic organisms through numerous large projects, and we have been involved in several fish reproduction and egg quality projects. Our cleaner fish activity is linked to the NTNU research programme "NTNU Oceans Pilot: Environmental interactions of sea-based aquaculture". NTNU is well equipped with labs for the analysis related to reproductive biology and physiology of fishes, including gene expression.

Technical University of Denmark (DTU): DTU Aqua - National Institute of Aquatic Resources is an institute at the Technical University of Denmark that conducts research, provides advice, educates and contributes to innovation in sustainable exploitation and management of aquatic resources. The institute investigates the biology and population ecology of aquatic organisms, aquatic physics and chemical processes, ecosystem structure and dynamics, taking account of relevant natural and anthropogenic drivers. We have significant experience in experimental and field studies of fish reproduction and labs are available for histological analysis.

AquaGen AS: AquaGen is a Norwegian breeding company running family breeding programs for Atlantic salmon, rainbow trout, tilapia, and lumpfish. AquaGen is a major supplier of genetically improved eggs to the international aquaculture industry. AquaGen is taking an active role to promote a responsible development of the aquaculture industry and is promoting the use of lumpfish as a non-chemical tool to control sea lice infestation in Atlantic salmon. AquaGen has successfully implemented US-based gender determination and maturation monitoring in its Atlantic salmon broodstock production, based on the results obtained in projects: The application of ultrasound technology in Atlantic salmon broodstock production (241398/O30) and Land based broodstock production of Atlantic salmon for whole year production of pathogen-free egg (235776/E40). This knowledge will now be applied in the CycloBreed project through WP2.

Namdal Rensefisk AS: Namdal Rensefisk is a lumpfish producer owned by Bjørøya Fiskeoppdrett AS, Marine Harvest AS, Nova Sea AS, Emilsen Fisk AS, Midt Norsk Havbruk AS, and AquaGen AS. By 2017, Namdal Rensefisk will provide its stakeholders with 5 mill lumpfish for sea lice control. Namdal Rensefisk is an active partner in the project CycLus 2015-2019 "Optimalisert bruk av rognkjeks i bekjempelse av lakselus i lakseoppdrett-biologi, fiskevelferd og effect som lusespiser i stor skala". In collaboration with AquaGen, Namdal Rensefisk is producing broodfish to produce genetically improved lumpfish eggs. Namdal Rensefisk will be an active partner in CycloBreed by providing staff and facilities for WP2.

Reference Group

The reference group was comprised of initially Olav Breck (MOWI), Dag Hansen (Arctic Cleanerfish) and Marco Schaer (Salmar settefisk). Later Olav Breck (MOWI) and Dag Hansen (Arctic Cleanerfish) were replaced by (Henriette Glosvik (MOWI) and Claudia Wittwer (Arctic Cleanerfish), respectively.

4 Project execution

WP1: Effects of short-term manipulation of photoperiod and temperature on the gonadal maturation, spawning and egg quality of lumpfish.

Temperature will be kept ambient (4-9°C) in all four tanks. Two of these tanks will be kept in short day light (SDL; 8L:16D) and other two in continuous day light (CDL) for three months. After 3 months, photoperiod for both of the SDL tanks will be changed to continuous light and one of the SDL tanks will be provided ambient water temperature (SDL0T) while the other will be provided ambient + 3°C (SDL3T). CDL tanks will be kept at continuous light but the temperature of one CDL tank will be kept at ambient temperature (CDL0T) while the other will be kept at ambient + 3°C (CDL3T). Blood and gonadal samples will be taken periodically for histological, gene expression and sex steroid analysis. Results of this short-term photo- and temperature manipulation will be later compared with the results of long-term photo- and temperature manipulation (WP2) in terms of gonadal development, spawning and egg quality.

WP2: Effects of long-term manipulation of photoperiod and temperature on the gonadal maturation, spawning and egg quality of lumpfish.

Task 2.1.

To evaluate the effects of photoperiod and temperature on the gonadal maturation, 1-year-old domesticated lumpfish juveniles (F1 generation) will be subjected to natural (NL - 2 tanks) and 9-month compressed (CL - 2 tanks) photoperiod starting from January 2017 (Fig. 1). Both groups will be kept at

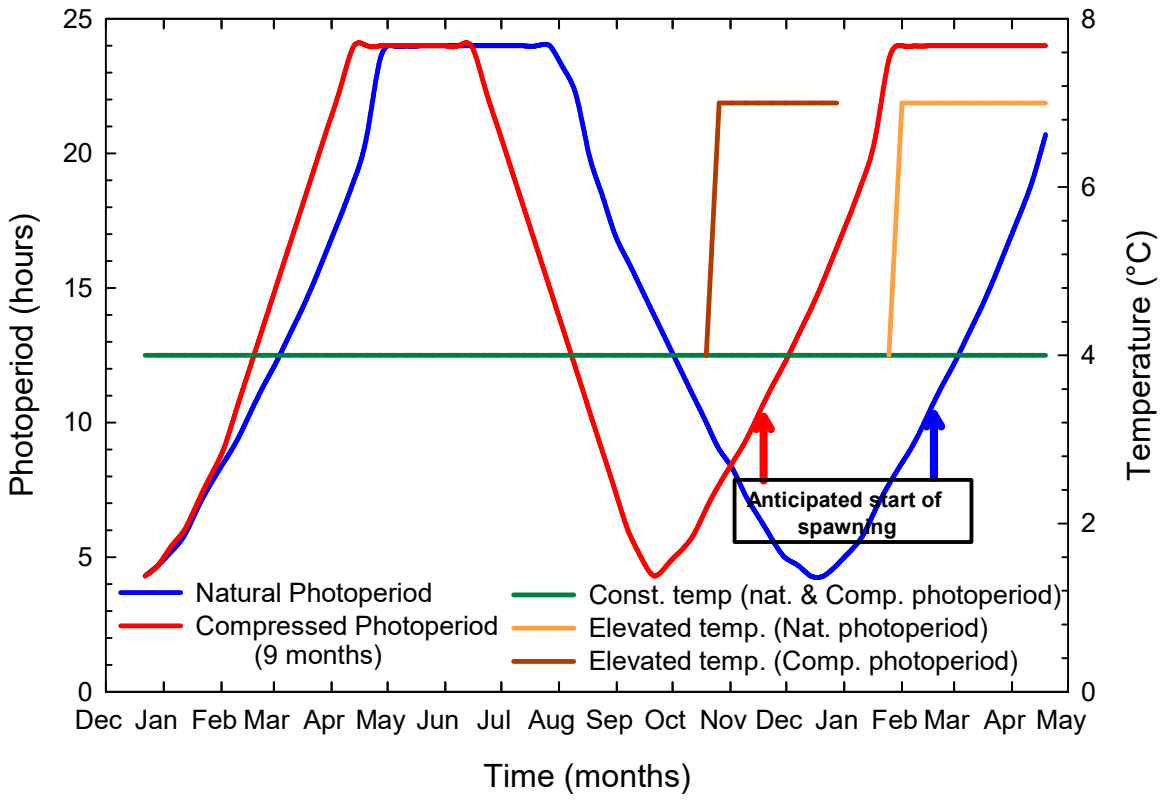

Fig. 1. Schematic plan of experimental set-up

a constant temperature of 4°C until close to final gonadal maturation (monitored through monthly

samplings of blood and gonads). Prior to the final maturation and ovulation, temperature in one tank from NL and CL will be gradually increased to 7°C while other two tanks will be kept at 4°C. This will provide 4 treatments: 1. NL 4°C; 2. NL 7°C; 3. CL 4°C and 4. CL 7°C. Results from treatments 1 and 3 will show the effects of photoperiod alone, under the condition of NL and CL while results from treatments 2 and 4 will show the effect of elevated temperature during final gonadal maturation. This set up will also give us an answer if lumpfish can be photo-manipulated (and temperature) to spawn year around. Once the spawning is completed, fish will be kept in the same tanks and the temperatures will be brought to 4°C again (1st/2nd quarter of 2018) and will be monitored until second spawning until 2019. Eggs and sperm will be stripped from the broodfish, fertilized, and incubated until hatch. No further rearing of larvae is planned in this project.

Task 2.2. Sampling: Monthly, blood (for sex steroids), gonads (histology – gonadal maturation), brain, pituitary, head kidney and liver (gene expressions) will be sampled and preserved in appropriate media until analysis in WP3, WP4 and WP5. Further, milt, eggs, embryos and newly hatched larvae will be sampled at appropriate time intervals for gamete and larval quality analysis.

Five major areas will be investigated utilizing a multidisciplinary approach and expertise within the consortium; WP1: To explore the short-term (5 months) effects of photoperiod and temperature on spawning of lumpfish; WP2: To explore the lumpfish sexual maturation and growth until spawning under natural and compressed photoperiod (Fig. 1) and the effect of elevated temperature during late gonadal development phase; WP3: Develop US-based staging protocols for gender determination and maturation monitoring, and the application of the protocols to synchronize and extend the stripping window of lumpfish.. This knowledge will be used to monitor individual males and females for sexual maturation (WP4) during the study period. WP4: Study the temporal changes in levels of plasma sex steroid during lumpfish gonadal maturation and the mechanisms governing gonadal maturation. WP5: Study the oocyte and testis development during sexual maturation, evaluate egg quality characteristics and explore molecular markers for egg quality in lumpfish.

Providing sufficient lumpfish for the industry requires a major boost in production of robust fish that can operate effectively throughout the salmon growth cycle. CycloBreed proposal will step up the knowledge of lumpfish reproductive biology and physiology and help to develop broodstock management protocols in relation to gonadal development and spawning, to enable year around production of lumpfish juveniles, to develop domesticated broodfish and to develop a selective breeding program. Overall, CycloBreed project will deliver valuable new insights into lumpfish reproductive physiology and its application.

Involved project partners are 1. Nofima AS, 2. NTNU 3. Technical University of Denmark (DTU) as R&D partners and 4. AquaGen AS 5. Namdal Rensefisk AS as industrial partners.

Expertise for WP1, WP2 and WP4 exists within Nofima and Velmurugu Puvanendran (responsible for WP1 and WP2), Helge Tveiten (responsible for WP4), Atle Mortensen and Øyvind Hansen have worked with lumpfish and other fish species reproductive biology over the past several years. Maren Mommens (responsible for WP3) has extensively worked with developing sex identification techniques in fish using ultrasound and her expertise will be used in WP3.

Expertise for WP5 will come from Elin Kjørsvik (responsible for WP5) and Jonna Tomkiewicz who have several years of experience in egg quality studies in marine fishes.

Nofima has two facilities 1. Centre for Marine Aquaculture Research and, 2. Aquaculture Research Station in Tromsø to conduct the experiment. Further, Namdal cleanerfish AS is also building a

broodstock facility and expected to be in operation in 2017. Nofima, NTNU and DTU has the necessary labs and equipment to analyse sex steroids, gene expressions and egg quality.

6 Findings, discussion, and conclusion

The results are given below are part of the six manuscripts (5.1 – 5.6) that was prepared by the PhD student Frank Mlingi. First five papers will be included in his thesis work, however, the last paper on egg quality will not be included due to the delays in analysing. Egg quality paper, however, will be published in scientific journal at a later stage.

6.1. Sexual maturation of farmed lumpfish *Cyclopterus lumpus*: Gonadal development and associated profiles of selected endocrine factors.

The first objective of Paper I was to histologically characterize gonadal development in lumpfish females and males. The second objective was to describe the changes in transcript levels of gonadotropin releasing hormones (GnRH); gonadotropin hormone receptors (GTHR); vitellogenin receptor (VTGR); selected steroidogenic pathway enzymes (CyP17a1, CyP19a1); gonadosomatic index and blood plasma levels of testosterone (T), 11-ketotestosterone (11-KT), and 17β-estradiol (E2) in relation to the stages of gonadal development.

This paper shows the processes of oogenesis and spermatogenesis, it also describes how the selected endocrine factors vary with the recruitment of successive stages of gonadal development in farmed lumpfish that were pooled from a six-month photoperiod and temperature experiment.

6.2. A short-to-continuous photoperiod integrated with temperature elevation can synchronize and advance sexual maturation in Lumpfish (*Cyclopterus lumpus*, L. 1758).

The main objective of Paper II was to evaluate the efficiency and reliability of ultrasound as a non-invasive tool in monitoring gonadal development of lumpfish during a normal reproductive cycle in a hatchery.

The paper shows how ultrasound was used to establish ultrasound-based maturation stages in lumpfish females and males. It also shows how the ultrasound-based maturation stages related to the histologically characterized gonadal stages, gonadosomatic index and sex steroids (T, 11-KT and E2).

6.3. A nine-month compressed annual photoperiod integrated with temperature elevation at the onset of final maturation advances sexual maturation and spawning in lumpfish (*Cyclopterus lumpus*, L. 1758) females.

The main objective of paper III was to study the effects of photoperiod and temperature on sexual maturation in female (vitellogenic) and male lumpfish in a six-month period.

This paper shows how sexual maturation in 18 months old lumpfish females and males, indicated by gonadosomatic index, gonadal histology, and sex steroids (T, 11-KT and E2), was affected by exposure to either a continuous photoperiod or a short-to-continuous photoperiod, both followed by temperature elevation by 3 ℃ at the onset of final maturation in females. The paper also describes the effects of photoperiod and temperature on growth performance.

6.4. Temperature rather than photoperiod effects is stable in controlling sexual maturation in lumpfish (*Cyclopterus lumpus*, L. 1758) males.

The main objective of Paper IV was to study the effect of photoperiod and temperature on the reproductive performance of female lumpfish over a period of 16 months.

This paper shows how the reproductive performance indicated by GnRHs, GTHRs, VTGR, steroidogenic pathway enzymes (CyP17a1 and CyP19a1), gonadosomatic index, gonadal histology and sex steroids (T, 11-KT and E2), and spawning of female lumpfish were affected following exposure to either a natural annual photoperiod or a nine-month compressed annual photoperiod combined with temperature elevation by 3 ℃ at the onset of final maturation. The paper also shows the effects of the photothermal manipulations on growth and fish condition.

6.5. Ultrasonic imaging as a means of monitoring sexual maturation in lumpfish.

The main objective of Paper V was to study the effect of photoperiod and temperature on the sexual maturation of male lumpfish over a period of 16 months.

This paper which comes from the same experiment with Paper IV shows how sexual maturation in male lumpfish indicated by GnRHs, GTHRs, steroidogenic pathway enzyme (CyP17a1), gonadosomatic index, gonadal histology and sex steroids (T and 11-KT) were affected following exposure to either a natural annual photoperiod or a nine-month compressed annual photoperiod combined with temperature elevation by 3 ℃ at the onset of final maturation in females. The paper also shows the effects of the photothermal manipulations on growth and fish condition.

6.6. The effects of photoperiod and temperature on lumpfish egg, embryo, and larval quality.

The first specific objective was to study differences in lipid/fatty acid and gene expression profiles in eggs, embryos and newly hatched larvae from females from different photothermal regimes. The second specific objective was to study the evolution of these biochemical and molecular parameters from prior to fertilization to hatching.

The paper shows how lipids/fatty acid and gene expression profiles were affected by the exposure of donor broodstock to different photothermal regimes. It also shows the variations in lipid/fatty acid profiles as well as differential gene expressions during the development from unfertilized eggs to newly hatched larvae.

Main results and discussion

Describing the lumpfish sexual maturation process (Paper I)

Lumpfish is an emerging aquaculture species, and its biology is not yet fully understood. Previous studies reported on spawning-related migrations in the wild (Davenport, 1985; Kennedy and Ólafsson, 2019), gonadal anatomy and estimates of the number of egg batches during spawning (Davenport and Lonning, 1983; Kennedy, 2018) and ovarian development (Pountney et al., 2020a). Despite such developments, there is still no information on the endocrine factors and the association of the ovarian development with endocrine changes. The testicular development and its association with endocrine changes are also unknown. Therefore, we aimed to improve on the existing basic knowledge on the reproductive biology of lumpfish by studying in detail the oogenesis and for the first time, spermatogenesis, and the associated profiles of gonadal growth (GSI) and selected endocrine factors. The endocrine factors were the genes encoding gonadotropin releasing hormones (GnRH2 and GnRH3); gonadotropin hormone receptors (FSHR and LHR); vitellogenin receptor (VTGR); and steroidogenic pathway enzymes (CyP17a1 and CYP19a1). Other endocrine factors were the plasma levels of sex steroids (T, 11-KT and E2). Lumpfish used for this study were pooled from an experiment (Experiment 1) that was run for 24 weeks (21st September 2017 to 8th March 2018). Sampling was done four times during the experiment, where measurements of body weight and gonad weight for GSI, blood collection for sex steroid radioimmunoassays, brain and gonad tissues collections for real-time

quantitative polymerase chain reaction (RT-qPCR) assays were conducted. Other gonad tissues were taken for histological studies of gametogenesis.

Like a previous study (Davenport, 1985), we observed a larger right ovary lobe compared to the left one which leaves space for the stomach and liver, in a matured female. Further, there were two distinct oocyte batches in the ovary from a macroscopic view. With reference to previous descriptions (Coward and Bromage, 1998; Kagawa, 2013; Macchi and Barrera-Oro, 1995; Setiawan et al., 2016; Taranger et al., 1999), we identified a total of 10 oogenic stages and grouped them into four ovarian development stages based on predominant oocyte/egg stages. The ovarian stages were pre-vitellogenic (perinucleolar, cortical alveolar and oil droplet oocytes), vitellogenic (primary, secondary and tertiary yolk oocytes), final maturation (maturing and matured oocytes), ovulation (ovulating and ovulated oocytes) stages. Interestingly, in one of the sampling points, two females were found to have more than two oogenic stages (Figure 1.)

