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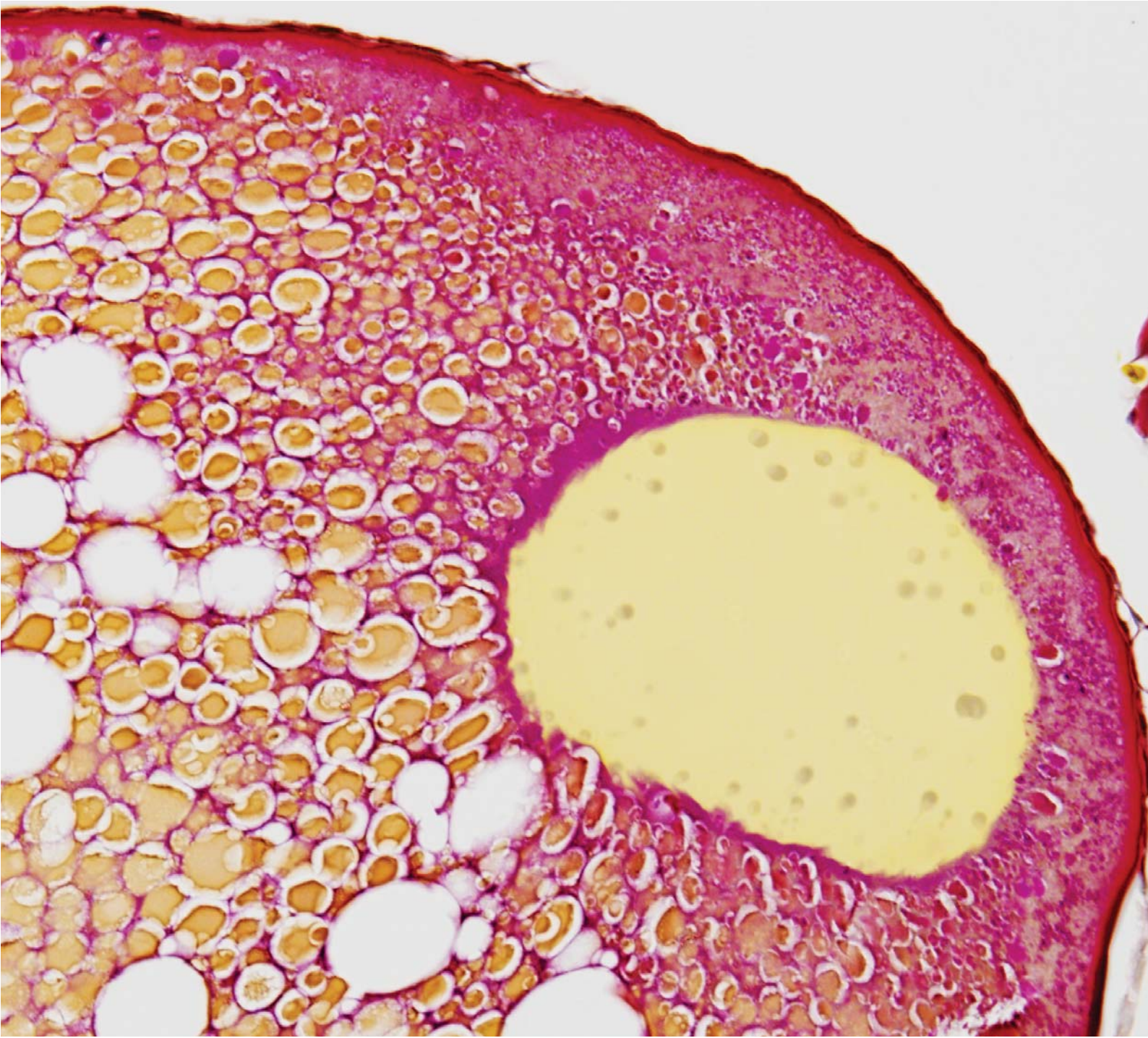

Reproductive Physiology of Female European Eel

PhD Thesis

Michelle Grace Pinto Jørgensen

Submitted: March 15, 2020

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Preface

The present thesis was submitted as partial fulfilment of the requirements for obtaining the joint Doctor of Philosophy (PhD) degree from Technical University of Denmark (DTU) and Norwegian University of Science and Technology (NTNU). The PhD project was performed at the National Institute of Aquatic Resources (host institute) and the Department of Biology at NTNU. The fellowship was awarded by DTU in support of the Nordic Five Tech Alliance collaboration on PhD education. In this context, the PhD studies were an integral part of two projects, namely "Eel Hatchery Technology for a Sustainable Aquaculture" (EEL-HATCH) and "Improve Technology and Scale-up Production of Offspring for European Eel Aquaculture" (ITS-EEL), which were financially supported by the Innovation Fund Denmark, grant no. 5184-00093B and 7076-00125B, respectively.

The PhD project was conducted between December 2017 and March 2020 under supervision of Dr. Jonna Tomkiewicz (principal supervisor, DTU Aqua), Professor Elin Kjørsvik (principal supervisor, NTNU), Professor Einar Eg Nielsen (co-supervisor, DTU Aqua) and Dr. Francesca Bertolini (co-supervisor, DTU Aqua). The experimental work was performed at the EEL-HATCH facility located at DTU Aqua in Hirtshals, within an integrated interdisciplinary team of researchers and industrial partners. Thus, the experiments provided an opportunity to conduct research as part of EEL-HATCH and ITS-EEL and thereby to generate knowledge to improve egg quality, as a step towards the overarching goal of closing the life cycle and creating a sustainable aquaculture of a critically endangered species, namely European eel.

External research stays took place at three locations: 1) NTNU, Trondheim, Norway, hosted by Professor Elin Kjørsvik, 2) School of Science, University of Greenwich, Medway Campus, Central Avenue, Chatham Maritime, Kent, United Kingdom, hosted by Senior Lecturer Joanna Miest, and 3) Laboratory BOREA, National Museum for Natural History, CNRS, Sorbonne University, Paris, France, hosted by Research Director Sylvie Dufour. The research stays at NTNU focused on planning and coordinating the studies of the PhD project and participating in courses relevant to the PhD project theme. The second research stay was at University of Greenwich, England. The tasks focused on learning to perform gene expression analyses using qPCR on collected samples and interpreting subsequent results. Finally, the third research stay with Sylvie Dufour and her research group that are acknowledged

for their expertise in molecular ontogeny and phylogeny was conducted to expand discussions on the endocrinology central to all studies in this PhD thesis.

During my PhD project, I completed three studies, each yielding a manuscript included in this thesis. These studies focused on induced vitellogenesis and ovarian development in European eel. Emphasis was on endocrine regulatory mechanisms between two key organs, ovary and liver, that advance oocyte development and vitellogenesis. The data were collected through experimental trials and analysed using an interdisciplinary methodology of molecular and morphological tools.

- **Paper I: Michelle G. P. Jørgensen,** Johanna S. Kottmann, Joanna Miest, Sylvie Dufour, Elin Kjørsvik, Jonna Tomkiewicz. Impact of carp pituitary extract at constant or increasing dose on ovarian development, expression of key genes and reproductive success in European eel, *Anguilla anguilla*. Submitted to *General and Comparative Endocrinology* (under review).
- **Paper II: Michelle G. P. Jørgensen**, Francesca Bertolini, Sylvie Dufour, Elin Kjørsvik, Jonna Tomkiewicz. Transcriptome profiles highlight activation of key ovarian functions during induced vitellogenesis in European eel. To be submitted to *Molecular Reproduction and Development.*
- **Paper III:** Francesca Bertolini, **Michelle G. P. Jørgensen**, Christiaan Henkel, Sylvie Dufour, Jonna Tomkiewicz. Unravelling the changes during induced vitellogenesis in female European eel through RNA-Seq: what happens to the liver? To be submitted to *PlosOne.*

In addition, I took part in a fourth study, linked to the theme of this PhD thesis, led by PhD student Johanna Sarah Kottmann (DTU Aqua) and contributed as co-author to the publication (included in her PhD thesis). This study focused on comparing salmon and carp pituitary extract hormonal treatments to induce ovarian development and vitellogenesis as well as evaluating subsequent effects on spawning success, egg quality and embryonic development partly through molecular changes. My contributions consisted of molecular laboratory work, including training in RNA extraction and execution of qPCR analyses as well as input to scientific discussions and developing the manuscript.

- Johanna S. Kottmann, **Michelle G. P. Jørgensen**, Francesca Bertolini, Adrian Loh, Jonna Tomkiewicz. Differential impacts of carp and salmon pituitary extracts on induced oogenesis, egg quality, molecular ontogeny and embryonic developmental competence in European eel. Submitted to *PLoS ONE.*

Through these studies, novel knowledge has been generated regarding female European eel reproductive physiology. Altogether, the studies expanded our understanding on endocrinology through the regulation of key genes during hormonally induced vitellogenesis in European female eels. Notably, the two studies (paper I and II) that investigated the changes in gene expression at the level of the transcriptome in the ovary and liver presented new insights into the reproductive physiology, not only in eel, but also generally in teleosts, which opens up for broader discussions on processes in and between the ovary and liver during reproductive development.

Kgs. Lyngby, March 15, 2020

Michelle Grace Pinto Jørgensen

English summary

The European eel is known for its complex life cycle and long migration from continental habitats in Europe and North Africa to their spawning area in the Sargasso Sea. Still, significant gaps in knowledge remain on their reproductive development, spawning and early life history. As for all vertebrates, gonadal development in fish is controlled through endocrine regulation on the brain-pituitary-gonad axis triggered by external and internal cues. In eels, a dual neuro-endocrine control on the brain-pituitary axis prevents sexual maturation in continental habitats. In captivity, this inhibition remains and consequently, farmed eels do not reproduce naturally. From a commercial perspective, this impedes closed cycle production and development of a sustainable aquaculture of this species that presently is critically endangered.

Nonetheless, gonadal development can be induced through hormonal treatments in captivity. For female eels, this is achieved using treatments with pituitary extracts from carp (CPE) or salmon. The extracts contain the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that stimulate the activity of two key organs, the ovary and liver, which are responsible for ovarian development and vitellogenesis. Furthermore, treatment with a maturation inducing steroid is needed to complete oocyte maturation and ovulation. Current hormonal treatments are suboptimal and the resultant reproduction success and egg quality are exceedingly variable. In this context, the focus of this PhD thesis was on studying characteristics of hormonally induced ovarian development and vitellogenesis in European eel with emphasis on the endocrine regulation in and between the ovary and liver. The thesis includes three interrelated experimental studies.

The objective of the first study was to expand insights into the endocrine regulation of induced ovarian development and vitellogenesis with focus on the ovary and liver, as well as subsequent reproductive success. Two commonly used CPE treatment protocols, using different doses (i.e. constant and a stepwise increasing), were compared to study potential dose dependent effects on the regulation of the developmental process. Results showed that both CPE treatments led to similar ovarian development and vitellogenesis as assessed biometrically and histologically, but rate of progression differed. A qPCR analysis showed that the expression of ovarian gonadotropin and steroid receptor genes, essential for gonad development, were upregulated during vitellogenesis using either CPE treatment and showed similar expression profiles regardless of CPE treatment dose. Correspondingly, the reproductive

success was not different between treatment groups. Three novel discoveries were made in this study: i) vitellogenin genes were expressed not only in hepatic tissue, but also in ovarian tissue, which indicated that the ovary is a secondary site of vitellogenin synthesis, besides the liver, ii) the receptor gene of FSH was expressed and upregulated in the liver, suggesting an FSH regulatory role here, and iii) heat shock protein 90 (Hsp90) was expressed and upregulated in the liver, which suggested a role in binding steroid receptors (a known Hsp90 function) during hepatic vitellogenesis. Altogether, these results increased our understanding on the complexity of vitellogenesis.

The objective of the second study was to elucidate transcriptomic responses to induced vitellogenesis in the ovary of CPE-treated eels. Changes in the ovarian transcriptome (the complete set on RNA in a sample) were elucidated using RNA-Seq, a high-throughput technique. RNA was extracted from ovarian tissue from eight female eels sampled prior to CPE treatment (week 0) and after nine weeks of CPE treatment given at a constant dose. During this period, the status of females changed from a previtellogenic to late vitellogenic developmental stage. RNA-Seq was performed to identify differentially expressed genes in ovarian tissue between females at these two stages. The analysis showed that key genes involved in processes such as vitellogenesis, steroidogenesis, vitamin uptake, ovulation and tissue growth were upregulated, while genes linked to early oocyte development were downregulated. Thus, biological processes and pathways regulated during induced ovarian development paralleled expression patterns observed in other teleosts. In accordance with study 1, genes for vitellogenins were highly upregulated, thus confirming that the ovary besides the liver likely is a site of local vitellogenin synthesis in eel.

The objective of the last study was to unravel the role of the liver during induced vitellogenesis in the same female eels as in the second study. The liver is essential for vitellogenesis, as it produces vitellogenins through sex steroid action. Moreover, the liver has an important role in reallocating resources for energy metabolism as well as for reproductive development. This is an effect of eels ceasing to feed in nature during silvering, when eels enter their migratory phase. These fasting conditions are simulated during induced vitellogenesis. In this study, RNA-Seq was performed using RNA extracted from livers to compare transcriptomes of female eels sampled in week 0 and week 9. Results showed that the liver grew in size during induced vitellogenesis, following a growth pattern reported in other female teleosts during reproductive development, however different from a typical pattern for fasting. At the molecular level, upregulated genes were mainly involved in processes related to energy management biosynthesis, transport and reproduction, whereas downregulated were linked to developmental processes, organ maintenance and the immune system. Within

the reproductive biological processes, pathways related to steroid production were upregulated. The analysis detected steroid production genes that have not previously been investigated in eel, such as lectin, mannose binding 1 (*lman1*) and nuclear protein 1 transcriptional regulator (*nupr1*). These findings may be important as benchmark for further investigations on hepatic vitellogenesis.

In conclusion, the three studies expanded our knowledge on the endocrine regulation during induced ovarian development and vitellogenesis through experimental work and interdisciplinary methodologies with focus on the ovary and liver. Firstly, insights were substantiated as hormonal treatments led to regulation of known genes and biological pathway during reproductive development. Furthermore, novel information about genes involved in reproductive processes in the ovary and liver expanded our understanding of the complex regulation in and between these two organs. Particularly, the new findings do not only present possibilities for further studies on the reproductive physiology in eel, but they also open up for broader discussions on the regulation of vitellogenesis in teleosts, as well as the applicability of high-throughput technologies in aquaculture research.

Dansk resumé

Den europæiske ål er kendt for sin komplekse livscyklus og lange migration fra kontinentale habitater i Europa og Nordafrika til deres gydeområde i Sargassohavet. Alligevel er store dele af deres livscyklus stadig ukendte inklusiv deres reproduktion, gydning og tidligste livsstadier. Som for alle hvirveldyr, er den reproduktive cyklus hos fisk kontrolleret af hormoner fra det endokrine system (hjerne-hypofyse-gonade-aksen), som desuden er under påvirkning af ydre og indre faktorer. Hos ål forhindres kønsmodningen i kontinentale habitater imidlertid af en dobbelt neuroendokrin kontrol på hjerne-hypofyse-aksen. Denne hæmning forhindrer også kønsmodning og naturlig formering i fangenskab. Fra et kommercielt perspektiv hindrer det en lukket cyklus produktion, samt udvikling af en bæredygtig akvakultur af denne art, som nu er kritisk truet.

I fangenskab kan udviklingen af gonader imidlertid induceres gennem hormonbehandling. Hos hunål opnås dette gennem behandling med hypofyseekstrakter fra karpe (CPE) eller laks. Ekstrakterne indeholder gonadotropinerne, follikelstimulerende hormon (FSH) og luteiniserende hormon (LH), og erstatter virkningen af fiskens egen hypofyse. Hormonerne stimulerer aktiviteten i to nøgleorganer, ovariet og leveren, og dermed follikeludviklingen og vitellogenesen. Dertil kommer en behandling med et modningsinducerende steroidhormon, som er nødvendig for slutmodning af oocyter, samt ovulation. De nuværende hormonbehandlinger er suboptimale, og den resulterende reproduktionssucces og ægkvalitet er derfor meget varierende. I denne sammenhæng var fokus i denne ph.d. afhandling at studere karakteristika ved hormoninduceret ovarieudvikling og vitellogenese hos den europæiske ål med vægt på den hormonale regulering i og mellem ovarie og lever. Afhandlingen omfatter tre sammenhørende eksperimentelle studier.

Formålet med det første studium var at øge indsigten i den endokrine regulering forbundet med induceret udvikling og vitellogenese med fokus på ovarie og lever, samt den efterfølgende reproduktionssucces. To almindeligt anvendte behandlinger med CPE med forskellig dosering (konstant dosis og trinvis stigende dosis) blev sammenlignet for at studere potentielle dosisafhængige effekter på reguleringen af udviklingsprocessen. Resultaterne viste, at begge CPE-behandlinger førte til sammenlignelig ovarieudvikling og vitellogenese, vurderet biometrisk og histologisk. Derimod forløb udviklingen med forskellig hastighed. En qPCR-analyse viste, at begge CPE-behandlingerne førte til en stigning i ekspressionen af gener for gonadotropin- og steroidreceptorer, hvilke er

essentielle for ovarieudvikling og vitellogenese. Ekspressionen viste også samme mønster for begge CPE-behandlingsdoser. I overensstemmelse hermed var reproduktionssuccesen ikke forskellig mellem CPE-behandlingsgrupper. I studiet, blev der endvidere gjort tre nye opdagelser: i) gener for vitellogenin blev som forventet udtrykt i levervæv, men også i ovarievæv, hvilket indikerer, at ovariet hos ål sekundært til leveren syntetiserer vitellogenin, ii) FSH-receptorgenet var udtrykt ikke kun i ovariet, men også i leveren, hvilket antyder en separat FSH-regulatorisk rolle her, og iii) genet for heat shock protein 90 (Hsp90) blev udtrykt og opreguleret i leveren, hvilket kan tyde på, at proteinet er involveret i binding af steroidreceptorer (en kendt Hsp90-funktion) under lever-vitellogenese. Tilsammen øger disse resultater vores viden om, samt forståelse af vitellogenesens kompleksitet.

Det følgende studium havde til formål at belyse den transkriptomiske respons i ovariet under CPE-behandling. Ændringer i ovarietranskriptomet, dvs. det komplette sæt RNA, kan belyses ved hjælp af RNA-Seq, en ”high-throughput” teknik. RNA ekstraheret fra ovarievæv fra otte hunål før CPE-behandling (uge 0) blev sammenlignet med væv efter ni ugers CPE-behandling ved konstant dosis. Gennem denne periode ændrede hunålene status fra pre-vitellogenese til et sent stadium i vitellogenesen. RNA-Seq blev udført for at identificere differentielt udtrykte gener i ovarievæv mellem hunner i disse to stadier. Analysen viste, at nøglegener involveret i processer såsom oocytudvikling, vitellogenese, steroidproduktion, vitaminoptagelse, ovulation og vækst af væv var opregulerede, mens gener relateret til tidlig oocytvækst var nedregulerede. Ekspressionsmønstre og reguleringen af de biologiske processer, som resultat af CPE-behandlingen, svarede således til observationer i andre teleoster. I overensstemmelse med det første studie viste analysen en stærk opregulering af genekspressionen for vitellogenin, hvilket støtter formodningen om, at ovariet har en lokal vitellogeninsyntese hos ål.

Formålet med et sidste studium var at udrede leverens rolle under den inducerede vitellogenese i de samme hunål som i det foregående studie af ovariet. Leveren er central for vitellogenesen, da den producerer vitellogeniner under påvirkning af steroider. Derudover har leveren også en vigtig rolle i reallokering af ressourcer til energimetabolisme og reproduktivudvikling. Dette er en effekt af, at ål ophører med at tage føde til sig, når de bliver til blankål, som er migrationsstadiet. Disse fasteforhold simuleres under induceret vitellogenese. I dette studie blev RNA-Seq udført for at sammenligne RNA ekstraheret fra levervæv af hunål, der blev prøvetaget i uge 0 og 9. Resultaterne viste, at leveren var vokset i størrelse under behandlingen, et vækstmønster som også er rapporteret for andre teleoster under reproduktivudvikling. På det molekylære niveau var opregulerede gener hovedsageligt involveret i processer relateret

til energi biosyntese, transport og reproduktion, mens nedregulerede gener var knyttet til udviklingsprocesser, organvedligeholdelse og immunsystemet. Inden for de reproduktive biologiske processer blev funktioner relateret til steroidproduktion opreguleret. Analysen påviste gener for steroidproduktion, der ikke tidligere er blevet undersøgt i ål, såsom lectin, mannose binding 1 (*lman1)* og nuclear protein 1 transcriptional regulator (*nupr1*). Disse opdagelser kan være betydningsfulde som benchmark i fremtidige undersøgelser af leverens vitellogenese.

Som konklusion udvidede resultaterne i de tre studier vores viden om den endokrine regulering under induceret reproduktivudvikling hos hunål ved hjælp af eksperimentelle forsøg og interdisciplinære analyser med fokus på ovariet og leveren. Først viste resultaterne at hormonbehandlingerne førte til en regulering af gener og biologiske processer, der er kendetegnende for den almindelige reproduktive udvikling hos teleoster. Samtidig bragte studiernes nye opdagelser ny information om gener involveret i reproduktive processer i ovarie og lever, hvilket har bidraget til vores viden om, samt forståelse af den komplekse regulering af og mellem disse to organer. Særligt introducerer disse studier ikke kun nye muligheder for undersøgelser af den reproduktive fysiologi hos ål, men åbner også for bredere diskussioner af den endokrine regulering hos teleoster, samt anvendelsen af ”high-throughput” teknologier i akvakulturforskning.

List of abbreviations

11-KT, 11-ketotesterone
20β-HSD, 20β-hydroxysteroid dehydrogenase
CPE, carp pituitary extract
DA, dopamine
DHP, 17α,20β-dihydroxy-4-pregnen-3-one
DOC, 11-deoxycorticosterone
E2, 17β-estradiol
FSH, follicle-stimulating hormone
FSHR, follicle-stimulating hormone receptor
GnRH, gonadotropin-releasing hormone
GnRHa, gonadotropin-releasing hormone antagonists
GtH, gonadotropin
H&E, hematoxylin and eosin
hCG, human chorionic gonadotropin
Hsp90, heat shock protein 90
IGF, insulin-like growth factor
LH, luteinizing hormone
LHR, luteinizing hormone receptor
MIS, maturational inducing steroid hormone
NGS, next generation sequencing
PAS, Periodic Acid-Schiff
PE, pituitary extract
qPCR, real-time polymerase chain reaction
RAS, recirculating aquaculture systems
RNA-Seq, RNA-Sequencing
SPE, salmon pituitary extract
TWGD, teleost whole genome duplication
VTG, vitellogenin

Synthesis

1. European eel

1.1. Life cycle

The anguillids are peculiar catadromous species, and among them the European eel (*Anguilla anguilla*) is well-known for its complex life cycle and enigmatic migration (Tesch, 1982). Their life cycle is divided into an oceanic and continental phase, each characterised by several morphologically distinct stages and interconnecting migration routes (Tesch, 1977; Figure 1). Thus, the European eel's life includes a 6000 km

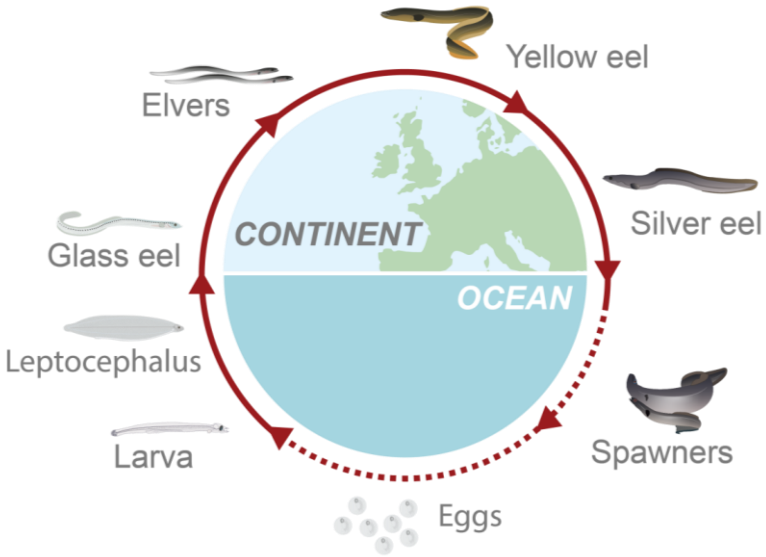

Figure 1. The life cycle of the European eel comprises a continental phase in Western European and North Africa and an oceanic phase during which they migrate to their spawning area in the Sargasso Sea. Graphic: Jeppe Hyttel Nedergaard, North Sea Science Park.

oceanic journey from continental freshwater habitats in Western Europe and Northern Africa to their native spawning area in the Sargasso Sea (Schmidt, 1923; Schmidt, 1925; McCleave, 2003; Miller et al., 2019; Figure 2). The eel's continental life focuses on foraging (Moriarty, 2003) and is the least elusive part of their life cycle, because their habitats are known, thus data collection is feasible. In contrast, exceedingly little is

known about their oceanic life stages, which involves their early life history, but also a key event, namely their reproduction. In fact, the eels' spawning area has only been established by discoveries of the earliest larval stages, while eggs, hatching larvae and adult specimens remain undiscovered (Schmidt, 1923; Schmidt, 1925; Tesch, 1977). Therefore, knowledge on the European eel's reproductive biology, both behaviour and physiology, is exclusively a result of experimental work.

Figure 2. The catadromous life of the European eel entails a long migration from continental habitats in Western European and North Africa to their spawning area in the Sargasso Sea. After spawning the hatched larvae will migrate to the continental habitats. Graphic: Jeppe Hyttel Nedergaard, North Sea Science Park.

The first stages of the eel's life take place in the ocean. Spawning and fertilisation of gametes are assumed to occur here leading to the development of embryos that eventually hatch as yolk-sac larvae. These early life stages have not yet been observed in nature; however, the appearance of late yolk-sac larvae and early feeding stages a century ago has delimited the spawning area in the Sargasso Sea (Schmidt, 1922, 1923, 1925). These larvae will develop into the characteristic leaf-shaped leptocephalus larval stage, unique to the Elopomorphs, and begin their migration towards the continental habitats (Mochioka, 2003; Otake 2003). Eventually, the leptocephali will approach

these habitats, whereupon they metamorphose into the first continental life stage, glass eels.

Glass eels are transparent, hence the name, yet as they travel through coastal areas and move toward continental habitats, they begin to gain pigment (Tesch, 1977; Tabeta & Mochioka, 2003). The pigmented juveniles are called elvers, and they inhabit lakes, rivers and coastal areas. As they grow in size, they develop into yellow eels, named after their characteristic pigmentation (Tesch, 1977; Moriarty, 2003). During the continental phase, eels forage and store energy as fat in the muscle, around the viscera and in the undeveloped gonads (Durif et al., 2009). Eventually, the yellow eels enter another transformation process called silvering (Aoyama & Miller, 2003). The name derives from the change of abdominal pigmentation from yellow to silvery, but the transformation includes many additional adaptations as they prepare for migration and return to their oceanic habitat (Boetius & Boetius, 1967; Tesch, 1977).

Despite the pigmentation change is the most conspicuous characteristic of silvering, significant morpho-logical and physiological adaptations to their ensuing oceanic life and migration take place (Sébert, 2003; Balm et al., 2007; Rousseau et al., 2009; Figure 3). Sensory systems are modified, including eyes, nostrils and lateral line system, which become more noticeable (Zacchei & Tavolaro, 1988; Durif et al., 2009). Internally, the swim bladder is modified (Durif et al., 2009, Sébert et al., 2009), and as the eels cease feeding while silvering the digestive system degenerates (Tesch, 1977). As a result, the liver becomes involved in the reallocation of fat and glycogen production necessary for the eels' survival during their long migration (Lewander et al., 1974; Durif et al., 2009). Physiologically, the osmoregulatory system is adapted to an increase in salinity, a presumed modification to migration in deeper waters (Fontaine, 1976; Durif et al., 2009). Finally, silvering enforces an endocrine barrier that prevents sexual maturation.

Figure 3. Silver eel. Photo credit: Sune Riis Sørensen, DTU Aqua.

While silver eels have slightly larger gonads compared to yellow eels, they remain pre-pubescent (Tesch, 1977; Durif et al., 2009). Indeed throughout silvering, sexually maturation and gonadal development are prevented at the brain-pituitary level by a dopaminergic inhibition (Dufour et al., 2005; Weltzien et al., 2009). Consequently, eels

cannot reproduce in their continental phase. To complete gametogenesis and gonad development, the inhibition must be removed at an unknown point along their migration or upon arrival at the Sargasso Sea, for spawning to commence (Dufour et al., 2003; Sébert et al., 2008). While hormonal induction of reproductive development in captivity has enhanced knowledge on the eels' reproductive development, the stimulus or stimuli that trigger sexual maturation in nature are entirely speculative (Weltzien et al., 2009).

The reproductive biology of European eel is not only enigmatic, but also challenging to study. During the past 20 years, there have been significant decreases in stocks of this species (Jacoby and Gollock, 2014), and the aquaculture industry's urge for a closed cycle production, has encouraged efforts into reproducing eels in captivity. However, protocols for captive breeding continue to be impeded by inadequate knowledge about the species' reproduction and early life stages. Consequently, domestication of the species has not yet been achieved.

1.2. Eel aquaculture and development of hatchery technology

Domestication of any species, aquatic as well as terrestrial, is essential for optimal farming and sustainable mass production. This is achieved through closed cycle production (Weber & Lee, 2014). However, for a number of teleost species, farming is presently capture-based, as wild-caught juveniles are used as the basis for rearing to a marketable stage (Ottolenghi, 2004). This method of farming has many disadvantages, such as unreliable supplies of juveniles as well as the inability to establish selective breeding programs. Closed cycle production is pertinent for European eel, a critically endangered species, where markets have been considerably reduced due to trade restrictions (CITES, 2007). Still, market prices for eel are high in Europe and Asia, as they are considered a delicacy. During the 1970-80s, resource efficient aquaculture technology was established using recirculating aquaculture systems (RAS) for outgrowing to a commercial size. The next step would be to establish hatchery technology, in order to change the production baseline from wild-caught glass eels to juveniles breed in captivity. This would meet customers' demands for sustainability, re-establish markets and enable selective breeding programs. Thus, a closed cycle production of European eel is of interest from a commercial perspective to expand production and markets, and it also serves the consumers' interest in a sustainable product. At the same time, it may contribute to the conservation of a critically endangered and ecologically important species (Tesch, 1982; van der Thillart et al., 2009).

With the aim of establishing a closed cycle production, researchers and industry have engaged in the development of hatchery technology for eel. In particular, insights into the reproduction and early life history of the Japanese eel (*Anguilla japonica*) and European eel have significantly been enhanced through a combination of experimental and field investigations (Aida et al., 2003; van der Thillart et al., 2009). Knowledge about all relevant life stages, their physiological requirements and interactions with the ambient environment is essential to establish breeding protocols, culture systems and practices (Tomkiewicz et al., 2019). Since the beginning of the 2000s, targeted research into the reproduction of European and Japanese eel as well as larval culture has increased information. This has advanced the field significantly, leading to the production of glass eels for the Japanese eel, while progress for the European eel continues to be challenged by the establishment of first feeding in the larval stage. However, improvements in all aspects of hatchery technology are still required, in order to obtain a sustainable and cost-efficient production of offspring.

Generally for anguillids, the first obstacle encountered in breeding is that they do not reproduce naturally in captivity, because they are arrested in reproductive development (Dufour et al., 2003; Sébert et al., 2008; Weltzien et al., 2009). Nevertheless, this can be counteracted through assisted reproduction using hormonal treatments to initiate and sustain gametogenesis (Mylonas et al., 2010). Experimental *in vitro* and *in vivo* studies have significantly expanded our understanding on the complex hormonal mechanisms that control eel physiology, yet assisted reproduction protocols are still necessary for breeding, and the subsequent egg quality remains variable. In view of this, the endocrine regulation of gametogenesis and gonad development continues to have relevance and it is an area, where valuable knowledge can be gained, particularly in relation to egg quality. Fortunately, the annotation of the eel genome (Henkel et al., 2012) has opened up for an array of molecular methodologies that can be used to fill critical gaps in knowledge within this field of research.

The present PhD thesis on the reproductive physiology of female European eel is part of the innovation projects “Eel Hatchery Technology for a Sustainable Aquaculture” (EEL-HATCH) and “Improve Technology and Scale-up production of offspring for European eel aquaculture” (ITS-EEL). Building on previous achievements, these projects aim at establishing larval production and hatchery technology targeting a closed cycle production of European eel in captivity. Within this framework, research targets different life stages and their requirements in captivity using a controlled experimental approach. The goal is to increase and fill gaps in knowledge in order to provide novel information, methodologies and technologies with focus on understanding eel biology and developing a sustainable production of European eel. The

approach and achievements in EEL-HATCH and ITS-EEL, as well as previous studies (Tomkiewicz et al., 2019), are summarised below.

One targeted research area is the production of broodstock feeds to meet the female nutritional requirements needed for producing eggs of high quality (Støttrup et al., 2013, 2016; da Silva et al., 2016). Protocols for hormonally induced gamete development as well as final follicular maturation have been tested; however, protocols are still suboptimal, specifically in relation to farmed broodstock (Okamura et al., 2014; Tomkiewicz et al., 2019). Moreover, efforts into developing techniques for optimal strip-spawning and handling of gametes have allowed for successful fertilisation of eggs (Sørensen et al., 2013; Butts et al., 2014). Enhanced production of viable eggs and larvae has led to the establishment of protocols for incubation of embryos and culture of larvae. Within this field of research, light regimes (Politis et al., 2014), microbial control (Sørensen et al., 2014), salinity (Sørensen et al., 2016; Politis et al., 2018a) and temperature (Politis et al., 2017; 2018b) have been investigated and standardised protocols developed. A major bottleneck is first feeding of larvae and factors affecting larval development and growth (Butts et al., 2016; Politis et al., 2018c). Hence, creating feed to sustain larvae to the first-feeding and finally glass eel stage is an essential step in establishing larval culture, growth into the leptocephalus stage and finally attaining the first glass eels for the European eel.

In spite of the progress in hatchery technology, optimising female hormonal treatments is a crucial focus area, because production of eggs of high and consistent quality persists to be an issue (Okamura et al., 2014; Tomkiewicz et al., 2019). Thus, fundamental knowledge on eel oogenesis, vitellogenesis and ovarian development is needed for optimising hormonal treatments and breeding protocols to achieve a predictable production of viable offspring.

2. Female reproductive biology

2.1. Reproductive strategies

Teleost fish species demonstrate an extreme variety of reproductive strategies (Smith & Wootton, 2015). Some species are semelparous and spawn only once in a lifetime. In contrast, most species are iteroparous, in other words, they have several breeding seasons during their lives (Murua & Saborido-Rey, 2003; Lowerre-Babieri et al., 2011). Furthermore, three distinct categories of ovarian development are recognised, explicitly synchronous, group-synchronous and asynchronous development (Murua & Saborido-Rey, 2003; Lowerre-Babieri et al., 2011; Lubzens et al., 2010). These development patterns also relate to spawning strategies, which differentiate females into batch spawners that develop oocytes and spawn several times during a season, and total spawners that release all eggs in one event (Murua & Saborido-Rey, 2003; Smith & Wootton, 2015). Thus, fish with synchronous oocyte development are total spawners, while fish with asynchronous development are batch spawners. Conversely, in group-synchronous development there are distinct cohorts of oocytes at different stages, which are consequently spawned in batches (Tyler & Sumpter, 1996; Murua & Saborido-Rey, 2003; Lubzens et al., 2010). Moreover, batch spawning species can have indeterminate or determinate fecundity (Lowerre-Babieri et al., 2011). The two types are separated based on whether oocyte recruitment is fixed at the onset of the reproductive season or if recruitment occurs continuously. These groups can additionally be divided into income or capital breeders depending on how the female provides energy to developing oocytes, where the former achieves this through continuous feeding, while the latter largely relies on stored energy in muscle or liver (Houston et al., 2007; Lowerre-Babieri et al., 2011).

Despite scarce information on anguillid reproduction, they are presumed to be semelparous and likely die after spawning. Often they have been described as total spawners, yet there is evidence that they are batch spawners with group-synchronous development (Suetake et al., 2003). Considering their long migration, there should be little doubt that anguillids are capital spawners with energy stored in the liver and around viscera (Durif et al., 2009). In fact, protein and lipid need to be allocated to the oocyte once the endocrine inhibition preventing sexual maturation is released, whereupon oogenesis and vitellogenesis naturally proceed (Böetius & Böetius, 1980; Adachi et al., 2003).

2.2. Endocrine regulation of oogenesis and vitellogenesis

Sexual maturation and reproduction in teleosts involve activation of neural pathways in the brain, which activate endocrine glands and hormonal regulation (Ho, 1987; Lubzens et al., 2010). This leads to development of gametes and eventually to spawning. For females, oogenesis is the development from oogonium to mature egg, and it involves primary growth that comprises previtellogenesis during which oocytes increase in size and often acquire lipid droplets as well as cortical alveoli, a second growth phase that entails steroid-driven vitellogenesis and massive growth, followed by final oocyte maturation and subsequent ovulation (Tyler & Sumpter, 1996; Patiño & Sullivan, 2002; Lowerre-Babieri et al., 2011).

Early in the female's life, the gametes are present as diploid oogonia in the ovaries. When the oogonia initiate meiosis, they enter the chromatin nucleolus stage and are then referred to as oocytes (Lowerre-Babieri et al., 2011). In primary growth, meiosis is temporarily arrested in prophase I in the oocyte that becomes surrounded by epithelial cells and undergoes ooplasmic growth (Tyler & Sumpter, 1996).

In the primary growth phase or previtellogenesis, the oocytes are characterised by the presence of a central germinal vesicle and the aforementioned epithelial layer composed of two cell types, namely inner granulosa and outer theca cells (Tyler & Sumpter, 1996). These changes are observable at a microscopy level, for instance through the use of histology or light microscopy (Lowerre-Barbieri, 2011). The oocytes grow in this phase and in many pelagic spawning species lipid droplets begin to appear in the ooplasm (Kagawa, 2013). As the oocyte develops, the lipid droplets enlarge, while concurrently accumulating and moving from the periphery of the oocyte membrane towards the nucleus (Reading & Sullivan, 2011). Previtellogenesis ends at the appearance of structures called cortical alveoli. Little is known about the synthesis and function of these structures. However, they have been associated with the Golgi apparatus and likely play a role in the impending fertilisation of the mature egg (Iwamatsu & Ohta, 1976, Selman et al., 1993). At this stage, the oocytes are ready to enter the secondary growth phase or vitellogenesis (Mommsen & Walsh, 1988), in response to environmental stimuli that activate neural pathways in the brain leading to the production of gonadotropin-releasing hormone from the hypothalamus (Donaldson et al., 1973; Ho, 1987).

Gonadotropin-releasing hormone acts on the pituitary gland initiating the secretion of two gonadotropin hormones known as follicle-stimulating hormone (FSH)

and luteinizing hormone (LH) that regulate gonad development (Donaldson et al., 1973; Yaron et al., 2003; Levavi-Sivan et al., 2010; Figure 4). The activation of the endocrine pathways results in commencement of vitellogenesis (Mommsen & Walsh, 1988; Yaron et al., 2003). This process involves receptor-mediated events on the brain-pituitary-ovary-liver axis, leading to production of vitellogenin in the liver, accumulation of yolk in the oocyte and oocyte growth (Ho, 1987; Reading & Sullivan, 2011; Figure 5).

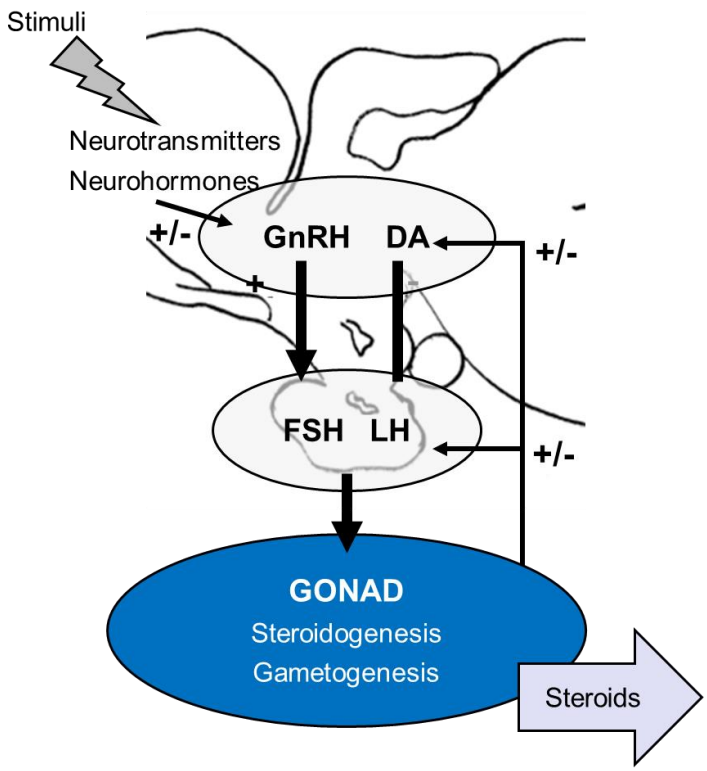

Figure 4. A schematic diagram of the brain-pituitary-gonad in fish, which controls gametogenesis. Internal and/or external stimuli activate neural pathways, which leads to the release of gonadotropin-releasing hormone (GnRH) from the brain. GnRH stimulates the pituitary gland to synthesise and release the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are transported to the gonad, whereupon they activate gametogenesis and steroidogenesis. The steroids are involved in the advancement of gametogenesis, but also in the regulation through feedback mechanisms on the brain-pituitary-gonad axis. In the brain, an inhibition by dopamine (DA) exists, which prevents the synthesis and release of FSH and LH from the pituitary gland.

In the early phase of vitellogenesis, FSH acts on its receptors (FSHR) in the oocyte resulting in the synthesis of the major estrogen 17β-estradiol (E2). E2 is produced by the catalyst aromatase in the granulosa cells of the oocyte from androgens (testosterone) supplied to the theca cells (Ho, 1987; Nagahama & Yamashita, 2008; Tokarz et al., 2015). While FSH induces the production of E2, LH and growth factors potentially regulate it (Patiño & Sullivan, 2002; Okuzawa, 2002). After production, E2 is transported via the blood to the liver, where it stimulates the synthesis of vitellogenins, precursors to yolk proteins (Reading & Sullivan, 2011).

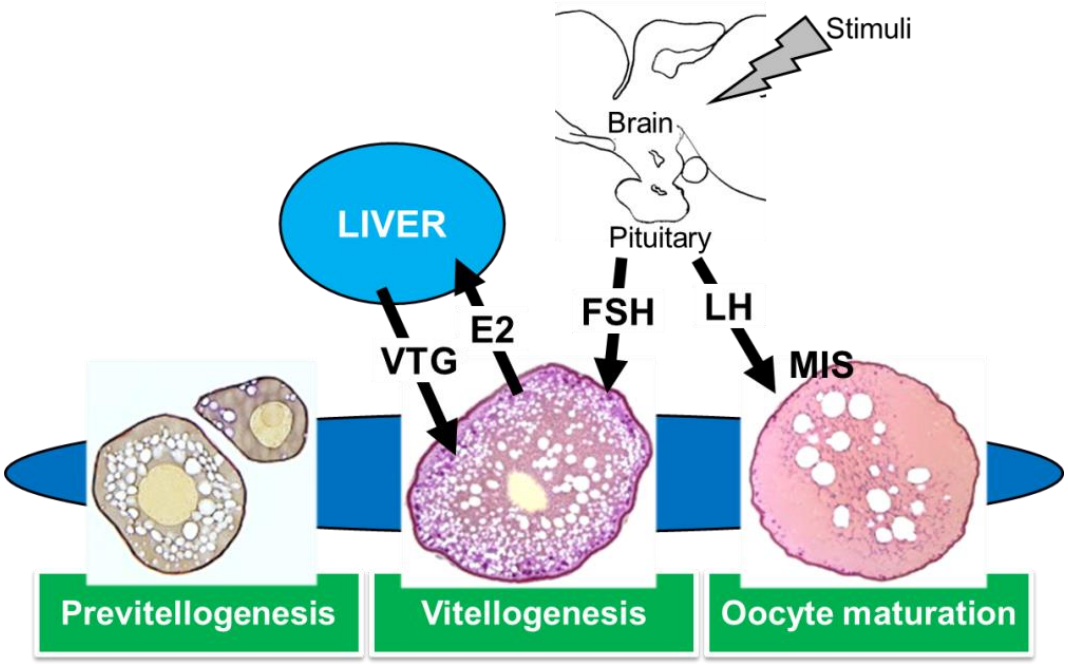

Figure 5. A schematic diagram of oocyte development in teleosts. During previtellogenesis meiosis is arrested. Internal and/or external stimuli stimulate the pituitary gland via neuro-endocrine action, which leads to synthesis and release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are transported to the ovary, whereupon they activate vitellogenesis and steroidogenesis. FSH binds to its receptors in the follicle and stimulates the synthesis of 17β-estradiol (E2), which is turn is transported to the liver through the blood. In the liver, E2 stimulates the synthesis of vitellogenin (VTG) that is transported via the blood to the oocyte, where it is endocytosed, processed and incorporated as yolk. LH binds to its receptor in the follicle leading to final oocyte maturation through the production of maturational inducing steroids (MIS) and resumption of meiosis. In final oocyte maturation, the nucleus migrates from the centre of the ooplasm to the animal pole and breaks down. Ultimately, the oocyte is released from its follicle; thereby, the mature egg is ovulated.

In the liver, E2 binds to nuclear estrogen receptors (Reading & Sullivan, 2011), which in teleosts exists as two subtypes, ESR1 and ESR2, and three in Actinopterygii (the ray-finned fish), ESR1, ESR2A and ESR2B (Nelson & Habibi, 2013). The liver's responsiveness to E2 is likely enhanced by other estrogens (Patiño & Sullivan, 2002). Once bound to a nuclear receptor, E2 induces the transcription of vitellogenins (Ho, 1987). The rate of hepatic synthesis depends on vitellogenin, which is found as two or more types in fish, and its sensitivity to E2 (Reading & Sullivan, 2011). After translation and post-translation modifications, vitellogenin is packaged in vesicles and secreted into the bloodstream (Mommsen & Walsh, 1988; Ho, 1987; Reading & Sullivan, 2011). Thereby, vitellogenin are transported from liver to ovary.

In the ovary, vitellogenin is selectively taken up from the capillaries by endocytosis via membrane vitellogenin receptors located in the granulosa cells (Reading & Sullivan, 2011). Upon entering the ooplasm vitellogenin is processed into yolk proteins. Thus, yolk is formed and as it accumulates in the ooplasm, the oocyte enlarges (Cerdà et al., 2008; Palstra & van den Thillart, 2009; Reading & Sullivan, 2011). Simultaneously, in species with lipid droplets, amassing and growth of these contribute to the enlargement.

Besides being involved in the biosynthesis of E2, the androgens themselves have been observed to play a role in oocyte growth. It is well known that androgens, particularly 11-ketotestosterone (11-KT), are essential for spermatogenesis in males (Schulz et al., 2010). Nevertheless, the presence of 11-KT has also been observed in several teleosts during vitellogenesis (Kazeto et al., 2011). Indeed, the first observation was in wild New Zealand freshwater eels (*Anguilla dieffenbachii* and *Anguilla australis*; Lokman et al., 1998). While its exact role in vitellogenesis remains unclear, is has been implicated in oocyte growth and lipid accumulation in anguillids (Rohr et al., 2001; Matsubara et al., 2003; Lokman et al., 2007; Sudo et al., 2012) as well as upregulating the expression of FSHR (Setiawan et al., 2012). Additionally, insulin-like growth factors (IGFs), likely also play key roles in oocyte growth (Rohr et al., 2001; Lokman et al., 2007). The underlying mechanisms of the IGF pathway are also not clearly understood, yet they are suspected of being involved in ovarian steroidogenesis during vitellogenesis in teleosts (Kagawa et al., 1994; Maestro et al., 1997).

In the last stages of vitellogenesis, the concentration of LH increases in many teleosts as they approach final maturation, thus the hormone likely has an effect at this stage (Patiño & Sullivan, 2002; Swanson et al., 2003). At the brain-pituitary level a dopaminergic inhibition of LH exists. The extent of the dopaminergic inhibition is species-specific and is under control of environmental or internal stimuli (Dufour et al.,

2005, 2010; Levavi-Sivan et al., 2010). During vitellogenesis, this inhibition increases until immediately before final maturation. The regulation of dopamine occurs through a feedback loop on the brain-pituitary-gonad axis, where E2 levels increase the inhibition in many teleosts (Senthilkumaran & Joy, 1995; Saligaut et al., 1999; Levavi-Sivan et al., 2005). When the inhibition is reduced, LH is released from the pituitary gland and final maturation is initiated followed by ovulation of mature eggs (Levavi-Sivan et al., 2010). Furthermore, there is some evidence that dopamine also has an effect on FSH production, but more research is needed on this (Vacher et al., 2000).

As LH binds to its receptor in the follicle there is a switch from steroidogenesis of primarily E2 to the production of maturational inducing steroid (MIS) hormones (Nagahama & Yamashita, 2008). Interestingly, the production of MIS is unique to teleosts. Comparable to the role of aromatase, 20b-hydroxysteroid dehydrogenase (20β-HSD) catalyses the synthesis of MIS in the granulosa cells from derivatives of progesterone in the theca cells (Tokarz et al., 2015). Just as IGFs have an effect on oocyte growth, they also facilitate maturational competence of follicles, i.e. enhance their responsiveness to MIS; however, presently there is no evidence on their specific functional activity (Patiño & Sullivan, 2002). Similarly, activins have been observed to influence maturational competence (Pang & Ge, 2002).

MIS mediate their effect by binding to their membrane receptors, in turn activating the maturation promoting factor in the oocyte (Patiño & Sullivan, 2002). Thus, MIS resumes meiosis in the oocyte (Bobe et al., 2003; Kagawa, 2013). In final maturation, the nucleus changes conspicuously, as it migrates from the centre of the ooplasm to the animal pole, where it eventually breaks down (Lubzens et al., 2010, Kagawa, 2013). This ultimately leads to the release of the oocyte from its follicle, in other words, ovulation of the unfertilised egg. Before the mature oocyte is ovulated from its follicle, all visible structures disappear and hydration takes place (Lowerre-Barbieri, 2011; Kagawa, 2013).

Now spawning can commence as the ovulated eggs are released from the female (Cerdà et al., 2008). In nature, spawning is typically triggered by environmental stimuli, which can be biotic factors, behavioural, pheromonal, etc., or by internal cues, such as fluctuations in hormone levels (Peter & Yu, 1997; Levavi-Sivan et al., 2010). Contrarily in captivity, the fish are removed from these cues and as a result natural spawning may become problematic. This also applies to the onset of gonadal development, which in many species is inhibited (Mylonas et al., 2010; Weber & Lee, 2014).

3. Assisted reproduction

3.1. Role in aquaculture

Terrestrial farming has a long tradition of being a significant source of food for humans, in contrast to aquaculture, which has only begun to gain global importance in recent decades. However, the role of aquaculture and the number of species of interest is rapidly increasing (FAO 2012; Moffitt & Cajas-Cano, 2014). Not only can aquaculture reduce pressure on overexploited fish stocks, but it also has great potential for contributing to the growing requirements of global food production (Weber & Lee, 2014). To realise these potentials, domestication is essential for optimal farming and mass production.

3.2. Assisted reproduction technologies

Teleosts have numerous and diverse reproductive strategies in nature controlled by internal and external cues (Murua & Saborido-Rey, 2003; Levavi-Sivan et al., 2010). Although few teleosts are able to complete their life cycle in captivity without external intervention, such as common carp (*Cyprinus carpio*) and salmonids, or by mimicking spawning cues for instance by providing nesting substrates for crucian carp (*Carassius* sp.) or channel catfish (*Ictalurus punctatus*), many species need assistance through hormonal intervention and handling (Bardach et al., 1972; Mylonas et al., 2010; Weber & Lee, 2014). Nonetheless, even with reproductive assistance, the resultant gamete quality is often low. Consequently, most fish farming remains capture-based to some degree by using wild broodstock to acquire higher offspring developmental success. In other cases, gamete quality is too low for successful offspring production and wild-caught juveniles are used as the basis instead (Ottolenghi et al., 2004; Weber & Lee, 2014). Capture-based farming is a major bottleneck in domestication; however, it may be reduced or even eliminated with assisted reproduction (Mylonas et al., 2010).

In captivity, most teleosts encounter reproductive dysfunctions (Bromage et al., 1992; Kagawa et al., 2005; Palstra et al., 2005; van Ginneken & Maes, 2005). Depending on species, these dysfunctions often arrest gamete development in previtellogenesis, vitellogenesis, final maturation or ovulation, but can also be failure to release mature gametes after successful gametogenesis (Bromage et al., 1992; Mylonas & Zohar, 2000; Zohar & Mylonas, 2001; Mylonas et al., 2007). As a result, breeding protocols need to be tailored to the species of interest to counteract their dysfunction at the appropriate reproductive developmental stage. To facilitate this, many assisted

reproduction approaches have been developed, resulting in an extensive array of options and products (Mylonas et al., 2010; Weber & Lee, 2014). In many cases, the dysfunctions can be solved with exogenous hormonal treatments to enable gametogenesis, while strip-spawning can be used to release mature gametes.

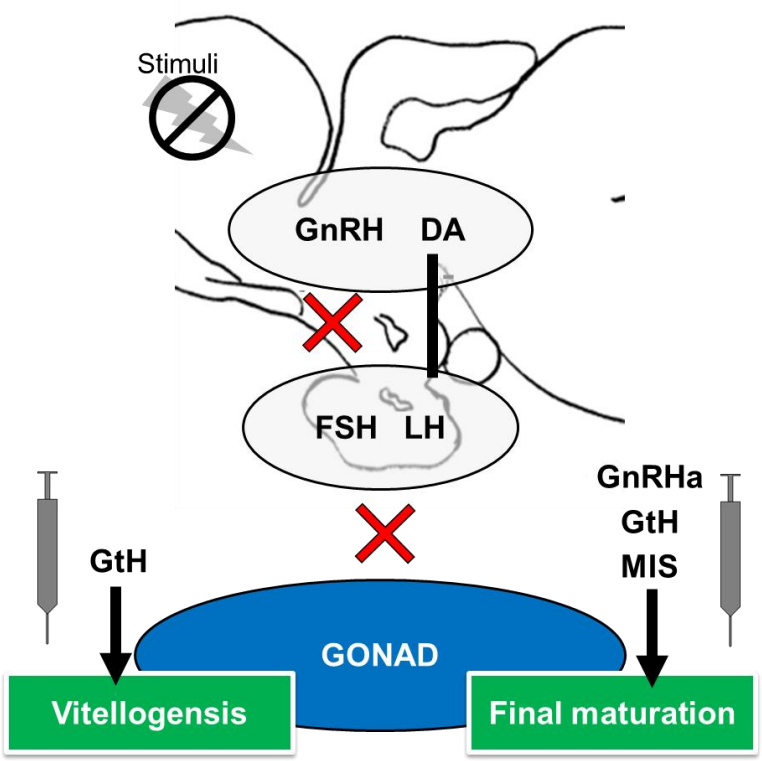

Figure 6. A schematic diagram of reproductive dysfunctions encountered in fish cultures and the exogenous hormonal treatments that can be applied. In captivity the internal and/or external stimuli that induce gametogenesis are not present, which often prevents captive reproduction. Dopamine (DA) typically inhibits the pituitary gland from synthesising and releasing the gonadotropins (GtH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Induction of vitellogenesis can be achieved using exogenous GtH, while final oocyte maturation can be induced using gonadotropin-releasing hormone antagonists (GnRHa), GtH and maturational inducing steroids (MIS).

Most females in captivity initiate vitellogenesis, in fact, this issue primarily pertains to anguillids. Failure to initiate vitellogenesis is a result of a dopaminergic inhibition at the brain-pituitary level, which prevents the release of sufficient levels of

the gonadotropins FSH and LH (Chang & Jobin, 1994). In nature, gonadotropin-releasing hormone induces gonadotropin release from the pituitary gland (Levavi-Sivan et al., 2010). Alternatively, vitellogenesis can be triggered by administering gonadotropins exogenously, typically with pituitary extract from carp (CPE) as or salmon (SPE) for anguillids (Kagawa et al., 2005; Palstra et al., 2005; Mylonas et al., 2010; Figure 6). However, administering heterologous hormones is not optimal. Therefore, research into developing recombinant gonadotropins has been conducted for example for female Japanese eels (Kamei et al., 2003; Kazeto et al., 2008). Lastly, gamete development needs to be sustained with regular injections in anguillids (Mylonas et al., 2010).

Failing to complete final maturation, is primarily due to insufficient LH release from the pituitary gland, since this hormone facilitates the secretion of MIS. In final maturation, the oocytes acquire maturational competence (Patiño & Sullivan, 2002), and failing to complete this phase can result in follicular atresia, i.e. breakdown of follicles (Weber & Lee, 2014). Therefore, single or multiple injections of SPE, CPE or human chorionic gonadotropin (hCG) are used in, for example, spotted sea bass (*Lateolabrax maculatus*) and pikeperch (*Sander lucioperca*), as they supply high amounts of LH (Donaldson, 1973; Lam, 1982; Donaldson & Hunter, 1983; Zohar & Mylonas, 2001; Mañanos et al., 2008; Figure 6). Alternatively, injections or implants gonadotropin-releasing hormone antagonists have been used in for instance turbot (*Scophthalmus maximus*) and yellowtail flounder (*Pleuronectes ferrugineus*) to actuate LH release from the pituitary gland (Mylonas et al., 1995; Mylonas & Zohar, 2000; Mylonas et al., 2010). For problematic species, such as anguillids, sustaining the level of gonadotropins is not always sufficient; consequently, final maturation and ovulation is triggered with exogenous MIS (Mylonas & Zohar, 2009; Weber & Lee, 2014).

Salmonids are examples of teleosts that successfully complete gametogenesis without external assistance (Bromage et al., 1992). However, both males and females are unable to spawn their mature gametes in captivity. In this case, gametes are strip-spawned and fertilisation is typically artificial (*in vitro*) to produce offspring (Zohar, 1988; Mylonas et al., 2010).

3.3. Assisted reproduction of eel

Current breeding protocols

Anguillids are among the most problematic species to breed in captivity, due to the dopaminergic inhibition as well as insufficient gonadotropin-releasing hormone (Dufour et al., 2003; Vidal et al., 2004). Despite these impediments, numerous studies have generated knowledge, which has advanced reproductive success substantially for the Japanese eel, but also considerably for the European eel (Okamura et al., 2014; Tomkiewicz et al., 2019). The template of breeding protocols is similar for all anguillids and involves both males and females. Spermatogenesis is successfully induced and completed with regular injections of hCG. Conversely, females are more problematic: 1) oocytes are locked in previtellogenesis unless vitellogenesis is initiated and sustained with regular injections of pituitary extract (PE), and 2) a final maturation protocol using exogenous MIS is required. Finally, strip-spawning is necessary to release mature gametes from males and females to produce zygotes.

Historical perspective

While reproductive success has been higher for Japanese eel, the initial investigations that elucidated features of the complex reproductive physiology of anguillids focused on European eel. Thus, the first experiments of European eel took place in 1930s, where Fontaine (1936) succeeded in inducing spermatogenesis in male eels using hCG from pregnant female urine. However, applying the same treatments on females proved unsuccessful. Three decades later, vitellogenesis in female eels was successfully induced and sustained with repeated injections of CPE given at a constant dose (Fontaine, 1964). Then in the 1980s, the first viable offspring were produced adapting the protocol of Fontaine, by using CPE administered at a stepwise increasing dose along with 11-deoxycorticosterone (DOC) as exogenous MIS (Bezdenezhnykh & Prokhorchik, 1983; Prokhorchik et al., 1986; 1987).

Around this period, Yamamoto and Yamauchi (1974) successfully induced vitellogenesis in Japanese eel with weekly injections of SPE. Despite previous attempts with CPE (Satoh et al., 1992), using SPE has been most successful in producing viable offspring following protocols by Yamamoto and Yamauchi (Yamamoto et al., 1974; Yamamoto & Yamauchi, 1974; Yamauchi et al., 1976; Yamauchi & Yamamoto, 1982; Yamauchi, 1990). To induce final maturation, injections of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) was implemented (Yamauchi, 1990; Ohta et al., 1996).

Besides European and Japanese eel, there have also been attempts at breeding other anguillids using adaptations of aforementioned protocols. Regularly injections of SPE are typically applied in protocols for the American eel (*Anguilla rostrate*; Oliveira & Hable, 2010) and the New Zealand freshwater eels (Lokman & Young, 2000).

Induction of gametogenesis

Despite many attempts at testing and optimising weekly PE injection protocols, success rates continue to be limited and egg quality highly variable (Okamura et al., 2014). Consequently, standard PE injection protocols have not been established for European eel and vary depending on the study. The basic principle is that PE injections are given regularly, typically once a week (Figure 7). Besides type of PE, usually SPE or CPE, the optimal dose and timing at which the regular injections are given continue to be explored in experimental protocols (Okamura et al., 2014). At present, experimental studies on European eel generally use a constant dose of CPE (Müller et al., 2003; Palstra et al., 2005; Pérez et al., 2011) or SPE (Pedersen, 2003; Tomkiewicz et al., 2019). While injections are commonly given once a week, Pedersen (2003) administered SPE twice a week and Palstra et al. (2010) administered four CPE injections weekly. Parallel with female induction and maturation, spermatogenesis is to this day induced with hCG in accordance with Fontaine's initial protocol from 1936.

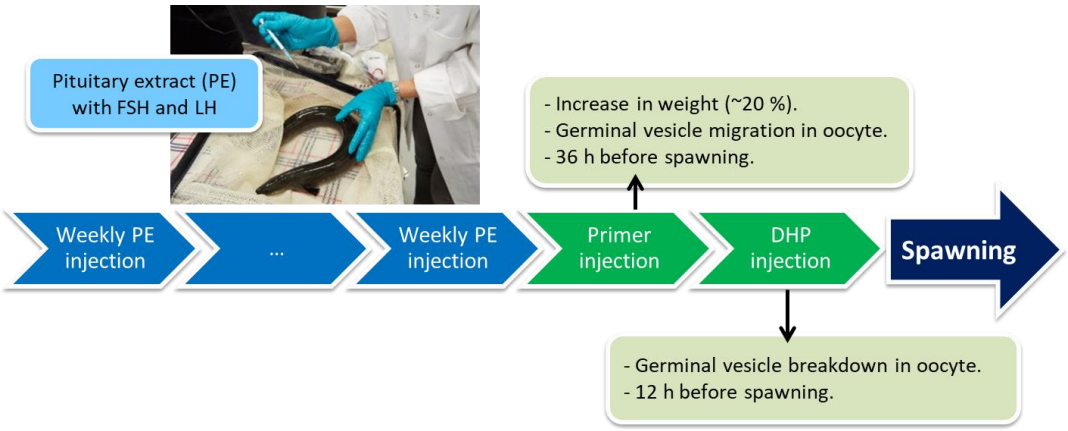

Figure 7. A schematic diagram of the assisted reproduction of European eel. To induce vitellogenesis weekly injections of pituitary extract (PE) are administered. Final maturation is induced with an additional PE injection, i.e. primer injection, followed by an injection of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) to induce ovulation. Photo credit: Sune Riis Sørensen, DTU Aqua.

Induction of final follicular maturation

After administering regular injections for a variable number of weeks, depending on the specific injection protocol, the females increase rapidly in weight and present signs indicating that they are approaching final maturation (Yamauchi, 1990; Ohta et al., 1996, 1997). Typically, they increase significantly in weight and through biopsies the oocyte stage can be determined (Figure 7). If the oocytes begin to show indications of germinal vesicle migration to the animal pole and breakdown, they have reached final maturation (Yamamoto et al., 1974; Yamamoto & Yamauchi 1974; Sugimoto et al., 1976; Palstra et al., 2005). However, weekly injections of PE seldom complete final maturation successfully and ovulation is not reached. Yet, this obstacle is overcome with a final maturation protocol. At this point, breeding protocols are less variable as final maturation is typically induced following the procedures first presented for the Japanese eel (Yamauchi, 1990; Ohta et al., 1996, 1997). Steps include a supplementary injection of PE, referred to as a primer injection, which sustains the ongoing oocyte development and prevents a potential halt. Depending on the oocyte developmental stage (Kagawa, 2003; Palstra et al., 2005), though typically 24 hours after the primer injection, an injection of MIS (usually DOC or DHP) is administered. Thus, final maturation is completed.

Strip-spawning of gametes and fertilisation

Approximately 12 hours after injection with MIS, ovulation proceeds and mature eggs are strip-spawned from females. Similarly, milt is stripped from males and used to fertilise eggs artificially, followed by incubation of zygotes to commence embryonic development and subsequent larval hatch (Tanaka, 2003).

4. Reproductive physiology of female European eel

The entire adult life history and reproductive biology of European eel remain entirely unknown in nature. Consequently, most aspects of the reproductive physiology of both male and female eels have been studied utilising hormonal induction of reproductive development. For females, knowledge particularly pertaining to the endocrine regulation of vitellogenesis and final maturation has accumulated during the past decades.

The impediment of natural sexual maturation in eel has been elucidated. It is a result of dual neuro-endocrine control, specifically a dopaminergic inhibition at the brain-pituitary level and insufficient gonadotropin-releasing hormone (Dufour et al., 2003, 2010; Vidal et al., 2004). This prevents the release of FSH and LH and as consequence impedes initiation of vitellogenesis. For oviparous vertebrates, vitellogenesis, oocyte maturation and ovulation advance through endocrine activity on the brain-pituitary-gonad-liver axis (described in Section 2.2.). In eels, this process can be initiated through PE injections (described in Section 3.3.), and the primary biological processes and pathways on this axis during induced development have been studied (Dufour et al., 2003; Weltzien et al., 2009).

Knowledge on the regulation of FSH is still scarce. Regarding LH, however, its release from the pituitary gland in eel is inhibited by dopamine and stimulated by gonadotropin-releasing hormone from the brain as for most teleosts (Dufour et al., 2003). In field studies, pituitary content of LH was observed to rise in European yellow eel and markedly increase at silvering (Marchelidon et al., 1999; Dufour et al., 2003). In captivity, PE treatment led to the synthesis of LH mRNAs in the pituitaries of European female eels as their ovaries developed and increased in size (Schmitz et al., 2005). Similarly, during induced ovarian development using sex steroids, LH synthesis increased in the pituitaries as a result of positive feedback mechanisms, particularly by E2 (Dufour et al., 1983a, 1983b, 1989). On the contrary, pituitary mRNA levels of FSH decreased with ovarian growth and treatment time (Schmitz et al., 2005).

PE treatments likely lead to exogenous FSH stimulating its receptors in the follicles, thus inducing E2 synthesis. In this context, such treatments resulted in increased plasma levels of E2 (Leloup-Hatey et al., 1986; Peyon et al., 1997). E2 will act on its receptors in the liver. Accordingly, genes for estrogen receptors have been observed expressed in the liver during induced development (Palstra et al., 2010; Parmeginani et al., 2015). E2 activity then actuates vitellogenin synthesis, congruent with detected expression of vitellogenin genes in the liver as a result of PE treatments

(Palstra et al., 2010; Parmeginani et al., 2015). Thereby vitellogenin plasma levels will increase, which was observed in female eels as a result of gonadotropin treatments (Peyon et al., 1997). In the ovary, vitellogenin is endocytosed into the developing oocytes through vitellogenin receptor activity (Reading & Sullivan, 2011). A new study has characterised a vitellogenin receptor in the liver of European eel (Morini et al., 2020). Altogether, induced vitellogenesis seemingly proceeds in accordance with the general theory described for teleosts in Section 2.2.