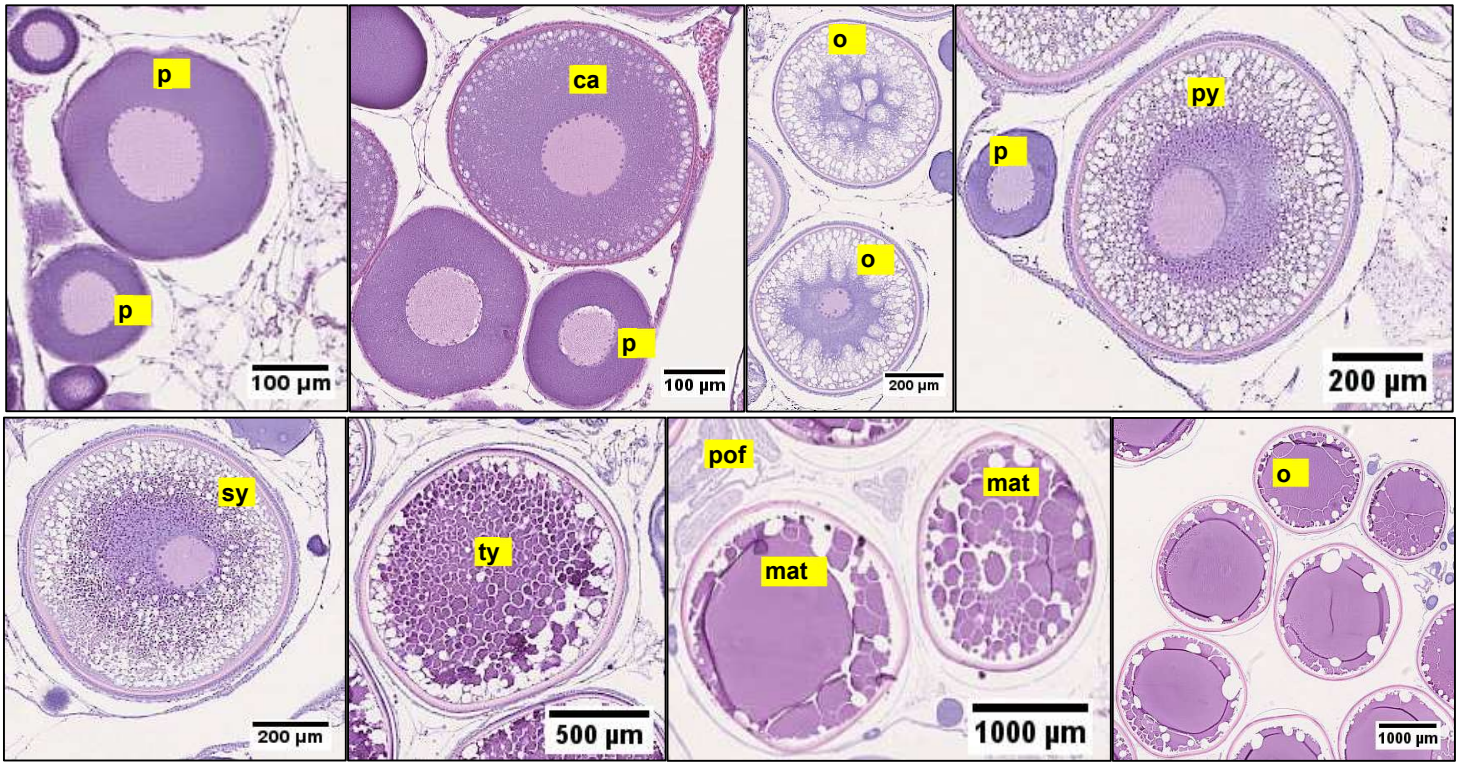

Figure 1 Morphologies of different oocyte stages in *C. lumpus*. Perinucleolus (pn), cortical alveoli (ca), oil droplet (od), primary yolk (py), secondary yolk (sy), tertiary yolk (ty), maturation (mat), matured (matd), ovulation (ov), post-ovulatory follicle (pof).

Accompanying the recruitment of more developed oogenic stages, was an increase in oocyte sizes that was steepest at the recruitment of matured oocytes. Oocyte diameters increased from 0.2 ± 2.1 x 10-3 mm to 1.7 ± 2.2 x 10-2 and volumes from 6.0 x 10-3 ± 2.0 x 10-4 mm3 to 3.2 ± 1.1 x 10-1 mm3 significantly ($P < 0.05$). The increase in oocytes diameters was accompanied with a significant ($P < 0.05$) decrease in the nucleus-oocyte diameter ratios from perinucleolar (0.5 ± 6.8 x 10-3 mm3) to secondary yolk (0.2 ± 1.6 x 10-2) oocytes. Similar changes in oocyte diameters were reported previously by Kennedy (Kennedy, 2018) and Pountney and co-workers (Pountney et al., 2020a) for the same species. The increase in oocyte size is linked to the growth of oocytes particularly during vitellogenesis, and the steep increase at final maturation is related to hydration (Lyman-Gingerich and Pelegri, 2007; Mañanós et al., 2008; Wallace and Selman, 1981). Our oocyte frequency distributions showed a general multimodal pattern with overlaps of diameters among the different stages, indicating that different oocyte

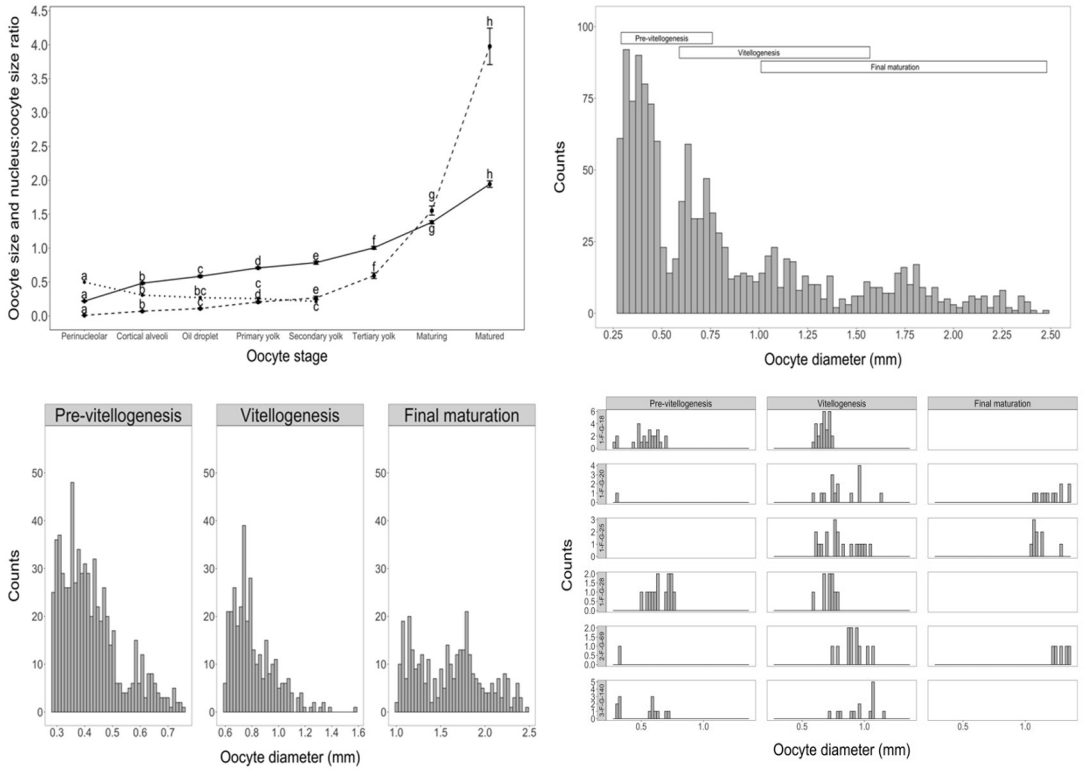

Figure 2 A: Oocyte size changes in relation to oocyte stages in *C. lumpus*. Increases in mean oocyte diameter (mm, solid line) and calculated oocyte volume (mm^3, dashed line) from perinucleolar to matured oocytes, and a decrease in mean nucleus:oocyte diameter ratio (dotted line) to secondary yolk (vitellogenic) oocytes. Different letters on top of or below error bars indicate significant differences in mean oocyte diameters, volumes and nucleus:oocyte diameter ratios between oocyte stages. N.B.: Measurements and calculations were done only from perinucleolar oocytes whose cytoplasms were not obstructed by the very large nuclei. **B and C:** Diameter frequency distribution of *C. lumpus* oocytes grouped according to developmental stages. PV = Pre-vitellogenesis, V = Vitellogenesis, FM = Final maturation. (**A**) Frequency distribution of all oocyte stages (1320 oocytes), the white shaded horizontal bars dermacate individual stages, (**B**) frequency distributions of oocyte diameters in each individual developmental stage (There are 330 oocytes per stage). **D:** Diameter frequency distribution of oocytes from six individual *C. lumpus* females. The frequency distributions are grouped according to developmental stages.

stages can be found in similar sizes (Figure 2). The presence of two distinct batches of oocytes in a mature female from the macroscopic view and the more than two oocyte stages accompanied by overlapping of oocyte sizes from the histological assessment suggest group-synchronous ovarian organization and asynchronous spawning strategy in lumpfish. Therefore, as a strategy, lumpfish females could be recruiting ovulated eggs in several batches during the spawning season (Murua and Saborido-Rey, 2003). This agrees with previous findings which estimated 4 to 8 batches during a two-week period (Davenport, 1985) but disagrees with a recent study which concluded with a maximum of two batches (Kennedy, 2018). The acknowledged lack of histological examinations that would elucidate the ovarian development organization in the latter study could be the reason of this disagreement.

Without significant changes, GnRH2 and GnRH3 gene expressions decreased from pre-vitellogenic to ovulation stages (Figure 3). In agreement with this suggestion is the finding on blue gourami (*Trichogaster trichopterus*) in which GnRH3 expression levels decreased from pre-vitellogenic to final maturation oocytes (Degani, 2014). Although this could suggest that the GnRHs are mostly needed at the initiation of the reproductive cycle, the initiation of feedback mechanisms from the gonads to the pituitary gland and the brain is more likely. Endocrine factors from the gonads therefore could be exerting negative feedbacks on the secretion of GnRHs. In support of this, sex steroids have been shown to exert complex positive or negative feedbacks at the brain and pituitary levels in teleosts which vary with the species and physiological state (Fontaine et al., 2020). The gene expressions of FSHR, LHR, VTGR and CYP19a1 increased significantly ($P < 0.05$) to their peak levels at final maturation and decreased afterwards.

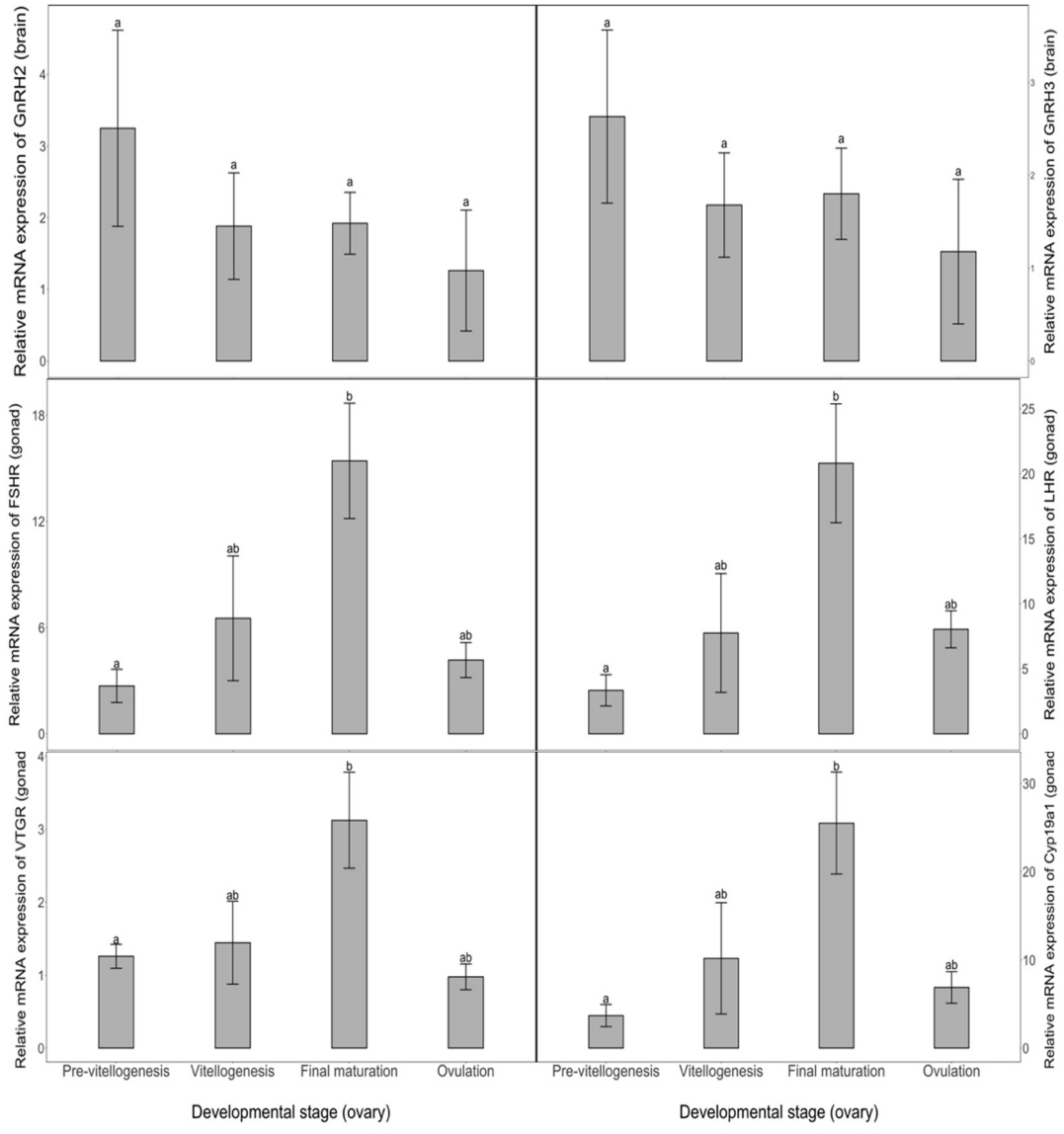

Figure 3 Gene expression levels of selected endocrine factors in relation to developmental stages in *C. lumpus* females. Gonadotropin-releasing hormone II (GnRH2, **A**); gonadotropin-releasing hormone III (GnRH3, **B**); Follicle stimulating hormone receptor (FSHR, **C**); Leuteinizing hormone receptor (LHR, **D**); vitellogenin receptor (VTGR, **E**); and cytochrome P450 19a1(CyP19a1 (aromatase), **F**), grouped according to predominating developmental stage. Different letters on top of error bars indicate significant differences in expression levels between stages.

GSI, T and 11-KT increased to their peak levels at ovulation while E2 increased significantly to final maturation and decreased slightly at ovulation (Figure 4).

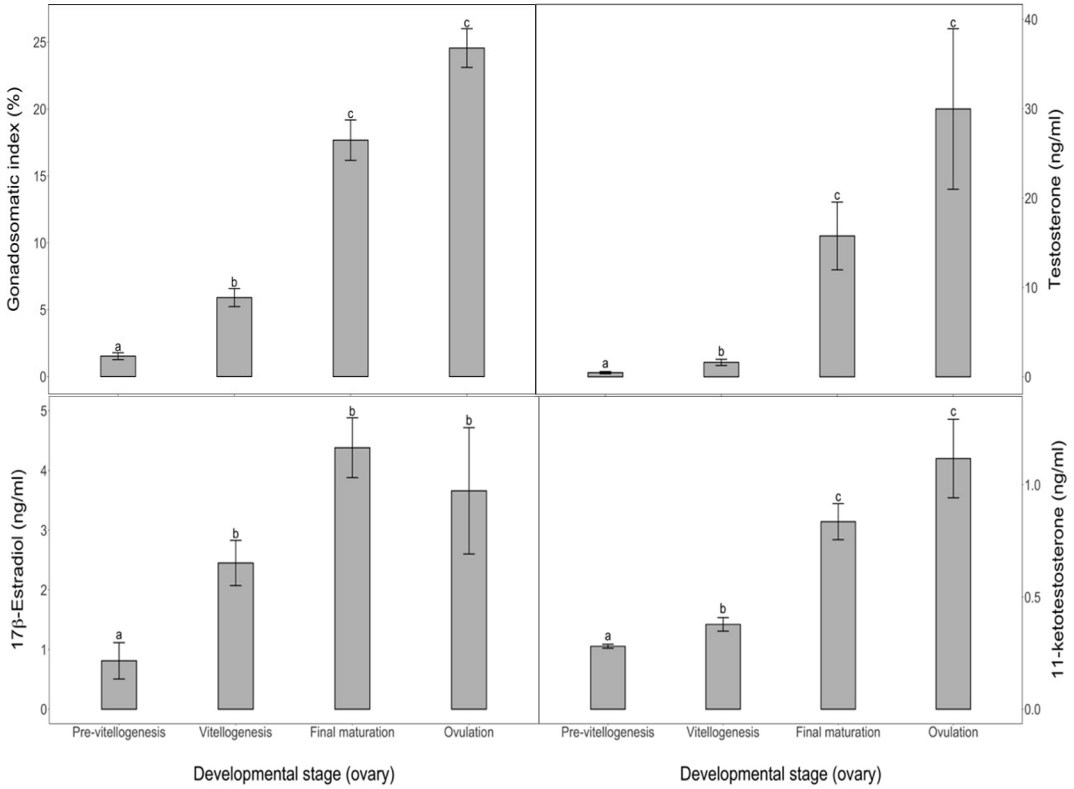

Figure 4 Ovary growth and plasma levels of sex steroids in relation to developmental stages in *C. lumpus* females. Gonadosomatic index (**A**), testosterone (**B**), 17β-Estradiol (**C**) and 11-ketotestosterone (**C**) grouped according to predominating developmental stages. Different letters on top of error bars indicate significant differences between developmental stages.

The parallel trends of FSHR and LHR which mediate the gametogenesis and steroidogenesis roles of the pituitary derived FSH and LH could indicate that the gonadotropins are synthesized simultaneously to play their specific biological roles for the different oocyte generations at the same time. Similar trends were demonstrated by the pituitary, gonadal and plasma concentrations of FSH and LH in the half-smooth tongue sole (*Cynoglossus semilaevis*) which has an asynchronous ovarian development (Shi et al., 2015). Additionally, in the group-synchronous multiple batch spawner Atlantic halibut (*Hippoglossus hippoglossus*) (Murua and Saborido-Rey, 2003), FSHR was abundant in primary growth while LHR was abundant in final maturation, analyses of which were obtained from follicle samples that were categorized based on diameter and histological evaluations in follicles that were grouped in different stages (Kobayashi et al., 2008). Therefore, we speculate that the trends of FHR and LHR in lumpfish would behave as those in the Atlantic halibut if the follicles were sampled based on their stages. Similar patterns demonstrated by the gonadal VTGR, CyP19a1 and E2 are also possible indicators of the presence of several generations of oocytes in the ovary at one time. E2 stimulates the liver to synthesize and secret vitellogenin (Kagawa, 2013), VTGR plays a binding role during sequestration of vitellogenin into the oocytes (Lubzens et al., 2010) while CyP19a1 encodes the enzyme that aromatizes T into E2

(Mills et al., 2014), and their patterns suggest that while predominant oocyte generation is in final maturation, there is recruitment of another into vitellogenesis leading to their increasingly higher levels at final maturation. Similarly, in addition to being positively correlated, CyP19a1 and E2 varied concomitantly in the Atlantic cod (*Gadus morhua*) (Johnsen et al., 2013). T and 11-KT increased up to and were highest at ovulation, suggesting that these androgens have specific roles during the late ovarian developmental stages which could range from exerting feedbacks at the brain and pituitary level to local biological roles in the gonads. Steroidogenic feedbacks on FSH and LH and the protective hormonal environment against atresia required by the follicles being continued by T after cessation of E2 production have been suggested (Kime, 1993; Okuzawa, 2002; Smith and Haley, 1988). Decreased aromatase activity has also been linked to increased levels of T in the plasma since T is released in the circulation as it is no longer needed E2 at this point (Campbell et al., 1976; Kagawa, 2013). Future research needs to elucidate the relationship between plasma T and E2 levels in relation to aromatase activity in staged follicles of lumpfish. In support of the trends of plasma sex steroids in the lumpfish females, in the Atlantic cod (Gadus morhua) blood plasma levels of E2 correlated with gonadosomatic index and were highest in spawning females (Dahle et al., 2003), in the sea bass (*Dycentrarchus labrax*) (Mañanós et al., 1997; Prat et al., 1990). There could also be fluctuations in E2 and T blood plasma levels during the spawning period in lumpfish however, due to the duration of our experiment we could not confirm this. For example, in the Atlantic halibut (*H. hippoglossus*) and European seabass (*D. labrax*) blood plasma levels of E2 and T exhibited fluctuations that were associated with recruitment of successive egg batches during the spawning period (Mañanós et al., 1997; Methven et al., 1992; Prat et al., 1990). However, the fluctuations may not be as prominent in T which continues to persist in peak levels during maturation and ovulation because its production in the thecal cells is not decreased (Nagahama, 1994). The changes in 11-KT that showed similar trends with T, may as well be attributed to interference of the 11-KT antibody with T. This may need to be confirmed through a cross-reactivity study of the 11-KT antibody.