PE treatments have also been showed to increase testosterone levels (Peyon et al., 1997). Besides being processed into E2 by aromatase in the follicles, studies on New Zealand freshwater eel (Rohr et al., 2001; Lokman et al., 2007) and Japanese eel (Tosaka et al., 2010) have suggested that androgens have a direct functional role. Specifically, androgens have been linked to lipid droplet accumulation in the oocyte during previtellogenesis and vitellogenesis. Furthermore, findings in an *in vitro* study on Japanese eel hepatocytes suggested that androgens work synergistically with E2 in the liver to produce vitellogenin (Asanuma et al., 2003). However, characterisation of the expression of androgen receptors in the ovary as well as liver, require additional research.

Receptors facilitating vitellogenesis are of particular interest in anguillids, because they belong to the group of teleosts that went through whole gene duplication (TWGD), an event where several genes were duplicated (Hoegg et al., 2004). In this context, paralog genes of the LH receptor (*lhcgr1* and *lhcgr2*) were discovered in European eel (Maugars & Dufour, 2015). Additionally, estrogen receptor paralog genes have been characterised, specifically of the nuclear sub-type ER2 (*esr2a* and *esr2b*) as well as the membrane receptor (*gpera* and *gperb*; Lafont et al., 2016). The precise functional roles of the paralogs are not known, but they are likely linked to vitellogenesis as the genes are expressed during this phase (Lafont et al., 2016; da Silva et al., 2018). Two androgen nuclear receptor paralog genes (*ara* and *arb*) have also been discovered in European eel (Peñaranda et al., 2014). Altogether, several paralog receptor genes have been characterised, but their functional roles and regulation in the ovary and liver during vitellogenesis need further clarification.

Besides reproduction, the liver is of interest during vitellogenesis from a metabolic and energetic perspective. Vitellogenesis likely takes place while the eels migrate to the Sargasso Sea, at which point they cease feeding (Tesch, 1977). Similarly, during hormonally induced maturation in captivity, eels will not be fed to simulate the natural circumstances. Therefore, controlled experiments allow for investigations into the role of the liver concerning energy metabolism and allocation of reserves that would

occur while migrating long distances (Lewander et al., 1974; Durif et al., 2009). In-depth studies using high-throughput molecular techniques, such as transcriptomics, could reveal physiological changes that take place in the liver as well as on the axis between it and the ovary during induced vitellogenesis.

Transcriptomics has not been widely applied for anguillids. In fact, to our knowledge only five studies have been published concerning European eel: 1) an evolutionary study investigating TWGD in anguillids (Rozenfeld et al., 2019a), 2) on pituitaries of pre-pubertal female silver eel (Ager-Wick et al., 2013), 2) on liver tissue in males before and after sexual maturation (Churcher et al., 2015), 4) on the effect of cold seawater treatment in male eels (Rozenfeld et al., 2019b), and 5) on ovarian tissue from a yellow eel, a pre-pubertal silver eel and a post-spawning matured eel (Burgerhout et al., 2016). The latter study on the ovarian transcriptomes compared immature and matured eels after spawning. However, information on the vitellogenic stage is still required to understand the regulatory mechanisms, which occur during induced ovarian development.

5. Methodologies

5.1. An interdisciplinary approach

The PhD project applied an interdisciplinary approach to study female responses to hormonal treatment using a synthesis of modern methodologies and knowledge integrated from other disciplines. In a controlled experiment, a comparative study on the effects of hormonal treatments was executed and it entailed regular tissue sampling for specific laboratory analyses. Initially, histology was used to expand knowledge on oocyte and ovarian development. Concomitant, molecular techniques, i.e. real-time polymerase chain reaction (qPCR) and RNA-Sequencing (RNA-Seq) were utilised to investigate the underlying regulation of vitellogenesis, oocyte growth and ovarian development. While the initial gene expression analysis using qPCR focused on specifically targeted ovarian and hepatic functions, RNA-Seq was applied to increase the number of investigated genes, which permitted the detection of key biological processes in the ovary and liver.

5.2. Histology

Histology is the study of biological tissues and cells on a microscopic level. As the structure of a tissue, cell and organelle is directly related to its function, histology can be used to understand the physiology of organisms (Bloom, 1952; Booth, 1983). The method employs histochemical stains to visualise different structures and components in tissues and is often used to study the cytological characteristics of different cell types. The resulting visualisation of microscopic structures is a valuable tool to examine the intersection of anatomy, physiology and molecular biology (Pawlina & Ross, 2018).

In studies on teleost reproductive characteristics, histology is typically used to accurately determine gonadal development, i.e. reproductive stage, of both males and females, which is required to assess for instance the reproductive strategy, timing of sexual maturation and reproductive cycle of fish populations (Lowerre-Barbieri et al., 2011). At the population level this information can be used to enhance stock assessments and sustainable management. Additionally, studying characteristics at the individual level can be an important tool in basic research aimed at understanding physiological processes, such as oogenesis. During oogenesis, oogonia and oocytes undergo significant morphological and cytological changes, such as increase in size as well as the appearance, enlargement, movement or disappearance of structures and organelles (Tyler & Sumpter, 1996; Lowerre-Barbieri et al., 2011). Simultaneously, the

stroma (connective tissue) of the ovary, abundantly supplied with blood capillaries, develops to support and sustain follicular development. Altogether, these adaptations can be used to differentiate oocyte and ovarian developmental stages in juvenile as well as adult females during their reproductive cycle.

The typical dyes utilised to visualise structures in tissues is hematoxylin and eosin, referred to as H&E stain. This method is quick, inexpensive and can reveal a considerable number of microscopic anatomical features (Booth, 1983; Titford, 2005). Hematoxylin is an alkaline dye that binds to DNA and RNA and as result stains the nuclei blue. In contrast, eosin is acidic and is used as a counterstain that binds to protein-rich structures, such as muscle fibres, collagen and red blood cells, and dyes them in a pink/red shade. The combined effect of the two dyes is that other structures are visualised in different shades between blue and pink (Figure 8A). H&E staining has limitations, because not all structures contain DNA, RNA or protein and are consequently not stained. This is the case for lipid droplets and cortical alveoli. The appearance of these cytological structures are linked to specific phases of primary growth in oogenesis, and are therefore used to accurately determine gonadal development in several teleosts, including eel (Lowerre-Barbieri et al., 2011). Since H&E staining does not differentiate lipid droplets or cortical alveoli visually, attempting to determine developmental stages in primary growth can be problematic. Nonetheless, staining methods with other dyes targeting additional molecules can be employed to overcome this issue (Alturkistani et al., 2016).

Periodic Acid-Schiff (PAS) is a three step staining method that uses periodic acid solution 0.5%, Schiff's reagent, Weigert's hematoxylin and metanil yellow. The method was developed to differentiate structures with a high amount of carbohydrate macromolecules, e.g. glycogen, glycoprotein and proteoglycans, found in, for example, connective tissues, mucus and basal membranes (Quintero-Hunter et al., 1991). Carbohydrate macromolecules are stained magenta when they react with Schiff's reagent, and by counterstaining Weigert's hematoxylin with metanil yellow, acidophilic components appear yellow or yellow-brown (Figure 8B). While PAS was aimed at visualising basal membranes, the technique also contrasts cortical alveoli, lipid droplets and yolk globules due to the additional step (Quintero-Hunter et al., 1991; Grier et al., 2009).

Altogether, histology allows for characterisation of the visible changes in developing oocytes. Combining this information with gene expression analyses has the potential to reveal the underlying regulation of physiological processes linked to the cytological and morphological changes.

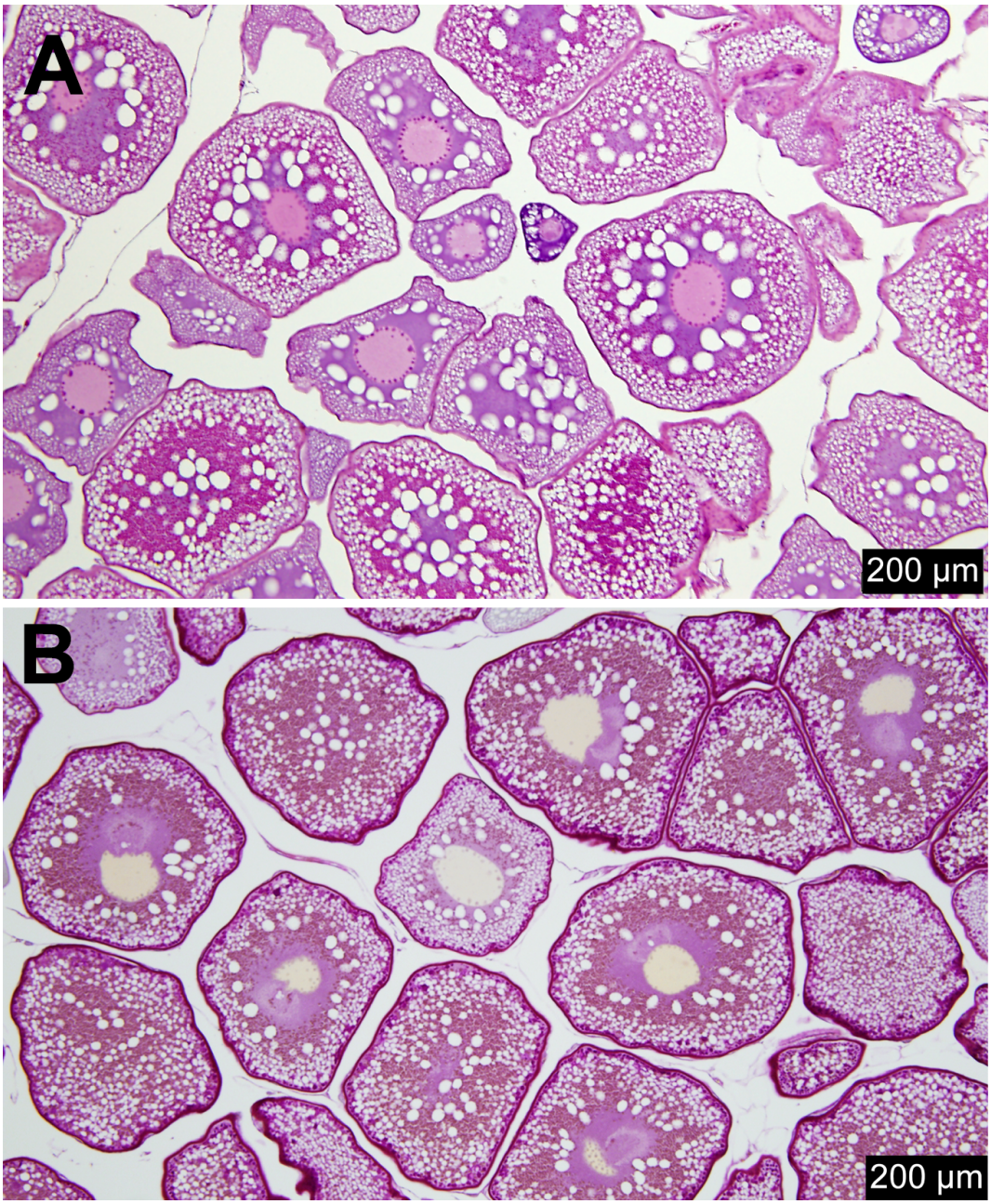

Figure 8. An overview of two histological staining techniques used to in ovarian tissue of two different female European eels at the same stage of ovarian developmental stage. A) H&E staining that uses hematoxylin (dyes DNA and RNA blue) and eosin (dyes protein red/pink). Cortical alveoli and lipid droplets are not stained and appear white. B) Periodic Acid-Schiff (PAS). Carbohydrate macromolecules are stained magenta due to a chemical reaction with Shiff's reagent and acidophilic components appear yellow or yellow-brown by counterstaining Weigert's hematoxylin with metanil yellow. Cortical alveoli appear magenta, lipid droplets are not stained and yolk globules appear yellow-brown. Photo credit: H&E, Jonna Tomkiewicz, DTU Aqua.

5.3. Molecular biology

Molecular biology is the study of the composition, structure and interactions of the cellular molecules DNA, RNA and proteins, which are involved in biological processes essential for the functions of cells, tissues and organs in an organism (Crick, 1970). The genome consists of DNA and is the genetic material of an organism (Kettman, 2001). The DNA is the template for the synthesis of RNA. The transcriptome comprises the complete set of RNA in an organism and represents the genetic activity, i.e. which genes are transcribed and at what level. The transcriptome includes coding RNA, called messenger RNA (mRNA), and a variety of non-coding RNAs. Generally, mRNA is characterised by a poly(A) tail (a tail of adenine bases) and is translated into protein. Furthermore, mRNA is the result of splicing, where non-coding regions of the gene sequence (the introns) are removed and the coding parts (the exons) are connected (Barciszewski & Clark, 2012). Non-coding RNAs have several regulatory functions, including modification of newly synthesised mRNA by small nuclear RNA (snRNA), transfer RNA (tRNA) that transfer amino acids to the ribosome during translation and ribosomal RNA (rRNA) that forms the ribosomes that translate the mRNA (Hombach & Kretz, 2016). Additionally, an increasing number of other non-coding RNAs continue to be identified and classified. These seem to have specific functions in regulating the development and physiology of organisms (Wang et al., 2018).

Measurements of gene expression can provide a partial or complete snapshot of the transcriptome in a cell or tissue, which can be used to investigate changes in genetic activity through comparative analyses. A common approach to this is to extract RNA from biological samples, followed by reverse transcription of RNA into complementary DNA (cDNA), a more stable molecule. The abundance of cDNA can then be measured using one of several methods available for gene expression quantification.

One method that has extensively been used to study gene expression is real-time polymerase chain reaction, also known as quantitative polymerase chain reaction (qPCR). This technique requires prior knowledge of the sequence of targeted genes, which limits its application. Analysis entails fluorescent dye or labelled probes to quantify the amount of PCR product (amplified cDNA fragments) in a sample (Arya et al., 2005). While the targeted fragment is amplified, the emitted fluorescence increases, and the abundance of targeted sequences can be quantified (Valasek & Repa, 2005). Recently, technologies such as the Fluidigm Biomark Dynamic Array have provided the option of quantifying a larger number of genes per analysis.

A recently developed technique allows high-throughput of gene expression analysis through next generation sequencing (NGS) that can process multiple DNA sequences in parallel (Heather & Chain, 2016). In the past, NGS was associated with high costs, but with time NGS techniques have become more efficient and affordable. In this context, RNA-Seq uses NGS to characterise and quantity RNA in a biological sample (Stark et al., 2019). In contrast to methods that use specific probes (such as qPCR) to detect transcripts, RNA-Seq is an untargeted approach (Chu & Corey, 2012). In other words, all transcripts can potentially be detected, regardless of whether their genomic sequences has been characterised (Wang et al., 2009). One of the most common RNA-Seq approaches, especially for non-model organisms, is mRNA-Seq, which focuses on mRNA. In mRNA-Seq, an enrichment of mRNA is performed after RNA extraction, typically using beads to capture the poly(A) tail of the mRNA. Then, sequenced fragments (reads) can be mapped to an existing reference genome with software that can split reads and locate them in the exons across the genome. If the quality of the reference genome is insufficient for the purpose of the study, the reads from RNA-Seq can be used for de novo sequence assembly. In other words, reconstruction of the sequenced transcriptome, wherein reads can then be mapped (Stein, 200; Chu & Corey, 2012). Whether reference-based or de novo, the number of mapped reads can be used for quantifying gene expression.

The availability of a preliminary assembly and annotation of the European eel genome (Henkel et al., 2012) has expanded the number studies investigating the expression of key genes during ontogeny. Presently, it has provided opportunities for high-throughput molecular investigations of gene expression at the transcriptome level to unravel regulation of biological processes in female European eel. Thereby, new information can be used to fill gaps in knowledge about the regulation of oogenesis, vitellogenesis and ovarian development.

6. Aims and objectives of the PhD thesis

6.1. Aims

The aim of this PhD thesis was to elucidate aspects of the reproductive physiology of female European eel by expanding knowledge on induced oocyte development, vitellogenesis and ovarian development. The primary focus was on the relationship between morphological and physiological changes associated with hormonally induced ovarian development with emphasis on the endocrine regulation in the ovary and liver, as both organs are involved in vitellogenesis.

The studies in this PhD thesis entailed design and completion of experimental work, application of interdisciplinary techniques for sample preparation and data analyses as well as bioinformatics. In the experiment, different hormonal treatment protocols were investigated in a comparative analysis. Samples collected from the experiment were then analysed utilising a synthesis of morphological and molecular methodologies in three studies to gain comprehensive understanding on the hormonal regulation of oocyte development and vitellogenesis as well as to observe indications of potential effects of assisted reproduction on egg quality.

6.2. Objectives

Study 1

The objective of the first study was to expand knowledge on the endocrine regulation of induced ovarian development and vitellogenesis in the ovary and liver, as well as to assess subsequent reproductive success. Two commonly used CPE treatment protocols, using different doses (increasing and constant), were compared to study potential dose dependent effects on the regulation of the developmental process. Female eels were randomly sampled before the onset of CPE treatments and at regular intervals during induced development. Ovarian development was assessed through biometry and histology at the morphological level, while expression levels of targeted genes encoding gonadotropin receptors, estrogen receptors, androgen receptors, vitellogenins and heat shock proteins were analysed using qPCR in both ovarian and hepatic tissues. Lastly, to study the reproductive success, fertilisation, hatch and deformity rates were analysed.

Study 2

The objective of this study was to elucidate transcriptomic responses of the ovary to induced vitellogenesis in female eels that received the constant CPE dose in study 1. Focus was on comparing two different ovarian developmental stages, specifically the previtellogenic and vitellogenic stage. RNA-Seq was performed on ovarian tissue sampled from females in these two developmental stages. A differential gene expression analysis, based on read counts that were mapped to the European eel reference genome, was used to identify key genes and biological processes influenced by the CPE treatment.

Study 3

The objective of the last study was to unravel the role of the liver during induced vitellogenesis in the same female eels as in study 2. The purpose was to elucidate the major biological changes that occur in the liver as a result of the CPE treatment with emphasis on vitellogenesis as well as fasting, as it is common practice to not feed eels during assisted reproduction. RNA-Seq data from hepatic tissue of female eels before induced ovarian development, i.e. in previtellogenesis, and in late vitellogenesis were used for a differential expression analysis and subsequent overrepresentation analysis. Thereby, key genes and biological processes linked to vitellogenesis and fasting could be identified.

7. Findings of the PhD thesis

7.1. Study 1

Impact of carp pituitary extract at constant or increasing dose on ovarian development, expression of key genes and reproductive success in European eel, *Anguilla anguilla*

Sexual maturation in European eel is prevented at the brain-pituitary level (Dufour et al., 2003; Vidal et al., 2004), which can be overcome by PE treatments to stimulate ovarian development and vitellogenesis (Mylonas et al., 2010). Thus, we compared two CPE treatments, specifically a constant dose and a stepwise increasing dose, administered weekly to farmed females in a controlled experiment (see experimental design in Figure 9). Firstly, we sampled ovarian and hepatic tissue before and at intervals during induced vitellogenesis to explore endocrine regulation, particularly through molecular mechanisms using qPCR. Corresponding ovarian samples were obtained to histologically record oocyte and ovarian development. Secondly, the females that were not sampled to study the advancement of induced ovarian development, continued treatments to final follicular maturation, ovulation and in vitro fertilisation to investigate reproductive success through fertilisation rate, hatch success and larval deformity rate.

Results showed that both CPE treatments led to similar ovarian development and vitellogenesis as assessed biometrically and histologically, but progression occurred at different rates. The expression of ovarian gonadotropin receptors (FSHR and LHR), essential for oocyte development and hepatic vitellogenesis were upregulated during CPE treatments. The steroid receptors, additional drivers of reproductive development, increased in expression levels in both the ovary and liver during induced development. Moreover, gonadotropin and steroid receptors showed similar expression profiles regardless of CPE treatment dose. Vitellogenin genes (*vtg1* and *vtg2*) were expressed in the liver at all sampling points with the highest increase in expression in week 9. In fact, it was the largest increase in expression out of 13 genes analysed in both the ovary and liver. Lastly, reproductive success did not differ significantly between treatment groups, in accordance with the similarities between CPE treatment groups observed during induced ovarian development and vitellogenesis.

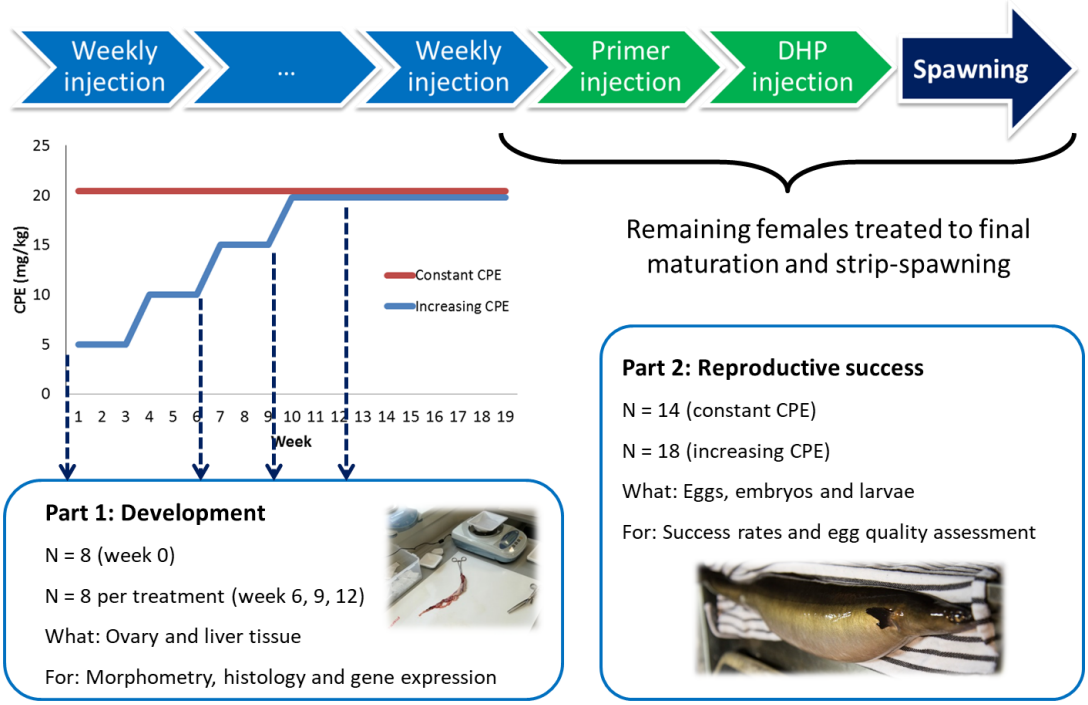

Figure 9. A schematic diagram of the experimental setup of study 1 on farmed female European eel. Part 1: Females were sampled in week 0, prior commencement of treatments. The experimental setup included weekly hormonal treatments with PE, during which females were regularly sampled (in week 6, 9 and 12). Part 2: Females that were not sampled in part 1, continued to receive weekly PE injections until they reached the stage of implementation of the final maturation treatment protocol (primer and DHP injection) to induce ovulation of eggs, which were then strip-spawned to allow for assessment of reproductive success. Photo credit: Sune Riis Sørensen, DTU Aqua.

In particular, three novel discoveries in this study have added to our fundamental understanding of the regulation of vitellogenesis as well as its complexity. Firstly, vitellogenin genes were expressed not only in hepatic tissue, but also in ovarian tissue, which indicated that the ovary is a secondary site of local vitellogenin synthesis, besides the liver. This suggestion was also based on the observed increase in vitellogenin expression as induced vitellogenesis progressed. Secondly, expression of the FSHR gene was upregulated in the liver, suggesting a FSH regulatory role here that needs further investigation. Finally, we found that heat shock protein 90 (Hsp90) was expressed and upregulated in the liver. Thus, Hsp90 may play a role in binding steroid receptors, a known function of this protein, during hepatic vitellogenesis. Altogether, these results bring new insights into the endocrine regulations of vitellogenesis, not only for eel, but for teleosts in general.

7.2. Study 2

Transcriptome profiles highlight activation of key ovarian functions during induced vitellogenesis in European eel

Changes in the ovarian transcriptome resulting from induction of vitellogenesis can be elucidated using RNA-Seq. This high-throughput technique expands the number of genes investigated simultaneously, and can thereby be used to generate new knowledge on the regulation of ovarian development.

A subset of samples originating from the experiment in study 1 was used in this study. The subset included extracted RNA from ovarian tissue sampled from eight female eels in week 0 prior to CPE treatment and after nine weeks of a constant dose CPE treatment (Figure 10). Histological criteria were used to document the stage of oocyte and ovarian development. RNA-Seq was performed on extracted RNA to identify differentially expressed genes between ovaries in a previtellogenic and late vitellogenic stage (sampled in week 0 and 9, respectively) and to detect genes and processes involved in oocyte and ovarian development.

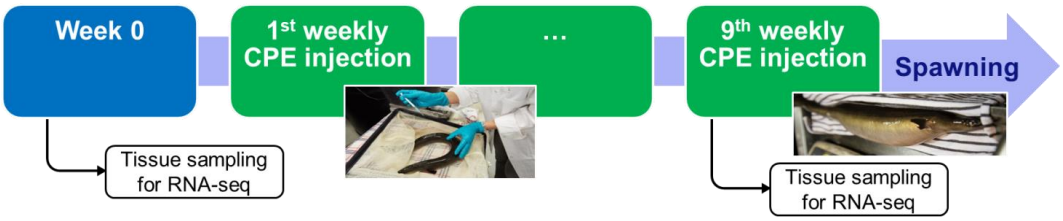

Figure 10. A schematic diagram of the sampling time points during the experimental trial of ovarian tissue from farmed female European eel for study 2. Photo credit: Sune Riis Sørensen, DTU Aqua.

In accordance with observed ovarian growth and progression of ovarian development, genes related to tissue growth, oocyte development, vitellogenesis and other ovarian functions were regulated from week 0 to 9. Specifically, genes related to early oocyte development were downregulated in ovaries in the late vitellogenic stage (week 9). Notably, *vtg1* and *vtg2* were the two most upregulated genes. This is in agreement with the findings in study 1, where these genes showed the largest change in expression, out of 13 genes analysed through qPCR, in the ovary. Other genes related to vitellogenesis were upregulated, including FSHR and LHR, one estrogen receptor, androgen receptors and genes related to steroidogenesis, e.g. aromatase, the catalyst that

synthesises E2 from androgens. Other genes related to oocyte development were also upregulated, including several activin receptors that enhance FSH synthesis in the oocyte, and IGF receptors and binding proteins, which mediate the effects of the IGF pathway. Genes for collagens and endothelial growth factors, related to tissue growth and angiogenesis, i.e. capillary network growth, were also upregulated, in accordance with observed ovary growth. A known vitellogenin receptor was downregulated, in agreement with previous observations (Perazzolo et al., 1999; Morini et al., 2020). Furthermore, aquaporins associated with osmoregulation after ovulation and retinol-binding protein 4-b linked to vitamin A uptake in the oocyte were upregulated.

Altogether, this study showed that CPE treatment activated and regulated a large number of genes and biological processes related to reproductive development in teleosts. This is the first time that RNA-Seq has been applied to elucidate changes during ovarian vitellogenesis in European eel, and the study has illuminated general effects of exogenous hormones introduced through PE injections. Finally, the analysis confirmed the expression and upregulation of vitellogenin genes in the ovary during vitellogenesis.

7.3. Study 3

Unravelling the changes during induced vitellogenesis in female European eel through RNA-Seq: what happens to the liver?

The liver plays a key role during oocyte development, as it is the producer of vitellogenin through E2 activity (Reading & Sullivan, 2011). Under natural conditions, eels cease feeding when they begin silvering and commence their migration (Tesch, 1977). To simulate this behaviour, eels are not fed during hormonally induced vitellogenesis. As a result, the liver has an additional role in reallocating resources to provide energy for swimming as well as in reallocating lipids, amino acids and other components necessary for gonadal development (Lewander et al., 1974; van Ginneken et al., 2005).

In this study, the analyses included a subset of liver samples originating from the experiment in study 1, using the same specimens as in study 2. Thus, the samples included RNA extracted from livers of female eels sampled in week 0 prior to treatment and after 9 weeks of CPE treatment. Liver growth was analysed in relation to ovarian development. As in study 2, an RNA-Seq analysis was performed on the RNA extract and data was used to identify differentially expressed genes in the livers of females before and after treatment, i.e. where ovaries were in a previtellogenic and vitellogenic stage, respectively.

Results showed that the liver grew during induced vitellogenesis. While an increase in liver size during vitellogenesis has been reported for several teleost species (Kaptaner et al., 2009; Jia et al, 2019), it is opposite expectations of fasting, where the liver would be expected to decrease in size (Larsen et al., 2001; Dias Junior et al., 2016). Thus, the increase in liver size is in accordance with expectations during vitellogenesis, but not with changes linked to food deprivation.

At the molecular level, upregulated genes were mainly involved in processes related to energy management biosynthesis, transport and reproduction, whereas downregulated were linked to developmental processes, organ maintenance and immune system (Figure 11). Within the reproductive biological processes, pathways related to steroid production were upregulated, specifically steroid metabolism, steroid biogenesis and response to estrogen stimulus. This is in agreement with the liver's role in the synthesis of vitellogenin (Ho, 1987; Reading & Sullivan, 2011). Moreover, *vtg1* and *vtg2* were the most upregulated genes in the liver. This is also consistent with findings in study 1, where these genes showed the highest increase in expression in the late

vitellogenic stage (week 9). Together with the vitellogenin genes, additional genes were grouped under the processes related to steroid production. This included genes not previously investigated in eel, such as lectin, mannose binding 1 (*lamn1*) that has been observed upregulated in the liver of E2 treated zebrafish (Levi et al., 2009) and nuclear protein 1 transcriptional regulator (*nupr1*). To our knowledge, there is no information on the involvement of these genes in fish reproduction. However, NUPR1 plays a role in the temporal expression of the beta subunit of LH during gonadotropin production in mice (Million Passe et al., 2008; Quirk et al., 2003).

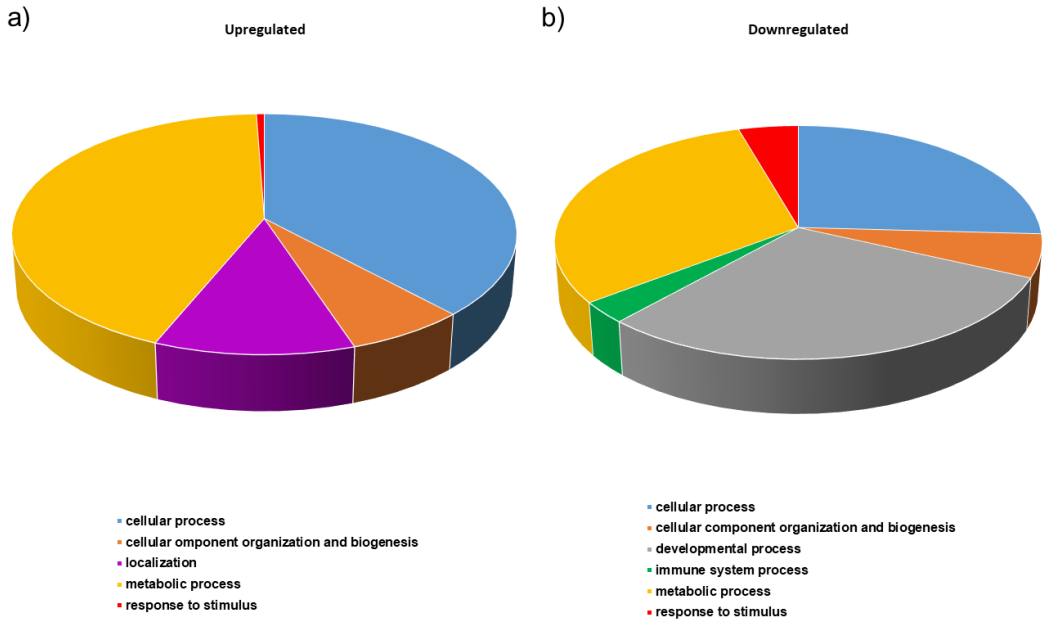

Figure 11. The proportion of regulated genes in European eel livers during induced vitellogenesis. Proportions represent significantly enriched terms belonging to a biological process, for upregulated and downregulated genes, with FDR less than 5%. Bertolini et al. (pg. 172).

Altogether, the results provide the first comprehensive overview (both at a phenotypical and at a high-throughput molecular level) of the changes that occur in the liver of European eel during induced vitellogenesis, where oocyte development occurs concurrently with fasting. The findings can be used as a benchmark for future investigations on vitellogenic processes.

8. Conclusions and perspectives

The three interlinked studies of this PhD thesis have increased insights into the reproductive physiology of female European eel by substantiated and expanded our knowledge on oocyte development, vitellogenesis and egg quality. The extensive experiment and regular sampling provided the basis for studying the progression of oogenesis meticulously. Through histology new details were added to the characterisation of oocyte development during previtellogenesis, vitellogenesis and final maturation. Furthermore, results from the molecular analyses have enhanced our knowledge about endocrine regulation and gene expression patterns in the ovary and liver during induced ovarian development and vitellogenesis. Altogether, the interdisciplinary approach used in this PhD thesis has allowed for one of the most comprehensive studies of vitellogenesis in European eel to date.

The consensus of the studies was that PE treatments to induce ovarian development in European eel led to observable changes at a morphological and molecular level. Oocytes showed progressive development, and both ovary and liver increased in size with time, indicating that oogenesis and vitellogenesis advanced as a result of the treatments. On a molecular level, expected regulation of specific genes and pathways essential for ovarian and vitellogenic functions was observed. For instance, gene expression of gonadotropin receptors increased in the ovary, while expression of steroid receptors increased in both the ovary and liver in correspondence with the observable development of the ovary and oocytes. Furthermore, vitellogenin expression was upregulated in the liver of females in the vitellogenic stage. In spite of the hormonal induction of vitellogenesis and ovarian development, the observed gene expression patterns in the three studies were comparable to observations previously made in other teleosts with and without hormonal induction.

Interestingly, novel discoveries introduced new perspectives on our knowledge of vitellogenesis as the ovary and liver may have more roles than previously presumed. One key finding in the qPCR analysis in study 1, was that vitellogenins were the most highly upregulated genes in the liver, but also in the ovary, in females in the vitellogenic stage. In agreement, through in-depth looks into the ovarian and hepatic transcriptome using RNA-Seq, vitellogenins were detected to be the most upregulated genes from previtellogenesis to vitellogenesis. Therefore, the consensus of the studies is that there potentially is a local production of vitellogenin in the ovary, a function which previously was assumed to be primarily hepatic. In the same line, the gene encoding FSHR was upregulated in the liver in study 1, suggesting that there is a direct FSH

regulation here besides in the ovary. In the same study, an upregulation of Hsp90 in the liver, led to the suggestion that this protein may be involved in vitellogenesis, because Hsp90 and other heat shock proteins are known to bind steroid receptors in the cell cytoplasm. Thus, our study opens up for further considerations and introduces new possibilities for future research into the endocrine regulation of vitellogenesis, not only in eel, but for teleosts in general.

An important consideration in these studies is that the PE treatment replaces the natural hormonal link between the pituitary gland and ovary on the brain-pituitary-ovary-liver axis. Therefore, studying the effects of this interference on the brain and pituitary gland would be of interest. An investigation into this may expand our understanding on the feedback loop on the axis. In connection with the findings in the presented studies, analysis of the activities on the feedback loop between brain, pituitary and ovary may add clarification as to why hormonal induction triggers vitellogenesis to the extent where it resembles expected regulation under natural induction.

The primary consensus in study 1 was that the two CPE treatments had similar effects on oogenesis, vitellogenesis and reproductive success. Explicitly, the gene expression of gonadotropin and steroid receptors were upregulated during vitellogenesis regardless of CPE treatment dose. Ultimately, the reproductive success of females did not differ between treatments and utilising a constant or increasing CPE dose both lead to viable offspring of similar quality. In spite of these results, there is a fundamental limitation when utilising PE treatments, as it introduces heterologous hormones into the eel. Thus, the likely species-specific requirements of European eel may not be fulfilled. Besides unknown gonadotropin levels, other substances could be present in CPE, which may affect endocrinology. An alternative treatment option is the use of recombinant hormones. Development of recombinant gonadotropins could be utilised to ensure saturation of required gonadotropin levels during development, which has been attempted for Japanese eel (Kamei et al. 2003; Kazeto et al., 2008). Additionally, optimising the response to PE treatments can potentially be achieved by applying steroid pre-treatment steps.

Pre-treatments can be applied to for instance enhance and synchronise development of oocytes. In species where oocytes develop asynchronously or in cohorts, final maturation occurs at intervals, and mature eggs are spawned in batches. Treating glass eels with E2 has been demonstrated to synchronise oocyte development and also to increase response competence to PE treatment (Tzchori et al., 2004; Ijiri et al., 2011; Okamura et al., 2014). Other studies on pre-treatments have examined the effect of androgens (11-KT) on oocyte development (Rohr et al., 2001; Matsubara et al.,

2003; Lokman et al., 2007; Sudo et al., 2012). In study 1 and 2 of this PhD thesis, the gene expression of androgen receptors in the ovaries indicated that 11-KT may have direct effect on oocyte growth in agreement with previous studies on Japanese (Matsubara et al., 2003; Sudo et al., 2012) and New Zealand freshwater eel (Rohr et al., 2001; Lokman et al., 2007). Furthermore, genes for androgen receptors were also detected expressed in the liver in study 1. In this context, an *in vitro* study on Japanese eel hepatocytes reported that 11-KT with E2 enhanced vitellogenin production, while 11-KT alone had no effect (Asanuma et al., 2003). Thus, treatment with 11-KT and E2 may have a synergistic effect on vitellogenesis in eel. Besides improving response to PE treatments, the role of 11-KT in oocyte development and vitellogenesis can be elucidated through gene expression analysis of androgen receptors as well as 11-KT responsive genes in the ovary and liver using techniques such as qPCR and RNA-Seq.

RNA-Seq facilitated the analyses of all expressed genes in ovary and liver, which increased our understanding of endocrine mechanisms of induced vitellogenesis in eels. The availability of a reference genome (Henkel et al., 2012) that can be used as template for reference-based RNA-Seq alignment offered opportunities, but also limitations in the analyses. One opportunity was the development of bioinformatics pipelines that provided comprehensive and accurate gene expression profiles in study 2 and 3. In the studies, the accuracy of these pipelines was validated with results from the qPCR analysis, but also intuitively from a biological perspective by identification of expected genes and expression patterns for biological processes. Nonetheless, the current genome assembly is highly fragmented. Consequently, several genes are split through different scaffolds, which has an effect on the *in silico* annotation. This had two major consequences in study 2 and 3: i) the mapping success was relatively low, and ii) the same gene may be mapped several times across the genome, which may result in a higher number of detected genes (not considering paralogs from TWGD) than the true number. A more complete version of the genome was recently reconstructed, but it is currently not available (Jansen et al. 2017). An alternative approach could be to create a reference transcriptome by de novo assembly with the same sequencing data analysed in study 2 and 3 and by using a similar bioinformatics methodology as in the RNA-Seq study by Rozenfeld et al. (2019a). Nevertheless, improvements in the European eel reference genome assembly and annotation would be beneficial for future molecular studies on European eel.

9. References

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Manuscripts

Paper I

Impact of carp pituitary extract at constant or increasing dose on ovarian development, expression of key genes and reproductive success in European eel, *Anguilla anguilla*

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Abstract

Successful captive breeding of European eel would allow for a closed cycle production. Yet, eels do not reproduce in captivity as gonad development is prevented at the brain-pituitary level. However, this can be overcome by treatments with fish pituitary extract (PE) to stimulate ovarian development. PE from carp (CPE) was administered weekly at constant or increasing dose to farmed broodstock females. The purpose was to investigate the effect of CPE dose on the endocrine regulation of induced ovarian development, vitellogenesis and subsequent reproductive success. Ovarian development was assessed through biometry and histology. At the molecular level, ovarian and hepatic expression of gonadotropin receptors (*fshr* and *lhcgr1*), estrogen receptors (*er1, er2a, er2b, gpera* and *gperb*), androgen receptors (*ara* and *arb*), vitellogenin (*vtg1* and *vtg2*) and heat shock proteins (*hsp70* and *hsp90*) was analysed through qPCR prior to and during development. In the remaining females, CPE treatments were pursued and follicular maturation induced by DHP to assess reproductive success by fertilisation, hatch and deformity rates. Results showed that both CPE treatments led to similar ovarian development and vitellogenesis, while progression occurred at different rates. Expression of ovarian gonadotropin receptors, hepatic vitellogenin as well as ovarian and hepatic steroid receptors were upregulated similarly by either treatment. Reproductive success was not significantly different between treatments. The gene expression analysis also revealed three novel findings: 1) vitellogenin expression and upregulation not only in the liver, but also in the ovary that suggests a secondary site of vitellogenin synthesis, 2) hepatic expression and upregulation of *fshr* that suggests a direct FSH regulatory role in the liver, and 3) hepatic expression and upregulation of *hsp90* that suggests a role of this protein in vitellogenesis.

Keywords

Oocyte histology; vitellogenesis; gonadotropin receptors; steroid receptors; heat shock proteins

1. Introduction

The European eel, *Anguilla anguilla*, is well-known for its catadromous life cycle. It entails immature eels migrating from continental habitats in Europe and North Africa to their spawning area in the Sargasso Sea and offspring drifting to the continents along oceanic currents (Schmidt, 1923; Munk et al., 2010). Furthermore, European eel is a high value species in aquaculture. Currently, eel farming is capture-based as wild-caught glass eels are used as basis for rearing to a marketable size. Albeit a closed cycle eel production is desirable, it is impeded by their complex life cycle. The first obstacle is successful captive breeding. Due to complex hormonal control mechanisms that relate to their migration, eels do not reproduce during their continental phase and neither in captivity. Specifically, sexual maturation and gonad development is hindered at the brain-pituitary level by insufficient gonadotropin-releasing hormone levels and dopaminergic inhibition, preventing the synthesis and secretion of gonadotropins (Dufour et al., 2003; Vidal et al., 2004). Consequently, present breeding protocols of eels in captivity utilise assisted reproduction to induce gametogenesis.

In fish, oogenesis is divided into primary and secondary oocyte growth. Primary growth (i.e. previtellogenesis) is characterised by arrest of meiosis, formation of an outer layer of follicle cells and lipid accumulation as droplets in the ooplasm (reviewed in Lubzens et al., 2010; Reading & Sullivan, 2011). The pituitary gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), induce secondary growth that leads to vitellogenesis (i.e. yolk formation in the oocyte) and sexual maturation (Dufour et al., 2003; Vidal et al., 2004). Comprehensively, FSH binds to its receptor and stimulates estrogen secretion, mainly 17β-estradiol (E2), from the follicle. In turn, E2 binds to its receptors in the liver resulting in production of vitellogenin. Vitellogenin is then transported to the ovary through the bloodstream and incorporated via vitellogenin receptors into the oocyte, where it is processed into yolk globules. These accumulate in the oocyte leading to massive growth (reviewed in Cerdà et al., 2008). Androgens also play key roles in oocyte growth both during the lipid droplet accumulation stage (Rohr et al., 2001; Lokman et al., 2007) and vitellogenesis (Asanuma et al., 2003). Once vitellogenesis is completed, production of progestogens, also known as maturational inducing steroid hormones (MIS), is stimulated by LH through its receptor in the follicle. MIS, such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP), reactivates meiosis ultimately leading to germinal vesicle breakdown and ovulation of the oocyte from its follicle (reviewed in Cerdà et al., 2008; Kazeto et al., 2011).

Assisted reproduction protocols have been established for inducing gonad development leading to viable egg and offspring production in eels, yet developmental success and

egg quality remain variable (Tomkiewicz et al., 2019). Repeated injections of pituitary extract (PE) from carp (CPE) and salmon (SPE) successfully initiate and sustain vitellogenesis as they contain the triggers, FSH and LH (reviewed in Mylonas et al., 2010; Okamura et al., 2014). Yet, injection protocols differ widely. Three parameters are often modified: type and dose of PE as well as time interval between injections (reviewed in Okamura et al., 2014). For European eel, maturation was first induced using a constant dose of CPE (Fontaine, 1964) and the first larvae were obtained using an increasing dose of CPE (Bezdenezhnykh & Prokhorchik, 1983; Prokhorchik et al., 1986, 1987). At present, studies on European eel typically use a constant dose of CPE (Müller et al., 2003; Palstra et al., 2005; Pérez et al., 2011) or SPE (Pedersen, 2003; Tomkiewicz et al., 2019). PE injections seldom resulted in successful final follicular maturation and ovulation, but this was solved with a final maturation step, which consisted of an additional injection of PE followed by an injection of DHP (Japanese eel, *Anguilla japonica*, Yamauchi, 1990; Ohta et al., 1997; European eel, Pedersen, 2003; Palstra et al., 2005).

Substantiating knowledge on PE induced ovarian development is essential to determine best practices, in order to produce eggs of predictable quality and offspring with high developmental success. Thus, our aim was to study effects of administering two different CPE protocols to induce vitellogenesis and ovarian development in farm-raised female broodstock of European eel. In a controlled experiment, a stepwise increasing CPE dose was compared with a constant dose. Since there is a progressive increase in gonadotropin production during vitellogenesis in other teleosts (reviewed in Lubzens et al., 2010), we hypothesised that females receiving an increasing CPE dose would have more physiological ovarian development, which in turn would improve egg quality. Effects of the treatments were assessed through biometric parameters and ovarian histology on eels sampled at various intervals. The underlying endocrinology was further elucidated through gene expression analysis of relevant hormone receptors and proteins in ovary and liver. Reproductive success was assessed through fertilisation rate, hatch success and larval deformity rate.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696) and fish were handled according to the European Union regulations

regarding protection of experimental animals (Dir 86/609/EEC). Efforts to reduce stress and handling of animals were implemented.

2.2 Fish husbandry and broodstock selection

European eels were raised from the glass eel stage at a commercial eel farm (Stensgård Eel Farm A/S, Randbøl, Denmark) in recirculating aquaculture systems (RAS) with ~23°C freshwater salted to 1-2 psu. The fish were fed DAN-EX 2848 (BioMar A/S, Brande, Denmark).

From the size of ~60 cm, females were then fed broodstock diets EH-5 or EH-6 for a period of 18 months (Butts et al., 2019). The lipid content and fatty acid composition of these diets was similar, whereas amino acid composition varied (i.e. arginine). Large females were selected for the experiment [n = 88, total length (TL) 76 ± 4 cm, body weight (BW) 892 ± 118 g] and transported in an aerated freshwater tank to the research facility EEL-HATCH, Technical University of Denmark, Hirtshals. The eels were randomly distributed into two separate RAS each with three 1150 L tanks (i.e. ~15 eels per tank) with freshwater salted to 10 psu (see below), a flow rate of ~15 L min^{-1} and temperature of 18-20°C. An equal number of females from each diet was distributed into each RAS. They were acclimated to saltwater (i.e. from 10 to 36 psu) over two weeks by adding natural seawater supplemented with Blue Treasure Aquaculture Salt (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Quindao, China). The light regime was 12 h light (at low light intensity; ~20 lux W) and 12 h dark.

Males (n = 120, TL 39 ± 2 cm, BW 122 ± 18 g) were transported under the same conditions as the females to the research facility and kept in two separate RAS each with four 485 L tanks (i.e. 15 fish per tank) at the same conditions as the females and similarly acclimated for two weeks.

2.3. CPE treatments of females and hCG treatment of males

After acclimation, individual eels were anaesthetised in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg L^{-1}, Sigma Aldrich, Darmstadt, Germany). TL (cm) and BW (g) were measured before tagging with a passive integrated transponder (PIT, 12×2 mm) tag in the dorsal musculature for ID.

Extracts were obtained by grinding dried carp pituitary glands (Ducamar Spain S.L.U, Bilbao, Spain) in NaCl solution (0.9%) and centrifuging the solution at 3600 RPM (Ohta et al, 1996, 1997). The supernatant was stored at –20°C until use.

Two female treatment groups were established, one per RAS. One treatment group (n = 38, TL 76 ± 3 cm, BW 900 ± 102 g) received weekly intramuscular injections at a constant dose of 20 mg kg^{-1} of CPE. The other group (n = 42, TL 75 ± 4 cm, BW 885 ±

131 g) received weekly intramuscular injections at a stepwise increasing dose of CPE (i.e. 5 mg kg^{-1} for the first three weeks, 10 mg kg^{-1} from week 4-6, 15 mg kg^{-1} from week 7-9, and 20 mg kg^{-1} from week 10). The experimental design is illustrated in Fig. 1. BW and TL before treatment did not differ between the two groups (BW, P = 0.345, Mann-Whitney U test; TL, P = 0.319, t-test).

Male spermatogenesis was induced using weekly intramuscular injections of 150 IU human chorionic gonadotropin (hCG, Sigma Aldrich) per fish (Pérez et al., 2000; Tomkiewicz et al., 2011).

2.4. Sampling

Eight female eels were randomly selected for sampling in week 0 (prior treatments), and eight eels per treatment in week 6, 9 and 12 (Fig. 1). The selected eels were euthanized in an aqueous solution of benzocaine (20 mg L^{-1}). TL and BW were measured. Gonad and liver were then dissected and weighed (g). Gonado-somatic index [GSI = (gonad weight / body weight) × 100] and hepato-somatic index [HSI = (liver weight / body weight) × 100] were calculated. Tissue samples were collected for histological and gene expression analyses.

2.5. Ovarian histological analysis

Tissue was sampled from the midsection of ovaries of fish dissected in week 0, 6, 9 and 12 and of spawned fish. Tissue was fixated in a 4% solution of formaldehyde (Hounisen, Skanderborg, Denmark) at room temperature. Fixed tissues were dehydrated, embedded in paraffin using a Shandon Excelsior ES histokinette (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Tissue-tek®TEC embedding system (Sakura Finetek, Alphen aan den Rijn, Netherlands) and sectioned at 5 µm using a Shandon Finesse®ME+ microtome (Thermo Fisher Scientific). Tissue samples were mounted on glass slides, stained with periodic acid solution 0.5% (Merck KGaA, Darmstadt, Germany), Schiff's reagent (Merck KGaA), Weigert's hematoxylin (Merck KGaA) and metanil yellow (Sigma Aldrich; Quintero-Hunter et al., 1991). Sections were photographed using a digital camera (Model DP71, Olympus, Center Valley, Pennsylvania, USA). The diameters of the 10 largest oocytes in each sample were measured using Olympus cellSens Entry 1.11 software. Only oocytes sectioned through the nucleus were measured.

Progression of oocyte development was categorised based on morphological characteristics described in previous studies on Japanese eel (Kayaba et al., 2001), European eel (Pérez et al., 2011; da Silva et al., 2016) and other teleosts (reviewed in Lowerre-Barbieri et al., 2011). The classification included the following stages:

perinuclear nucleoli oocytes (PN), pre-lipid droplet oocytes (PLD), previtellogenic lipid droplet oocytes (LD1-4), vitellogenic oocytes (VT1-4), germinal vesicle migration oocytes (GVM), germinal vesicle breakdown oocytes (GVBD), hydrated oocytes (HYD) and postovulatory follicles (POF). A specific aim was to identify the occurrence of cortical alveoli (CA) using the staining protocol by Quintero-Hunter et al. (1991). LD and VT stages were further subdivided to explore gene expression patterns (Table 1). Following this classification scheme, each female was assigned to a reproductive stage based on the most advanced oocyte stage present in the ovarian sample.

2.6. Gene expression analysis

Tissues (~0.01 g), sampled from the midsection of ovary and from the middle of the liver of fish dissected in week 0, 6, 9 and 12, were preserved in RNA-later (Qiagen, Hilden, Germany) at 4°C for 24 h and stored at –20°C until analyses of gene expression. RNA was extracted following the protocol NucleoSpin® RNA (Macherey-Nagel, Düren, Germany). RNA purity (260/280 = 2.17 ± 0.04, 260/230 = 2.26 ± 0.12) was evaluated through spectrophotometry using Nanodrop One (Thermo Fisher Scientific) and the RNA concentration was measured using Qubit (Thermo Fisher Scientific). Genomic DNA was removed from the extracted RNA using the PerfeCta® DNase I (RNase-free) kit (Quanta Biosciences, Hilden, Germany). Then 450 ng of total RNA was transcribed to cDNA using the qScript™ cDNA synthesis Kit (Quanta Biosciences).

Expression of gonadotropin receptors (*fshr* and *lhcgr1*), nuclear estrogen receptors (*esr1*, *esr2a* and *esr2b*), membrane estrogen receptors (*gpera* and *gperb*), androgen receptors (*ara* and *arb*), VTG genes (*vtg1* and *vtg2*) and heat shock proteins (*hsp70* and *hsp90*) in ovarian and hepatic tissues was analysed through real-time polymerase chain reaction (qPCR) and compared between treatment groups and over time. Specific primers were selected based on previous qPCR work on European eel (Table 2). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes as their expression profiles were the same regardless of treatment, sampling week and tissue.

Gene expression in ovarian and hepatic tissues was analysed through qPCR Biomark™ HD system (Fluidigm, South San Francisco, Californien, USA) in 96.96 IFC using four qPCR replicates per sample. A pre-amplification step of cDNA was done following the Fluidigm protocol (PN 100-5875). Samples were diluted 1:5 before being loaded onto the arrays and the primers were loaded at a concentration of 50 µM. The arrays were run according to Fluidigm 96.96 IFC protocol (PN 100-9792) with a Tm of 60°C.

The coefficient of variation (CV) of the four qPCR replicates was calculated and gene transcripts were considered stable if CV < 4% (Hellemans et al., 2007). The relative

quantity of target gene transcripts was normalised to the geometric mean of reference genes. Gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method with the week 0 group average as reference (Livak & Schmittgen, 2001). Expression of each gene was given in arbitrary units and week 0 was set as 1.

2.7. Final follicular maturation, fertilisation and offspring performance

Remaining females progressed to final maturation and strip-spawning (Ohta et al., 1996, 1997; Tomkiewicz et al. 2012). Females were weighed at each weekly CPE injection to follow ovarian development. When females increased in BW by ~10-20%, an ovarian biopsy was performed, and if oocytes had reached Stage 4 (i.e. nucleus migration in transparent oocyte) according to Palstra et al. (2005), a CPE primer injection (20 mg kg^{-1}) was administered. Approximately 24 h later, when the oocytes reached Stage 6 (i.e. nucleus breakdown and few large lipid droplets) according to Palstra et al. (2005), DHP (2 mg kg^{-1}; DHP crystalline, Sigma Aldrich) was injected at several places along the ovary. This induced final follicular maturation and ovulation, and strip-spawning could be performed after ~12-15 h. Eggs were stripped into dry plastic trays by gently pressing the female's abdomen. Ovulated eggs were weighed and fertilised according to a standardised protocol (Butts et al., 2014; Sørensen et al., 2016).

Milt from 4-5 spermiating males was pooled and the spermatocrit and mobility was assessed following Sørensen et al. (2013). The milt was diluted in an immobilising medium (Asturiano et al., 2004; Peñaranda et al., 2010) and added to the unfertilised eggs in a predetermined ratio according to Butts et al. (2014). To activate sperm, artificial seawater was prepared using reverse osmosis filtration (Vertex Puratek 100 gpd RO/DI, Vertex Technologies Inc., Huntington Beach, California, USA), salted with Blue Treasure (Qingdao Sea-Salt Aquarium Technology Co., Ltd.) to 36 psu and kept at 20°C before being added to the mixture of eggs and milt dilution (Butts et al., 2014).

After fertilisation, the eggs were kept at 20 ºC in 15 L of the prepared artificial seawater and the percentage of floating eggs (i.e. viable eggs) was estimated after 1 h (Butts et al., 2014; Sørensen et al., 2016). Fertilisation rate was estimated after 4 h when the first cleavages are observable. Five replicate samples of ~100 eggs were photographed using a digital camera (Digital Sight DS-Fi1, Nikon Corporation, Tokyo, Japan) connected to an optical microscope (Eclipse 55i, Nikon Corporation) at 20x magnification and eggs with cleavage were counted to calculate fertilisation rate (the number of fertilised eggs divided by the total number of eggs).

In order to determine larval hatch rate and deformity rate, ~100-200 floating eggs were collected in triplicates according to da Silva et al. (2018). The eggs were incubated in darkness at 18°C in flasks (Nunc® 75 cm^2 flasks, non-treated with ventilated caps, Thermo Fisher Scientific) with 250 ml of artificial seawater (36 psu) mixed with

ampicillin (50 mg L^{-1}) and rifampicin (50 mg L^{-1}) for ~60 h. After hatch, the larvae were euthanised using tricaine methanesulfonate (MS-222, Sigma Aldrich) at a concentration of 15 mg L^{-1} and hatch rate was calculated (the number of hatched larvae divided by the total number of eggs, multiplied with fertilisation rate). Then larvae with deformities were counted to calculate deformity rate (the number of deformed larvae divided by the total number of larvae).

2.8. Statistical analyses

All statistical analyses were performed using R version 3.3.2 (R Core Team, 2019). Level of significance was set at 0.05. Assumptions of variance homogeneity, analysed through Levene's test, and normality of residuals were fulfilled for all parametric tests. The data were transformed if residuals deviated from normality with an optimal power transformation computed by the Box-Cox function (package MASS). Non-parametric tests were used if assumptions could not be fulfilled. Results from eels of diets EH-5 and EH-6 did not differ and were therefore combined in the analyses.
Kruskal-Wallis Chi-squared test was performed on the histological data, followed by a pairwise comparison using Wilcoxon rank-sum test to observe significant differences between groups. Oocyte diameter, GSI and HSI were analysed with a Kruskal-Wallis test and Dunn's test was applied post hoc to detect significant differences between the interaction of treatments and sampling week as well as between most advanced oocyte stage as determined through histology. Two-way ANOVAs were used to analyse the effect of CPE treatments at the different sampling weeks on gene expression. One-way ANOVAs were used to analyse oocyte developmental stage effect on gene expression. Post hoc Tukey's tests were used to detect significant differences between groups. Chi-squared test was used to analyse the difference in number of successful strip-spawnings and the number of females with hatched larvae between treatment groups.

3. Results

3.1. CPE induction of ovarian development

3.1.1. Progression of oogenesis

Weekly CPE injections, using a constant or increasing dose, successfully induced ovarian development in all female eels, as evident in the increase in GSI, which reached a value of ~22 after 12 weeks in both treatment groups as compared to 1.4 in week 0 (Table 3A).

The ovarian development was assessed by histological changes that allowed us to detail the progression of oogenesis (Fig. 2-3). During primary growth (previtellogenesis), oocytes accumulated lipid droplets that increased in size and number with oocyte growth (LD1-3; Fig. 2A). Gradually, this led to the formation of larger lipid droplets around the nucleus and numerous smaller peripheral droplets (LD4). At this stage, the staining revealed magenta vesicles among lipid droplets in the peripheral ooplasm of LD4 oocytes marking the first appearance of CA (Fig. 2B and 3A). At the onset of secondary growth, small yolk globules in the peripheral ooplasm among lipid droplets and CA characterised early vitellogenic oocytes (VT1; Fig. 2B-C). During vitellogenesis, yolk globules gradually increased in size and number (VT2; Fig. 2D), progressively filling the ooplasm from oocyte membrane to nucleus (VT3; Fig. 2D). The most advanced vitellogenic stage showed initiation of lipid droplet fusion and coalescence of yolk globules (VT4; Fig. 2E). Concurrently, CA enlarged at the periphery of the ooplasm (Fig. 3B-C). During final follicular maturation, germinal vesicle migration (GVM) and germinal vesicle breakdown (GVBD) were observed, while yolk globules and lipid droplets coalesced, eventually ending in oocyte hydration (HYD).

Different oocyte stages were present simultaneously in the same ovarian tissue forming cohorts of specific developmental stages (Fig. 2). Prior to CPE treatments, ovaries were dominated by different stages of previtellogenic oocytes as well as adipocytes (Fig. 2A). During vitellogenesis, groups of previtellogenic as well as vitellogenic oocytes at different stages were present, while adipocytes diminished (Fig 2B-E). Likewise, different groups of developing oocytes were present among postovulatory follicles (POF) after successful strip-spawning (Fig. 2F).

The mean diameter of the largest oocytes increased with time reaching a value of ~600 µm in both treatment groups after 12 weeks as compared to 171 µm in week 0 (Table 3A). The mean oocyte diameter significantly increased with oocyte stages (Kruskal-Wallis Chi-squared = 1166.5, DF = 6, $P < 0.001$; Table 3B). The increases in oocyte diameter paralleled increments in GSI with oocyte stages (Kruskal-Wallis Chi-squared = 1151.1, DF = 6, $P < 0.001$; Table 3B).

HSI increased with time from 0.8 in week 0 to 1.2-1.3 in week 12 in both treatment groups, reflecting the activation of vitellogenic and metabolic liver functions (Table 3A). HSI significantly increased with oocyte stages (Kruskal-Wallis Chi-squared = 854.63, DF = 6, $P < 0.001$; Table 3B).

3.1.2. Comparison of CPE treatment dose

GSI increased progressively from week 0 to 12, yet at different rates for the two CPE treatments (Kruskal-Wallis Chi-squared = 1173.2, DF = 6, $P < 0.001$; Table 3A).

In week 0 (Fig. 2A), the majority's most advanced oocyte stage was LD3, while LD4 was observed in one female. In week 6 (Fig. 2B), all females from the constant and increasing group had progressed into early vitellogenesis (VT1). In week 9, females in the increasing group were in early stages of vitellogenesis (VT1-VT2; Fig. 2C), whereas females in the constant group were further in vitellogenesis (VT2-VT3; Fig. 2D). After 12 weeks (Fig. 2E), both the constant and increasing group were in late vitellogenesis (VT4) with some females approaching final follicular maturation (GVM). Thus, progression of ovarian development differed between treatment groups (Kruskal-Wallis Chi-squared = 49.335, DF = 6, $P < 0.001$; Fig. 4). The increasing group was delayed in week 9, but caught up with the constant group by week 12. Post hoc pairwise tests revealed four significantly different groupings based on the most advanced oocyte stage, specifically: i) week 0, ii) both treatment groups in week 6 and the increasing treatment in week 9, iii) the constant treatment in week 9 and iv) both treatment groups in week 12.

The mean diameter of the largest oocytes increased at different rates for the two treatment groups (Kruskal-Wallis Chi-squared = 1184.0, DF = 6, $P < 0.001$; Table 3A). A post hoc pairwise analysis showed that mean oocyte diameter increased at each sampling week in both treatment groups ($P < 0.05$). These significant increments paralleled the ones observed for GSI ($P < 0.05$; Table 3A).

HSI increased at different rates for the two treatment groups (Kruskal-Wallis Chi-squared = 827.45, DF = 6, $P < 0.001$; Table 3A). A post hoc pairwise analysis showed that HSI significantly increased from week 0 to 6 and 6 to 9 in both treatment groups, as well as from week 9 to 12 in the increasing group, but not in the constant group, which had reached the maximum value in week 9 ($P < 0.05$).

3.2. Effect of CPE treatments on gonadotropin receptor expression

As expected, both gonadotropin receptors *fshr* and *lhcgr1* were expressed in the ovary (Fig. 5). Remarkably, *fshr* expression was also detected in the liver, but likely at lower abundance as suggested by a CT difference of 8 between ovary and liver mean values in week 0.

The expression of ovarian *fshr* increased significantly from week 0 to 6 (constant group, 2.7-fold compared to week 0, $P < 0.001$; increasing group, 4.3-fold compared to week 0, $P < 0.001$). It increased further in the constant group from week 6 to 9 (8.9-fold compared to week 0, $P = 0.002$) and became stable. No further changes in *fshr* expression were observed in the increasing group. When correlated with ovarian development, *fshr* was stable during previtellogenesis, increased significantly in VT1 (3.1-fold compared to week 0, $P = 0.006$) and was stable onwards.

Expression of *lhcgr1* increased significantly from week 0 to 6 (constant group, 3.5-fold compared to week 0, P < 0.001; increasing group, 6.5-fold compared to week 0, P < 0.001) and week 6 to 9 in the constant group (21.9-fold compared to week 0, P = 0.006), whereas the increasing group was stable. From week 9 to 12, *lhcgr1* expression increased significantly in the increasing group (10.9-fold compared to week 0, P = 0.030), whereas expression in the constant group was stable. A significant difference between treatment groups was observed in week 9, as the constant group had the largest increase (21.9 versus 4.4-fold compared to week 0, P = 0.010). When correlated with ovarian development, *lhcgr1* expression was lowest in previtellogenesis, significantly higher in late vitellogenesis and overlapped in stages LD4-VT2.
In the liver, the expression of *fshr* significantly increased from week 0 to 6 in the increasing group (6.8-fold compared to week 0, P = 0.010) and from week 6 to 9 in the constant group (13.2-fold compared to week 0, P = 0.035) with no additional changes observed with time in either treatment. A significant difference between treatment groups was observed in week 6, as expression increased more in the increasing group (6.8 versus 1.6-fold compared to week 0, P = 0.048). In correlation with ovarian development, expression increased significantly in VT1 (4.2-fold compared to LD3, P = 0.039) and remained high with maximum levels observed in VT3 (11.0-fold compared to LD3). Expression of *lhcgr1* was undetectable in the liver at all sampling points.

3.3. Effect of CPE treatments on steroid receptor expression

3.3.1. Estrogen receptor expression

All estrogen receptors were expressed in the ovary and liver, except *gpera* that was not detected in the liver (Fig. 6).
In the ovary, *esr1* expression did not change significantly with time for either treatment or when correlated to ovarian developmental stage.
Expression of ovarian *esr2a* and *esr2b* showed different tendencies. Expression of *esr2a* showed a significant increase from week 0 to 6 (constant group, 2.6-fold compared to week 0, P = 0.006; increasing group, 4.1-fold compared to week 0, P < 0.001) followed by a gradual decrease, but without substantial significant changes. Similarly, when correlated with ovarian development, expression of *esr2a* increased significantly from LD4 to VT1 (2.4-fold compared to LD3, P = 0.037), but was in general similarly expressed throughout.
Expression of ovarian *esr2b* tended to decrease with time leading to significant differences in the increasing group in week 9 (0.4-fold compared to week 0, P = 0.026) and 12 (0.1-fold compared to week 0, P = 0.001), but only in week 12 in the constant

group (0.2-fold compared to week 0, P = 0.012). In agreement, *esr2b* expression was highest in early ovarian developmental stages and progressively decreased.
Expression of ovarian *gpera* increased from week 0 to 6 (constant group, 2.5-fold compared to week 0, P = 0.014; increasing group, 2.0-fold compared to week 0, P < 0.001), then gradually decreased with a significant drop observed from week 9 to 12 (constant group, 0.4-fold compared to week 0, P = 0.002; increasing group, 0.3-fold compared to week 0, P = 0.005). When correlated with ovarian development, *gpera* expression was generally stable with slight variations.
In contrast, expression of ovarian *gperb* increased sharply from week 0 to 6 (constant group, 6.5-fold compared to week 0, P = 0.037; increasing group, 5.8-fold compared to week 0, P < 0.001) followed by stable or slightly declining levels. This was also evident when correlated to ovarian development, as a sharp increase was observed in VT1 (5.1-fold compared to week 0, P = 0.015) followed by stable levels.
In the liver, expression of *esr1* increased progressively with maximum levels in week 12 (constant group, 37.1-fold compared to week 0; increasing group, 53.5-fold compared to week 0). Similarly, expression according to ovarian developmental stage gradually increased with the highest levels observed in late vitellogenesis and GVM.
Expression of *esr2a* in the liver had a similar expression profile as *esr1*, but with smaller fold-increases. The highest levels were observed in week 12 (constant group, 6.5-fold compared to week 0; increasing group, 7.7-fold compared to week 0). In agreement, a slight gradual increase in expression was observed when correlated with ovarian developmental stage.
Hepatic *esr2b* expression also showed a similar expression profile as *esr1* and *esr2a*, but at lower fold increases. The highest increase was in week 9 for the increasing group (2.7-fold compared to week 0) and week 12 for the constant group (2.6-fold compared to week 0). When correlated with ovarian developmental stage, the pattern was the same as for *esr2a*.
Expression of hepatic membrane *gpera* was not detected at any sampling points. Concerning *gperb*, no significant changes were observed.