In males, only internal observations were conducted, and we observed germ cells and blood vessels in the testicular tissue (Figure 5). The testicular organization of germ and somatic cells in lumpfish is cystic which is typical for fish and amphibians (Uribe et al., 2014). In a teleost testis, germ cells develop synchronously in cysts where they are enclosed by Sertoli cells, together making up the germinal epithelium (Koulish et al., 2002; Nagahama, 1994; Schulz and Miura, 2002). The germ cells were identified as spermatogonia (sg), spermatocytes (sc), spermatids (st) and spermatozoa with reference to previous descriptions (Leal et al., 2009; Miura et al., 1991; Schulz et al., 2010, 2005; Uribe et al., 2014).

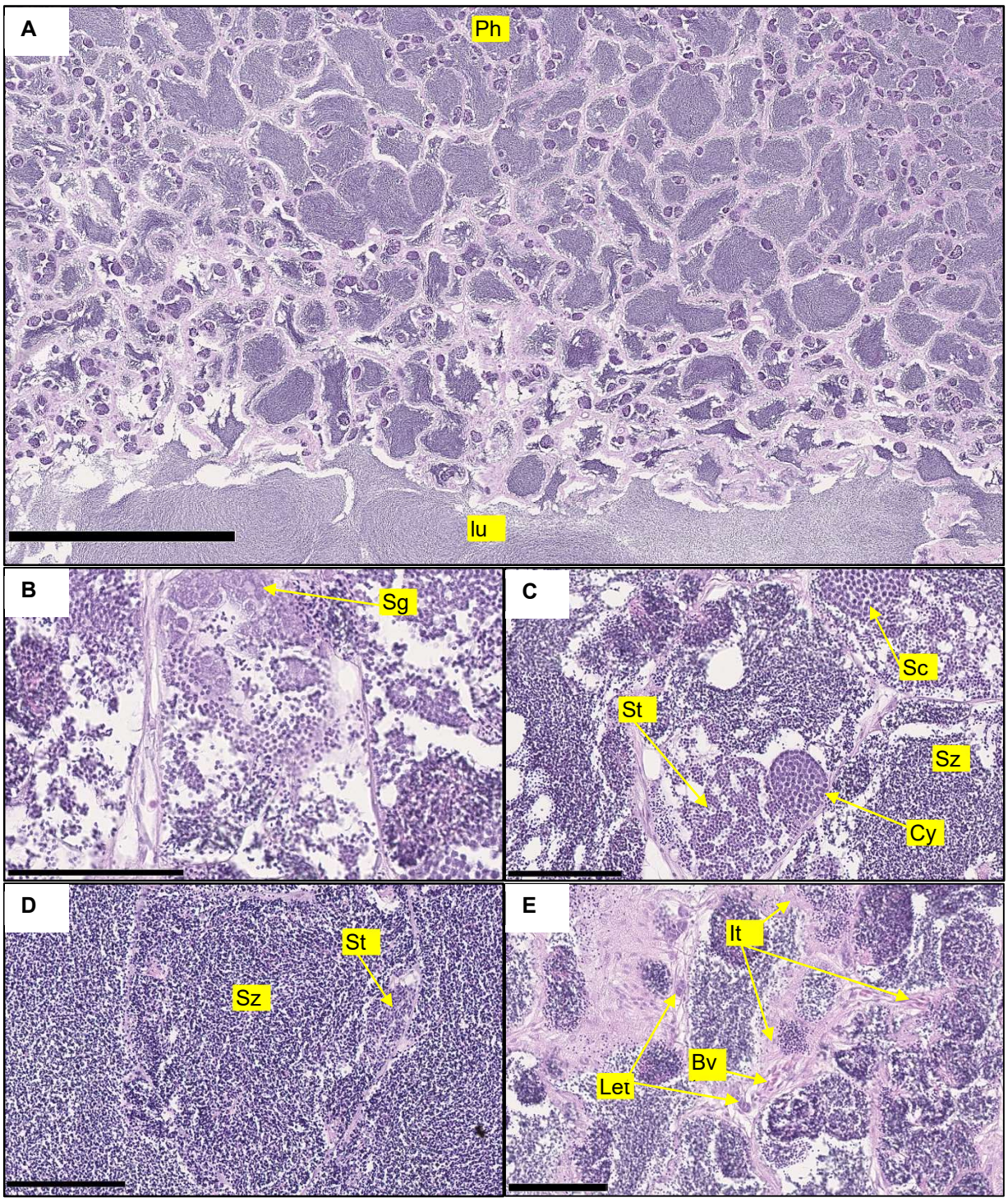

Figure 5 Internal morphologies of a *C. lumpus* testis and germ cells. (**A**) A transverse section of the lumpfish testis, from the periphery (Ph) to the lumen (lu). There are spermatogenic cells in cysts (Cy) (Spermatogonia (Sg)/Spermatocytes (Sc)/ Spermatids (St)) towards the periphery, and in the lumen there are free spermatozoa (Sz). (**B-E**) Enlarged sections showing all spermatogenic stages and interstitial tissues (It) (blood vessels (Bv) and Leydig cells (Le) observed in the It). Scale bars: **A** = 1 mm, **B-E** = 100 µm.

These germ cells are highly conserved in fish spermatogenesis that involves three major phases, proliferative/spermatogonial phase; meiotic/spermatocytary phase; and spermiogenic/differentiation phase (Nóbrega et al., 2009). With reference from previous studies (García-López et al., 2006; Koya et al., 2003), we renamed these germ cells as early (spermatogonia), mid (spermatocytes), late (spermatids) and functional maturation (spermatozoa) testicular developmental stages for convenience. Males were then grouped into the different testicular developmental stages based on the predominancies of the different spermatogenic stages.

Interestingly, contrary to females, GnRH2 and GnRH3 expressions in males increased with the recruitment of more developed testicular developmental stages (Figure 6). This phenomenon is a likely indication that GnRH2 and GnRH3 in lumpfish are expressed differently in the two sexes. In support of this, it has been reviewed that the feedback mechanisms from the gonads to the pituitary gland and brain can also be different between sexes of the same fish species (Fontaine et al., 2020). Similar to our findings, increasing GnRH2 and GnRH3 levels with increasing sexual maturation have been reported in the blue gourami (Levy et al., 2009) and turbot (Scophthalmus maximus) (Zhao et al., 2018). FSHR and LHR increased from early development to their peak levels at mid development and decreased afterwards (Figure…). On the other hand, in salmonids, plasma FSH levels increased to peak levels just before and decreased at spermiation while plasma LH levels were very low in the early stages but increased notably in spermiating or running males (Campbell et al., 2003; Gomez et al., 1999; Prat et al., 1996). Non-salmonids showed similar trends to the salmonids as well as LH being high from early spermatogenesis until the spawning period and decreasing at regression while FSH increases with spermatogenesis (Gen et al., 2003; Hassin et al., 2000).

The difference between our findings and those from other is probably due to different regulation mechanisms in different species as discussed earlier for the females. However, further research is needed to elucidate the patterns of circulating plasma levels of these gonadotropins in lumpfish. While it is widely accepted that FSH regulates Sertoli cell activities and LH regulates the Leydig cell sex steroid production, their biological activities are less clearly separated in fish (Schulz et al., 2010, 2001). It has been shown that FSHR expresses preference for FSH and LH, although very high concentrations of LH are needed implying that this could be restricted to periods with peak LH plasma levels, similarly to the LHR preference for FSH (Schulz et al., 2010). CyP17a1 showed a general increase to late development and decreased afterwards (Figure 6), a pattern which supports the role of this key gene in androgen biosynthesis whose activity seems to be highest at the late development in line with androgen peak levels (discussed next). In support of this, a pattern of CyP17a1 which resembled that of 11-KT in was also demonstrated in the males of Atlantic salmon (Kleppe et al., 2017).

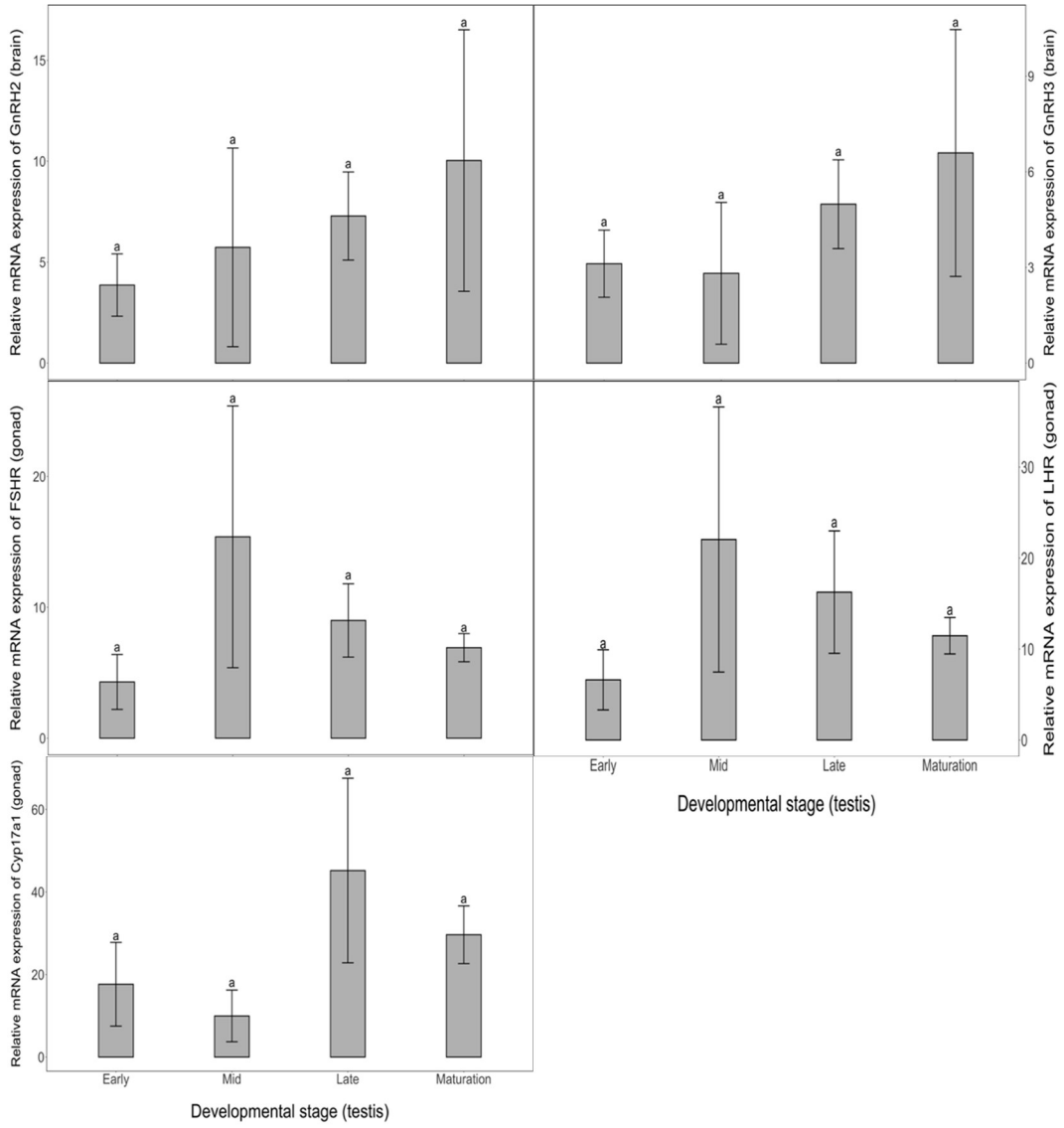

Figure 6 Gene expression levels of selected endocrine factors in relation to developmental stages in *C. lumpus* males. Gonadotropin-releasing hormone II (GnRH2, **A**); gonadotropin-releasing hormone III (GnRH3, **B**); Follicle stimulating hormone receptor (FSHR, **C**); Leuteinizing hormone receptor (LHR, **D**); and cytochrome P450 17a1(CyP17a1, **E**), grouped according to predominating developmental stages. Different letters on top of error bars indicate significant differences in expression levels between stages.

GSI, T and 11-KT increased to their peak levels at late development and decreased afterwards (Figure 7). Similar trends of T and 11-KT in relation to testicular development were reported in the Atlantic cod and Atlantic halibut (Dahle et al., 2003; Weltzien et al., 2002), generally these androgens increase gradually during spermatogenesis and decrease at spermiation (Schulz et al., 2010; Woods, L. et al., 2008). Androgens in male fish can support either the whole spermatogenesis or certain steps such as spermatogonial multiplication, spermatocyte formation, maturation, spermiation or initiation of puberty (Schulz et al., 2010; Schulz and Miura, 2002). Further, T is also reported to act via feedback mechanisms which can compromise FHS-dependent (besides LH) signaling and steroidogenesis. It is in the later stages when LH becomes increasingly important in regulating the androgen production, as well as sperm maturation (acquisition of motility) by influencing the production of progestins (Schulz and Miura, 2002).

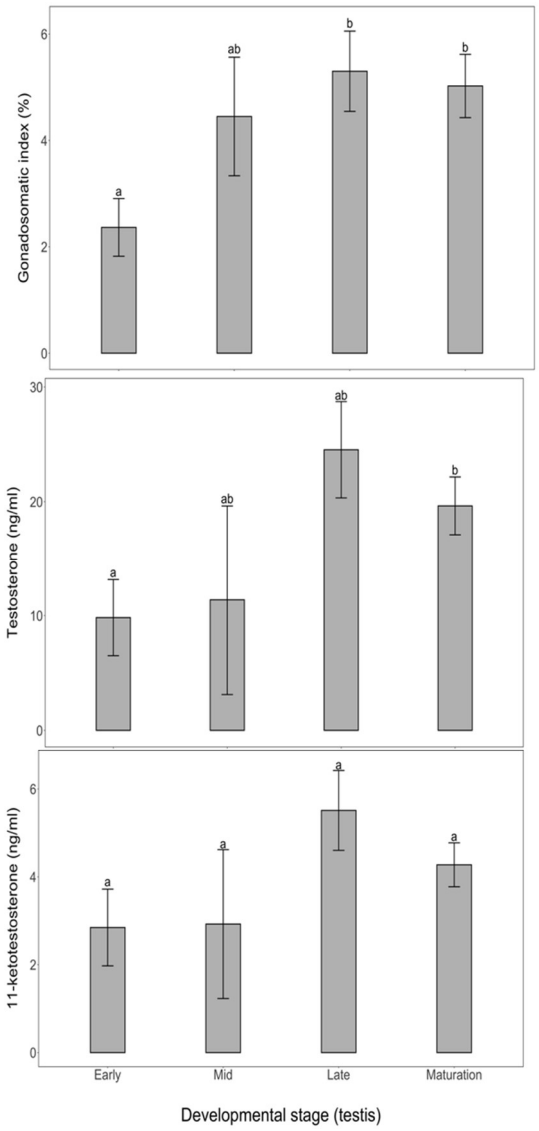

Figure 7. Testicular growth and mean blood plasma levels of androgens in relation to developmental stages in C. lumpus males. Gonadosomatic index (**A**), testosterone (**B**) and 11-ketotestosterone (**C**) grouped according to predominant developmental stages. Different letters on top of error bars indicate significant differences.

In both females and males, GSI and sex steroids were significantly positively correlated, higher correlation coefficients were in females. The positive correlations indicate that these parameters show similar patterns during the reproductive development in lumpfish. Also, the dependence of E2 on T as a precursor in aromatization could be explained by the positive correlations between the two steroids. In agreement to our findings, in other fish species, GSI, T, 11-KT and E2 showed positive correlations, also they increase and are high in spawning fish but decrease in spent fish (Dahle et al., 2003; Harmin et al., 1995; Methven et al., 1992).

Ultrasound based maturation monitoring in lumpfish (Paper V)

For efficient juvenile production, monitoring of the gonadal state is important since it leads to accurate prediction of ovulation for example as well as accurate timing of inducing changes in the reproductive development (Ortenburger et al., 1996). In this study, we aimed to establish ultrasound as a non-invasive method for monitoring sexual maturation in lumpfish under farm conditions. We pooled lumpfish females and males from an experiment (experiment 2) that was aimed at evaluating the effects of photoperiod and temperature on the reproductive performance. During routine samplings, the ultrasound (Esoate

Alpha) observations were conducted on anesthetized fish alongside collections of gonads for gonadosomatic index (GSI) estimation, gonad tissues for histological classification of gonad stages and blood for sex steroid (testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2)) analyses.

By using the observed ultrasound-based gonad features, we grouped females and males into F0 to F5 and M0 to M5, respectively, which represented immature to spent stages (Figure 8A). The progression of these ultrasound-based maturation stages was associated in part with changes in secondary sexual characteristics where: belly size increase; redening of the urogenital pore; and oozing of eggs were detected in females, body colour change; body shape changes and running milt were detected in males. The development of secondary sexual characteristics in fish is linked with the readiness to spawn since they appear at sexual maturity, indeed they are under the influence of sex steroids (Wheeler et al., 2020). In relation to histology, the ultrasound-based maturation stages followed the recruitment of more developed ovarian and testicular stages. F0, F1 and F2 were predominated with pre-vitellogenic and vitellogenic stages, while F3 (76 %) and F4 (67 %) were predominated with the final maturation stage and F5 represented a spent female. In males, M0, M1 and M2 were predominated by early and mid-stages while M3 and M4 represented mostly functional maturation and M5 represented spent testes (Figure 8B). Although GSI, histomorphology and the sex steroids as conventional methods can accurately determine the maturation status of the fish, they could be less preferred than ultrasound due to their undesirable features such as sacrificing valuable or even non-representative individuals, stressing the fish by frequent handling, limitation to providing maturation status information on an individual only once and cost (Næve et al., 2018b; Ortenburger et al., 1996; Swenson et al., 2007).

In females, the progression of ultrasound-based maturation stages followed the recruitment of more developed ovarian stages determined through histology. Ovary histology sections from females categorized as F0 and F1 were predominated by pre-vitellogenic oocytes, F2: vitellogenic oocytes, while F3 and F4: final maturation oocytes (F3 = 76 %, F4 = 67 %). Two females were categorized as F5, the ovary histology sections were predominated by pre-vitellogenic oocytes and ovulated eggs in one and the other, respectively (Figure 10A).

In males, the ultrasound-based maturation stages also followed the recruitment of more developed testicular stages. Testis sections from males categorized as M0 were predominated by spermatogonia, M1 and M2: spermatocytes, while M3, M4, and M5 were predominated by spermatozoa (M3 = 57 %, M4 = 87 %, M5 = 100 % (1 male)) (Figure 10B).