3.3.2. Androgen receptor expression

Androgen receptors (*ara* and *arb*) were also expressed in both tissues (Fig. 7).
Expression of ovarian *ara* increased from week 0 to 6 (constant group, 1.7-fold compared to week 0, P = 0.005; increasing group, 2.2-fold compared to week 0, P = 0.001). The highest increase was observed in the constant group in week 9 (3.0-fold compared to week 0), but levels decreased significantly from week 9 to 12 to the same level as observed in week 6 (1.6-fold compared to week 0, P = 0.020). In week 9, treatment groups differed significantly (3.0 versus 1.5-fold compared to week 0, P =

0.030). In relation to ovarian development, expression was generally stable with slightly (not significantly) higher levels in VT3 (2.7-fold compared to LD3).
A similar expression profile was observed for ovarian *arb*, with a significant increase from week 0 to 6 (constant group, 1.8-fold compared to week 0, P = 0.004; increasing group, 3.0-fold compared to week 0, P < 0.001) followed by stable or slightly decreasing levels. When correlated with ovarian development, no changes were observed in *arb* expression.
In the liver, *ara* expression progressively increased, with a significant increase in week 6 (constant group, 2.2.-fold compared to week 0, P < 0.001; increasing group, 3.7-fold compared to week 0, P < 0.001) and maximum levels in week 9 (constant group, 5.8-fold compared to week 0, P < 0.001; increasing group, 6.0-fold compared to week 0, P = 0.030). According to ovarian developmental stage, a similar progressive increase in levels was observed.
An increase in hepatic *arb* expression was also observed, but with less amplitude. In week 6, expression increased significantly (constant group, 1.5-fold compared to week 0, P = 0.028; increasing group, 2.2-fold compared to week 0, P = 0.002) with no further changes. When correlated with ovarian development, *arb* expression was similar at all stages.

3.4. Effect of CPE treatments on vitellogenin expression

As expected, *vtg1* and *vtg2* were expressed in the liver (Fig. 8). Unexpectedly, their expressions were also detected in the ovary at lower abundances as suggested by a CT difference of 4 for *vtg1* and 1.7 for *vtg2* between liver and ovary mean values in week 0.
In the ovary, *vtg1* and *vtg2* expression was the same from week 0 to 6, but increased significantly from week 6 to 9 in the constant group (*vtg1*, 26.6-fold compared to week 0, P < 0.001; *vtg2*, 44.2-fold compared to week 0, P < 0.001) and increasing group (*vtg1*, 1.9-fold compared to week 0, P = 0.003; *vtg2*, 17.8-fold compared to week 0, P = 0.049), followed by a significant drop in *vtg2* expression from week 9 to 12 in the constant group (6.1-fold compared to week 0, P = 0.007). No further changes were observed in the increasing group. The expression of *vtg1* significantly differed between treatment groups in week 9, as expression had increased more in the constant group (26.6 versus 1.9-fold compared to week 0, P = 0.026). When correlated with ovarian development, *vtg1* and *vtg2* expression levels were higher in VT3 (24.1 and 47.3-fold compared to week 0, respectively) and similar in the other stages.
In the liver, expression of *vtg1* and *vtg2* showed different profiles than in the ovary and with substantially larger fold changes. A significant increase in hepatic *vtg1* expression was observed from week 0 to 6 (constant group, 18.1-fold compared to week 0, P < 0.001; increasing group, 10.9-fold compared to week 0, P < 0.001). Expression

increased further from 6 to 9 (constant group, 542.4-fold compared to week 0, P < 0.001; increasing group, 84.8-fold compared to week 0, P = 0.005) and treatment groups differed here as expression had increased more in the constant group (P = 0.027). Concerning *vtg2*, expression increased from week 0 to 6 (constant group, 8944.1-fold compared to week 0, P < 0.001; increasing group, 10696.2-fold compared to week 0, P < 0.001). A second significant increase was observed from week 6 to 9 in the constant group (37034.1-fold compared to week 0, P = 0.002).
In correlation with ovarian development, *vtg1* expression was lowest in LD3, gradually changed in LD4-VT2 and was highest in VT3-GVM. Expression of *vtg2* significantly increased in VT1 (10290.4-fold compared to LD3, P < 0.001) and remained high until GVM.

3.5. Effect of CPE treatments on heat shock protein expression

Heat shock protein (*hsp70* and *hsp90*) expression was detected in both tissues (Fig. 9). Likely, abundance was lower in the liver as suggested by CT differences of 6.6 for *hsp70* and 2.6 for *hsp90* between ovary and liver mean values in week 0.
For *hsp70*, no variation in expression was observed in either tissue. Variation was also not observed in *hsp90* expression in the ovary. In contrast, an increase was observed in hepatic *hsp90* expression in CPE treated eels. The increase was significant from week 6 to 9 (P < 0.001) with maximum levels in week 12 (constant group, 7.0-fold compared to week 0; increasing group, 4.8-fold compared to week 0). When correlated with ovarian development, hepatic *hsp90* expression was lowest in stages LD3-VT1 and highest in VT3-GVM.

3.6. Reproductive success

Weekly CPE treatments were maintained for the remaining females of the constant (n = 14) and increasing group (n = 18) until they reached final follicular maturation and ovulation (Table 4). The first female in the constant group was strip-spawned in week 10, most of the others in weeks 12-13 and the last one in week 14 (Fig. 10). For the increasing group, the first eels were stripped-spawned in week 13 and the other eels during the two following weeks. Altogether, the increasing group had received less CPE to reach final follicular maturation (170-210 versus 240-260 mg kg^{-1} CPE). In total, 2 eels from the constant group did not ovulate leading to 86 and 100% of females successfully ovulating for the constant and increasing group, respectively, but the difference was not significant (Chi-squared = 2.743, DF = 1, P = 0.098). Likewise, the percentage of females that produced larvae, 42% for the constant and 72% for the increasing group, did not significantly differ (Chi-squared = 2.801, DF = 1, P = 0.094).

No significant difference between treatment groups was observed for mean egg weight (t = -0.610, DF = 13.397, P = 0.552) or percent floating eggs (t = -1.644, DF = 16.978, P = 0.119). Furthermore, no significant differences were observed in fertilisation rate **(**t = -1.529, DF = 24.218, P = 0.139) or hatch rate (t = 1.590, DF = 17.023, P = 0.130). Lastly, the proportion of larval deformities was similar for the constant and increasing group (t = 0.099, DF = 3.496, P = 0.927).

4. Discussion

4.1. CPE induction of ovarian development, ovulation and reproductive success

In the present study, previtellogenic females progressed similarly through vitellogenesis, yet at different rates depending on CPE treatment dose. CPE treatments are well documented to induce vitellogenesis in teleosts, including European eel (reviewed in Mylonas et al., 2010; Okamura et al., 2014).

Before commencement of treatments (week 0), the females were in previtellogenesis and in early vitellogenesis in week 6. However in week 9, the constant dose group had progressed further into vitellogenesis as evident in larger GSI, HSI, oocyte diameter and more advanced oocyte stages. From week 10 and onwards, the increasing group received a weekly CPE dose of 20 mg kg^{-1}; in other words, both treatment groups now received the same dose. This dose increment allowed the increasing group to reach the same stage of development as the constant group in week 12.

The last stage of previtellogenesis, marking the transition into vitellogenesis, was characterised by the appearance of CA. Irrespective of treatment dose, CA appeared after CPE treatments commenced (one week 0 female had CA). The results indicate that CA were generally produced concurrently with induction of vitellogenesis. This is in agreement with a previous study on coho salmon, *Oncorhynchus kisutch*, which linked the appearance of CA with FSH and estrogens (Campbell et al., 2006). Additionally, *fshr* expression has been observed to increase at the CA stage in zebrafish, *Danio rerio*, (Kwok et al., 2005). Although little is known about the role of CA in teleosts, they are suggested to be involved in fertilisation, at which point they exocytose their content into the perivitelline space (Iwamatsu & Ohta, 1976; Motta et al., 2005). Hence, the observable changes in CA in the present study are likely in preparation for the approaching fertilisation of the mature egg.

Anguillid gonad development can arrest if gonadotropin production is not sufficient to sustain vitellogenesis. FSH and LH may act on different developmental stages of oocytes in eel and the latter is crucial for final follicular maturation (reviewed in Cerdà

et al., 2008; Kazeto et al., 2011). If gonadotropin levels in CPE do not mimic natural levels during ovarian development, egg quality could be affected. Replicating natural hormonal levels is a challenge in the eel, since there is no access to naturally matured eels for comparison. Similarly, there is no information on the natural duration of full sexual maturation for European eel. Using an increasing CPE treatment may still be more suitable as increasing levels of gonadotropins have been observed during vitellogenesis in other fish species (reviewed in Lubzens et al., 2010). Besides lack of knowledge on gonadotropin levels, other pituitary hormones could be present in CPE, which may affect the endocrine regulations and reproductive success.

Nevertheless, females responded successfully to both CPE treatments, reached final follicular maturation and ovulated. Females in the constant group reached final maturation from week 10 to 14. The increasing group started reaching final maturation three weeks later (week 13), but were all strip-spawned over a shorter period (up to week 15). In the present experiment, as compared with previous studies on farmed-reared European eels, the overall response rates of both treatment groups were higher, and time to ovulation shorter. Success rates of PE treatments are highly variable and response typically occurs after 11-29 weeks when using one weekly injection depending on PE type and dose (reviewed in Okamura et al., 2014).

Our results suggest that the amount of CPE does not affect the rate of transition into early vitellogenesis, since both groups reached VT1 in week 6. Nevertheless, it likely affects the rate at which vitellogenesis progresses, since in week 9 the constant group had reached VT2-VT3, whereas the increasing group was still in VT1-VT2. The constant treatment may have provided an excess of CPE as both groups reached the same level of development after 12 weeks. Ultimately, the reproductive success of females did not differ between treatments and utilising a constant or increasing CPE dose both lead to viable offspring of similar quality.

4.2. Regulation of ovarian gonadotropin and steroid receptors

Analysing gene expression in ovarian tissue is in itself complex, as it is measured across follicles, oocytes, adipocytes and ovarian stroma. In the present study, the complexity is further expanded, because of the asynchronous ovarian development. As a result, expression is an average of the transcriptional activity of all present oocyte stages and surrounding tissues. Nevertheless, the observed differences between treatment groups in ovarian gonadotropin and steroid receptor expression were in accordance with the morphological changes observed during development.

In various teleosts, FSH is important early in vitellogenesis and LH is associated with later stages in preparation for final follicular maturation (Swanson et al., 2003). Their functions are mediated through gonadotropin receptors, FSHR and LHR. In mammals,

the interaction between gonadotropins and their respective receptor are highly specific, in other words, there is little cross-activation. Gonadotropin-receptor binding may be less selective in some teleosts. For instance, FSHR has been observed to bind both gonadotropins in several species, while LHR was more specific of its respective hormone LH (reviewed in Levavi-Sivan et al., 2010). Accordingly, experiments on eel recombinant gonadotropins and gonadotropin receptors showed high specificity of LHR towards LH, while a small cross-reaction of homologous (eel) LH on FSHR was observed (Kazeto et al., 2008). CPE as well as SPE were shown to cross-react with both eel recombinant FSHR and LHR (Kazeto et al., 2008; Minegishi et al., 2012). This indicates that heterologous (carp or salmon) gonadotropins are able to activate both eel FSHR and LHR.

Both *fshr* and *lhcgr1* expression levels were upregulated at the transition into vitellogenesis and remained high in the following stages. Jeng et al. (2007) also found that PE upregulated *fshr* and *lhr* in Japanese eel. In zebrafish, *fshr* increased from previtellogenesis and peaked in mid-vitellogenesis followed by a decrease, whereas *lhr* peaked in full-grown oocytes (Kwok et al., 2005). Similar patterns have been observed in yellowtail, *Seriola quinqueradiata*, in which *fshr* peaked in early vitellogenesis and *lhr* peaked in later stages (Rahman et al., 2003). In our study, the expression profiles of *fshr* and *lhcgr1* are rather similar, suggesting a role of LH not only in full-grown oocytes, but also in early vitellogenic stages. In a previous study on previtellogenic European eel, an increase in LH also suggested an early role of this gonadotropin in oogenesis (Aroua et al., 2005).

Estrogen nuclear and membrane receptors have been characterised for several teleosts, including Nile tilapia, *Oreochromis niloticus* (Chang et al., 1999), channel catfish, *Ictalurus punctatus* (Patiño et al., 2000), gilthead seabream, *Sparus aurata* (Socorro et al., 2000) and European eel (Lafont et al., 2016). While in mammals there are two nuclear sub-types ERα (*esr1*) and ERβ (*esr2*), for European eel and other teleosts, the latter is present as two paralogs (*esr2a* and *esr2b*) as a result of teleost whole gene duplication (TWGD). In addition, two membrane paralogs (*gpera* and *gperb*) have been identified in European eel (Lafont et al., 2016) and European sea bass, *Dicentrarchus labrax* (Pinto et al., 2018), also a result of TWGD.

In the present study, nuclear and membrane estrogen receptors were expressed differently during ovarian development. Expression of *esr1* did not change during ovarian development, while moderate changes in *esr2a*, *esr2b* and *gpera* expression were observed. The most significant change was the increase in ovarian expression of *gperb* at the transition into vitellogenesis. As in the present study, *esr1* was stable in Japanese eel treated with catfish PE; however, *esr2* expression was also stable (Jeng et al., 2012). Expression of *esr1* was higher in the remaining ovary of European eels that

had ovulated than in the ovary of immature eels, yet no changes were observed in the other nuclear or membrane estrogen receptors (Lafont et al., 2016). Estrogens are essential for oogenesis and their activity is mediated through their receptors, but their specific roles in fish are still far from being understood. A role of *gperb* in the E2-induced meiotic arrest has been proposed in zebrafish (Peyton & Thomas, 2011) and common carp, *Cyprinus carpio* (Majumder et al., 2015). Our study indicates that its expression increases at the initiation of vitellogenesis.

Two androgen nuclear receptors (*ara* and *arb*) are present in the eel as a result of TWGD. Both were expressed in the ovary at relatively stable levels at all oocyte developmental stages, but with a small increase during vitellogenesis. In agreement with our findings, the expression of both ovarian androgen receptors increased during vitellogenesis in PE treated European eel (Peñaranda et al., 2014) and Japanese eel (Jeng et al., 2012). Androgens have previously been implicated in oocyte development in teleosts, such as coho salmon (Fitzpatrick et al., 1994) and Atlantic cod, *Gadus morhua* (Kortner et al., 2009). In fact, studies on shortfinned eel, *Anguilla australis*, and Japanese eel have suggested that androgens are directly related to lipid droplet accumulation in the oocyte during previtellogenesis and vitellogenesis (Rohr et al., 2001; Lokman et al., 2007; Tosaka et al., 2010).

4.3. Regulation of vitellogenesis

A well-known step in the progression of vitellogenesis is FSH binding to its receptor in the ovarian follicular cells triggering E2 synthesis. E2 released in the bloodstream acts on the liver via its receptors and stimulates hepatic vitellogenin synthesis (reviewed in Cerdà et al., 2008; Kazeto et al., 2011).

As expected, vitellogenin was expressed in the liver. Both hepatic *vtg1* and *vtg2* expression largely increased as the females transitioned into early vitellogenesis, further increased in mid-vitellogenesis and plateaued. This upregulation is in agreement with previous studies on European female eels treated with either an increasing CPE dose (Palstra et al., 2010) or a constant dose (Parmeginani et al., 2015).

The three nuclear estrogens receptors were upregulated from the initiation of vitellogenesis in the liver in CPE treated females. The expression of *esr1* continued to increase reaching highest levels in mid-vitellogenesis to GVM. An increase in *esr1* was previously observed in the livers of matured female eels that had ovulated as compared to immature eels (Lafont et al., 2016). Our present results differ to findings in similar studies on induced maturation of European eel that observed either stable expression of *esr1* (Parmeginani et al., 2015) or an initial increase followed by a drop (Palstra et al., 2010). The precise functional roles of the three nuclear estrogen receptors are still to be

investigated, but they are likely all involved in hepatic vitellogenin expression as reviewed by Nelson and Habibi (2013).

Contrary to the nuclear estrogen receptors, *gperb* was stable in the liver throughout development, whereas *gpera* was not detected. In sea bass, low hepatic expression was reported of *gpera* and *gperb*, particularly of the former, in comparison with other tissues (Pinto et al., 2018). Both paralogs have been detected in the liver of European eel, where *gperb* was lower in matured females that had ovulated than in immature eels (Lafont et al., 2016).

Expression of both androgen receptors (*ara* and *arb*) increased as the CPE treated females entered vitellogenesis. Then expression of *arb* plateaued, while expression of *ara* continued to progressively increase. An *in vitro* study on Japanese eel hepatocytes, reported that 11-ketotestosterone (11-KT) with E2 enhanced vitellogenin production, while 11-KT alone had no effect (Asanuma et al., 2003). Our results also suggest a role of androgens and their receptors in hepatic vitellogenesis, but investigations are necessary on their functional role and interaction with estrogens.

Unexpectedly, *fshr* expression was detected in the liver, whereas *lhgcr1* was not. Furthermore, *fshr* expression increased at the onset of vitellogenesis. To our knowledge hepatic *fshr* expression has not been reported in teleosts before. This finding suggests a direct effect of FSH on the liver. A previous study reported the expression of *fshr* in hepatocytes of rat, a non-oviparous species (Mancinelli et al., 2009). In the rat, its role was suggested to be part of an autocrine loop regulating proliferation of cholangiocytes (i.e. epithelial cells of the bile duct). Our finding in the eel opens new research perspectives on whether *fshr* may have a similar action as in the rat or a role in the liver linked to the synthesis of vitellogenin or other hepatic functions.

An additional unexpected finding was the expression of vitellogenin in the ovary. Specifically, expression of both *vtg1* and *vtg2* peaked in mid- to late vitellogenesis and dropped afterwards. This profile was different from that in the liver, where the expression remained high until GVM. Vitellogenin synthesis is generally considered a typical liver function in oviparous species. However, previous studies on other species, such as teleosts and shrimps, have as in ours reported its expression in other tissues, such as the ovary itself. Indeed, in shrimps, *Penaeus semisulcatus* and *Metapenaeus ensis*, the ovary plays a key role in the synthesis of vitellogenin (Avarre et al., 2003; Tsang et al., 2003). In teleosts, vitellogenin genes were expressed in several tissues, including the ovary, of zebrafish (Wang et al., 2005) and turbot, *Scophthalmus maximus* (Xue at al., 2018). In ovaries of turbot, expression increased significantly between previtellogenic oocytes and oocytes that had nearly completed vitellogenesis. A mutual observation in the studies on teleosts was that the hepatic production was substantially larger, which was also evident in this study. Our and aforementioned studies highlight

the question of local vitellogenin synthesis in the ovary. Furthermore, it introduces the possibility of local regulation of vitellogenin expression in the ovary mediated by FSH and estrogens.

Finally, heat shock proteins are found in all studied organisms and are known to be useful indicators of response to stress (reviewed in Iwama et al., 1998). Nonetheless, they have an additional role in vertebrates regarding steroid receptors of estrogens, androgens and progestogens (Smith, 1993; reviewed in Pratt & Toft, 1997). Specifically, Hsp40, Hsp70 and Hsp90 are proposed to create "foldosomes" that bind free steroid receptors in the ooplasm preventing entry into the nucleus until the steroid hormone is present and available for binding (Hutchison et al., 1994). The steroid hormone, bound to its receptor, is released from the complex and can enter the nucleus. Presently, we revealed that the expression of *hsp90* was specifically upregulated in the liver of CPE treated eels. No variation was observed in the ovary. This allows us to propose a new role for Hsp90 in hepatic vitellogenesis as heat shock proteins may interact with steroid receptors in the liver.

Conclusion

Using a constant or increasing CPE dose on female European eels led to similar induction of vitellogenesis and oogenesis. Both CPE treatments resulted in eggs of similar quality as well as viable offspring. By analysing gene expression our understanding of endocrine mechanisms in vitellogenesis was substantiated. As expected, CPE upregulated the expression of ovarian gonadotropin receptors as well as ovarian and hepatic steroid receptors. The expression of *vtg1* and *vtg2* was observed not only in the liver, but also in the ovary, implicating the ovary as a secondary site of vitellogenin synthesis. Remarkably, hepatic expression and upregulation of *fshr* suggests a novel role of FSH in the direct regulation of the liver. Lastly, the expression and upregulation of *hsp90* in the liver suggest a role of this protein in hepatic vitellogenesis as heat shock proteins are known to create complexes with associated steroid receptors.

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Tables

Table 1. Developmental phases and oocyte stages categorised according to cytological characteristics of oocytes or postovulatory follicles.

Phase	Stage	Cytological characteristics
Previtellogenesis	PN/PLD	Perinuclear nucleoli (PN) and pre-lipid droplet oocytes (PLD). Homogeneous ooplasm.
	LD1	Lipid droplet oocytes (LD). First appearance of a small number of lipid droplets.
	LD2	Lipid droplets are scattered around the ooplasm close to the oocyte membrane.
	LD3	Increased number of lipid droplets forming a ring in the ooplasm.
	LD4	Abundant lipid droplets increasing in size, from oocyte membrane to nucleus.
Vitellogenesis	VT1	First appearance of small yolk globules in the periphery of the ooplasm.
	VT2	Increased number of yolk globules among lipid droplets.
	VT3	Abundant yolk globules increasing in number and size, and filling the ooplasm from the oocyte membrane to nucleus.
	VT4	Yolk globules filled the ooplasm and surrounded the nucleus. Initiation of lipid droplets fusion and coalescence of yolk globules.
Final follicular maturation	GVM	Germinal vesicle migration (GVM) towards the animal pole. Further fusion of lipid droplets and coalescence of yolk globules.
	GVBD	GVM to the animal pole completed and germinal vesicle breakdown (GVBD) initiated. Complete fusion of lipid droplets.
	HYD	Hydrated oocyte (HYD) with no visible structures in the ooplasm.
Post spawning	POF	Postovulatory follicle (POF). Convoluted follicle cells in different stages of degradation.

Table 2. Sequences of European eel, *Anguilla anguilla*, primers used for amplification of genes by qPCR. Full gene name, abbreviation, accession number, primer sequences and reference for primers are given for each gene.

Full name	Abbreviation	Accession no.	Primer (5′-3′) (F: Forward; R: Reverse)	Reference
Follicle stimulating hormone receptor	*fshr*	LN831181	F: CCTGGTCGAGATAACAATCACC R: AATCTTGGAGAAATCAGGCAGT	Maugars & Dufour 2015
Luteinizing hormone receptor	*lhcgr1*	LN831182	F: GCGGAAACACAGGGAGAAC R: GGTTGAGGTACTGGAAATCGAAG	Maugars & Dufour 2015
Estrogen receptor alpha (nuclear)	*esr1*	CUH82767	F: GCCATCATACTGCTCAACTCC R: CCGTAAAGCTGTCGTTCAGG	Lafont et al. 2016
Estrogen receptor beta (nuclear)	*esr2a*	CUH82768	F: TGTGTGCCTCAAAGCCATTA R: AGACTGCTGCTGAAAGGTCA	Lafont et al. 2016
Estrogen receptor beta (nuclear)	*esr2b*	CUH82769	F: TGCTGGAATGCTGCTGGT R: CCACACAGTTGCCCTCATC	Lafont et al. 2016
Estrogen receptor (membrane)	*gpera*	CUH82770	F: CAACTTCAACCACCGGGAGA R: TGACCTGGAGGAAGAGGGACA	Lafont et al. 2016
Estrogen receptor (membrane)	*gperb*	CUH82771	F: AACCTGAACCACACGGAAA R: TGACCTGGAAGAAGAGGGACA	Lafont et al. 2016
Androgen receptor alpha	*ara*	FR668031	F: CGG AAG GGA AAC AGA AGT ACC R: AGC GAA GCA CCT TTT GAG AC	Peñaranda et al. 2014
Androgen receptor beta	*arb*	FR668032	F: CGC TGA AGG AAA ACA GAG GT R: CAT TCC AGC CTC AAA GCA CT	Peñaranda et al. 2014
Vitellogenin 1	*vtg1*	EU073127	F: GACAGTGTAGTGCAGATGAAG R: ATAGAGAGACAGCCCATCAC	Parmeggiani et al. 2015
Vitellogenin 2	*vtg2*	EU073128.1	F: GATGCTCCCCTAAAGTTTGTG R: AGCGTCCAGAATCCAATGTC	Parmeggiani et al. 2015
Heat shock protein 70	*hsp70*	AZBK01685255	F: TCAACCCAGATGAAGCAGTG R: GCAGCAGATCCTGAACATTG	Politis et al. 2017
Heat shock protein 90	*hsp90*	AZBK01838994	F: ACCATTGCCAAGTCAGGAAC R: ACTGCTCATCGTCATTGTGC	Politis et al. 2017
Elongation factor 1-alpha	*ef1a*	EU407824	F: CTGAAGCCTGGTATGGTGGT R: CATGGTGCATTTCCACAGAC	Politis et al. 2017
Beta-actin	*β-actin*	DQ286836	F: AGCCTTCCTTCCTGGGTATG R: GTTGGCGTACAGGTCCTTAC	Parmeggiani et al. 2015

Table 3. Biometric changes during induced vitellogenesis. Gonado-somatic index (GSI), hepato-somatic index (HSI), mean diameter of the 10 largest oocytes in a histological section according to (A) CPE treatment groups and time of sampling and (B) ovarian developmental stages. All measurements are given as mean ± SD; n = number of females. Values labelled with different letters are significantly different ($P < 0.05$, Dunn's test).

A	Treatment	Sampling	n	GSI	HSI	Oocyte diameter (µm)
		Week 0	8	1.4 ± 0.3^{a}	0.8 ± 0.1^{a}	171.04 ± 7.92^{a}
	Constant	Week 6	8	5.1 ± 1.1^{b}	0.9 ± 0.1^{b}	292.67 ± 29.50^{b}
	Increasing		8	4.6 ± 0.4^{b}	0.8 ± 0.1^{b}	288.50 ± 16.56^{b}
	Constant	Week 9	8	17.9 ± 6.7^{c}	1.3 ± 0.3^{c}	505.31 ± 63.66^{c}
	Increasing		7	8.7 ± 2.0^{d}	1.0 ± 0.2^{d}	416.92 ± 37.45^{d}
	Constant	Week 12	8	22.4 ± 4.5^{e}	1.3 ± 0.2^{c}	596.82 ± 59.27^{e}
	Increasing		8	22.3 ± 5.8^{e}	1.2 ± 0.2^{c}	577.07 ± 56.48^{e}
B	Stage	Sampling	n	GSI	HSI	Oocyte diameter (µm)
	LD3	Week 0	7	1.4 ± 0.1^{a}	0.8 ± 0.1^{a}	174.63 ± 7.07^{a}
	LD4	Week 0	1	1.7^{a}	0.8^{ab}	191.49^{a}
	VT1	Week 6	19	5.3 ± 1.3^{b}	0.9 ± 0.1^{b}	317.60 ± 43.53^{b}
	VT2	Week 9	5	9.0 ± 1.7^{c}	1.0 ± 0.2^{c}	440.14 ± 36.11^{c}
	VT3	Week 9	7	17.6 ± 6.6^{d}	1.3 ± 0.2^{d}	536.14 ± 57.48^{d}
	VT4	Week 12	9	22.1 ± 5.0^{e}	1.2 ± 0.2^{e}	607.83 ± 59.75^{e}
	GVM	Week 12	7	19.8 ± 3.0^{e}	1.2 ± 0.2^{e}	606.43 ± 59.83^{e}

Table 4. Overview of the number of females and the reproductive success of CPE treatment groups. The proportions of successful strip-spawnings and of females that produced larvae are given in percent and number of females in parentheses. Female weight, length, egg weight, floating egg layer and offspring success rates are given in mean ± SD.

	Constant	Increasing
n	14	18
Length (cm)	77 ± 4	75 ± 4
Initial weight (g)	924 ± 106	888 ± 129
Successful strip-spawnings [% (n)]	86 (12)	100 (18)
Strip-spawned females with hatched larvae [% (n)]	42 (5)	72 (13)
Egg weight (g)	324.8 ± 83.8	333.6 ± 55.8
Floating layer (%)	51 ± 37	70 ± 26
Fertilisation rate (%)	0.3752 ± 0.2738	0.5284 ± 0.2944
Hatch rate (%)	0.0234 ± 0.0277	0.0832 ± 0.1582
Deformity rate (%)	0.6396 ± 0.3515	0.6084 ± 0.2231

Figures

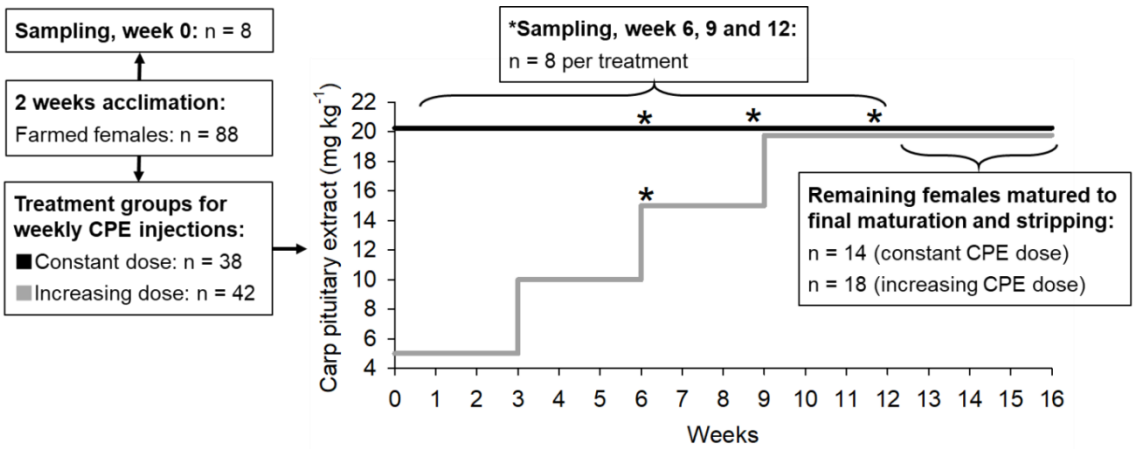

Fig. 1. Schematic overview of the experimental setup and sampling of farm-raised female European eels at the experimental facility. After two weeks of seawater acclimation, an initial control group (week 0, n = 8) was euthanized and sampled. The other females (n = 80) were divided into two treatment groups that received a weekly injection of carp pituitary extract (CPE) either at a constant dose (n = 38) or stepwise increasing dose (n = 42). Treated females were euthanized and sampled after injection 6, 9 and 12 (n = 8/treatment/sampling). Remaining females continued to receive weekly injections until they reached final maturation and subsequent strip-spawning.

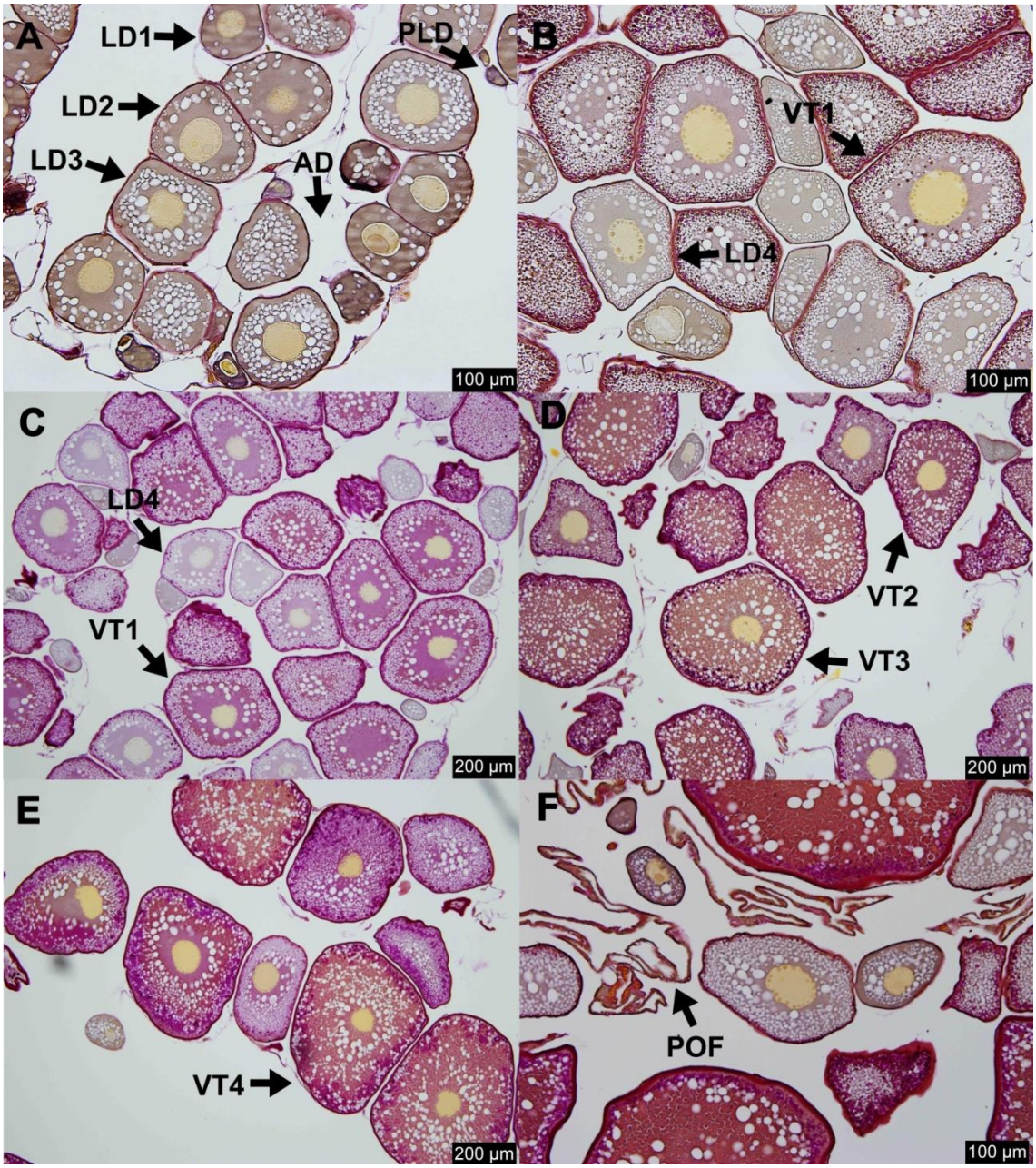

Fig. 2. Sections of ovarian tissue illustrating progression of oocyte development in European eel. (A) Week 0 (GSI = 1.5), (B) week 6 (constant CPE dose, GSI = 5.3), (C) week 9 (increasing CPE dose, GSI = 8.1), (D) week 9 (constant CPE dose, GSI = 11.5), (E) week 12 (constant CPE dose, GSI = 20.7), and (F) after strip-spawning (increasing CPE). AD = adipocyte, PLD = pre-lipid droplet oocyte, LD1-4 = lipid droplet oocytes stages 1 to 4, VT1-4 = vitellogenic oocytes stages 1 to 4, POF = post-ovulatory follicle. Staining: Periodic acid Schiff's hematoxylin counterstained by metanil yellow.

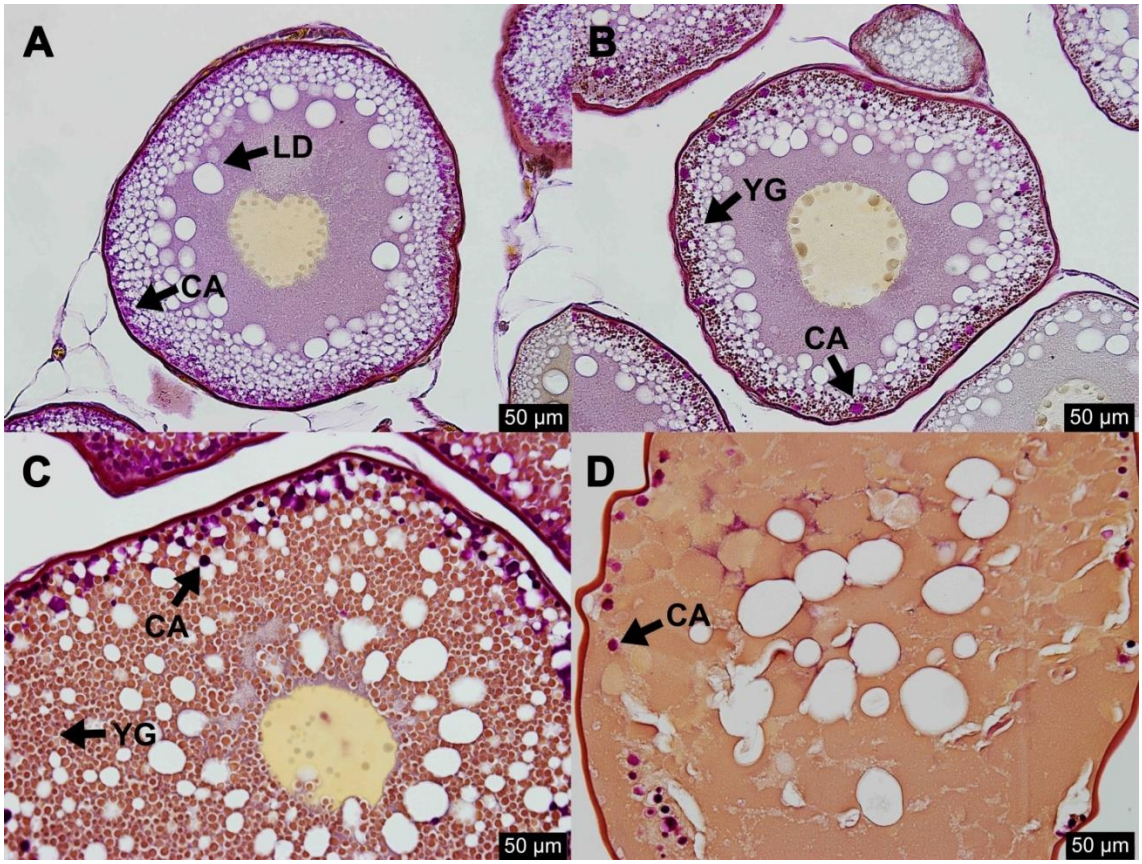

Fig. 3. Sections of ovarian tissue illustrating the presence of cortical alveoli in the cytoplasm at different oocyte developmental stages in European eel. (A) Late lipid droplet stage (LD4; increasing CPE dose, GSI = 4.2), (B) early vitellogenic stage (VT1; increasing CPE dose, GSI = 4.5), (C) mid vitellogenic stage (VT2; constant CPE dose, GSI = 11.5) and (D) hydrating oocyte (constant CPE dose, GSI = 22.2). CA = cortical alveoli, LD = lipid droplet, YG = yolk globule. Staining: Periodic acid Schiff's hematoxylin counterstained by metanil yellow.

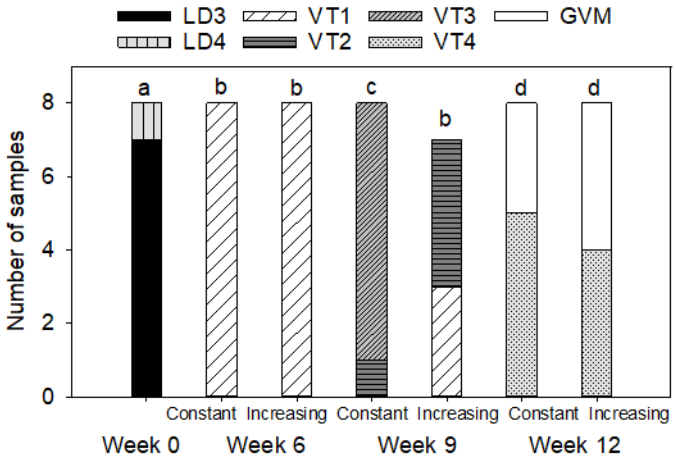

Fig. 4. Proportion of oocyte stages according to treatment and time. Number of female European eels categorised according to the most advanced oocyte developmental stage present ovarian samples obtained in week 0, and per CPE treatment group in week 6, 9 and 12. Columns labelled with different letters are significantly different ($P < 0.05$, Wilcoxon rank-sum test). LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage.

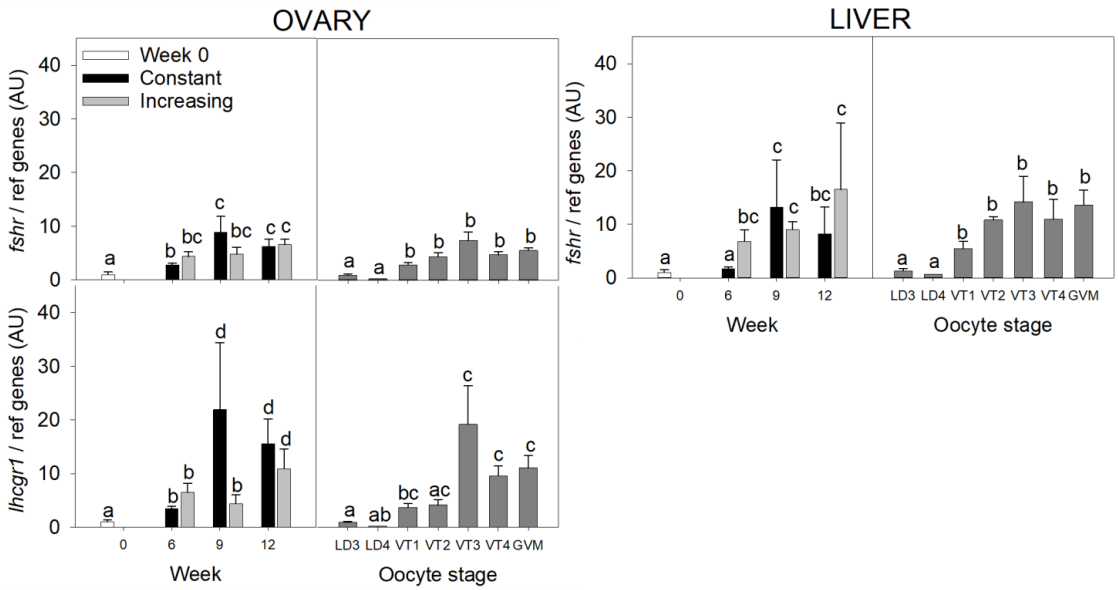

Fig. 5. Relative gene expression of gonadotropins, follicle stimulating hormone receptor (*fshr*) and luteinizing hormone receptor 1 (*lhcgr1*), in ovary and liver samples of female European eel measured by qPCR according to either treatment group (i.e. constant or increasing CPE dose) or ovarian developmental stage. Data are presented as fold change in arbitrary units (AU) according to the $2^{-\Delta\Delta CT}$ method, error bars represent SEM and different letters indicate significant differences ($P < 0.05$, Tukey test). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes. Abbreviations: LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage. See number of samples in Table 3.

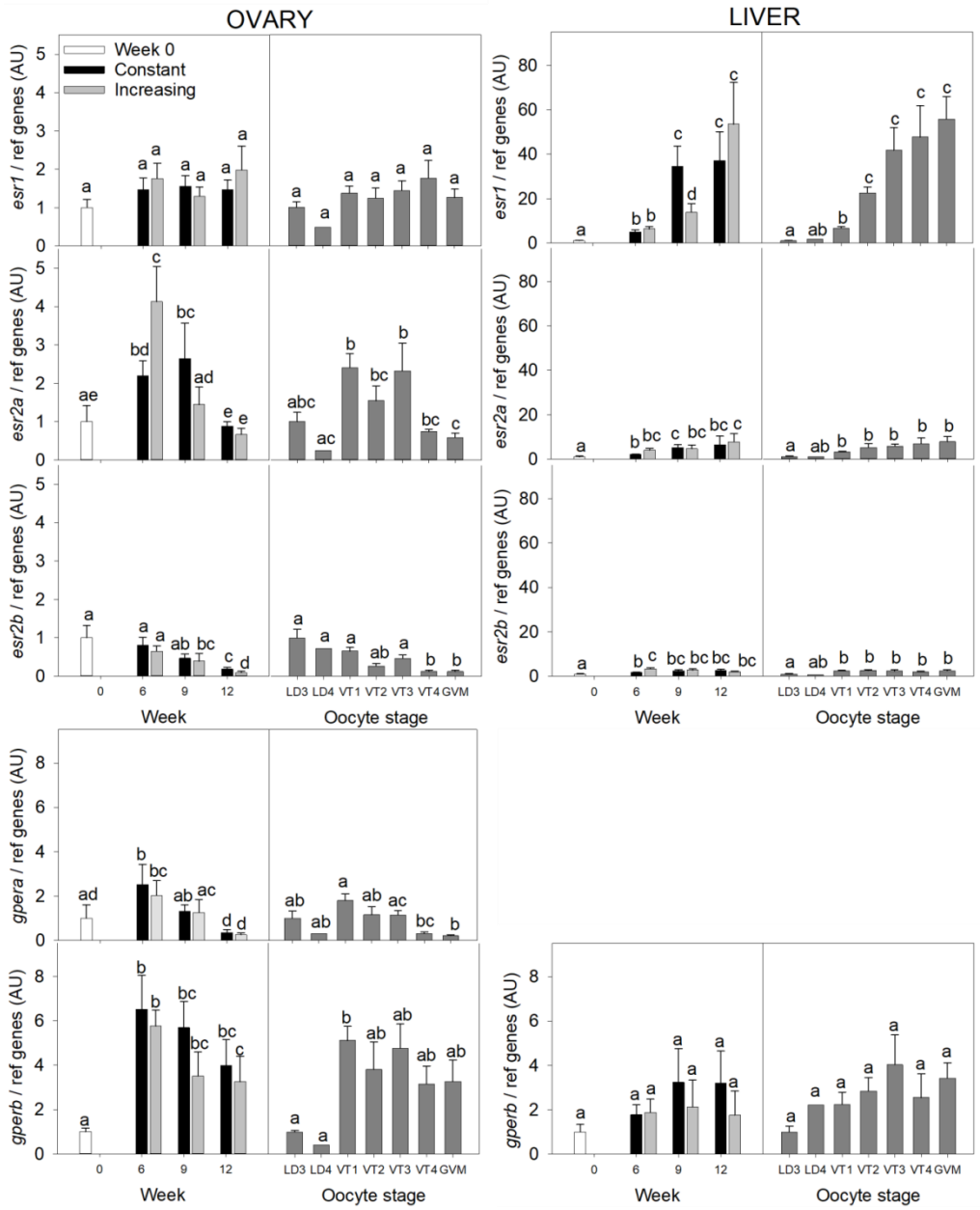

Fig. 6. Relative gene expression of nuclear (*esr1*, *esr2a* and *esr2b*) and membrane estrogen receptors (*gpera* and *gperb*) in ovary and liver samples of female European eel measured by qPCR according to either treatment group (i.e. constant or increasing CPE dose) or ovarian developmental stage. Data are presented as fold change in arbitrary units (AU) according to the $2^{-\Delta\Delta CT}$ method, error bars represent SEM and different letters indicate significant differences ($P < 0.05$, Tukey test). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes. Abbreviations: LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage. See number of samples in Table 3.

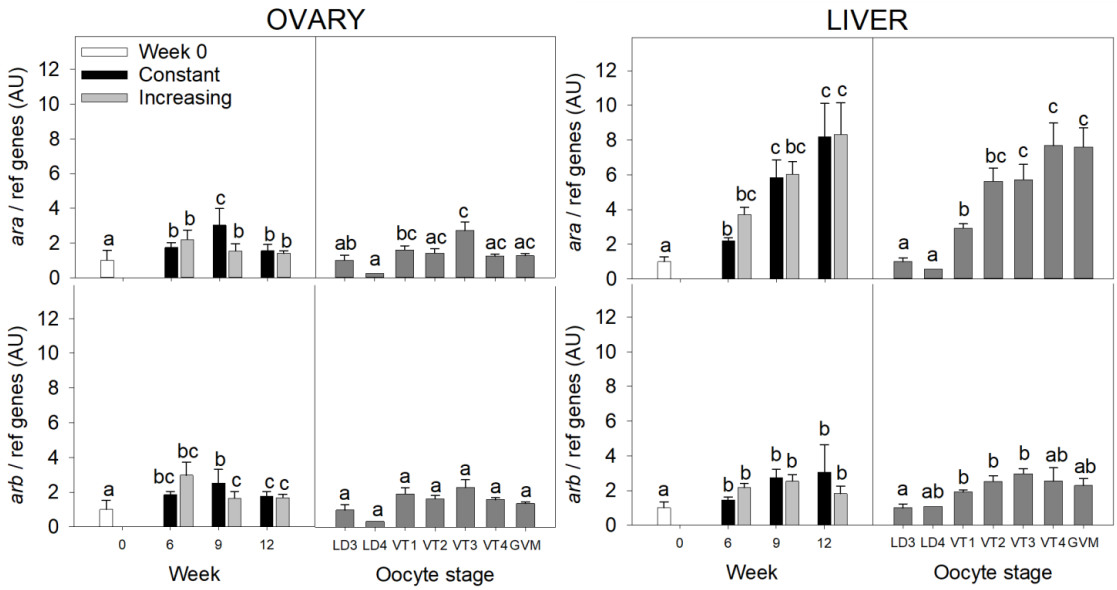

Fig. 7. Relative gene expression of androgen receptor alpha (*ara*) and beta (*arb*) in ovary and liver samples of female European eel measured by qPCR according to either treatment group (i.e. constant or increasing CPE dose) or ovarian developmental stage. Data are presented as fold change in arbitrary units (AU) according to the $2^{-\Delta\Delta CT}$ method, error bars represent SEM and different letters indicate significant differences ($P < 0.05$, Tukey test). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes. Abbreviations: LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage. See number of samples in Table 3.

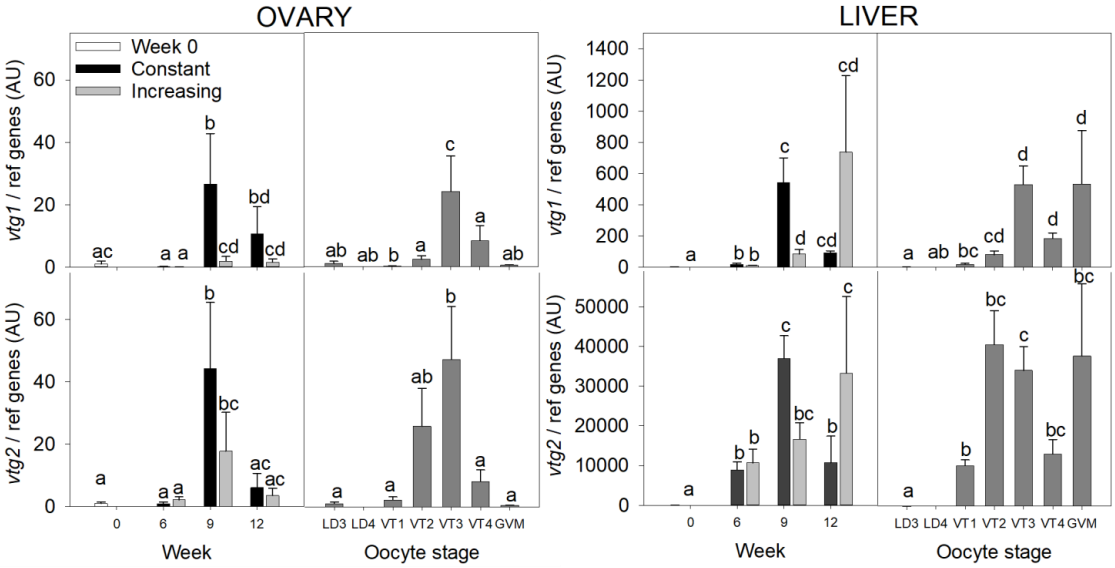

Fig. 8. Relative gene expression of vitellogenin 1 (*vtg1*) and 2 (*vtg2*) in ovary and liver samples of female European eel measured by qPCR according to either treatment group (i.e. constant or increasing CPE dose) or ovarian developmental stage. Data are presented as fold change in arbitrary units (AU) according to the $2^{-\Delta\Delta CT}$ method, error bars represent SEM and different letters indicate significant differences ($P < 0.05$, Tukey test). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes. Abbreviations: LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage. See number of samples in Table 3.

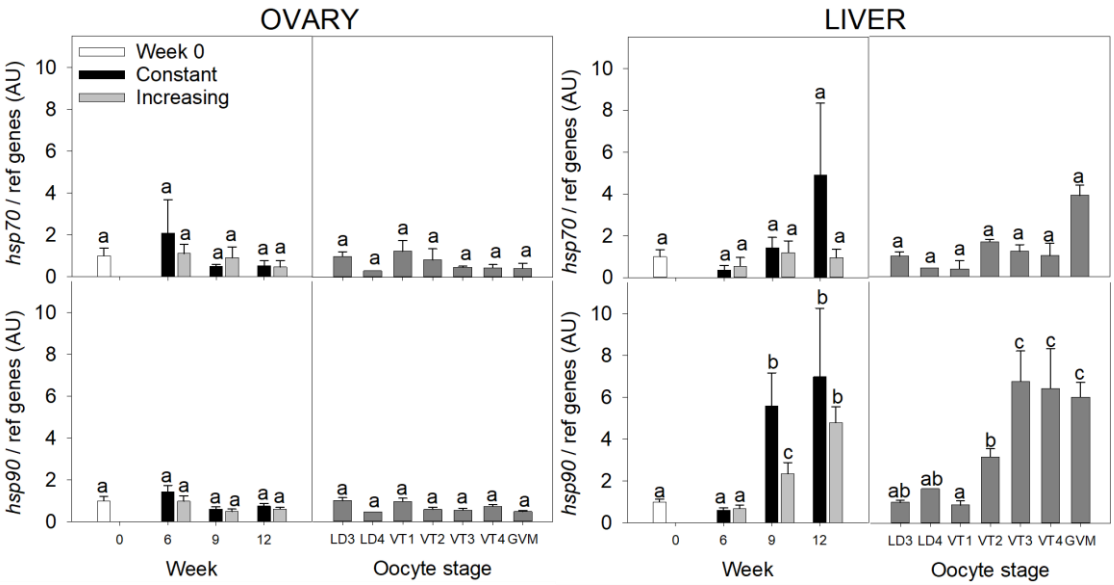

Fig. 9. Relative gene expression of heat shock protein 70 (*hsp70*) and 90 (*hsp90*) in ovary and liver samples of female European eel measured by qPCR according to either treatment group (i.e. constant or increasing CPE dose) or ovarian developmental stage. Data are presented as fold change in arbitrary units (AU) according to the $2^{-\Delta\Delta CT}$ method, error bars represent SEM and different letters indicate significant differences ($P < 0.05$, Tukey test). Elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*) were used as reference genes. Abbreviations: LD = lipid droplet stages 3 and 4, VT = vitellogenic stages 1 to 4, GVM = germinal vesicle migration stage. See number of samples in Table 3.

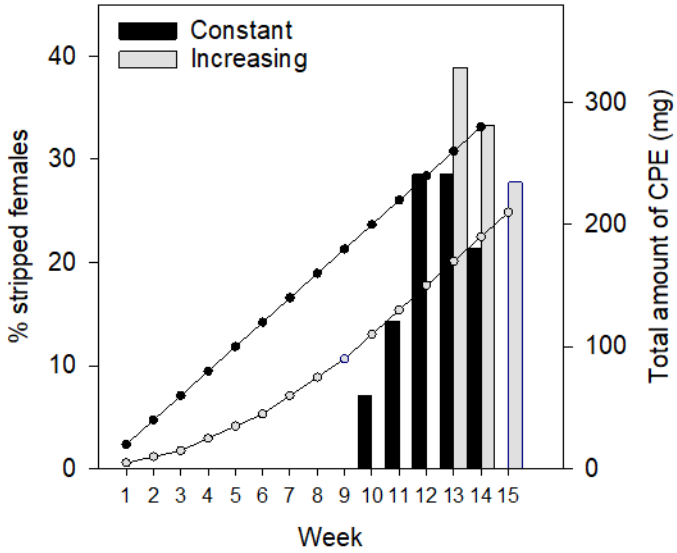

Fig. 10. Percentage of successfully stripped female European eels (left y-axis) for each week (bars) and total amount of CPE (mg) administered per female (right y-axis) at a given week (lines) according to treatment.

Manuscripts

Paper II

Transcriptome profiles highlight activation of key ovarian functions during induced vitellogenesis in European eel

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Abstract

In all vertebrates including teleosts, sexual maturation and reproduction is regulated by endocrine activity on the brain-pituitary-gonadal axis. Yet, in the enigmatic case of European eel, *Anguilla anguilla*, the reproductive development is inhibited by complex neuroendocrine control at the brain-pituitary level prior migration to their spawning area in the Sargasso Sea. Our aim was to elucidate transcriptomic responses to induced vitellogenesis using carp pituitary extract (CPE) in the ovary of female eels. Ovarian tissue samples from females before induction (previtellogenesis) and after 9 weeks of CPE treatment (late vitellogenesis) were compared using RNA-Seq. The analysis showed an upregulation of genes related to vitellogenesis including receptors for gonadotropins, steroids, insulin-like growth factors, activins and enzymes for steroidogenesis. Vitellogenins were the most upregulated genes suggesting local production in the ovary. Genes connected to early oocyte development, such as zona pellucida proteins, were downregulated, whereas genes for aquaporins involved in ovulation, a vitamin A carrier, and fatty acid-binding proteins linked to atresia were upregulated. In accordance with the observed ovarian growth, genes related to tissue growth were also upregulated. Altogether, this study showed that CPE treatment activated and regulated a large number of genes and biological processes related to reproductive development in teleosts.

Keywords

Anguilla anguilla; assisted reproduction; gonadotropins; oogenesis; RNA-Seq

1. Introduction

As for all vertebrates, sexual maturation and reproduction in fish is controlled by the brain through hormonal action on the brain-pituitary-gonadal (BPG) axis. In oviparous animals, including most teleosts, the BPG axis in females is commonly referred to as the brain-pituitary-gonadal-liver axis (BPGL-axis), because the liver is responsible for synthesises of yolk proteins that are needed for oocyte development and production of viable eggs (Hara et al., 2016). This is known as vitellogenesis and the process commences at the onset of the reproductive cycle. In the ovary, the transition of oocytes from a previtellogenic to vitellogenic stage differentiates two growth phases, namely primary and secondary oocyte growth (Tyler & Sumpter, 1996; Patiño & Sullivan, 2002; Grier et al., 2009; Franca et al., 2010).

During previtellogenesis, membranous organelles proliferate, mRNAs accumulate in the ooplasm and in marine pelagophil teleosts, lipid droplets often appear (Grier et al., 2009; Lowerre-Barbieri et al., 2011; Selman & Wallace, 1989; Grier et al., 2009; Kagawa, 2013). At the end of previtellogenesis, cortical alveoli are formed within the ooplasm of many species (Selman & Wallace, 1989), marking the transition into secondary growth (Mommsen & Walsh, 1988).

During vitellogenesis, the oocyte develops and accumulation of yolk globules in the ooplasm is governed by endocrine activity on the BPGL-axis (Reading et al., 2017). The first step of vitellogenesis is activation of neural pathways in the brain through environmental and internal stimuli leading to the production of gonadotropin-releasing hormones (GnRH) by the brain. GnRH acts on the pituitary gland initiating the secretion of two gonadotropins, hormones controlling gonad development, known as follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In female teleosts, FSH stimulates the secretion of estrogens from the follicle through its receptor (FSHR). The estrogens then bind to their receptors in the liver leading to the production of vitellogenin, a precursor to yolk. Vitellogenin is then transported to the ovary via the bloodstream and begins to accumulate as yolk in the oocyte resulting in massive growth accompanied by thickening of the zona pellucida, leading to the final maturation stage (Ho, 1987; Reading et al., 2017).

During oocyte maturation, the nucleus (germinal vesicle) migrates to the animal pole, where the micropyle is located. Particularly, LH is involved in the maturation process and subsequent ovulation. The hormone binds to its receptor (LHR) in the follicle initiating the release of progestogens, also known as maturational inducing steroids (MIS). MIS reactivates meiosis, ultimately leading to ovulation of eggs and spawning (Cerdá et al., 2008; Kazeto et al., 2011).

In nature, sexual maturation and reproductive development are generally induced by external and internal cues. In the case of eels, sexual maturation is arrested at a pre-pubertal stage when they initiate their enigmatic migration to the spawning area in the Sargasso Sea (Tesch, 1977, 1982). Neuroendocrine mechanisms that impede sexual maturation have been identified to be insufficient GnRH and dopaminergic inhibition at the brain-pituitary level, which disables the secretion of FSH and LH (Dufour et al., 1988; Vidal et al., 2004). In experimental work, sexual maturation of female eels can be induced through repeated hormonal injections of piscine pituitary extracts (PE), as a exogenous source of FSH and LH, thereby counteracting the dopaminergic inhibition (Kagawa et al., 2003; Mylonas et al., 2010; Okamura et al., 2014). Common practice to induce vitellogenesis is through weekly PE injections for a period of 11-29 weeks until the most advanced oocytes initiate final maturation (Okamura et al., 2014). To complete final maturation and for ovulation to proceed MIS must be administered exogenously (Yamauchi, 1990; Ohta et al., 1996, 1997). The endocrine and regulatory mechanisms on the BPGL-axis during induced maturation in Japanese eel, *Anguilla japonica*, European and shortfinned eel, *Anguilla australis* have extensively been studied (Dufour et al., 2003; Weltzien et al., 2009). Nevertheless, many questions remain as neither the neural inhibitory mechanisms nor the precise regulation of oocyte development during induced vitellogenesis are fully understood.

Recent research has expanded knowledge on the complexity of this chain-of-events and extended regulatory mechanisms to include genes that traditionally have been associated with other biological processes. In the eel, previous studies have for instance implicated androgens and insulin-like growth factors (IGFs) in influencing oocyte growth (Rohr et al., 2001; Lokman et al., 2007). By analysing the ovarian transcriptome of vitellogenic females, multiple endocrine regulations can be further elucidated, similar to studies in other fish, such as Nile tilapia, *Oreochromis niloticus* (Tao et al., 2013), olive flounder, *Paralichthys olivaceus* (Fan et al., 2014) and Atlantic cod, *Gadus morhua* (Kleppe et al., 2014). One study on the ovarian transcriptomes of European eel has been conducted, which compared immature and post-spawning matured eels (Burgerhout et al., 2016).

In this context, we aimed at experimentally investigating the regulation of oocyte and ovarian development at the transcriptome level using RNA-Seq. Therefore, we compared transcriptomes of ovarian tissue sampled from females before PE treatment and after nine weeks of treatment, thus targeting a late vitellogenic stage at the latter sampling point.

2. Results

2.1. Ovarian development

The weekly carp pituitary extract (CPE) injections induced ovarian development in female eels as evident by an increase in gonado-somatic index (GSI) from 1.5±0.3 in week 0 to 16.7±6.6 in week 9 ($p < 0.001$; Table 1). The histological analysis showed that females progressed from previtellogenesis in week 0 to a late vitellogenic stage after 9 weeks of CPE treatments (Table 1; Figure 1). Thus, the most advanced oocytes in the ovaries were in a previtellogenic stage, i.e. lipid droplet stage 3 (LD3) and 4 (LD4), before CPE induction (Figure 1A). Less developed previtellogenic oocytes were present simultaneously, including pre-lipid droplet oocytes (PLD) and lipid droplet stages 1-2 (LD1-2), and adipocytes were abundant between oocytes. In week 9, the most advanced oocytes were in a mid or late vitellogenic stage (Figure 1B). Vitellogenic oocytes were characterised by a significant increase in volume, in comparison with previtellogenic oocytes, as well as abundance of yolk globules and large lipid droplets in the ooplasm (Figure 1B-C). All specimens were in vitellogenic stage 3 (VT3), except one female that was in stage 2 (VT2). Less developed vitellogenic oocytes (VT1) and previtellogenic oocytes (PLD and LD1-4) were also present, while adipocytes were no longer visible.

In agreement with GSI and histology, a principal component analysis (PCA) plot based on the RNA-Seq analysis showed that samples from week 0 and 9 clustered into two separate groups (Figure 2; Table 1). This differentiation was observed in spite the asynchronous oocyte development at both sampling points (Figure 1A-B). In week 0, the samples clustered closely with the exception of one, i.e. female 6. The oocytes in this female were at a more advanced stage (LD4) compared to the other females (LD3) in week 0 (Table 1). The scatter of week 9 samples was consistent with the variations observed in GSI (Table 1).

2.2. Mapping statistics and differential expression

After sequencing and filtering each sample, approximately 30,400,000*2 paired reads were produced. For week 0 and 9 groups, respectively 55.9±1.3% and 52.9±1.4% reads were uniquely mapped to the eel reference genome (Table 2) (Henkel et al., 2012), which are comparable to the mapping results reported by Burgerhout et al. (2016) in an RNA-Seq study on the ovaries of the same species and using the same European eel reference genome.

Similar to observations in GSI, histology and PCA plot, a clear differentiation between transcriptomes from week 0 and 9 was observed in the differential expression analysis.

After removing 193 DEGs with the same gene symbol, which showed opposite log_2-fold changes, 3,355 significantly differentially expressed genes (DEGs) were detected out of which 2,195 were upregulated and 1,660 were downregulated (Figure 3). The complete list of DEGs can be found in Table S1. Several DEGs of interest that are known to be involved in oocyte and ovarian development as well as vitellogenesis were detected. For instance, vitellogenin 1 and 2 (*vtg1* and *vtg2*) associated with vitellogenesis were the most upregulated in the analysis. The third highest upregulated gene was collagen alpha-2 chain (*co1a2*). Furthermore, DEGs known to be involved in reproductive processes and steroidogenesis, for example estrogen receptor 2 beta (*esr2b*), aromatase (*cyp19a1*) and arylsulfatase family member j (*arsj*) were upregulated. Similarly, several activin receptors (*acvr1b*, *acvr1c*, *acvr2a* and *acvr2b*) were upregulated, which have been suggested to be involved in oocyte development. Aquaporins (*aqp1*, *aqp4* and *aqp8*), serine protease 35 (*prss35*), metalloprotease-disintegrin genes (*adam15*, *adam17*, *adam19* and *adamts3*) and angiotensin-converting enzyme 2 (*ace2*) were upregulated and these genes have been linked to ovulation. In contrast, other DEGs were downregulated, such as very low-density lipoprotein receptor (*vldlr*) that is a vitellogenin receptor as well as zona pellucida sperm-binding proteins (*zp1*, *zp3* and *zp4*) that are components of the outer layer of the egg.

2.3. DEGs in the overrepresentation analysis

Through the overrepresentation analysis, 260 gene ontology (GO) biological processes were identified and a complete overview is found in Table S2. Several of the biological processes were involved in ovarian development, such as reproduction, tissue development, embryonic development, embryonic development ending in egg hatching and lipid metabolic processes (Figure 4). In this study, we primarily focused on these processes. Furthermore, several DEGs that were related in function to others, but not to the GO process, were identified as well.

The GO biological process reproduction contained 15 up- and 33 downregulated DEGs (Figure 4). The former included well-known genes involved in reproduction such as follicle-stimulating hormone receptor (*fshr*), luteinizing hormone receptor (*lhcgr1*), androgen receptor alpha (*ara*) and beta (*arb*), progesterone receptor type 2 (*pgr2*) and cytochrome P450 (*cyp17a1*). Downregulated genes included PIWI-like protein 2 (*piwil2*), growth and differentiation factor 9 (*gdf9*) and bone morphogenetic factor 15 (*bmp15*).