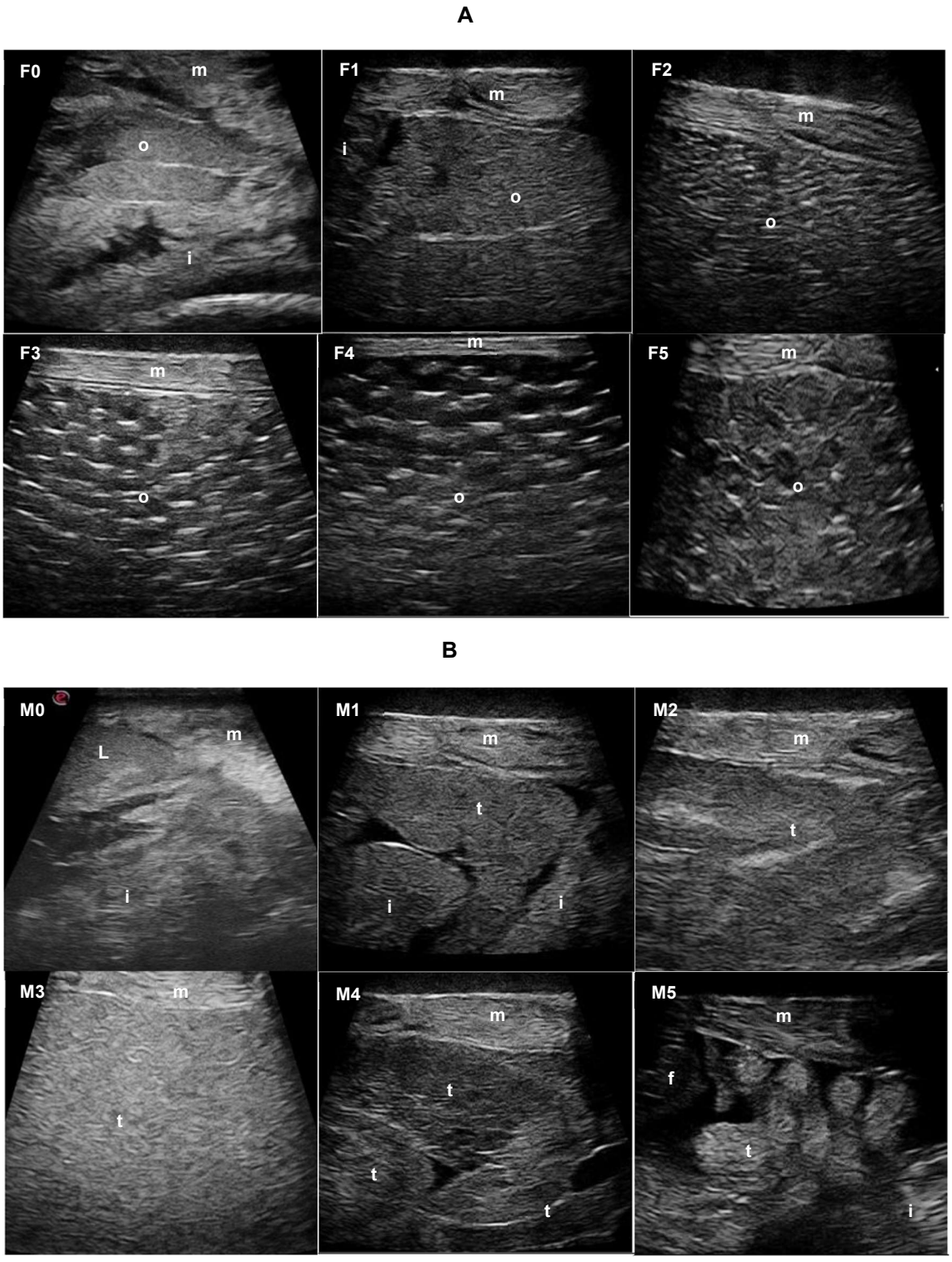

Figure 9 Ultrasound views of lumpfish ovaries (**A**) and testes (**B**). F0 to F5 are ultrasound-based maturation stages from immature to spent ovaries, and M0 to M5 are ultrasound-based maturation stages from immature to spent testes. Apart from ovaries (o) and testes (t), intestines (i), liver (L) and muscles (m) are also shown.

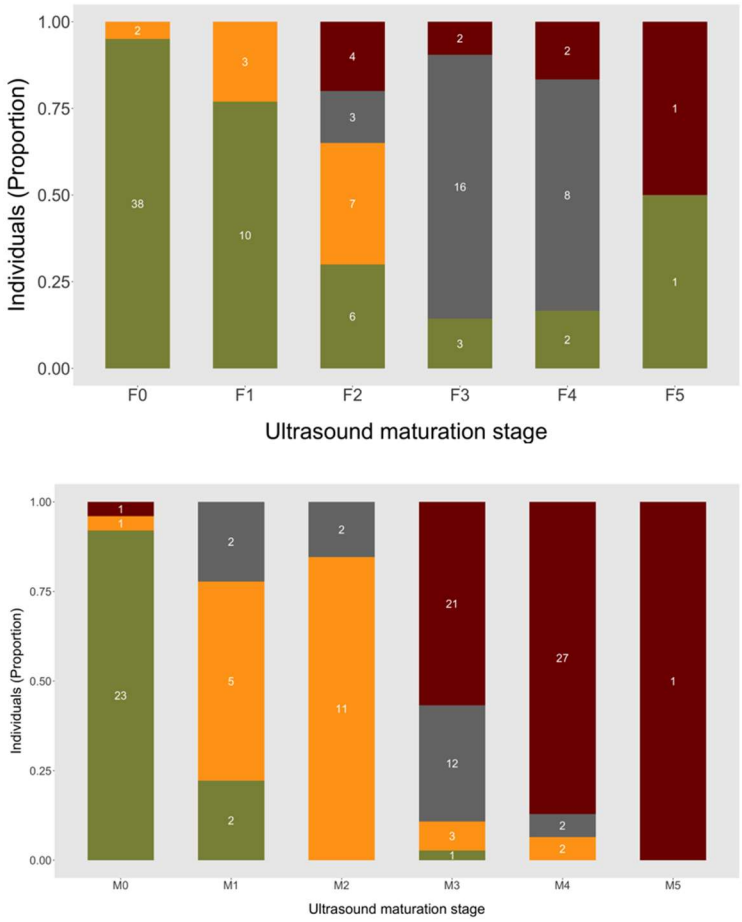

Figure 10 Progressions of ultrasound-based maturation category in relation to predominant ovarian (**A**) and testicular (**B**) stages from histology. Values inside bars show the number of females/males in each ultrasound-based maturation category with a particular gonadal stage comprising an area fraction ≥ 50 %.

The ultrasound-based maturation stages related to GSI, T, 11-KT and E2 in a manner that increased towards F3 and decreased later in females. In males, there was an increase up to M4 males and a decrease afterwards (Figure 11). Using these relationships, we established F3 and M3/M4 as arbitrary thresholds which can be used to identify matured females and males to eliminate the need for using ultrasound through most of the rearing period. This is crucial since one could focus on the period close to spawning. We however insist that the suggested thresholds should be adapted with caution in particular with regards to timing since maturation is prone to variations in growth rates as observed in the Atlantic cod (Armstrong et al., 2004). By incorporating the conventional methods in our study, we have shown that ultrasound can efficiently be used to monitor sexual maturation in lumpfish. Further, to ensure optimal success, it is arguably important for the hatchery worker to be adequately equipped with the skills and knowledge on the ultrasound as well as the reproductive anatomy of the fish (Hliwa et al., 2014; Næve et al., 2018b).

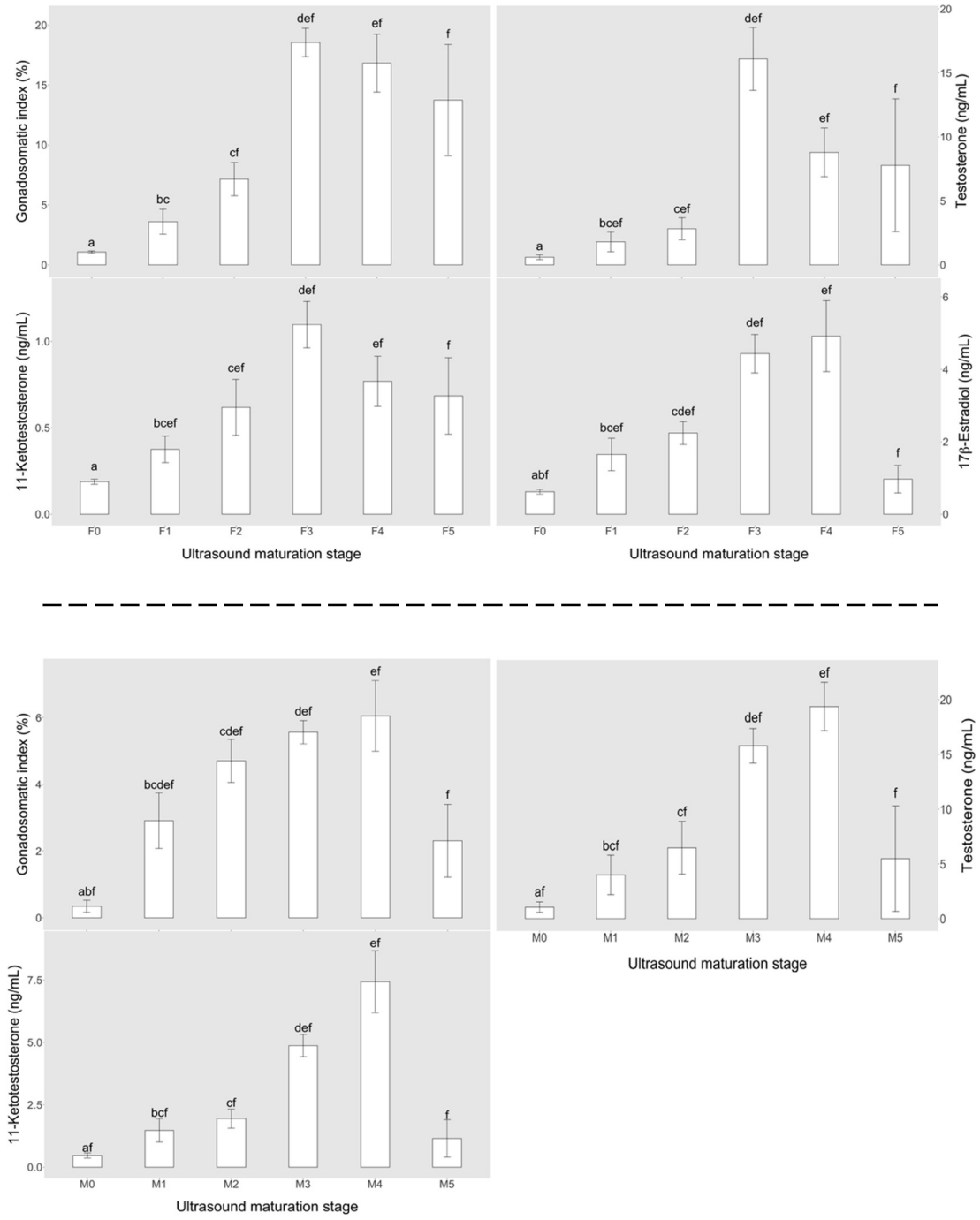

Figure 11. Gonadosomatic index and sex steroid levels at different ultrasound-based maturation categories in lumpfish females (top) and males (bottom). Each ultrasound category is assigned a unique letter (F0 & M0 = a, F1 & M1 = b, F2 & M2 = c, F3 & M3 = d, F4 & M4 = e, F5 & M5 = f), thus where an ultrasound-based maturation category has the same letter as the next ultrasound category, it implies that difference between the two is not statistically significant.

Photothermal effects on the reproductive performance of lumpfish (papers II, III, IV and VI).

The control of sexual maturation in lumpfish under farm conditions is still in its infancy. Until now it is yet to be clearly understood how photoperiod and temperature affect sexual maturation, spawning and egg quality. Thus, the aim of this section to study the response of the reproductive performance of adult and juvenile lumpfish to photoperiod manipulations followed by temperature elevation at the onset of final maturation in females. We conducted two experiments: In Experiment 1 (Papers I and II) (21st September 2017 to 8th March 2018), 18 months old lumpfish females and males (adults) were exposed to continuous (L:D = 24:0 for 24 weeks) and short-to-continuous (L:D = 8:16 for 17 weeks and then L:D = 24 for 7 weeks) photoperiods each with two tanks. This was followed by temperature elevation in one tank from each photoperiod at the onset of final maturation in females (at 17 weeks). In Experiment 2 (Papers III, IV, V and VI) (30th January 2018 to 1st June 2019), 12 months old lumpfish females and males (juveniles) were exposed to simulated natural annual, and nine-month compressed annual photoperiods at ambient temperatures also each with two tanks. This was also followed by temperature elevation in one tank from each photoperiod at 11 months and 13 months in the compressed annual and the natural annual photoperiods, respectively.

Body weight (BW) and condition factor (K)

In females, Paper II (Experiment 1) shows that BW and K varied significantly ($P < 0.05$) with time under the short-to-continuous photoperiod. BW and K were higher in the continuous and the short-to-continuous photoperiods, earlier and later, respectively. Following temperature elevation, BW was higher in the high temperature groups than in the ambient temperature groups from the same photoperiod while differences in K were not clear. In females therefore, growth is favoured under continuous photoperiod. Similarly, higher growth of lumpfish females under continuous photoperiod was reported by Imsland and co-workers (Imsland et al., 2018b). Since the fish were maintained under continuous photoperiod from hatching, the higher BW in the short-to-continuous photoperiod later could be related to compensatory growth as a result of the change from a photoperiod with a dark phase to continuous illumination (Ali et al., 2003). Paper III (Experiment 2) shows an increase in BW with time, while K fluctuated towards and decreased slightly during the spawning period. Initially, BW was mostly higher in the natural annual photoperiod but later it was in the compressed annual photoperiod. Compressing the annual photoperiod could be the cause of the later higher BW in this photoperiod while the decrease in K could be due to spawning as documented previously that K in lumpfish is low during their natural spawning season in the wild (Kennedy, 2018; Moring, 2001). In contrast to Paper II, Paper III shows higher BW in the ambient temperature groups compared to their high temperature counterparts, and an accelerated decrease in K in the high temperature groups. These observations can however be attributed to spawning (discussed later) which was advanced in the compressed annual photoperiod leading to lower BW and K. Decrease in K in relation to spawning was also observed in lumpfish previously (Imsland et al., 2019), also a negative correlation between growth and sexual maturation was observed in the Atlantic cod (*G. morhua*) (Taranger et al., 2006).

Males were generally smaller than females (Papers II and IV (Experiment 2)) similar to earlier observations that reported lumpfish males to be smaller than females, a feature that is more notable in spawning individuals (Davenport, 1985; Goulet et al., 1986b; Imsland et al., 2019, 2018b). Paper II shows that both BW and K increased and decreased in the continuous and short-to-continuous photoperiods, respectively. Interestingly, the photoperiodic effects were different from females in the same experiment, BW and K were higher in the short-to-continuous photoperiod earlier and later they were higher in the continuous photoperiod groups at the same temperatures. These observations suggest that the photoperiodic effects on BW and K in lumpfish are probably perceived differently between the sexes. The higher BW in the short-to-continuous photoperiod could be associated with growth compensation in males also, that probably started earlier following stagnation that resulted

from earlier sexual maturation (discussed later) (Ali et al., 2003; Taranger et al., 2006). Therefore, although growth in males is suppressed after reaching maturity, exposure to the short-to-continuous photoperiod might have stimulated more growth. The temporal variation in K can also be related to spawning as discussed for the females, where the loss in weight due to spawning and thus a decrease in K is a possible reason. In Paper IV, it is shown that BW increased towards and decreased during the spawning period as demonstrated in the females (Paper III). K on the other hand showed a general decrease throughout the experimental period, a different phenomenon from that in females from the same experiment. The decrease in K can be related to sexual maturation which starts earlier in males as well as the subsequent spawning, the latter being already highlighted earlier in females.

Gonadosomatic index (GSI)

In females, Paper II shows a general significant ($P < 0.05$) increase in GSI with time that was more notable in the short-to-continuous photoperiod compared to the continuous photoperiod. Here we speculate that the photoperiodic changes within the short-to-continuous photoperiod were perceived as those occurring during the change from autumn to spring. In Paper III, there was also a general increase in GSI which was more notable in the compressed annual photoperiod earlier compared to the natural annual photoperiod (Figure 12). These observations suggest that compressing the annual photoperiod leads to advanced ovarian growth compared to the natural annual photoperiod. With temperature elevation, in Paper II it is shown that GSI was higher in the high temperature groups compared to the ambient temperature groups from the same photoperiod. In Paper III, it is shown that the high temperature groups attained higher GSI's early on, later the GSI's were lower than in the ambient temperature groups from the same photoperiod. These observations suggest that temperature elevation accelerates the changes in GSI. Similarly, higher temperatures were associated with higher ovarian growth in the Atlantic cod (Kjesbu et al., 2010).

In males, Paper II shows a general decrease that was significant ($P< 0.05$) in the short-to-continuous photoperiod. Paper IV shows an increase in GSI towards the spawning period during which there was a decrease. The differences between the two papers are likely attributable to the age of the fish where those in Paper II were older and were found to be in functional maturation at the start of the experiment (discussed later). The increase and decrease in GSI could be related to development plasticity of testes manifested by the weight and volume changes occurring seasonally (Schulz et al., 2010). In Paper II, it is shown that at ambient temperature, GSI was higher and lower, earlier, and later, respectively in the short-to-continuous photoperiod compared to the continuous photoperiod. With temperature elevation, the changes in GSI were advanced in the high temperature groups. In Paper IV, GSI was mostly higher in the natural annual photoperiod compared to the compressed annual photoperiod. Following temperature elevation, GSI was higher in the high temperature compressed annual photoperiod compared to its ambient temperature counterpart as opposed to the natural annual photoperiod in which it was higher in the ambient temperature group. Therefore, under the short-to-continuous photoperiod and the compressed annual photoperiod, GSI changes are likely to be advanced, and further accelerated when there is temperature elevation. Under the continuous photoperiod, free running is likely (Imsland et al., 2019) while under the natural annual photoperiod, the fish are perceiving the simulated photothermal regimes as those occurring in nature hence the reproductive process has a rather natural rhythm (Bromage et al., 2001).

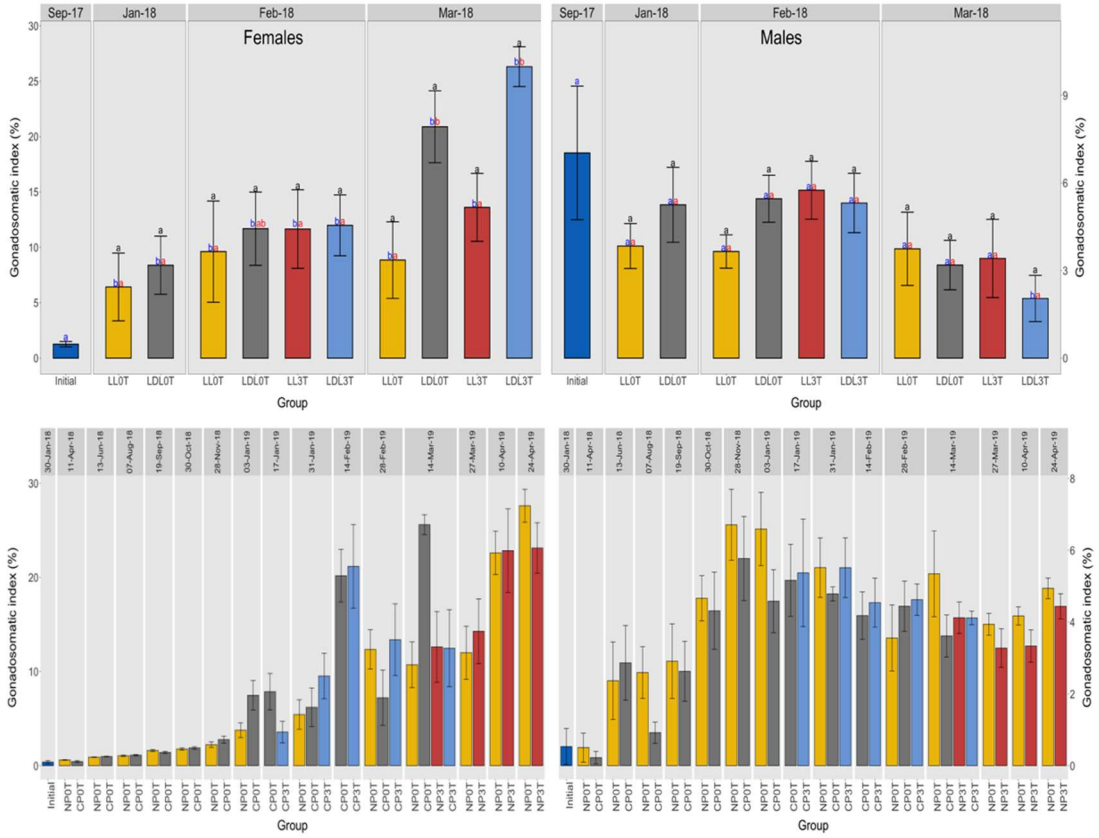

Figure 12. Gonadosomatic indices in *C.lumpus* females (left) and males (right) from **Experiment 1** (**A**) and **Experiment 2** (**B**), exposed to different photothermal regimes (treatments). **Experiment 1:** LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature. **Experiment 2:** NP0T = natural annual photoperiod at ambient temperature, CP0T = compressed annual photoperiod at ambient temperature, NP3T = natural annual photoperiod at elevated temperature, CP3T = compressed annual photoperiod at elevated temperature.

Gonadal development

In females, Paper II shows that the recruitment of more developed stages of ovarian development was advanced in the short-to-continuous photoperiod than in the continuous photoperiod (Figure 13). With temperature elevation, there seemed to be an acceleration of the recruitment in both photoperiods which was manifested by the recruitment of earlier or more developed stages. Further, at the end of the experiment, the final maturation and ovulation stages were in larger proportions in the short-to-continuous photoperiod groups which contained either vitellogenic or pre-vitellogenic oocytes as earlier stages to the continuous photoperiod groups. These observations suggest that ovarian development in lumpfish can be advanced and more synchronized under the short-to-continuous photoperiod or a photoperiod with a dark phase. Recent studies on lumpfish have reported spawning without a clear pattern in females exposed to continuous photoperiods (Imsland et al., 2019, 2018b), which is most likely due to asynchronous development of oocytes. Paper III shows long pre-vitellogenic (6 months) and vitellogenic (7 months) periods in relation to the final maturation and ovulation stages (Figure 13). The lengths of these long periods of ovarian growth are associated to their contributions to the fully yolked oocytes entering final maturation. The pre-vitellogenic and vitellogenic periods are stages of ovarian follicle growth, during which major lipid and protein nutrients required for embryonic

development are stored in the oocyte (Reading et al., 2017). During the long teleost pre-vitellogenic period, there is synthesis of organelles and molecules such as neutral lipids needed for later stages (Kagawa, 2013; Reading et al., 2017). In the long vitellogenic period there is an estrogen-dependent synthesis and secretion of vitellogenin from the liver which is then incorporated in the oocytes and cleaved to generate multiple egg yolk proteins that are stored in the ooplasm. During the same period, estrogen also induces the synthesis and secretion of choriogenin an oogenic protein forming the inner layer of the egg envelop (Hara et al., 2016). It can thus be seen that the ovarian follicle growth period results in fully yolked oocytes that subsequently undergoes final maturation and ovulation, and the resultant oocyte growth effect is substantial, comprising up to 80-90 % of the dry mass of an ovulated egg (Reading et al., 2017). It however should be noted that these periods may not adequately represent the exact timing of the different stages because our observations were based on the sampling intervals which might have not captured ovarian development in its entirety. In support, although it is proposed that ovary development in lumpfish needs at least 8 months in first spawning individuals, the exact time is still unknown (Kennedy, 2018). The possible advancing effect of the compressed annual photoperiod was manifested at the first appearance of final maturation oocytes the proportion of which was higher than in the natural annual photoperiod. Following temperature elevation, there was a higher recruitment of more developed stages in the high temperature group compared to the ambient temperature group within the natural annual photoperiod. Similarly, higher oocyte recruitment was attributed to warmer temperatures while arrested onset of vitellogenesis was attributed to low temperature in the Atlantic cod (Kjesbu et al., 2010; Yoneda and Wright, 2005). In the high temperature compressed annual photoperiod, the recruitment of more developed stages was low compared to other groups. Since spawning (discussed later) was advanced in this group, it is likely to be the cause of the lower recruitment of the more developed ovarian stages. Such observations suggest that ovarian development in farmed lumpfish can be advanced by compressing photoperiod integrated with elevation of temperature. In support, previous findings on advanced spawning in lumpfish (Imsland et al., 2019), Atlantic cod (Norberg et al., 2004) and striped trumpeter (Latris lineata) (Morehead et al., 2000) following compression of the natural photoperiods are indications that sexual maturation was also advanced.

Paper II shows that males were already in the functional maturation stage from the start of the experiment. Among the treatment groups, the largest proportion of functional maturation stage was in the ambient temperature continuous photoperiod, indicating that spermiation was highest in this group. With temperature elevation, the recruitment of the functional maturation stage was lower in the high temperature group. Within the short-to-continuous photoperiod, the recruitment of the functional maturation stage was also lower, it was mostly lower in the high temperature group compared to the ambient temperature group. Such observations suggest that there is possibility of free running of the circadian clock under the continuous photoperiod while under the short-to-continuous photoperiod, testicular development can be phased. On the contrary, in the Atlantic cod, continuous exposure to constant light resulted in inhibition of spermatogenesis and high apoptotic germ cell loss (Almeida et al., 2009). Paper IV shows that spermatozoa were already recruited four months after starting the experiment. Prior to temperature elevation, fractions of spermatozoa were larger in the natural annual photoperiod compared to the compressed annual photoperiod. Following temperature elevation, the high temperature compressed annual photoperiod recruited the largest fractions of spermatozoa. The spermatozoa fractions between the high and ambient temperature groups within the natural annual photoperiod exchanged between being larger and smaller at different time points. It therefore can be argued that elevation of temperature can be more effective in influencing testicular development in lumpfish under the compressed annual photoperiod compared to the natural annual photoperiod. Further, in contrast to previous findings where maturation in males (spawning coloration and running milt) to a large extent did not seem to be affected by photoperiodic manipulations (Imsland et al., 2019, 2018b), our findings suggest that photoperiod and temperature manipulations have the potential to phase sexual maturation in male lumpfish and hence improve the efficiency in the management of broodstock of both sexes. The suggested free running rhythm in both females and males of lumpfish

due to exposure to the continuous photoperiod is supported by the suggestion that exposure of fish to continuous photoperiod results in disruption of the melatonin rhythm. This leads to: altered circadian variations of the reproductive hormones hence free running, and inhibition or delaying of the onset of gametogenesis (Bayarri et al., 2009; Davie et al., 2007b; Karlsen et al., 2014).

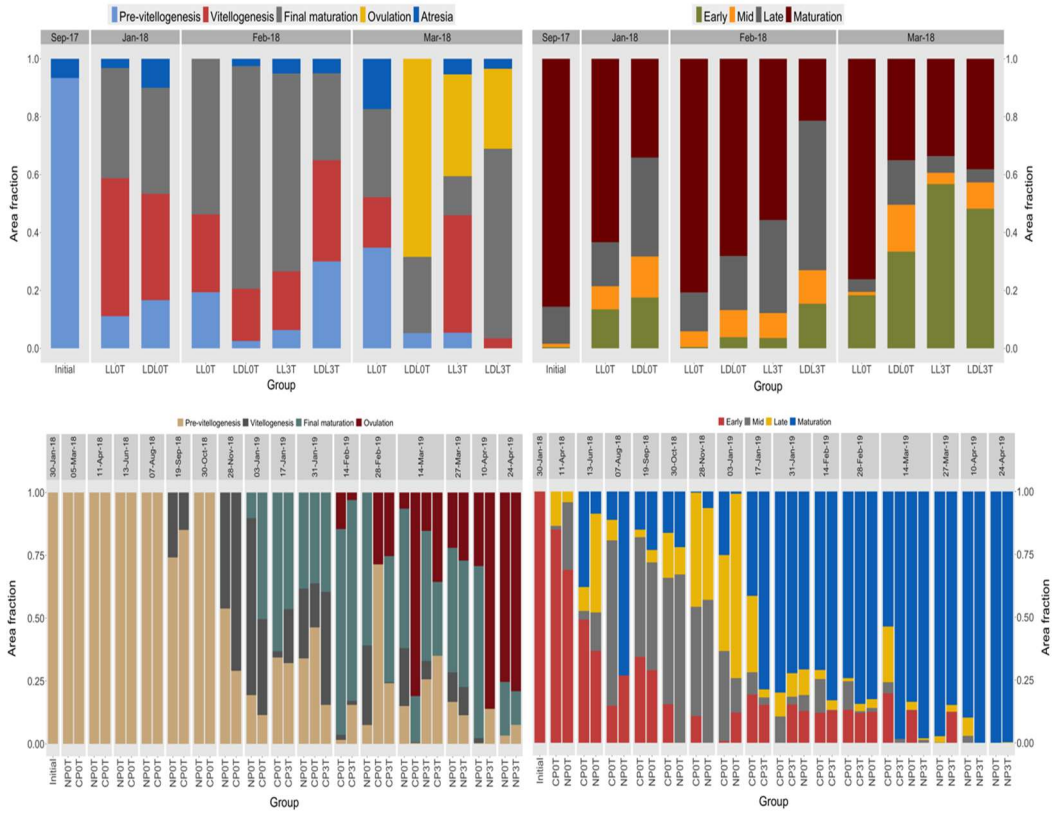

Figure 13. Mean area fractions (in proportions) in *C.lumpus* females (left) and males (right) from **Experiment 1** (**A**) and **Experiment 2** (**B**), exposed to different photothermal regimes (treatments). **Experiment 1:** LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature. **Experiment 2:** NP0T = natural annual photoperiod at ambient temperature, CP0T = compressed annual photoperiod at ambient temperature, NP3T = natural annual photoperiod at elevated temperature, CP3T = compressed annual photoperiod at elevated temperature.

Gonadotropin releasing hormones (GnRHs)

In females, GnRH2 and GnRH3 fluctuated in parallel without a clear pattern in Paper II. They were both upregulated in the continuous photoperiod compared to the short-to-continuous photoperiod throughout (Figure 14). Temperature elevation accelerated the changes in the GnRHs. The GnRH upregulation in lumpfish females within the continuous photoperiod could indicate that continuous light causes increased signalling of GnRHs possibly affecting the adjacent processes in a manner that does not show a particular pattern.

In males, GnRH2 and GnRH3 were significantly downregulated in all treatment groups compared to the start of the experiment as shown in Paper II. This observation could be linked to the early maturation in males, as males were already in functional maturation at the start of the experiment a condition which could be associated with high GnRH signalling. In agreement to this, in Paper I, it is shown that GnRH2 and GnRH3 regulation in males increased with the recruitment of more developed testicular stages. At ambient temperatures, the GnRHs were upregulated in the short-to-continuous photoperiod compared to the continuous photoperiod earlier, later they were almost similar. With temperature elevation, these GnRHs were upregulated in the high temperature continuous photoperiod compared to the high temperature short-to-continuous photoperiod earlier, later they were also almost similar. The GnRHs were significantly upregulated in the high temperature groups compared to the ambient temperature groups from the same photoperiods at the end of the experiment. These observations indicate that GnRH2 and GnRH3 in male lumpfish are influenced by photothermal manipulations, moreover, the effects of temperature could be different in different photoperiods. Alteration of the seasonality of GnRHs regulations following exposure to artificial photoperiods has also been reported in the European seabass (Espigares et al., 2017; Martins et al., 2015; Rodríguez et al., 2004). In the Atlantic cod females, the activity of GnRH3 was accelerated following a change from natural to continuous photoperiod compared to continuous exposure to a continuous photoperiod and a change from continuous to natural photoperiod (Hildahl et al., 2013). In the pejerrey (*Odontesthes bonariendis*), higher expressions of GnRH2 and GnRH3 occurred in fish exposed to a long photoperiod (16:8, L:D) compared to those exposed to a short photoperiod (8:16, L:D) regardless of water temperature (Miranda et al., 2009).

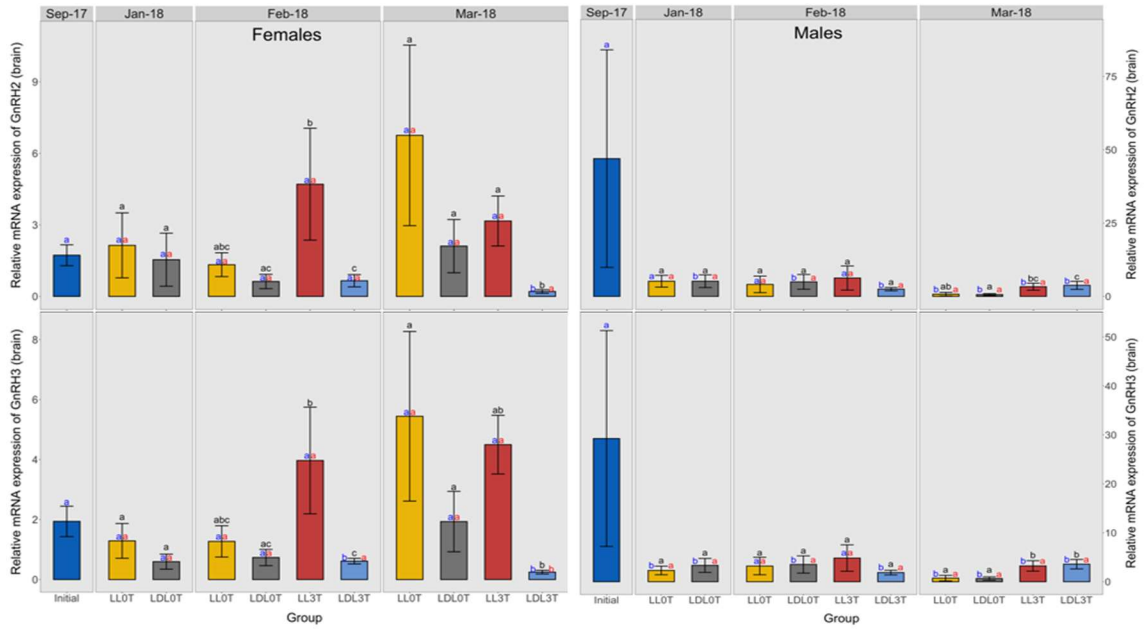

Figure 14. Temporal changes in relative mRNA expressions of GnRH2 and GnRH3 in *C.lumpus* females (left) and males (right) exposed to different photothermal regimes in **Experiment 1**. LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature.

Gonadotropin hormones and their receptors (GTHs and GTHRs)

FSHR and LHR in females fluctuated by increasing and later decreasing as shown in Paper II. At ambient temperatures, FSHR and LHR were upregulated in the short-to-continuous photoperiod than in the continuous photoperiod. Following temperature elevation, FSHR and LHR were higher and lower, earlier, and later, respectively in the continuous photoperiod compared to the short-to-continuous photoperiod (Figure 15). Within the photoperiods, temperature elevation caused different effects where upregulation occurred in the continuous photoperiod throughout while in the short-to-continuous photoperiod, upregulation and downregulation were observed earlier and later, respectively. These observations suggest that the regulations of the gonadotropin receptors respond differently to varying photoperiods, and differently affected by the same temperature change in different photoperiods. Previous research has also demonstrated that photoperiod and temperature changes affect factors related to gonadotropin hormone receptors: In the Atlantic cod, FSHβ and LHβ expressions were lower in females exposed to continuous photoperiod compared to those exposed to natural photoperiod (Cowan et al., 2012). On the other hand, exposure of Atlantic cod females to a natural photoperiod followed by a continuous photoperiod advanced the shifts in FSHβ and LHβ (Karlsen et al., 2014). In the pejerrey (*Odontesthes bonariendis*), higher expressions of the gonadotropin subunits FSHβ and LHβ occurred in fish exposed to a long photoperiod (16:8, L:D) compared to those exposed to a short photoperiod (8:16, L:D) regardless of water temperature (Miranda et al., 2009).

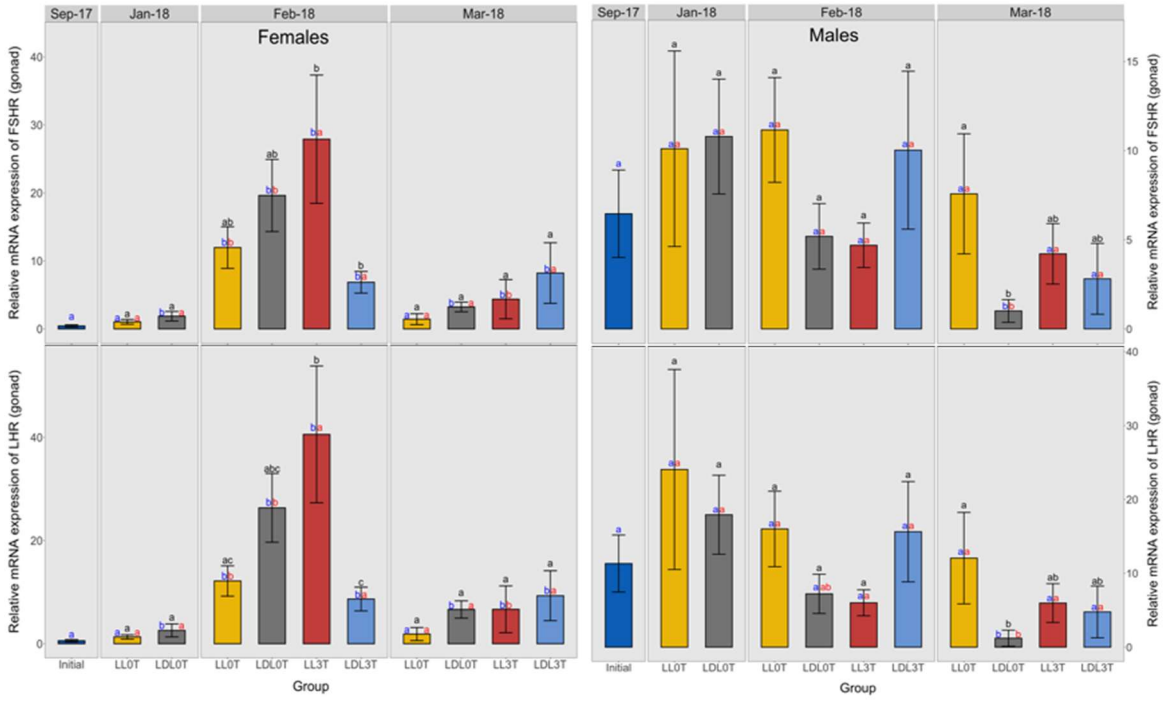

Figure 15. Temporal changes in relative mRNA expressions of FSHR and LHR in *C.lumpus* females (left) and males (right) exposed to different photothermal regimes in **Experiment 1**. LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature.