Within the GO process tissue development, the majority of DEGs were upregulated, 118, while 60 were downregulated (Figure 4). Collagen type VI (*col6a1*, *col6a2* and *col6a3*), vascular endothelial growth factor C (*vegfc*) and vascular endothelial growth factor receptor 3 (*flt4*) were upregulated. The two latter, COL6 transcripts were

associated with this GO term, whereas the former was not. Several genes that are linked with insulin-like growth factors were upregulated, specifically insulin-like growth factor binding proteins (*igfbp2a, igfbp3, igfbp7* and *igf2bp3*), insulin-like growth factor 1 receptor (*igf1r*) and insulin receptor (*insr)*. Transcripts *igfbp3* and *igfbp7* were detected in the overrepresentation analysis, while the others are related in function. Lastly, genes that have been linked with oogenesis were upregulated in the analysis, including a nuclear receptor subfamily 5 group a member 2 (*nr5a2*) as well as fatty acid-binding protein 3 and 7 (*fabp3* and *fabp7*). Though *fabp3* and *fabp7* are related in function, the latter was not associated with this GO term.

DEGs that play a role in the GO process embryonic development included 111 up- and 68 downregulated (Figure 4). Several of these genes overlapped with a second GO pathway relating to embryonic development ending in egg hatching, where 50 up- and 37 downregulated were identified. Upregulated DEGs of interest included genes that were also detected in the GO process tissue development, specifically *fabp3* and *igfbp3*.

In total, 61 and 40 DEGS were respectively up- and downregulating in the GO process lipid metabolic processes (Figure 4). Upregulated DEGs genes belonging to the cytochrome P450 enzyme family, namely cytochrome P450 family 17 subfamily A member 1 (*cyp17a1*) and cholesterol side-chain cleavage enzyme (*cyp11a1*). Other upregulated DEGs were included retinol-binding protein 4-b (*rbp4b*) and 17β-hydroxysteroid dehydrogenase 1 (*hsd17b1*). Furthermore, hydroxysteroid dehydrogenase 8 (*hsd17b8*) is related in function to *hsd17b1*, but was not associated with the GO term.

2.4. Validation of gene expression

To validate the DEGs discovered in the RNA-Seq analysis, the expression of eight genes were measured through qPCR. These included seven DEGs that in the differential expression analysis were upregulated from week 0 to 9 (*vtg1*, *vtg2*, *fshr*, *esr2b*, *ara*, *arb* and *lhcgr1*) and one that was downregulated (*rps18*). The selected genes showed the same expression pattern regardless of method, as evidenced by the highly significant linear correlation between RNA-Seq and qPCR data ($R^2 = 0.91$, $p = 0.00023$; Figure 5).

3. Discussion

3.1. Morphological and tissue adaptations during ovarian development

In female eels, Induction of vitellogenesis results in a conspicuous increase in ovary size, often measured through GSI, and occurs concomitant with morphological changes (Palstra & Thillart, 2009). The increase in ovary size in fish is largely a result of oocyte

growth (Grier et al., 2009; Kagawa, 2013). In correspondence, the increase in GSI from week 0 to 9 in the present study related to the progression of the most advanced oocytes from previtellogenic to vitellogenic stages. Along with the developing oocytes, the supporting tissue also grows. Particularly, there is an expansion of the connective tissue and capillary network (i.e. angiogenesis) as the ovary grows (Redmer & Reynolds, 1996; Reynolds & Redmer, 1998). In this context, *co1a2* was the third highest upregulated transcript in our differential expression analysis and it encodes collagen, the primary component in connective tissue. Similarly, *col1a1* was found expressed in the developed ovaries of PE treated European eels (Burgerhout et al., 2016). Besides upregulation of additional collagen transcripts (*col6a1*, *col6a2* and *col6a3*), genes related to endothelial growth (*vegfc* and *flt4*) also increased in expression level from week 0 to 9. In fact, VEGF is known to regulate angiogenesis in the ovary of vertebrates (Redmer & Reynolds, 1996; Reynolds & Redmer, 1998). During induced ovarian development, the regulation of genes for growth of supporting tissues tends to follow the observed increase in oocyte development and GSI, as generally anticipated.

3.2. Activation of genes and pathways during ovarian development

Accompanying the morphological changes of ovaries, immense transcriptional activity occurs during vitellogenesis, where processes are activated and the expression of genes increases, resulting in RNA accumulation (Mazabraud et al., 1975; Cerdá et al., 2008; Franca et al., 2010; Davidson, 2012). Congruently, a substantial change in transcriptional activity was evident in the present study, and the transition from previtellogenesis to vitellogenesis was reflected in the detection of a large number of DEGs between the two groups. The overrepresentation analysis identified several GO biological processes related to reproduction. Moreover, other DEGs not that were not part of these processes, but are known to be relevant in other species, were detected. For example, the two most upregulated genes in this study, i.e. vitellogenins (*vgt1* and *vgt2*), were not related to a GO process, although they are linked to vitellogenesis. Thus, investigated DEGs were associated with pathways and processes such as early oocyte development, oocyte vitamin uptake, vitellogenesis, steroidogenesis, ovulation and atresia.

The transition from the first growth phase (previtellogenic) to the second (vitellogenic) is accompanied by a change in expression of specific genes related to oocyte development (Lubzens et al., 2010). In the present study, transcript levels of *piwil2* were downregulated from the early oocyte stage (previtellogenic) to the vitellogenic stage. In accordance, PIWI proteins are important in germline development, and transcript levels have been observed to be higher in the earlier stages of oocyte development in zebrafish, *Danio rerio* (Ziv et al., 2008). Similarly, expression levels of *gdf9* and *bmp15*

were downregulated at week 9. The corresponding proteins, GDF9 and BMP15, which are part of the TGF-β family, have previously been implicated in previtellogenic oocyte growth in mammals as well as in some teleosts, including zebrafish (Liu & Ge et al., 2007), Altantic cod (Kleppe et al., 2014) and sea bass, *Dicentrarchus labrax* (Halm et al., 2008). In fact, a decrease in the expression of these proteins has been observed with increased gonadotropin receptor expression (Lubzens et al., 2010; Pfennig et al., 2015). In the present study, *gdf9* and *bmp15* expression profiles followed the patterns observed in previous studies.

Following early oocyte growth, gonadotropin and associated steroid hormone activities initiate and advance vitellogenesis (Donaldson et al., 1973; Yaron et al., 2003). Overall, steroidogenesis is under control of the gonadotropins, FSH and LH, and their activity is mediated through their respective receptors, FSHR and LHR (Levavi-Sivan et al., 2010). Generally, FSH is considered important for oocyte development in the early stages of vitellogenesis, while LH primarily is associated with final maturation in preparation for ovulation and spawning (Nagahama & Yamashita, 2008). In the present study, gonadotropin receptors *fshr* and *lhcgr1* were highly upregulated from previtellogenesis to vitellogenesis. This pattern is similar to observations in yellowtail, *Seriola quinqueradiata* (Rahman et al., 2003), zebrafish (Kwok et al., 2005) and Japanese eel (Jeng et al., 2007).

When FSH binds to its receptor, the synthesis of the major estrogen 17β-estradiol (E2) is induced. E2 is synthesised by the enzyme aromatase (CYP19A1) from androgens (testosterone) in the follicular cells (Tokarz et al., 2015). Accordingly, *cyp19a1* was upregulated in week 9. An upregulation of *cyp19a1* has often been observed during vitellogenesis, e.g. in rainbow trout, *Oncorhynchus mykiss* (Tanaka et al., 1992), Nile tilapia (Chang et al., 1997b), red seabream, *Pagrus major* (Gen et al., 2001) and Japanese eel (Jeng et al., 2012). Additional key players in steroidogenesis were upregulated in females in week 9, such as *cyp11a1* and *cyp17a1*, 17β-hydroxysteroid dehydrogenases (*hsd17b1* and *hsd17b8*) and liver receptor homolog-1 (*nr5a2*). CYP and HSD enzymes have previously been observed upregulated in several teleost species during ovarian development (Uno et al., 2012; Tokarz et al., 2015). Our findings are in accordance with the previous study by Burgerhout et al. (2016) showing that the expression of *cyp17a1* and *nr5a2* was higher in matured than immature female European eels. Interestingly, the activity of *nr5a2* has previously been linked to CYP17A1 in human cells (Yazawa et al., 2009). Additional overlaps with the study of Burgerhout et al. (2016) include the regulation of *hsd17b1* and an asylsulfatase (*arsj*). Sulfatases activate steroids, among other substances, from inactive steroid sulfates (Reed et al., 2005, Purohit et al., 2011).

While it is well-known that estrogens are key players in vitellogenesis in the liver, they also influence oocyte development through their receptors here. The functional roles of the different estrogen receptor sub-types are still unclear. One nuclear estrogen receptor subtype paralog (*esr2b*) was found upregulated in this study. The presence of estrogen nuclear receptor sub-types in the ovary has previously been described for Nile tilapia (Chang et al., 1999), channel catfish, *Ictalurus punctatus* (Patiño et al., 2000) and gilt-head seabream, *Sparus aurata* (Socorro et al., 2000) as well as in the European eel (Lafont et al., 2016). In Japanese eel, the expression of ovarian *esr2* did not significantly change during PE induced vitellogenesis, but the study did not specifically refer to either the *esr2a* or *esr2b* paralog (Jeng et al., 2012).

Both androgen receptors paralogs (*ara* and *arb*) were upregulated in the present study. Similar observations have been made for both Japanese and European eel matured with PE (Jeng et al., 2012; Peñaranda et al., 2014). Androgens have been linked to oogenesis in several fish, including coho salmon, *Oncorhynchus kisutch* (Fitzpatrick et al., 1994) and Atlantic cod (Kortner et al., 2009). More specifically, a role in lipid droplet accumulation during oogenesis has been suggested in shortfinned and Japanese eel (Rohr et al., 2001; Lokman et al., 2007; Tosaka et al., 2010).

After synthesis, E2 is transported through the blood to the liver, where it binds to receptors, thus inducing vitellogenin production. Then, vitellogenin is transported from liver to ovary and is sequestered from capillaries via membrane vitellogenin receptors (Prat et al., 1998; Hiramatsu et al., 2004; Reading et al., 2017). In this study, an ovary-specific vitellogenin receptor (VTGR), also named very low-density lipoprotein receptor (*vldlr*), was downregulated. Comparable observations have been observed in the oocytes of other fish. In European eel, *vldlr* was expressed in yellow eel, decreased in silver eels and were stable in hormonally treated eels (Morini et al., 2020). In rainbow trout, VTGR transcripts were also most abundant in previtellogenic oocytes and the authors suggested that VTGRs are recycled in oocytes of fish during the vitellogenic growth phase (Perazzolo et al., 1999).

Notably, the top two highest upregulated genes in the ovaries were vitellogenins (*vtg1* and *vtg2*), the synthesis of which is typically only associated with the liver. In previous studies on zebrafish (Wang et al., 2005) and turbot, *Scophthalmus maximus* (Xue at al., 2018) vitellogenin genes have been observed expressed extrahepatically, including in ovarian tissue. In fact, in turbot an upregulation of vitellogenin genes was observed from previtellogenesis to vitellogenesis, in agreement with our findings. In another study, we looked at gene expression of *vtg1* and *vtg2* in ovarian samples across several time points (week 0, 6, 9 and 12; Jørgensen et al., submitted). In this previous study, the vitellogenins had the highest expression in week 9 and showed the largest fold-change in the ovary out of 13 analysed genes using qPCR. In the present RNA-Seq analysis, we

could increase the number of investigated genes between week 0 and 9 to several thousands. Consequently, we could observe that vitellogenin genes were still the most upregulated, supporting the suggestion of local vitellogenin production in the ovary.

In addition to steroid hormones, the gonadal peptide hormones activins are important for oocyte development (Ge, 2005). Activins are produced by the ovary and their activity is mediated through activin receptors. In the present study, an upregulation of several activin receptors (*acvr1b*, *acvr1c*, *acvr2a* and *acvr2b*) was observed in vitellogenic females. In vertebrates, activin is a known enhancer of FSH synthesis (Ethier & Findlay, 2001), which was also specifically observed in European eel (Aroua et al., 2012). In zebrafish, an upregulation of activin type II receptors was associated with oocyte development (Pang et al., 2002).

Several other receptors and proteins have been observed to advance oocyte development, besides the described key players. For instance, receptors and binding proteins related to insulin-like growth factors (IGFs) were upregulated, including IGF1 receptor (*igf1r*), binding proteins (*igfbp*) and insulin receptor (*insr*). IGF-I has been associated with final maturation in red seabream (Kagawa et al., 1994), oocyte development in coho salmon and Atlantic cod (Campbell et al., 2006; Kleppe et al., 2014), and oocyte growth in eel (Lokman et al., 2007). In addition to IGFR1, INSR is able to bind IGFs (Steffensen et al., 2018). Interestingly, IGFBP3 is suspected of increasing the oocytes' response competence to MIS (Kamangar et al., 2006). Thus, the observed upregulations are in accordance with the role played by the IGF system in oocyte development.

Besides accumulation of yolk to ensure resources for embryonic development after fertilisation, vitamin transport into the oocyte is crucial (Lubzens et al., 2010). During embryogenesis, vitamin A plays a role in regulating transcription (Balmer & Blomhoff, 2002). Uptake of vitamin A into the oocyte is associated with lipoprotein transport and vitellogenin is another known carrier between the liver and ovary during vitellogenesis (Levi et al., 2008). In this context, a known carrier of vitamin A, retinol-binding protein 4-b (*rbp4b*), was upregulated from week 0 to 9. In the ovaries of rainbow trout, *rbp4* was expressed at similar levels in juveniles, vitellogenic and post-vitellogenic females (Levi et al., 2008). Interestingly in pigs, the expression of *rbp4* increased with follicle size (Rao et al., 2019). Furthermore, in the same study expression also increased significantly in ovarian tissue treated with FSH and LH, leading the authors to suggest that that RBP4 plays a role in follicular growth.

The layer between the follicle cells and the plasma membrane of the oocytes is called the zona pellucida (ZP) and is comprised of ZP proteins (Modig et al., 2007). In teleosts, the ZP is usually comprised of two to four different ZP proteins (Litsher & Wassarman, 2018). We found three genes encoding ZP proteins downregulated in week

9 females. In previous studies in European and Japanese eel, *zp* expression was observed to decrease from previtellogenesis to late vitellogenesis (Sano et al., 2010; Mazzeo et al., 2013). The transcripts have been also detected in previtellogenesis and vitellogenesis of gilthead seabream (Modig et al., 2006) and carp, *Cyprinus carpio* (Chang et al., 1996, 1997a). A noteworthy point is that for many fish, ZP protein synthesis occurs in the liver under E2 control, whereas for others the ovary is the place of synthesis, as observed presently. In view of this, Mazzeo et al. (2013) characterised the expression of *zp* genes in the ovary and liver of European eel and determined that the former was the primary site of ZP synthesis, which is also the case for the Japanese eel (Sano et al., 2010).

Following the final stages of vitellogenesis, LH activity leads to MIS-induced final follicular maturation that involves resumption of meiosis as well as nuclear migration and breakdown (Cerdá et al., 2008; Kazeto et al., 2011). MIS activity is mediated through membrane progesterone receptors (Thomas et al., 2002) that were not detected in our study, where the most advanced oocytes were in late vitellogenic stage. However, a nuclear progesterone receptor (*pgr2*) was upregulated. The regulation in expression of the various paralogs of nuclear and membrane progestin receptors has been investigated during spermatogenesis in male European eels (Morini et al., 2017), but data in female eels are still needing. The mRNA of nuclear PGR has previously been detected in the ovaries of zebrafish (Chen et al., 2010) and Atlantic salmon, *Salmo salar* (Chen et al., 2011). Because the receptor was localised in the follicular cells of zebrafish oocytes, it was suggested that it affects ovarian follicular functions, but does not partake in final maturation (Hanna et al., 2010).

When final maturation is completed, spawning can commence. As most marine fish, in nature the female eels likely release their mature gametes to the environment for external fertilisation. Consequently, there are mechanisms that prepare the oocytes for the change in osmotic conditions when spawned. One mechanism is water uptake, which is mediated by aquaporins and is critical for hydration as well as for increasing buoyancy of the oocytes (Fabra et al., 2005; Chauvigné et al., 2011). Congruently, aquaporins were upregulated in vitellogenic females in the present study. Genes encoding aquaporins have previously been observed upregulated in vitellogenesis in gilthead seabream (Fabra et al., 2006). Furthermore, genes, such as serine protease 35 (*prss35*), metalloprotease-disintegrins, members of the tumor necrosis factor (TNF) family, angiotensin-converting enzyme 2 (*ace2*) and fibroblast growth factors were upregulated in the present study. These have previously been observed to be involved in ovulation in salmonids (Lubzens et al., 2010).

While many detected DEGs were related to promoting growth or preparing for impending events, there were also some connected to atresia, i.e. breakdown of follicles.

Atresia is a hormonally regulated apoptotic process that can ensue if the hormonal signalling is disrupted during ovarian development (Saidapur, 1978). In this context, two fatty acid-binding proteins (FABPs) were upregulated in the analysis (*fabp3* and *fabp7*). FABPs are typically associated with lipid and lipoprotein metabolism; however, they have also been linked to atresia (Agulleiro et al., 2007). In zebrafish, the *fabp3* was detected in the ovary and liver and was most abundant during previtellogenesis and decreased with maturation (Liu et al., 2003), in agreement with present findings.

Conclusion

In this study, the upregulation of a large number of genes during induced vitellogenesis was observed. Despite artificial induction with PE, the subsequent activation of genes, biological processes and associated pathways paralleled observations in other species. This included the upregulation of genes related to vitellogenesis, such as gonadotropin receptors, steroid receptors and genes related to steroidogenesis. A key finding was that vitellogenins were the most upregulated genes. Thus, it seems that the ovary produces vitellogenins, besides the liver, which expands the present knowledge on the ovary-liver axis. Moreover, other genes related to oocyte development were upregulated, including several IGF receptors and binding proteins as well as activin receptors. Genes related to ovulation and vitamin uptake were also upregulated, such as aquaporins and retinol-binding protein 4-b, respectively. In contrast, genes related to early oocyte development were downregulated in vitellogenic females. Lastly, collagens and endothelial growth factors, related to tissue growth and angiogenesis, were upregulated in accordance with an observed increase in GSI. Together, the findings in this study can serve as benchmark for further transcriptomic investigations on the regulation of vitellogenesis in eels, but can also generally contribute to insights in teleosts.

4. Materials and methods

4.1. Ethics statement

All experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696) and fish were handled according to the European Union regulations regarding protection of experimental animals (Dir 86/609/EEC). Efforts to reduce stress and handling of animals were implemented.

4.2. Fish rearing and hormonal treatment

Farmed female eels reared in freshwater at a commercial eel farm (Stensgård Eel Farm A/S, Randbøl, Denmark) were transported in an aerated freshwater tank to the research facility EEL-HATCH at the Technical University of Denmark, Hirtshals. The eels (n = 38, total length 76 ± 136 3 cm, body weight 900 ± 102 g) were equally distributed into three 1150 L tanks in a recirculating aquaculture system and acclimatised to saltwater (from 0 to 36 psu) over two weeks by gradually adding natural seawater supplemented with Blue Treasure Aquaculture Salt (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Quindao, China). Temperature was maintained at 20°C. Light followed a 12 h day and 12 h night regime with low light intensity ~20 lux W and 30 min transition time.

After acclimation, individual eels were anaesthetised in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg L^{-1}, Sigma Aldrich, Darmstadt, Germany) and tagged with a passive integrated transponder (PIT, 12×2 mm) tag in the dorsal musculature for ID. Eight female eels were randomly selected for sampling prior CPE treatment, i.e. week 0 (total length 75 ± 4 cm, body weight 862 ± 100 g). For the remaining females, weekly intramuscular injections were initiated using a constant dose of 20 mg kg^{-1} of CPE according to initial body weight (Ducamar Spain S.L.U, Bilbao, Spain) to induce ovarian development. Dried carp pituitaries were grinded, diluted with physiological NaCl, centrifuged, and the supernatant was stored at –20°C before use (Ohta et al, 1996, 1997). After 9 weeks of treatment, eight females were randomly selected for sampling, i.e. week 9 (total length 75 ± 2 cm, body weight 901 ± 62 g).

4.3. Sampling for analyses

The sampled eels, i.e. week 0 (n = 8) and week 9 (n = 8), were euthanized by submergence in an aqueous solution of benzocaine (20 mg L^{-1}) for 5-10 min. Total body weight (g) and length (cm) were measured. The ovary was then dissected and weighed (g), GSI [(gonad weight / body weight) × 100] was calculated and tissue samples were collected at both sampling times.

Tissue was sampled (~0.01 g) from the midsection of ovaries and preserved in RNA-later (Ambion Inc.) at 4°C for 24 h and –20°C until qPCR and RNA-Seq analysis. Ovarian tissue samples for histology were obtained from the same location and fixed in 4% solution of formaldehyde (Hounisen, Skanderborg, Denmark) at room temperature.

4.4. Histological analysis

The ovarian tissue samples were dehydrated, embedded in paraffin using a Shandon Excelsior ES histokinette (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a Tissue-tek®TEC embedding system (Sakura Finetek, Alphen aan den Rijn,

Netherlands) and sectioned at 5 µm using a Shandon Finesse®ME+ microtome (Thermo Fisher Scientific). Tissue samples were mounted on glass slides and stained with periodic acid solution 0.5% (Merck KGaA, Darmstadt, Germany), Schiff's reagent (Merck KGaA), Weigert's hematoxylin (Merck KGaA) and metanil yellow (Sigma Aldrich) (Quintero-Hunter et al., 1991). All histological sections were photographed using a digital camera (Model DP71; Olympus, Center Valley, Pennsylvania). Evaluation of ovarian development was based on the most advanced oocyte stage present in a histological sample and identification was based on morphological characteristics described in Jørgensen et al. (submitted). In short, the classification included three types of oocyte, namely pre-lipid droplet oocytes (PLD), lipid droplet oocytes (LD1-4) and vitellogenic oocytes (VT1-3. LD1 are characterised by a small number of lipid droplets in the ooplasm, which increase in number (LD2), begin to form a ring around the nucleus (LD3) and eventually become abundant and increase in size, while cortical alveoli appear in the periphery of the ooplasm (LD4). The first vitellogenic oocyte stage (VT1) is characterised by the appearance of yolk globules in periphery of the ooplasm. As the yolk globules increase in number and size (VT2), they gradually begin to fill the ooplasm from oocyte membrane towards the nucleus (VT3). Following this classification scheme, each female was assigned to a reproductive stage based on the most advanced oocyte stage present in the ovarian sample.

4.5. Transcriptome analysis

RNA was extracted following the protocol NucleoSpin® RNA (Macherey-Nagel, Düren, Germany). RNA purity (260/280 = 2.17 ± 0.04, 260/230 = 2.26 ± 0.12) was evaluated through spectrophotometry using Nanodrop One (Thermo Fisher Scientific).

Paired end mRNA sequencing of the samples was performed at Novogene (Beijing, China) through Illumina Hi-Seq 2500 platform (Illumina Inc., San Diego, Californien, USA). Raw reads are available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under the accession number PRJEB37003. Read quality of each sample was evaluated using FastQC v.0.11.4 (Andrews, 2005) and adapter residuals, low quality regions, short reads were trimmed with Trimmomatic v.0.38 (Bolger et al., 2014) using the following parameters: HEADCROP:9 MINLEN:36 SLIDINGWINDOW:4:15. Only mate-paired trimmed reads were considered for downstream analyses. They were aligned against the European eel reference genome (https://doi.org/10.18710/L7GO8T) with TopHat2 v.2.0.13 (Kim et al., 2013) guided with the GFF file with known transcripts predicted by Henkel et al. (2012) and using standard parameters. Uniquely mapped reads were then retained using Samtools v.1.2 (Li et al., 2009). The transcript integrity number (TIN, Wang et al. 2016) was calculated for each transcript from the uniquely mapped reads using the software tin.py in the

RSeQC package using default parameters and then averaged across each sample (Wang et al., 2012, 2016). Week 0 and 9 fish had similar mean TIN values of 56.1±0.1 and 56.9±0.2, respectively.

Read count was performed with HTSeq v.4.4.0 (Anders et al., 2015) based on the uniquely mapped reads to each predicted transcript and using strict parameters for handling misalignments (i.e. "-m intersection-strict --stranded=no"). Differentially expressed genes (DEGs) of the remaining samples were identified with the R package DESeq2 (Love et al., 2014), where significant DEGs were retained. A PCA was calculated based on the regularised log-transformation of read count, and sample 12 (week 9) was removed from the differential expression analysis, as it appeared to be an outlier. The assembled genome still included redundant genes, therefore, DEGs with the same symbol but with opposite sign in $\log_2$-fold change were not considered. The expression threshold for differential expression analysis was $\log_2$-fold change $0.5 \geq$ or ≤ -0.5 and adjusted $p < 0.01$.

An overrepresentation analysis for GO biological processes was performed on the list of significant DEGs using the database Panther Classification System v.14.1 (http://www.pantherdb.org/) with *Danio rerio* as the reference organism. The significance level was set at FDR ≤ 0.05.

4.5.1. Validation

Genomic DNA was removed from the same extracted RNA as used for the RNA-Seq analysis using the PerfeCta® DNase I (RNase-free) kit (Quanta Biosciences, Hilden, Germany). Then 450 ng of total RNA was transcribed to cDNA using the qScript™ cDNA synthesis Kit (Quanta Biosciences).

Expression of selected genes was used to validate RNA-Seq results for all samples. These genes included follicle-stimulating hormone receptor (*fshr*), luteinizing hormone receptor (*lhgcr1*), nuclear estrogen receptor 2 beta (*esr2b*), androgen receptors (*ara* and *arb*), vitellogenin genes (*vtg1* and *vtg2*) and 40S ribosomal protein S18 (*rps18*) (Table 3). Two reference genes, elongation factor 1-alpha (*ef1a*) and beta-actin (*β-actin*), were included in the analyses as they were stable across samples and between sampling weeks in the qPCR analysis and not significantly differentially expressed in the RNA-Seq analysis.

Gene expression was preformed through qPCR Biomark™ HD system (Fluidigm, South San Francisco, Californien, USA) in 96.96 IFC using four technical replicates. A pre-amplification step of cDNA was done following the Fluidigm protocol (PN 100-5875). Samples were diluted 1:5 before being loaded onto the arrays and the forward and reverse primers were loaded at a combined concentration of 100 µM. The arrays were run according to Fluidigm 96.96 IFC protocol (PN 100-9792) with a Tm of 60°C.

The coefficient of variation (CV) of technical replicates was calculated and genes were considered stable if CV < 4% (Hellemans et al., 2007). The relative quantity of target gene transcripts was normalised to the geometric mean of stable reference genes. Gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method with the control average as reference (Livak & Schmittgen, 2001). The $\log_2$-fold change was calculated for each gene. These values were then correlated to the corresponding $\log_2$-fold change values from the RNA-Seq analysis in R version 3.3.2 (R Core Team, 2019) using the ggscatter (package "ggpubr").

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Tables

Table 1. Overview of the most advanced oocyte stage in a histological sample and gonado-somatic index (GSI) for European eel females sampled in week 0 and 9.

Sampling time	Female	Oocyte stage	GSI
Week 0	1	LV3	1.6
	2	LV3	1.4
	3	LV3	1.4
	4	LV3	1.1
	5	LV3	1.0
	6	LV4	1.7
	7	LV3	1.8
	8	LV3	1.5
Week 9	9	VT3	11.5
	10	VT3	18.1
	11	VT3	16.4
	12*	VT3	8.6
	13	VT3	26.6
	14	VT2	9.7
	15	VT3	26.9
	16	VT3	16.1

*Outlier in the differential expression analysis and removed from the analysis

Table 2. Overview of the mapping statistics for European eel ovarian transcriptomes from females in the previtellogenic (sampled in week 0) and late vitellogenic stage (sampled after 9 weeks of treatment).

Sampling time	Sample	Mapped reads (%)	Uniquely mapped reads (%)
Week 0	1	57.7	56.06
	2	58.6	53.74
	3	57.7	55.98
	4	59.3	57.48
	5	58.9	57.23
	6	58.6	56.90
	7	56.0	54.42
	8	56.8	55.20
Week 9	9	54.6	53.11
	10	55.0	53.48
	11	56.3	54.69
	12*	53.8	52.30
	13	51.5	50.04
	14	54.7	53.17
	15	53.7	52.12
	16	56.1	54.37

*Outlier in the differential expression analysis and removed from the analysis

Table 3. Sequences of European eel primers used for amplification of genes by qPCR. Full gene name, abbreviation, accession number, primer sequences and reference for primers are given for each gene.

Full name	Abbreviation	Accession no.	Primer (5′-3′) (F: Forward; R: Reverse)	Reference
Follicle stimulating hormone receptor	*Fshr*	LN831181	F:CCTGGTCGAGATAACAATCACC R: AATCTTGGAGAAATCAGGCAGT	Maugars&Dufour 2015
Luteinizing hormone receptor	*lhcgr1*	LN831182	F: GCGGAAACACAGGGAGAAC R: GGTTGAGGTACTGGAAATCGAAG	Maugars&Dufour 2015
Estrogen receptor (nuclear)	*esr2b*	CUH82768	F: TGTGTGCCTCAAAGCCATTA R: AGACTGCTGCTGAAAGGTCA	Lafont et al. 2016
Androgen receptor alpha	*ara*	FR668031	F: CGGAAGGGAAACAGAAGTACC R: AGCGAAGCACCTTTTGAGAC	Peñaranda et al. 2014
Androgen receptor beta	*arb*	FR668032	F: CGCTGAAGGAAAACAGAGGT R: CATTCCAGCCTCAAAGCACT	Peñaranda et al. 2014
Vitellogenin 1	*vtg1*	EU073127	F: GACAGTGTAGTGCAGATGAAG R: ATAGAGAGACAGCCCATCAC	Parmeggiani et al. 2015
Vitellogenin 2	*vtg2*	EU073128.1	F: GATGCTCCCCTAAAGTTTGTG R: AGCGTCCAGAATCCAATGTC	Parmeggiani et al. 2015
40s ribosomal S18	*rps18*	GBXM01005349	F: TGACCGATGATGAGGTTGAG R: GTTTGTTGTCCAGACCGTTG	Politis et al. 2017
Elongation factor 1-alpha	*ef1a*	EU407824	F: CTGAAGCCTGGTATGGTGGT R: CATGGTGCATTTCCACAGAC	Politis et al. 2017
Beta-actin	*β-actin*	DQ286836	F: AGCCTTCCTTCCTGGGTATG R: GTTGGCGTACAGGTCCTTAC	Parmeggiani et al. 2015

Figures

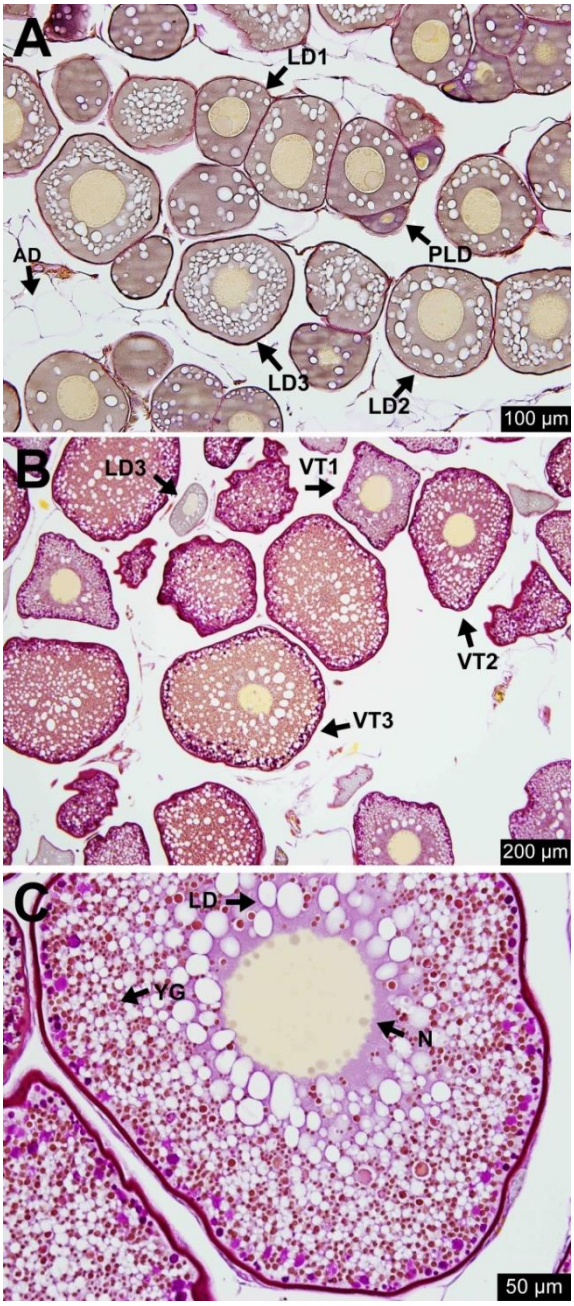

Figure 1. Histological overview of European eel ovarian tissue from females in the previtellogenic (A; sampled in week 0; GSI = 1.5; left) and vitellogenic stage (B and C; sampled after 9 weeks of treatment; GSI = 11.5; right). AD, adipocyte; PLD, pre-lipid droplet oocyte; LD1-3, lipid droplet oocytes stages 1 to 3; VT1-3, vitellogenic oocytes stages 1 to 3; LD, lipid droplet; YG, yolk globule, N, nucleus. Staining: Periodic acid Schiff's hematoxylin counterstained by metanil yellow.