In males, Paper II shows that FSHR and LHR fluctuated with time, showing significant ($P < 0.05$) downregulations towards the end. At ambient temperatures, the GTHRs were upregulated in the continuous photoperiod compared to the short-to-continuous photoperiod. With temperature elevation, they were upregulated in the high temperature group within the short-to-continuous photoperiod. Within the continuous photoperiod, temperature elevation resulted in their upregulations throughout. As mentioned above for females, expressions of FSHβ and LHβ in Atlantic cod males were also higher in the natural photoperiod compared to the continuous photoperiod (Cowan et al., 2012). In another study, a delay in the peak of blood plasma LH was observed in photoperiods other than the natural photoperiod in males of Sea bass (Rodríguez et al., 2004).

Steroidogenic pathway enzymes (CyP19a1 and CyP17a1)

The regulation of CyP19a1 in females fluctuated by increasing and later decreasing at the end of the experiment as shown in Paper II. CyP19a1 was upregulated in the short-to-continuous photoperiod compared to the continuous photoperiod at ambient temperatures. Following temperature elevation, CyP19a1 regulation was higher and lower, earlier and later, respectively in the continuous photoperiod compared to the short-to-continuous photoperiod. Within the photoperiods, temperature elevation caused different effects where upregulation occurred in the continuous photoperiod throughout while in the short-to-continuous photoperiod, there were upregulation and downregulation earlier and later, respectively.

In males, Paper II shows that CyP17a1 fluctuated with time, showing significant ($P < 0.05$) downregulations towards the end. At ambient temperatures CyP17a1 was upregulated in the continuous photoperiod compared to the short-to-continuous photoperiod. With temperature elevation CyP17a1 was upregulated in the high temperature group within the short-to-continuous photoperiod while in the continuous photoperiod, temperature elevation resulted in CyP17a1 being upregulated only at the end.

Such findings indicate that there is a potential for photoperiod and temperature to interact in their influence on the activities of the steroidogenic pathway enzymes CyP19a1 and CyP17a1 in lumpfish.

Vitellogenin receptor (VTGR)

The regulation of VTGR in females fluctuated by increasing and later decreasing at the end of the experiment as shown in Paper II. VTGR was upregulated in the short-to-continuous photoperiod compared to the continuous photoperiod at ambient temperatures (Figure 16). Following temperature elevation, VTGR regulation was higher and lower, earlier and later, respectively in the continuous photoperiod compared to the short-to-continuous photoperiod. Within the photoperiods, temperature elevation caused different effects where upregulation occurred in the continuous photoperiod throughout while in the short-to-continuous photoperiod, there were upregulation and downregulation earlier and later, respectively. There is therefore a possibility that photoperiod and temperature interact to influence the expression of VTGR in lumpfish females.

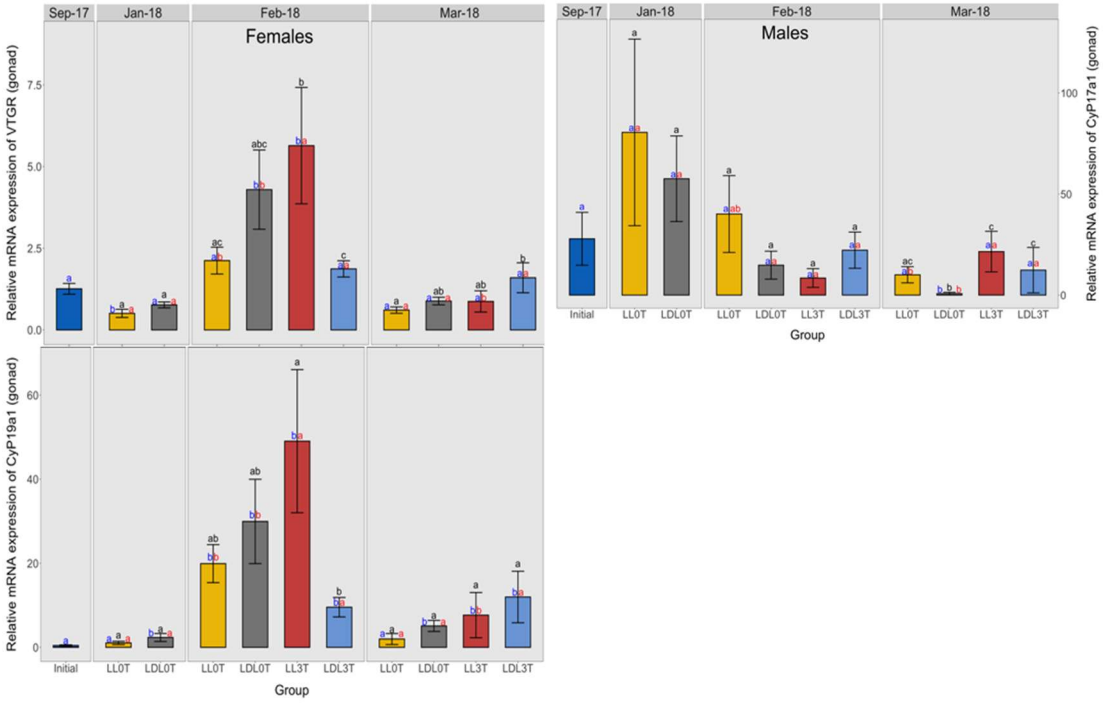

Figure 16. Temporal changes in relative mRNA expressions of VTGR and CyP19a1 in females (left), and CyP17a1 in males (right) of *C.lumpus* exposed to different photothermal regimes in **Experiment 1**. LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature.

Sex steroids (testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (E2))

In females, Paper II shows an overall significant ($P < 0.05$) increase in blood plasma levels of T, 11-KT and E2 with time. All sex steroids were generally higher in the short-to-continuous photoperiod than in the continuous photoperiod. With temperature elevation, there were lower blood plasma levels of T in the continuous photoperiod contrary to the short-to-continuous photoperiod in which T was higher earlier but later it was similar in the ambient and high temperature groups (Figure 17). 11-KT blood plasma levels were higher and lower, earlier and later, respectively within the high temperature short-to-continuous photoperiod, while in the continuous photoperiod, it was the opposite. E2 was also higher and lower, earlier and later within the continuous photoperiod in the high temperature group, while in the short-to-continuous photoperiod, temperature elevation resulted in higher E2 blood plasma levels throughout. Generally, the short-to-continuous photoperiod advanced the sex steroid profiles compared to the continuous photoperiod while temperature elevation accelerated the temporal changes in these profiles. Paper III shows that T and 11-KT increased towards the spawning period during which they decreased within the compressed annual photoperiod. On the other hand, in the natural annual photoperiod there was a general gradual increase notably at ambient temperatures. A possible explanation could be that, compressing the annual photoperiod advances the temporal changes in the androgens an effect that could lead to paralleled changes in the adjacent processes. Just before temperature elevation T and 11-KT were significantly ($P < 0.05$) higher in the compressed annual photoperiod. Following temperature elevation, T and 11-KT were higher in the high temperature groups than their ambient temperature counterparts earlier. Later, T and 11-KT were higher in the ambient temperature groups. This adds on the argument that temperature secondarily affects the levels of

circulating androgens in lumpfish by accelerating their temporal changes. E2 also showed an increase, but this was detected earlier than T and 11-KT. There was a decrease during the spawning period that was also earlier than T and 11-KT. This is due to the role of E2 in vitellogenesis where it stimulates the liver to synthesize and secret vitellogenin, hence it peaks before the androgens, also because testosterone is the precursor to estradiol, levels of this androgen are low when estradiol is needed in high concentrations and high when there is loss of aromatase activity in the ovarian follicles during final oocyte maturation (Chen et al., 2021; Kagawa et al., 1983). Earlier, E2 was mostly higher in the compressed photoperiod than in the natural annual photoperiod. Further, with temperature elevation E2 was higher in the high temperature groups but later it was lower compared to the ambient temperature groups from the same photoperiod. As discussed for the androgens, the compression of the annual photoperiod and the elevation of the ambient temperature are the likely causes of the advanced changes in E2. These observations show that sex steroids in lumpfish females are affected by photoperiod and temperature manipulations, hence potentially affecting the adjacent processes on which they are the final effectors. In females of other species, photothermal manipulations have also been shown to cause shifts in the cyclic patterns of blood plasma sex steroid levels. The cyclic patterns of E2 and T in females of Atlantic cod were shifted due to exposure to either compressed or extended annual photoperiod cycles compared to the normal 12-month annual photoperiod cycle (Norberg et al., 2004). In the haddock, blood plasma levels of T and E2 in females were low following exposure to continuous light (Davie et al., 2007a). Exposure of female Atlantic cod to a short-day photoperiod resulted in a more rapid increase in blood plasma levels of T and E2 in females compared to a normal photoperiod (Skjæraasen et al., 2004).

In males, Paper II shows that T and 11-KT fluctuated culminating with a decrease at the end of the experiment. The fluctuation of both androgens was most notable in the ambient temperature continuous photoperiod group. The levels of these androgens exchanged between being higher and lower between temperature groups within photoperiods and between photoperiod groups within temperatures. In general, temperature elevation resulted in higher T within the short-to-continuous photoperiod throughout. In the continuous photoperiod, T was lower and higher, earlier and later, respectively in the high temperature group. 11-KT was lower and higher, earlier and later, respectively in the high temperature groups than in the ambient temperature groups from the same photoperiod. Paper IV shows that T and 11-KT increased with time towards and decreased during the spawning period. Prior to temperature elevation, T was most of the time notably higher in the compressed annual photoperiod than in the natural annual photoperiod. Following temperature elevation, T was higher and lower earlier and later in the high temperature compressed annual photoperiod group than in the ambient temperature counterpart. In the natural annual photoperiod, T was higher in the high temperature group than in the ambient temperature counterpart throughout. In both photoperiods, temperature elevation resulted in 11-KT being higher and lower, earlier and later, respectively in the high temperature groups compared to the ambient temperature group from the same photoperiod. As in females, the cyclic patterns of plasma sex steroid levels in males of other species are altered due to photothermal manipulations. In the Atlantic halibut, exposure to continuous light resulted in higher T and 11-KT levels (Imsland et al., 2009; Norberg et al., 2001). In the haddock, continuous light on the other hand resulted in low blood plasma levels of T (Davie et al., 2007a). In the Atlantic cod males, exposure to a short-day photoperiod resulted in a rapid increase in blood plasma levels of T and 11-KT (Skjæraasen et al., 2004). The role of elevated temperature on advancing the profiles of sex steroids has also been reported other fish species such as winter flounder (Harmin et al., 1995) and Atlantic cod (Tveiten, 2008).

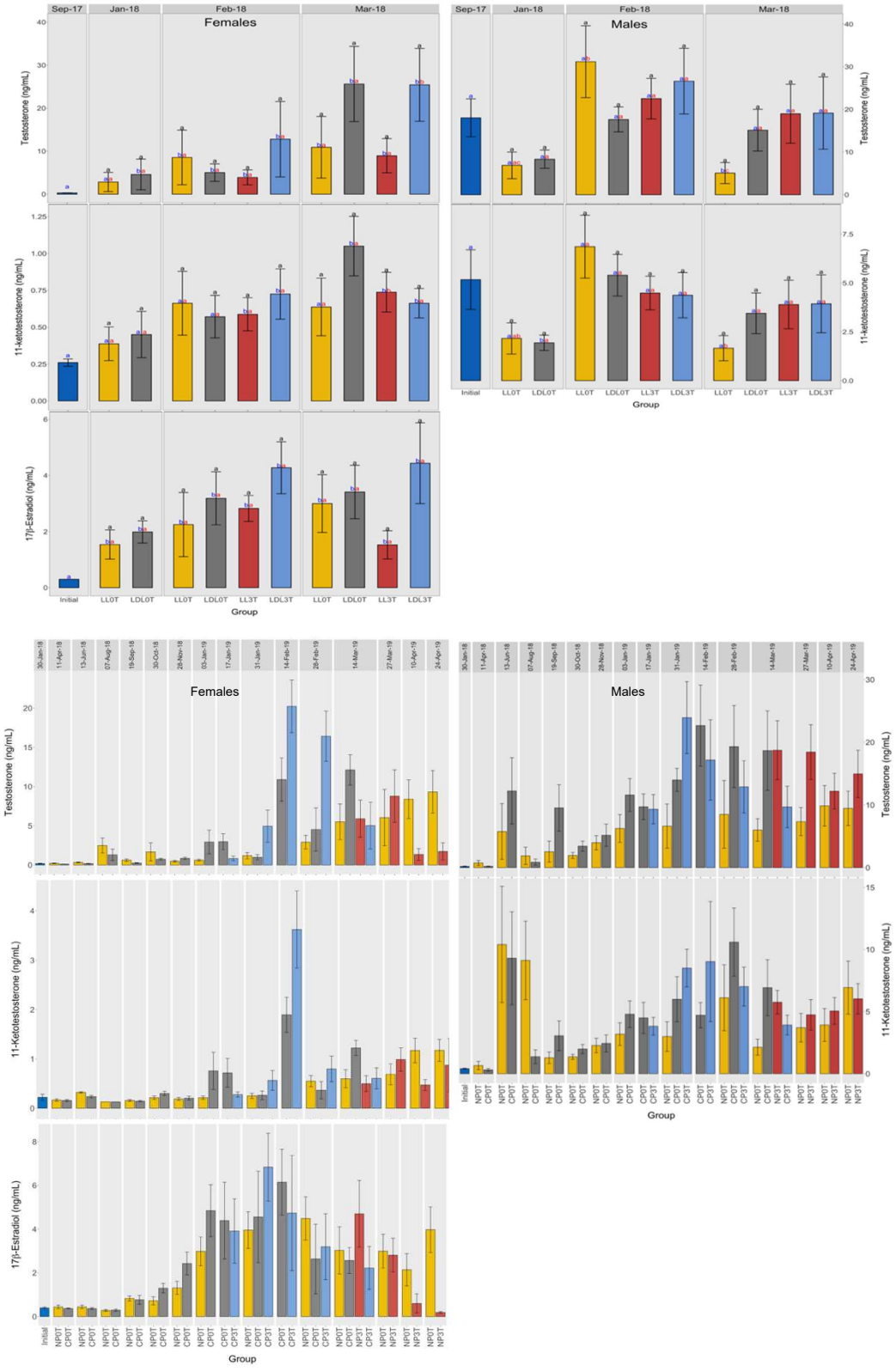

Figure 17. Temporal changes in blood plasma levels of sex steroids in *C.lumpus* females (left) and males (right) from **Experiment 1** (**A**) and **Experiment 2** (**B**), exposed to different photothermal regimes (treatments). **Experiment 1:** LL0T = continuous photoperiod at ambient temperature, LDL0T = short-to-continuous photoperiod at ambient temperature, LL3T = continuous photoperiod at elevated temperature, LDL3T = short-to-continuous photoperiod at elevated temperature. **Experiment 2:** NP0T = natural annual photoperiod at ambient temperature, CP0T = compressed annual photoperiod at ambient temperature, NP3T = natural annual photoperiod at elevated temperature, CP3T = compressed annual photoperiod at elevated temperature.

Spawning

Spawning events were observed earlier in the compressed annual photoperiod, earliest at high temperature. Later, spawning was detected in the natural annual photoperiod firstly at ambient temperature and later at high temperature. The delay of temperature elevation could be the cause of the delayed spawning in the high temperature natural photoperiod compared to its ambient temperature counterpart. Recent studies on lumpfish have reported shifts in spawning following compression of the natural photoperiods and further showed that spawning can be more predictable under compressed photoperiods than under the natural photoperiod (Imsland et al., 2019, 2018b). In addition to compression of natural photoperiods, other photoperiods than the natural photoperiod for the species have been used to shift spawning in other fish species (Carrillo et al., 1989; Mañanós et al., 1997; Van Der Meeren and Ivannikov, 2006). The effect of temperature is also undoubted as manifested in the compressed photoperiod. We detected that, despite improving the number of egg batches and egg weight by compressing the natural annual photoperiod, temperature elevation affected the two photoperiods differently, where the number of egg batches and weight were notably higher in the natural annual photoperiod compared to the compressed annual photoperiod. Linking the spawning observations to other factors discussed before, the compression of the annual photoperiod and elevation of temperature created a chain of events in the brain-pituitary axis that culminated with advanced spawning and the adjacent implications on egg batches and weights in the females (Figure 18). A recent study showed that, the spawning period in lumpfish at 6 ℃ can be twice as long as that at 9 ℃, under a 24-hour low intensity light (Pountney et al., 2020a). Water temperature can exert a strong influence on the spawning rhythm although its effect is largely argued to be secondary to photoperiod (Brown et al., 2006). Temperature-related changes in spawning and other parameters such as fecundity have been reported in other species (Brown et al., 2006; Devauchelle et al., 1988; Kjesbu, 1994). Therefore, temperature elevation accelerates the final maturation and ovulation, leading to the relatively short spawning period. Previously, it was recommended that, when using environmental cues to control reproductive development, the consideration of both photoperiod and temperature is important (Pankhurst and King, 2010). Our observations on spawning raise interesting questions on potential photothermal effects on egg quality.

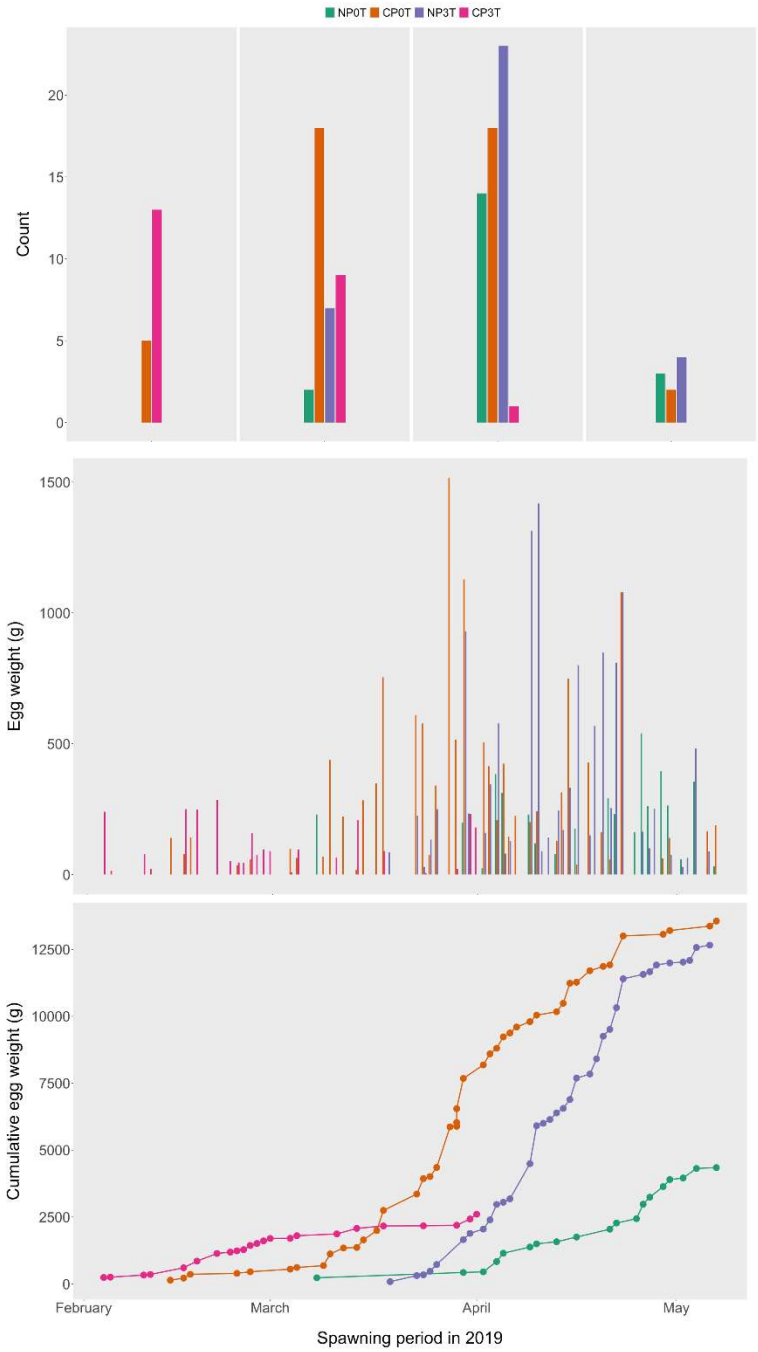

Figure 18. The number of spawns and weights of spawned eggs in *C. lumpus* females from different treatment groups.

Photothermal effects on lumpfish egg and larval quality (Paper VI)

In this study, we examined whether photothermal manipulations cause differences in morphological parameters of egg quality. For this, we studied the dynamic molecular regulation of embryonic development (unfertilized eggs – newly hatched larvae) and examined the relationship between morphological and the molecular parameters of egg quality.

Oocyte and testis development – histology and gene expressions: We examined the gonad morphology and calculate fish condition, gonadosomatic index (GSI), and fecundity. Gonad tissues were histologically examined for maturation indexing of oocytes and sperm cells. Gene expression from gonads, liver and pituitary were analysed using validated RT-qPCR, where mRNA transcript levels of the different GnRHs, GTHs, and their receptors were related to plasma sex steroid concentrations as well as to the stage of ovarian and testis development obtained from histological sections. Thus, it was able to assess all levels of the BPG-axis (in the same animal), and how environmental changes may be mediated within this regulatory system.

Maternal genes and gene expression patterns in eggs: We use RNA-sequencing as a promising tool for differentiating between the maternal gene pool in high- and low-quality eggs. Samples of unfertilised eggs, at early cleavage and early somite formation were used for comparative analyses. In addition, qPCR analysis was used for the most interesting genes, to confirm the data from the RNA-seq analyses.

Figure 19. Setup of the incubation. From each tank/group four females were used for sampling for egg quality. Three sub-samples (triplicates) from each female were incubated as shown in the pictures. In triplicates, sampling for gene expression was done at unfertilized eggs (UE), early gastrula (EG) and newly hatched larvae (NHL).

Morphological parameters

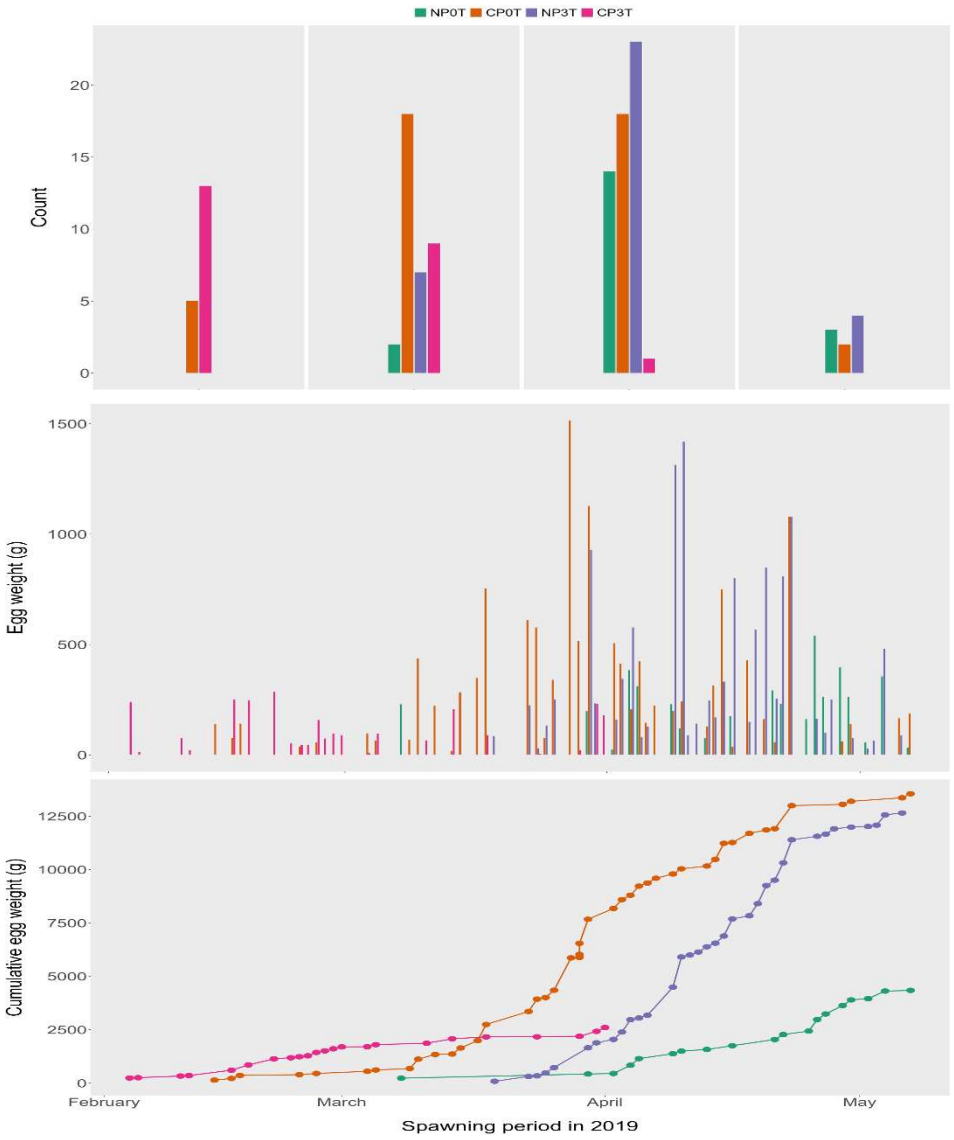

Figure 20 Egg batches, egg batch weight and cumulative egg weight.

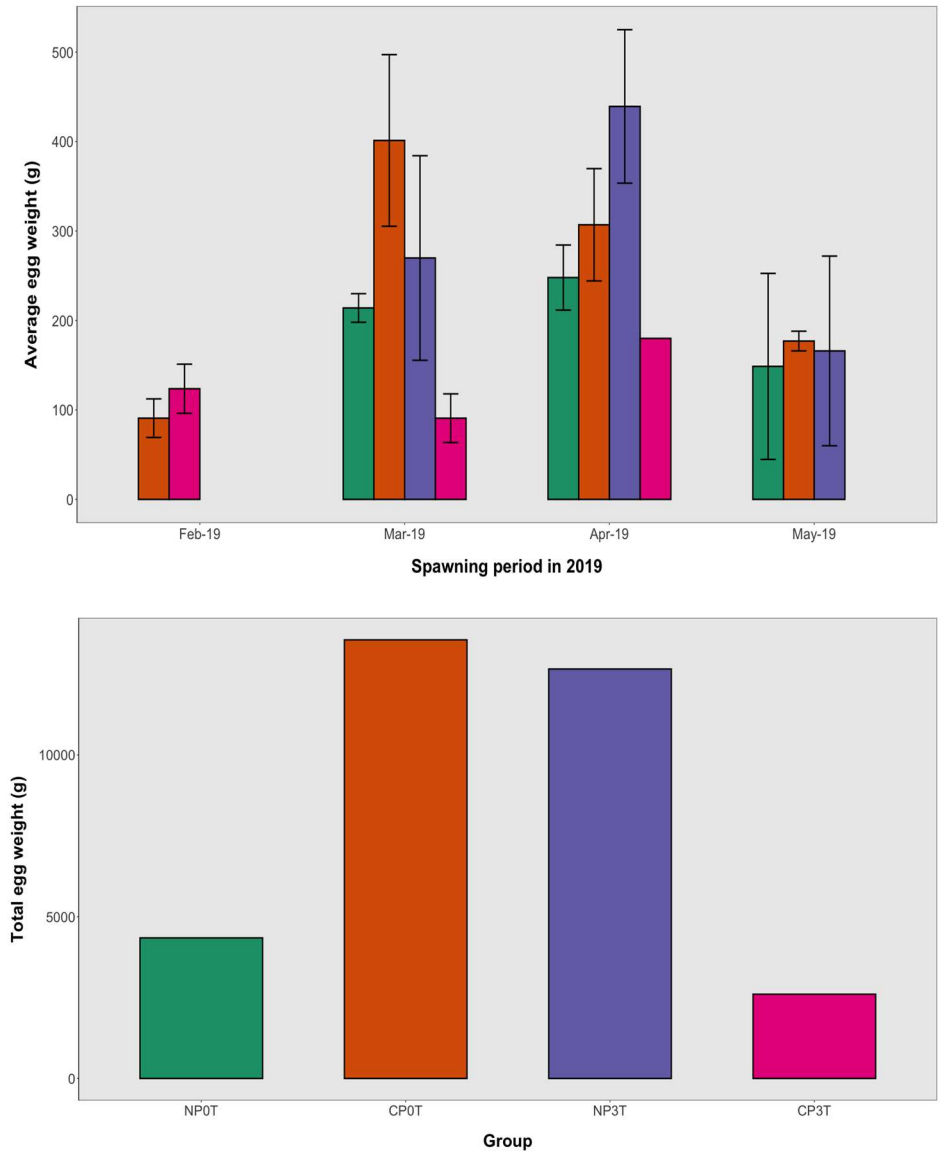

Figure 21 Average egg weight and total egg weight in each group.

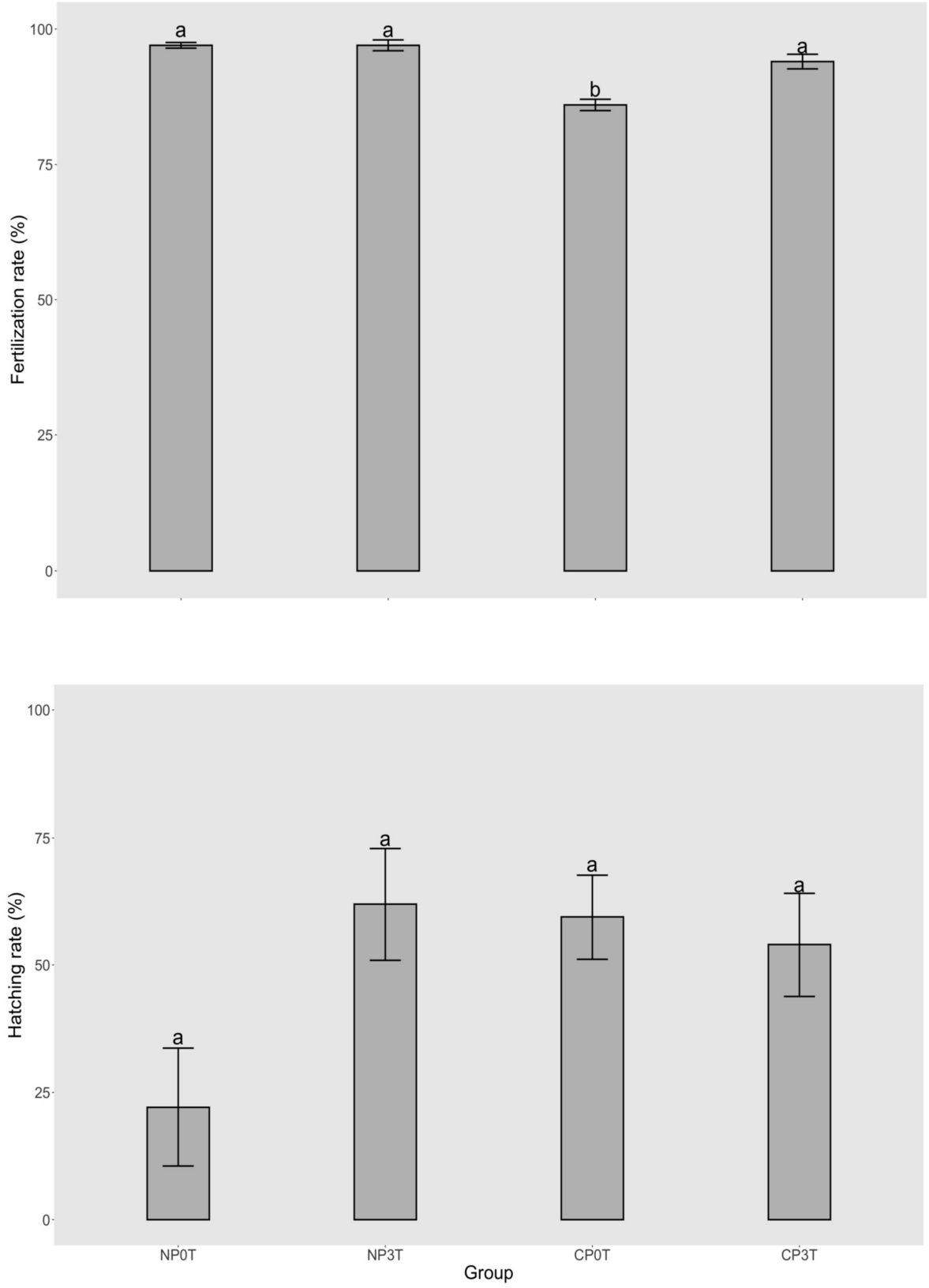

Figure 22. Fertilization and hatching rates

Gene Expression

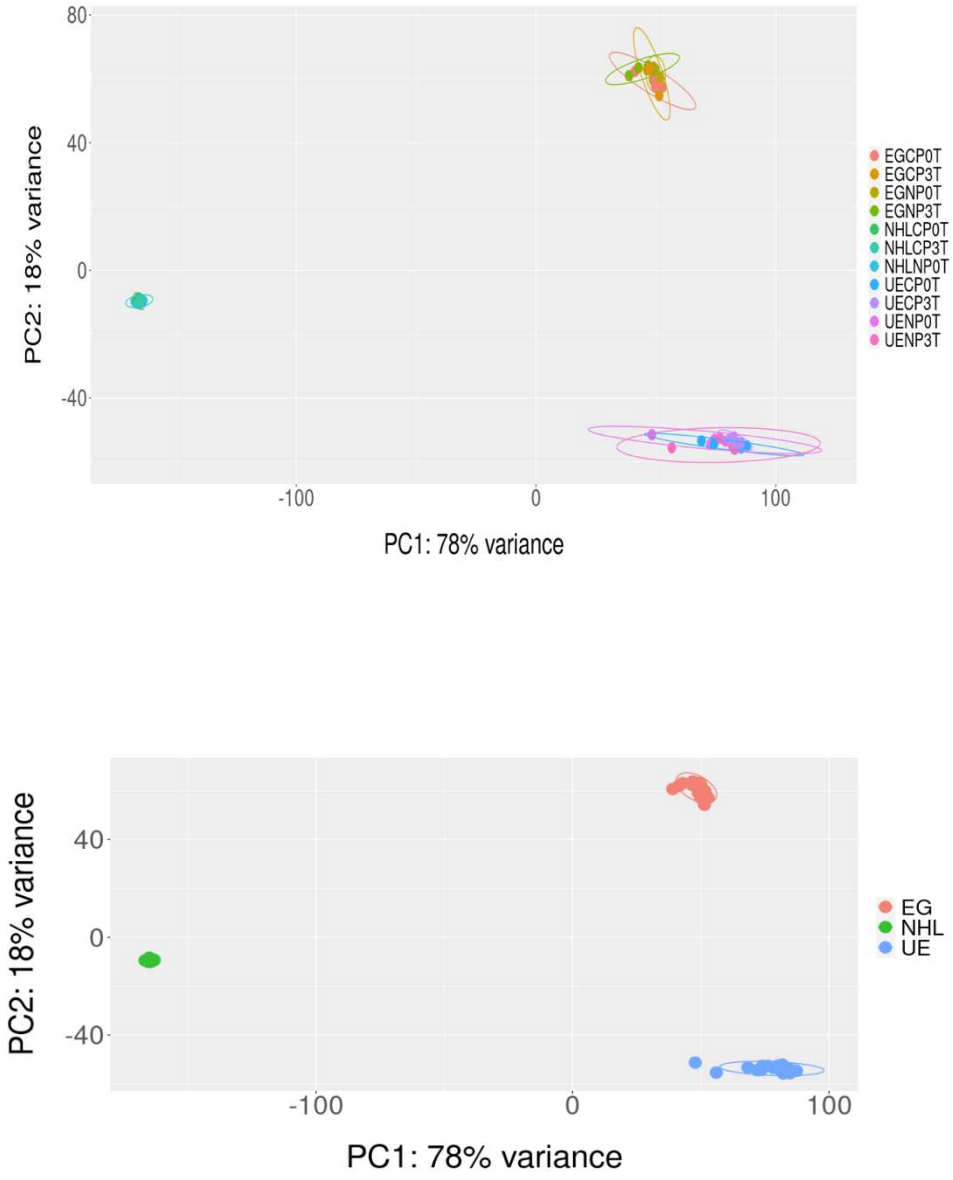

Figure 23 PCA of all stages and treatment groups (top) and only stages (bottom). There are three clear clusters in terms of development/incubation stages.

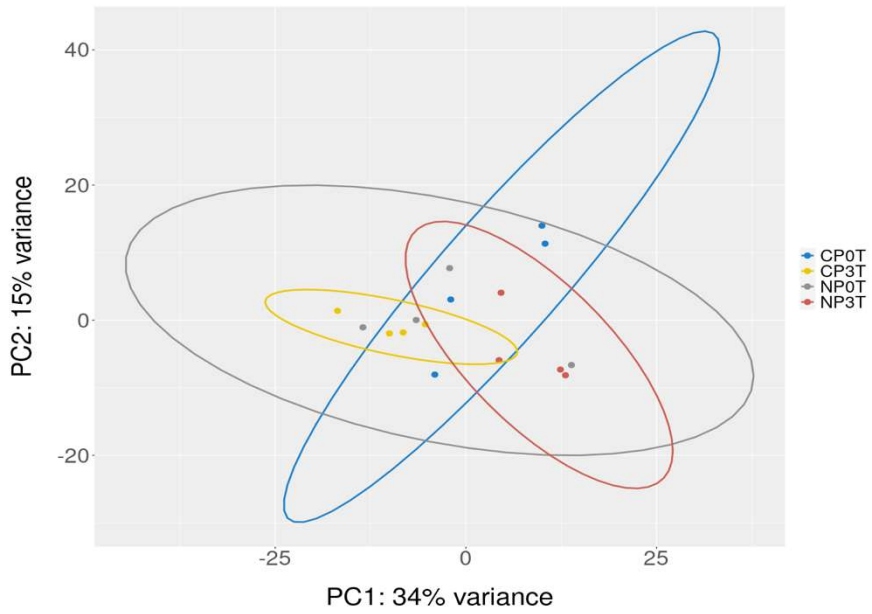

Figure 24 PCA of samples of unfertilized eggs from the different treatment groups.

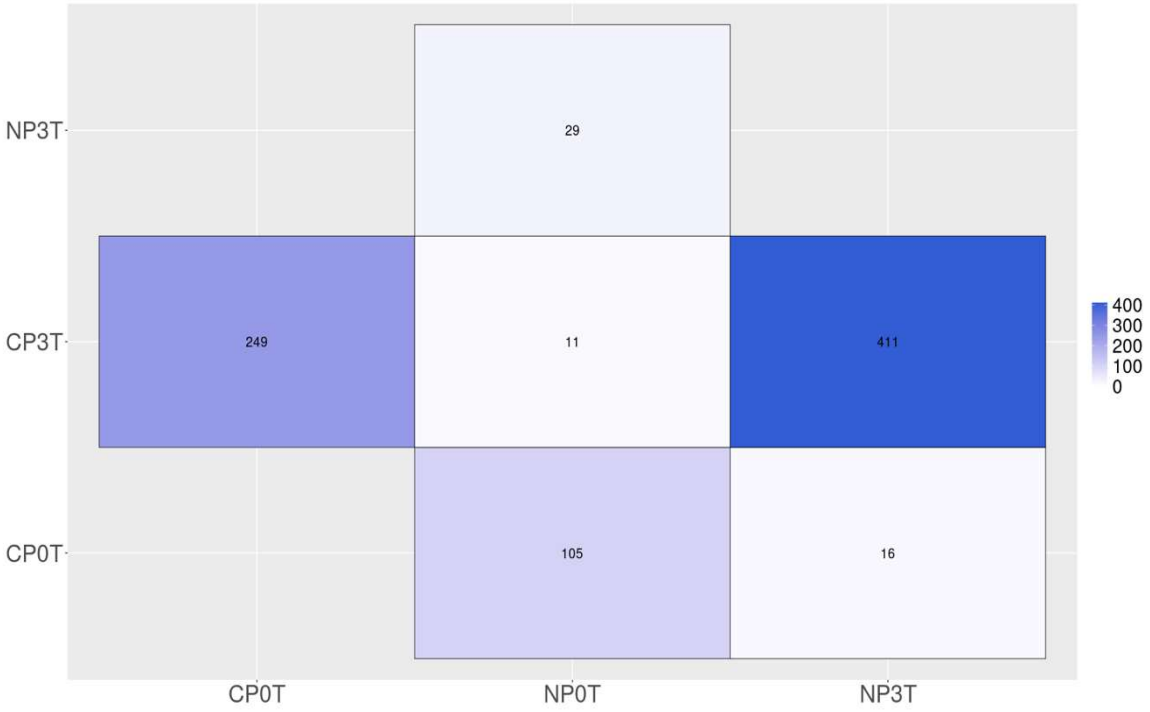

Figure 25: Number of differentially expressed genes between treatment groups for unfertilized eggs. Each cell indicates the number of differentially expressed genes for the respective intersecting row and column. The spectrum of colors relates to the magnitude of the differentially expressed gene counts.

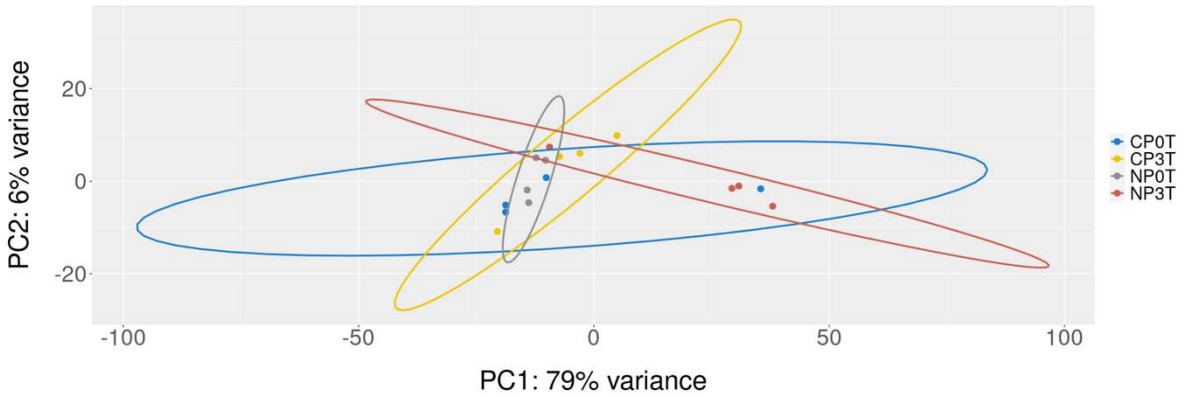

Figure 26. PCA of samples of early gastrula from the different treatment groups.

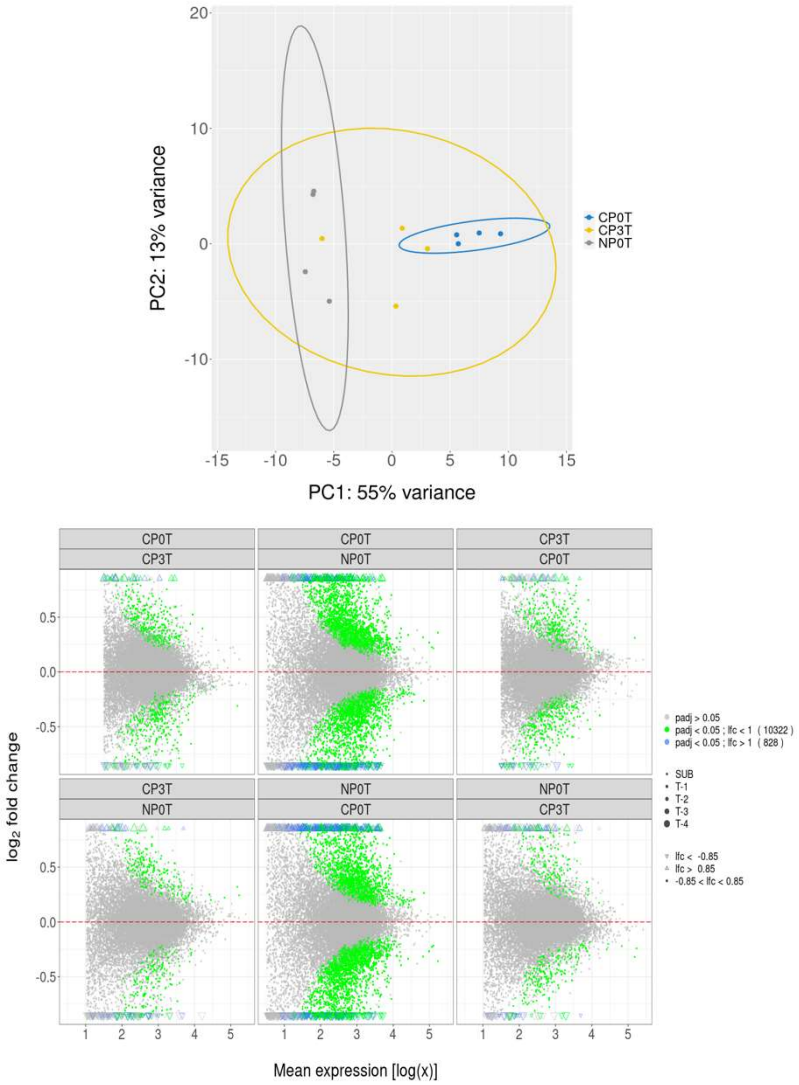

Figure 27.1 PCA (top) of samples of newly hatched larvae from the different treatment groups. **MA plot (bottom) of newly hatched larvae samples, pairwise comparisons of the different treatment groups:** Fold-change vs mean counts. Blue nodes on the graph represent statistically significant LFCs which are greater than a given value than a user-defined LFC parameter. Green nodes indicate statistically significant LFCs which are less than the user-defined LFC parameter. Gray nodes are data points that are not statistically significant. Numerical values in parantheses for each legend color indicate the number of transcripts that meet the prior conditions. Triangular shapes represent values that exceed the viewing area of the graph. Node size changes represent the magnitude of the LFC values (i.e. larger shapes reflect larger LFC values). Dashed lines indicate user-defined LFC values.

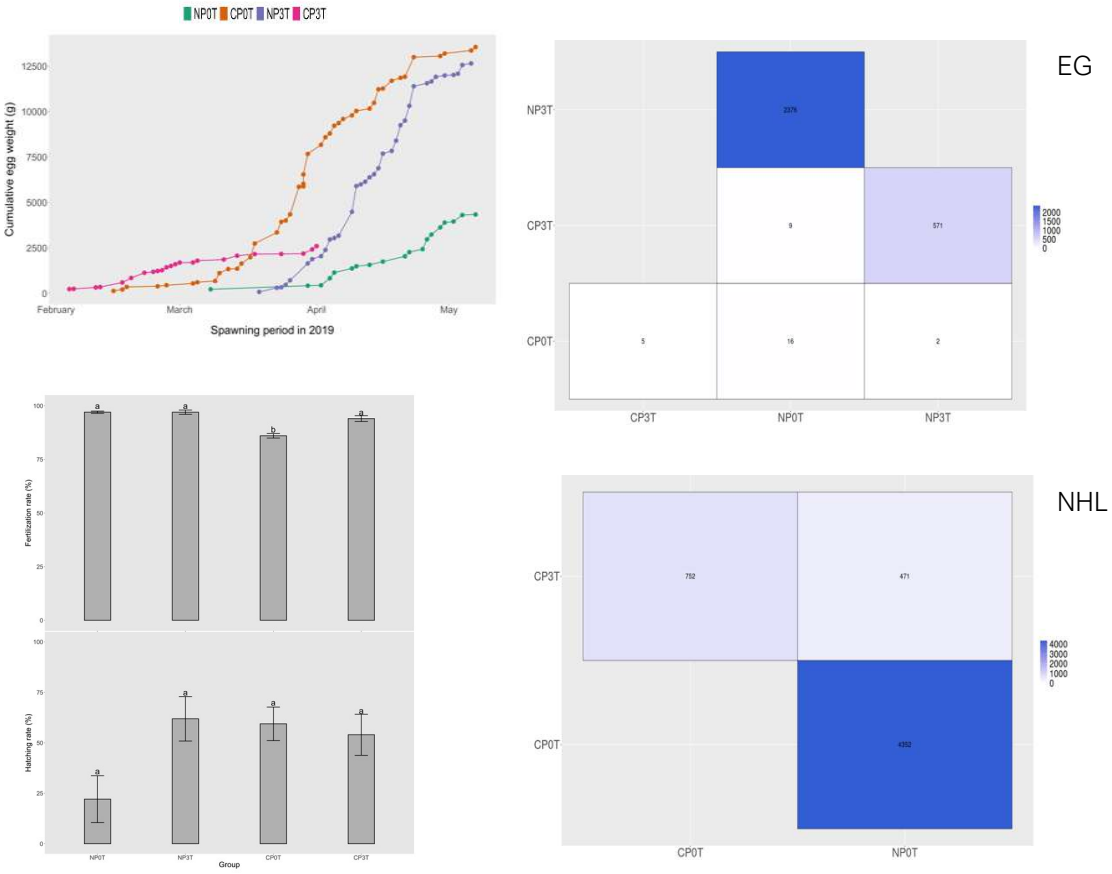

Figure 28. Relationship between morphological and molecular parameters as indicators of egg/embryo quality based on their characteristics in different treatment groups. Molecular parameters are the differentially expressed genes (DEGs) during early gastrula and newly hatched larvae stages.

There were four treatment groups (NP0T, CP0T, NP3T and CP3T) that were based on photoperiod and temperature manipulations. From these treatment groups, sampling for egg quality was conducted, where unfertilized eggs, early gastrula and newly hatched larvae were collected to study gene expressions. Fertilization and hatching rates were also determined.

Numbers of egg batches and egg weights increased towards April and decreased later. Spawning started in the CP groups with higher number of batches in CP3T. While the number of batches decreased in CP3T, it increased in the NP groups but remained almost constant in CP0T until April after which they decreased. Cumulatively CP0T had the highest egg weights followed by NP3T. In general, there is advanced spawning in CP groups, and temperature elevation caused even earlier spawning in the CP, cumulative and total egg weights were highest in CP0T, but that in NP3T was close. Compressing the photoperiod resulted in advanced spawning and higher egg production, elevation of temperature in NP resulted in higher egg production. Compared to NP3T, the CP groups displayed lower fertilization and hatching rates. The lowest hatching rate was in NP0T and the highest was in NP3T.

There were clearer differences in gene expression between development stages than between the treatment groups. Between the development stages, the highest number of differentially expressed genes was within the comparison between unfertilized eggs and newly hatched larvae. Between the

treatment groups, gene expression within unfertilized eggs was not different. Gene expressions within early gastrula and newly hatched larvae however resulted in clusters of the samples from the different treatment groups.

There was aadvanced spawning in CP3T and highest cumulative egg weight in CP0T that was closely followed by NP3T. Lower fertilization and hatching rates in CP0T and CP3T compared to NP3T (CP3T had much lower HR and at EG it had larger number of DEGs with NP3T than CP0T with NP3T). Lowest hatching rate in NP0T (at early gastrula, NP0T and NP3T have highest number of DEGs). At NHL NP0T and CP0T (higher hatching than in CP3T and NP0T) had the largest number of DEGs, followed by the comparison between CP3T and CP0T. It is likely that there are positive correlations between the morphological parameters and the gene expressions. It is also highly likely that photothermal manipulations cause variations in gene expressions although this is yet to be confirmed with further analyses.

7. Assessment of the results and implementations.

Our study which is the first to integrate photoperiod and temperature control, contributes to the efforts of demonstrating hour environmental manipulations could be used as a management strategy to ensure a year-round availability of lumpfish juveniles for stocking in salmon cages for the control of sea lice.

1. Results from 6.1 - 6.4 has provided the basic knowledge of the reproductive biology and physiology of Lumpfish under varying environmental conditions (photoperiod and temperature) and increased the knowledge of the underlying mechanisms involved. This knowledge of basic reproductive biology of lumpfish has adopted by our industry partners in their breeding activities in their facilities.

2. Results from 6.3 & 6.4 showed that lumpfish broodstock can be photo-manipulated to spawn different times of the year and increase in temperature prior to ovulation can synchronize the spawning. Compressing the natural photoperiod resulted in temporal shifts in levels of sex steroids and advanced gonad development, leading to earlier spawning. This knowledge has been currently implemented by our industry partners AquaGen and Namdal Rensefisk.

3. Results from 6.5 showed that gonadal development of lumpfish (less than 50g to adults) can be monitored non-invasively and the gender can be identified with above 90% accuracy. This is a huge benefit and important tool for the breeding companies, in this case our industrial partner AquaGen, in maintaining a proper sex ratio (increased females) in their breeding program. Currently AquaGen has implemented this tool in their breeding program for lumpfish.

8. Main findings and deliverables

1. A detailed description of lumpfish reproductive biology has been described in terms of gonadal development using histological analysis and physiology using sex steroid development.

2. Demonstrated that spawning of lumpfish can be shifted using photomanipulation and spawning can also be synchronized by increasing the temperature prior to ovulation.

3. Developed a non-invasive gender identification protocol during juvenile stage (less than 50 g) to adults using the ultrasound. With this protocol male and female juvenile lumpfish can be separated at an earlier stage and this early-stage gender identification is important for breeding program. This early selection optimizes the use of the broodstock facility since fewer males are needed compared to females for egg production. Further maturation monitoring is useful for the timing of cryopreservation and egg production.

9. Dissemination

9.1. Conference Presentations

1. Puvanendran did a presentation at Sats Marint 2018 conference in Bergen. Title: Status of developing a selective breeding program for lumpfish.

2. Frank presented the results at the Ocean Week in Trondheim in May 2019.

3. Frank Mlingi et al. also presented the work from cylcobreed at the 7th International Workshop on the Biology of Fish Gametes in Rennes, France held from 2nd - 6th September 2019.

4. Frank Mlingi oral presentation of the results from the WP1 (experiment 1) at the European Aquaculture Conference in Berlin, Germany in October 2019. He won best student poster presentation award.

5. Maren Mommens et al. 2019. Non-invasive sex determination in lumpfish (Cyclopterus lumpus l.) using ultrasound technology. 7th International Workshop on the Biology of Fish Gametes, 3-6 Sept. 2019, Rennes, France.

6. Maren Mommens et al. presented a poster of the ultrasound results at the European Aquaculture Conference in Berlin, Germany in October 2019.

7. Puvanendran presented the project results at the Rognkjeks Forum (NCE Aquaculture) held in Bodø on 26 & 27 November 2019.

8. Frank did a presentation “Sexual maturation and its response to photoperiod and temperature manipulations in domesticated lumpfish females” at the Havbruk 2020 in June 2020.

9. Frank Mlingi oral presentation at the European Aquaculture Conference in Madeira, Portugal in October 2021.

9.2. Scientific Manuscripts (Planned)

1. Frank Mlingi et al. Sexual maturation of farmed lumpfish *Cyclopterus lu*mpus: Gonadal development and associated profiles of selected endocrine factors. Completed MS. Soon to be submitted to Aquaculture.

2. Frank Mlingi et al. A short-to-continuous photoperiod integrated with temperature elevation can synchronize and advance sexual maturation in Lumpfish (*Cyclopterus lumpus*, L. 1758). Completed MS. Soon to be submitted.

3. Frank Mlingi et al. A nine-month compressed annual photoperiod integrated with temperature elevation at the onset of final maturation advances sexual maturation and spawning in lumpfish (*Cyclopterus lumpus*, L. 1758) females. Completed draft MS available. To be submitted to Plus One.

4. Frank Mlingi et al. Temperature rather than photoperiod effects is stable in controlling sexual maturation in lumpfish (*Cyclopterus lumpus*, L. 1758) males. Completed draft MS available. To be submitted to Plus One.

5. Frank Mlingi et al. Ultrasonic imaging as a means of monitoring sexual maturation in lumpfish (*Cyclopterus lumpus*). Completed MS. Soon to be submitted to Aquaculture.

6. Frank Mlingi et al. Transcriptomic profiling of egg quality in lumpfish in relation to broodstock exposure to photothermal manipulations.

9.3. News articles

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4. In www.Fishfarmingexpert.com. Lumpfish researcher wins student prize in Berlin. 8 October 2019. https://www.fishfarmingexpert.com/article/lumpfish-researcher-wins-student-spotlight-award-in-berlin/

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