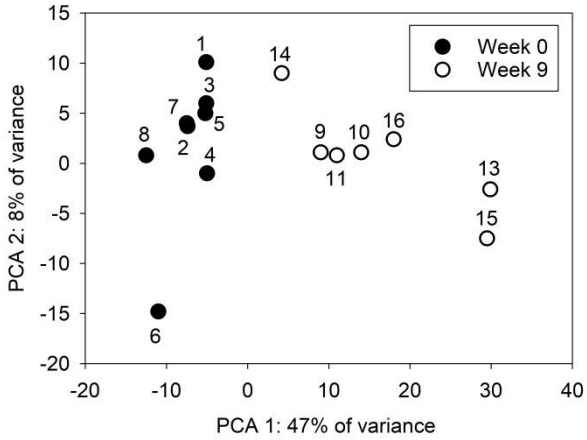

Figure 2. PCA plot of the differential expression analysis for European eel ovarian transcriptomes from previtellogenic (sampled in week 0) and late vitellogenic females (sampled after 9 weeks of treatment).

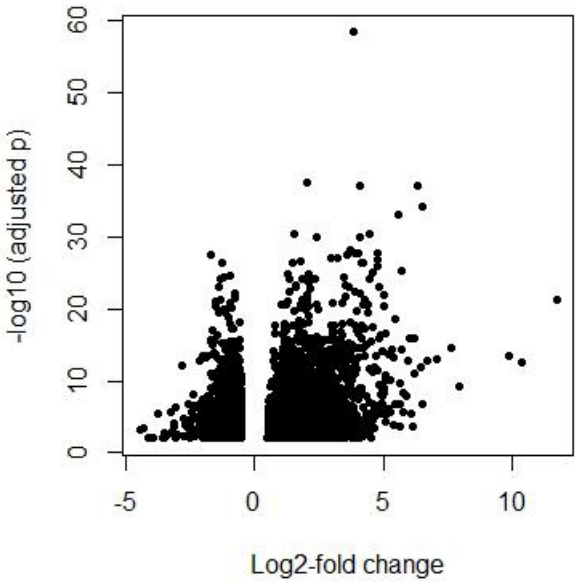

Figure 3. Volcano plot of the differential expression analysis for European eel ovarian transcriptomes from previtellogenic (sampled in week 0) and late vitellogenic females (sampled after 9 weeks of treatment). Each dot represents a gene. The x-axis reports the $\log_2$-fold change, while the y-axis reports the –log10 of the adjusted *p*-value. Genes with a positive $\log_2$-fold change were upregulated, while genes with a negative $\log_2$-fold change were downregulated. The expression threshold was $\log_2$-fold change $0.5 \geq$ or ≤ -0.5 and adjusted $p < 0.01$.

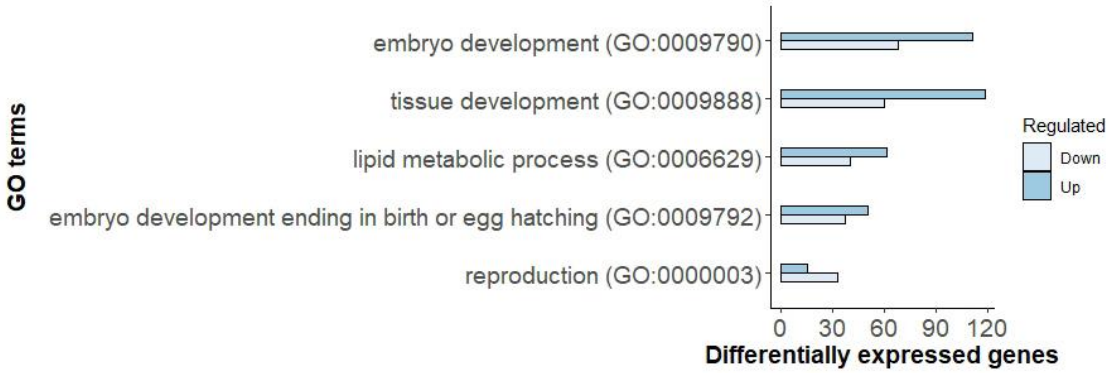

Figure 4. Overview of up- and downregulated DEGs for selected gene ontology (GO) biological processes for female European eel. Ovarian transcriptomes from females in the previtellogenic (sampled in week 0) and late vitellogenic stage (sampled after 9 weeks of treatment) were compared. GOs were identified through an overrepresentation analysis with *Danio rerio* as the reference organism using the Panther Classification System v.14.1 (http://pantherdb.org/) and the number of differentially expressed genes (DEGs). The expression threshold for DEGs was $\log_2$-fold change $0.5 \geq$ or ≤ -0.5 and adjusted $p < 0.01$, and the significance level was set at FDR ≤ 0.05 for GOs.

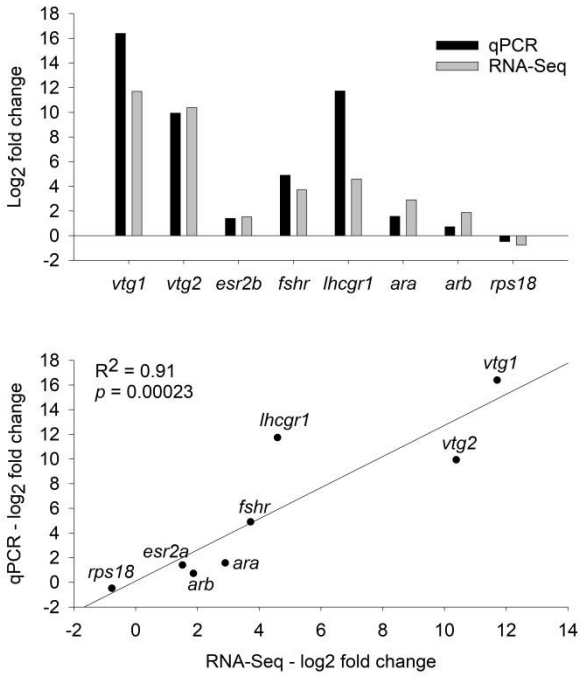

Figure 5. Validation of RNA-Sequencing (RNA-Seq) results in female European eel, *Anguilla anguilla* by quantitative real-time PCR (qPCR). Data from qPCR and RNA-Seq were compared and correlated for eight differentially expressed genes, seven of which were upregulated (*vtg1*, *vtg2*, *fshr*, *lhcgr*, *esr2*, *ara* and *arb*) and one was downregulated (*rps18*) in females in the previtellogenic (sampled in week 0; GSI = 1.5; left) and late vitellogenic stage (sampled after 9 weeks of treatment). Log_2-fold change values for qPCR results were correlated to RNA-Seq results ($R^2 = 0.91$, $p = 0.00023$).

Supplementary tables

Table S1: Complete list of significantly differentially expressed genes (DEGs) detected in the differential expression analysis. Annotation numbers are based on the assembly. For each DEG, $\log_2$-fold change, P-value, homologous uniprot name and gene symbol is reported.
Data available at:
https://drive.google.com/drive/folders/1f8HxgjjtU3Brc-KYVXmjG9nx94zJ3PMa?usp=sharing

Table S2: Complete list of significantly enriched gene ontology (GO) biological processes. For each GO biological process identified in the overrepresentation analysis the following was reported: the number of genes in the reference species, the number of genes detected from the differential expression analysis, the expected number genes, the fold enrichment, raw P-value and FDR. GOs were identified through an overrepresentation analysis with *Danio rerio* as the reference organism using the Panther Classification System v.14.1 (http://pantherdb.org/) and the number of differentially expressed genes (DEGs). The expression threshold for DEGs was $\log_2$-fold change $0.5 \geq$ or ≤ -0.5 and adjusted $p < 0.01$, and the significance level was set at FDR ≤ 0.05 for GOs.
Data available at:
https://drive.google.com/drive/folders/1f8HxgjjtU3Brc-KYVXmjG9nx94zJ3PMa?usp=sharing

Manuscripts

Paper III

Unravelling the changes during induced vitellogenesis in female European eel through RNA-Seq: what happens to the liver?

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Abstract

The life cycle of European eel (*Anguilla anguilla*), a catadromous species is complex and enigmatic. In nature, during the silvering process prior to their long spawning migration, reproductive development is arrested and they cease feeding. In studies of reproduction using hormonal induction, eels are equivalently not feed. Therefore, in female eels that undergone vitellogenesis, the liver plays different essential roles being involved both in vitellogenin synthesis and in reallocating resources for the maintenance of vital functions, performing the transoceanic reproductive migration and completing reproductive development. The present work aims at unravelling the major transcriptomic changes that occur in the liver during induced vitellogenesis in female eels. mRNA-Seq data from 16 animals (8 before induced vitellogenesis and 8 in their 9th week of hormonal treatment) was generated and differential expression analysis was performed comparing the two groups. This analysis detected 1,328 upregulated and 1,490 downregulated transcripts. Overrepresentation analysis of the upregulated genes included biological processes related to biosynthesis, response to estrogens, mitochondrial activity and localization, while downregulated genes were enriched in processes related to morphogenesis and development of several organs and tissues, including liver and immune system. Among key genes, the upregulated ones included vitellogenin genes (*VTG1* and *VTG2*) that are involved in vitellogenesis, together with *ESR1* and two novel genes not previously investigated in European eel (*LMAN1* and *NUPR1*), which have been linked with reproduction in other species. Moreover, several up-regulated genes, such as *CYC1*, *ELOVL5*, *KARS* and *ACSS1* are involved in the management of the effect of fasting and *NOTCH*, *VEGFA* and *NCOR1*, were linked with development, autophagy and liver maintenance in other species. These results will increase the understanding of the molecular changes that occur in the liver during vitellogenesis in this complex and distinctive fish species, bringing new insights on European eel reproduction and broodstock management.

Key words

Anguilla anguilla; RNA-Seq; liver; vitellogeneis; fasting

Introduction

The life cycle of European eel (*Anguilla anguilla*), a catadromous species, is complex and spans over a wide range of geographical area with highly diverse habitats. European eels spend their life stages from glass eel to yellow eel, the so-called continental-phase, in freshwater and coastal marine areas of Europe and northern Africa, for a period ranging from five to 20 years. After this period, a process called silvering prepares the future spawners (silver eels) for their oceanic reproductive migration that will lead them to their spawning areas in the Sargasso Sea [1], [2]. However, silver eels are still sexually immature when they leave the continental habitats and their development remains blocked at this prepubertal stage as long as they remain in their continental habitats [3], [4]. In fact, both a low stimulation by gonadotropin-releasing hormone neurons and a strong dopaminergic inhibition maintain the production of pituitary gonadotropins at a low level, thus preventing the eels to complete the sexual maturation process presumably until they reached the Sargasso sea [5]. During silvering, several phenotypical and physiological changes occur: for example, the abdominal skin color changes from yellow to silver, the pectoral fins darken and the eyes increase in size [6]. At this stage, they stop feeding, the digestive tract regresses, while several metabolic changes occur [7]–[9]. Consequently, European eels need to reallocate and use existing reserves for the migrating to their spawning areas and their reproductive development.

Since the 1980's, the European eel stock has drastically declined, due to climate change, obstacles to migrations and reduction of habitats, pollutants, and fisheries activities [10], [11], the latter a result of being a highly valuable niche product in several European countries (Netherlands, Italy, and Denmark among others; FAO Fishery Statistics, 2002). Consequently, the species is now ranked critically endangered [12]. This has led to increased efforts in closing the life cycle of European eel in captivity, in the attempt to relieve the pressure from fisheries and to develop a sustainable aquaculture, which is also happening to the closely related anguillid species, the Japanese eel *Anguilla japonica* [13]. New advancements in induced sexual maturation have led to the development of effective assisted reproduction strategies using hormonal treatments to induce gamete development [14] in European eel, enabling production of viable embryos and larvae entering the feeding larval stage [15]. In the attempt to mimic the natural conditions during reproduction experiments, as a common and consolidated practice eels are not fed [16].

Whether it is induced in captivity or occurring naturally in their native habitat, vitellogenesis in female European eel, like other teleosts is regulated by the hypothalamus-pituitary-gonad-liver axis, where the liver plays several key roles. One of the liver's major roles is to provide key substances required by the developing oocyte,

particularly vitellogenin that is a precursor to yolk proteins. The biosynthesis of vitellogenin is mainly controlled by the gonadotropin follicle-stimulating hormone (FSH) that stimulates the synthesis by ovarian follicle cells of 17β-estradiol (E2), which in turn stimulates the production of vitellogenin through the interaction of E2 with its receptors in the liver (reviewed by [17]. Vitellogenin is then transported to the ovary through the bloodstream and accumulates in the developing oocyte as yolk globules [18]. Moreover, as the eels do not feed, the liver is involved in the reallocation of resources for energy metabolism and glycogen production for survival, trans-oceanic migratory swimming activity and completing reproductive development [19].

Transcriptomic changes in the liver during reproductive development remains little investigated in European eel. So far, one study that targeted male spermatogenesis and 14,913 transcripts, detected expression changes of several genes involved in key biological pathways, e.g. lipid metabolism, fatty acid synthesis and transport, mitochondrial function, steroid transport and bile acid metabolism [20]. The technological advancements and the availability of the first genome assembly annotation [21] provide the opportunity to increase the number of such types of high-throughput analysis, such as RNA-Seq analysis in this species. This allowed to conduce broader gene expression investigations that has lead, for example, to the first characterizations and differentiations of the pituitary and ovary gland among yellow and silver eels [22], [23].

The aim of this work was to unravel the transcriptomic changes that occur in the liver of female European eels during induced vitellogenesis, comparing immature stage to pre-spawning stage.

Materials and Methods

Eel maturation and sample collection

All experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2015-15-0201-00696) and fish were handled according to the European Union regulations regarding protection of experimental animals (Dir 86/609/EEC).

Farmed female eels (n = 38) were reared in freshwater at a commercial eel farm (Stensgård Eel Farm A/S, Randbøl, Denmark) and were transported in an aerated freshwater tank to the research facility EEL-HATCH, Technical University of Denmark. The eels were placed in three tanks of 1150 L each connected to recirculating aquaculture system. The eels were acclimatized to saltwater (i.e., from 0 to 36 psu) over two weeks by adding natural seawater supplemented with Blue Treasure Aquaculture

Salt (Qingdao Sea-Salt Aquarium Technology Co., Ltd., Quindao, China). Water temperature was maintained at ~20°C and light in a 12 h light and 12 h dark light regime with low light intensity (~20 lux W) throughout the experiment.

After two weeks acclimation, eight female eels (total length 75 ± 4 cm, body weight 862 ± 100 g) were randomly selected for sampling prior hormonal treatment (i.e., week 0). The remaining eels were anesthetized individually in an aqueous solution of benzocaine (ethyl p-aminobenzoate, 20 mg L^{-1}, Sigma Aldrich) and tagged with a passive integrated transponder (PIT, 12×2 mm) tag in the dorsal musculature for ID. Vitellogenesis was induced in these eels through weekly intramuscular injections with a constant dose (20 mg/kg) of carp pituitary extract (CPE; Ducamar Spain S.L.U) as part of an experimental study (Jørgensen et al., 2020 in review). One week after the 9th injection (i.e., week 9), eight females (total length 75 ± 2 cm, body weight 901 ± 62 g) were randomly selected for sampling. All selected eels were euthanized by submergence in an aqueous solution of benzocaine (20 mg L^{-1}) for 5-10 min.

From each of the 16 sacrificed eels, hepatic tissue samples was collected and stored in RNA later at 4°C for 24 h and then –20°C until extraction. In addition, ovarian tissue for histological analyses was preserved in 4% sodium-buffered formalin (Hounisen, Skanderborg, Denmark) and stored at room temperature. Moreover, phenotypic parameters such as body length, body, gonad and liver weight was recorded and hepatosomatic index (HSI), and gonadosomatic index (GSI) [24] were calculated as follows:

HSI = liver weight /body weight*100

GSI = gonad weight / body weight *100

Correlation among HSI and GSI was calculated with the ggpubr R package (https://CRAN.R-project.org/package=ggpubr).

Histological analysis for determination of oocyte and ovarian developmental stage

Fixed tissues were dehydrated, embedded in paraffin using a sectioned at 5 μm using standard procedures. Sections were mounted on glass slides, stained with a 0.5% periodic acid solution, Schiff's reagent, Weigert's hematoxylin and metanil yellow (Quintero-Hunter et al., 1991). Progression of oocyte development was categorised based on morphological characteristics of the most advanced oocytes according to Jørgensen et al. (in review) with emphasis on previtellogenic stages (lipid droplet stages LD1-4) and vitellogenic stages (VGT1-3).

RNA-Seq data generation, processing, alignment and read count

RNA was extracted from hepatic tissue with NucleoSpin® RNA (Macherey-Nagel, Germany) according to the manufacturer's instruction. RNA purity and quantity (260/280 = 2.17 ± 0.04, 260/230 = 2.26 ± 0.12) were evaluated through spectrophotometry using Nanodrop (Thermo Fisher Scientific, USA). Then, 2 µg of each RNA sample were sent to Novogene (Beijing, China) to check for RNA integrity (RIN) through Bioanalyzer (Agilent Technologies, Santa Clara, CA), that assessed RIN > 8.2 for all samples. Paired end 150bp mRNA sequencing was then performed using Illumina HiSeq 2500 platform following the manufacturer's instruction (Illumina Inc, USA).

Quality of the generated reads was assessed through FastQC 0.11.4 [25]. The company pre-trimmed reads from the adapters, but a further trimming was performed with Trimmomatic 0.38 [26] with the following parameters: HEADCROP:9 MINLEN:36 SLIDINGWINDOW:4:15. Only mate-paired trimmed reads were considered for downstream analyses. Trimmed reads were aligned to the European eel draft reference genome [21] using Tophat 2.0.13 [27] and including the GFF file with known transcripts available at https://doi.org/10.18710/L7GO8T. Uniquely mapped reads were then retained using Samtools 1.2 [28]. HTSeq-0.6.1 [29] was used to count reads for each gene with parameters "-m intersection-strict --stranded=no".

Differential expression and over representation

Differential expression among week 0 and week 9 was analysed with the R Package Deseq2 [30]. Principal Component Analysis (PCA) was calculated based on the regularized log-transformation of read count with the same software. Differentially expressed genes (DEGs) with Padj < 0.01 was chosen as a threshold, according to what has been previously utilized by [23] in a differential expression analysis of ovary tissues of the same species. Retained genes were then filtered as the status of the assembled genome is not complete and some genes may be redundant. Therefore, every gene that showed opposite sign of Log2 fold change was removed.

Statistical overrepresentation test was performed with upregulated and downregulated gene separately using Panther (http://www.pantherdb.org/), considering Gene Ontology (GO) biological processes as annotation set and *Danio rerio* as reference gene set. Only GO terms with FDR P < 0.05 were considered.

Validation of RNA-Seq analysis

A further DNase treatment with PerfeCta® DNase I (RNase-free) kit (Quanta Biosciences, Germany) was performed on the previously extracted RNA samples. Then,

approximately 450 ng of total RNA was retrotranscribed to cDNA using the qScript™ cDNA synthesis Kit (Quanta Biosciences, Germany). A total of six genes were selected (*VTG1, VTG2, ESR1, HSP90, IGF1* and *IGF2*) as well as two reference genes (Cytochrome c oxidase subunit I (*COX1*) and beta-actin (*β-actin*) that did not show significant changes in the mRNA-Seq analysis. Primers are listed in Table 1. Gene expression was performed with qPCR Biomark™ HD system (Fluidigm) in 96.96 IFC using four technical replicates. A pre-amplification step of cDNA was done following the Fluidigm protocol (PN 100-5875). Samples were diluted 1:5 before being loaded onto the arrays and the forward and reverse primers were loaded at a combined concentration of 100 µM. The arrays were run according to Fluidigm 96.96 IFC protocol (PN 100-9792) with a Tm of 60°C. The relative quantity of target gene transcripts was normalized to the geometric mean of the two reference genes. Gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method with the control average as reference [31], then log fold change was calculated as follow:

$$\text{Log fold change} = \text{LOG}\ \frac{\overline{2^{-\Delta\Delta CT}\ week\ 9}}{\overline{2^{-\Delta\Delta CT}week\ 0}}$$

Correlation among fold changes of the genes of the two approaches (RNA-Seq and qPCR) was calculated with the ggpubr R package (https://CRAN.R-project.org/package=ggpubr).

Results

Phenotypic and RNA-Seq overview

The distribution of HSI across samples is shown in Fig 1a. The average HSI was 0.78 (± 0.10) for week 0 and 1.25 (± 0.24) for week 9 (increase of 80.59%, with a higher degree of variability in week 9). The increase in HSI was highly correlated to the increase in GSI (r= 0.93 P=2.9e-07; Fig 1b) accompanying a transition of oocyte and ovarian developmental stage from previtellogenic (seven females in PLV3 and one in PLV4) to late vitellogenenic (one female in VGT2 and seven in VGT3).

The sequencing and filtering produced approximately 25,600,000*2 paired reads for each sample (Table 2). Approximately 57 % (±2.89) of these reads uniquely mapped against the eel reference genome, and these results are in line with what was reported by [23] in a RNA-Seq analysis in ovaries of the same species and with the same reference genome. The PCA plot based on the RNA-Seq analysis showed that samples from week 0 and 9 clustered into two separate groups (Fig 2). These variations may be related to differences in the individual response to the weekly injection of CPE to induce vitellogenesis.

Differential expression

From 34,208 annotated genes, a total of 61 with the same symbol and significantly differentially expressed showed opposite signs in Log2fold change values and were therefore removed from the analysis as they may represent a problem in the annotation. After this filtering step, a total of 1,328 upregulated and 1,490 downregulated genes were detected (Fig 3 and Table S1). The most highly upregulated genes were vitellogenin genes (*VTG1*, and *VTG2*), with Log2fold change of 11.50 and 11.47, respectively.

Some of the genes differentially expressed have been previously detected in relation to European eel male reproductive development [20]. Common genes detected included serine palmitoyl transferase 2 (*SPTLC2*), elongation of very long chain fatty acids 1 (*ELOVL1*) and diacylglycerol O-acyltransferase 1 (*DGAT1*), which were downregulated in both studies. Conversely, mid1-interacting protein 1-like (MID1IP1L), acetyl-coenzyme A acetyltransferase 1-like (*ACAT1*), ATP-binding cassette sub-family D member 3 (*ABCD3*), ATP-synthases, glutamine synthetase, NADH dehydrogenase and hydroxysteroid dehydrogenase protein 2-like (*HSDL2*) and sulfotransferase 6B1-like gene (*SULT6B1*) were upregulated in both studies.

Overrepresentation analyses

The PANTHER classification system was used for overrepresentation analyses of the differentially expressed transcripts and to classify them according to biological processes, based on the function of the encoded protein in the context of a larger network of proteins that interact to accomplish a process at the cellular or organism level. Here, processes that came from upregulated and downregulated genes showed differences.

The analysis conducted on the upregulated genes (Fig 4, Table S2) showed the presence of several processes related to biosynthesis (33 terms), such as lipids, cholesterol and organic compounds, isopentenyl diphosphate, aromatic compounds, nucleotides and nucleosides. Another process was related to mitochondrion activity reorganizations/energy generation and only present in upregulated genes (e.g. ATP biosynthesis and proton/electron transport) with 10 terms. A total of four biological processes were related to steroid production, including estrogen (steroid metabolism/biogenesis, response to estrogen stimulus) subcategories. This process included genes such as *VTG1* and *VTG2* (as mentioned above), Estrogen receptor 1 (*ESR1*), Lectin, Mannose Binding 1 (*LMAN1*) and Nuclear Protein 1, Transcriptional Regulator (*NUPR1*). Similarly, the GO biological processes related with localization were only present in the upregulated genes, including energy coupled proton transport

and oxidative phosphorylation. Several of these upregulated genes were included in more that one biological process. Among those, Cytochrome C1 (*CYC1*) that was present in 65 biological processes, the hepatic fatty acid elongase-5 (*ELOVL5*) that was present in 58 biological processes, lysyl-tRNA synthetase (*KARS*) and Acyl-CoA synthetase short-chain family member 1 (*ACSS1*) that were included in 55 biological processes, and Coenzyme A (CoA) synthase *(Coasy)*, that was present in 46 biological processes.

Among the downregulated genes (Fig 4, Table S3), development and morphogenesis of organs (e.g. liver, hematopoietic, digestive system), tissues and cells (e.g. axon, type B pancreatic cell, aorta), were an important part of the GO biological processes (49 terms), not detected with the analysis performed in the upregulated genes. Moreover, a unique set of term for the pathways of downregulated genes was the negative regulation of different general processes (18 terms) such as nitrogen compounds, transcription, biosynthesis of macromolecules and RNA. Other terms that were only overrepresented in the downregulated genes were immune system development and processes. Notch receptor 1a (*NOTCH1* or *NOTCH1A*) was present in 85 of the downregulated biological processes, Vascular endothelial growth factor (*VEGFA*) was present in 72 biological processes and Nuclear receptor corepressor 1 (*NCOR1*) was present in 51 processes.

Gene expression validation

To validate the DEGs discovered through RNA-Seq analysis, the expression of eight genes were measured through qPCR. These included four genes that were upregulated in week 9 (*VTG1, VTG2, HSP90* and *ESR1*), two that were significantly downregulated (*IGF2* and *IGF1*) and two genes that were not significantly differentially expressed and that were used as reference genes (*EF1A* and *β-actin*). The selected genes showed similar expression pattern regardless of method. In fact, correlation among the differential expression pattern in high (R= 0.98, p = 0.00063; Fig 5), which validates the accuracy of the RNA-Seq analysis.

Discussion

RNA-Seq analysis can provide an overview at the whole transcriptome level of genetic activities in several organisms under different environmental or physiological changes. This is particularly useful to understand the molecular mechanisms of animals with a unique physiology such as the European eel. Together with the Japanese and American eels, they are among the few fish that do not eat during vitellogenesis. This unusual aspect is interesting in the perspective of future management strategies of broodstock, as

the efforts to complete the life cycle and rear these species in captivity are increasing [13], [32]. Moreover, eel represents a unique model to study vitellogenesis in such a distinctive condition. In female eels, the liver plays key roles as it is involved in the mobilization of reserves, including protein and lipids, to be processed and used for egg yolk formation and to supply energy necessary for maintenance and, in nature, for swimming to the Sargasso sea and then spawn [33].

At a phenotypical level, the size of liver in teleosts tends to increase during vitellogenesis, as it has been shown in several fish species e.g.[34], [35]. By contrast, the hepatosomatic index tends to decrease during lack of food/fasting, when vitellogenesis is not involved [36], [37]. In nature, no significant differences in liver size have been detected among female yellow eels and eels in the prepubertal silvering stages of the European eel [7], in agreement with observations in Japanese eels [38]. Our data showed that, despite the absence of food during the trial, during induced maturation the dimension of liver increase, almost doubling its initial value. The increase in HSI during induced maturation is in agreement with previous studies in the European eel [39]. Moreover, our results showed a highly significant positive correlation between HSI and GSI.

When we looked at the differential expression analysis, a high-number genes were significantly different among week 0 and week 9. Some of the upregulated and downregulated DEGs were in common with the study performed on male eels [20]. These genes are mainly related to energy management and biosynthesis of metabolites that may represent common requirements for energy and metabolite management and survival in this species. In agreement with [20], there is a distinction of the patterns of upregulated and downregulated genes, as upregulated genes were mainly involved in biosynthetic processes, localization and process related to reproduction, whereas downregulated were linked to developmental processes, organ maintenance and immune system.

Reproduction: Response to Estrogen

The brain-pituitary-gonadal-liver axis plays a crucial role in the reproductive processes of oviparous female teleosts. Here, the main role of liver is to produce vitellogenin, a glycolipoprotein that is deposited to the egg in the ovary through the bloodstream [17]. Increased transcription levels of vitellogenin genes in the liver have already been observed in several works on induced vitellogenesis of European eel [40], [41]. Our results agree with these findings and provide clear evidence that after 9 weeks, the vitellogenin genes (*VTG1* and *VTG2*) have the highest increase in expression among all the other genes assayed.

The overrepresentation analysis indicated that, together with the vitellogenin genes, other genes are grouped under the GO biological process response to estrogen. The group counted seven genes including *ESR1*, *LMAN1* and *NUPR1*. *ESR1* gene is well known, as it is the nuclear receptor that binds E2 to initiate the synthesis of vitellogenin [17], [41]. The *NUPR1* gene encodes a chromatin-binding protein that is involved in the adaptation to stressor events. It has been investigated in the liver of zebrafish in relation to the response to chemical compounds [42]. So far, no information on the involvement of this gene in fish reproduction is available. However, *NUPR1* plays a role in the temporal expression of the beta subunit of luteinizing hormone, during gonadotropin development in mice, as shown by knockout studies [43], [44]. In teleosts, luteinizing hormone regulates oocyte maturation, where it leads to resumption of meiosis until metaphase II in oocytes. Moreover, luteinizing hormone acts on follicle cells to stimulate the synthesis of progesterone that will activate meiosis in the oocyte [45]–[48]. Therefore, *NUPR1* may represent an interesting target for further investigation in teleost vitellogenesis.

As *LMAN1*, an analysis among 2,523 transcripts and different vitellogenic stages in zebrafish, showed evidence that this gene is also upregulated in the liver of E2 treated males [49]. As in our study, Levi and colleagues reported the upregulation of *VTG1* and *ESR1* and included in the estrogen receptor GO term as well as genes that were detected in our study, yet not under this biological process. These genes are Reticulon *1 (RTN1)*, Prefoldin Subunit 6 (*PFDN6*) and Nitric Oxide Synthase Interacting Protein (*NOSIP*) genes that in our analysis were upregulated as well as Low Density Lipoprotein Receptor (*LDLR*) and Heterogeneous Nuclear Ribonucleoprotein H1 (*HNRNPH1*) that were downregulated.

In some teleosts, the liver is also important for producing zona pellucida (ZP) proteins that will constitute the zona radiata of the egg, which is a complex membrane forming around the ooplasm of teleost fishes egg, providing physical protection and protecting the egg against being fertilized more than one spermatozoa (polyspermy) [50]. In gilthead seabream, all mRNA isoforms of ZP genes have been detected expressed in the liver [51]. The most recent available European eel genome annotation that we used in our analysis presented four ZP genes annotated and a ZP-like gene, but they were not differentially expressed in the timeframe analyzed. These results were in agreement with a previous study in the European eel on two ZP genes analyzed over four time points in the liver (i.e., week 0, 4, 8 and 12), where no significant differential expression was detected [52]. These results and ours support the indication that for European eel, the liver does not play a main role in the production of ZP proteins during vitellogenesis.

Metabolism

Biosynthetic processes and energy

Liver is responsible for many energy metabolic processes, including metabolic protein synthesis, degradation, and fatty acids biosynthesis [53]. Despite liver growth phenotypically followed the vitellogenic pattern, several DEGs and related biological processes were linked to response to fasting conditions. For example, *CYC1* was the upregulated gene that was present in the highest number of enriched biological processes, particularly nucleotide/nucleoside metabolic process and ATP metabolism. This gene seems to be involved in adaptation to high temperatures in Redband trout [54] and is important for liver adaptation in prolonged fasting in humans [55]. *ELOVL5* that in our work was included in biological processes such as biosynthesis of several molecules, plays an important role in long-chain monounsaturated and polyunsaturated fatty acid synthesis. *ELOVL5* activity is regulated during development by diet, hormones and drugs. Furthermore, *ELOVL5* activity may affect hepatic glucose production during fasting [56]. *ELOVL5* was also highly expressed in the livers of laying hens and increased rapidly after sexual maturity [57]. *Coasy* plays a central role in cellular metabolism in agreement with our enrichment analysis, where it was enriched in biosynthetic processes of aromatic compounds, carbohydrate derivatives, nitrogen compounds, heterocyclic compounds and nucleotides. A downregulation of *Coasy* in zebrafish expressed mainly in the liver, muscles and head, affected neural and vascular development from early life stages [58].
Other genes that in our work were included in biosynthetic processes were *KARS*, differentially expressed in the muscle of trout when fasting [59] and *ACSS1*, important for maintaining normal body temperature during fasting and for energy homeostasis in mice [60].

Morphology and development

Food deprivation affects morphogenesis and development of several organs, as these processes needs to be shut down to reallocate resources to produce energy for surviving and, in the case of eel, migration and vitellogenesis. In our analysis, downregulated genes were mainly included in development or morphogenesis of several organs including the liver, immune system, digestive system and sensory organs. The overrepresentation analysis may indicate a downregulation of genes that are important for the maintenance of several organs. It would be interesting to understand if the downregulation of genes related to these processes in the liver has an impact on the maintenance of other organs as suggested by the overrepresentation analysis (e.g. the

gastrointestinal tract that in eel shrinks during fasting) but to our knowledge no information is yet available.

In teleosts as in other vertebrates including mammals, the liver acts as an endocrine gland involved in body growth. Under the stimulatory effect of pituitary growth hormone, the liver produces Insulin growth factors (IGF), which are released in the bloodstream and exert mitogenic activities on various tissues such as muscle and skeleton (for review: [61]). As shown in salmonids, fasting induces a decrease in liver IGF1 transcripts and arrest in body growth, while refeeding leads to a rise in liver IGF1 transcripts (e.g. [62]). The downregulation of the liver expression of IGF1 and IGF2 observed in our study highlights the inhibition of the "body growth function" of the liver during eel sexual maturation.

In our work, *NOTCH1* was downregulated and was overrepresented in biological processes such as morphogenesis and development of several tissues and organs. This gene is involved in several processes, including animal organ development, chordate embryonic development and regulation of cell fate specification. In human, misregulation of this gene can cause liver cancer [63]. Activation of Notch signaling requires a direct contact between cells expressing Notch ligands and cells expressing Notch receptors [64]. It is interesting to observe that its receptor, jagged canonical Notch ligand 1b (*JAG1B*) was also downregulated in this analysis. In zebrafish, the inhibition of Jagged-mediated Notch signaling in the liver affects biliary tract development and generates multi-organ defects [65].

VEGFA, that was downregulated in our study, was mainly present in biological processes related to morphogenesis and development. In fact, this gene stimulates the proliferation of sinusoidal endothelial cells and hepatocytes during liver regeneration [66].

Liver was identified as one of the most important immune relevant organs in mammals and fish [53], [67]. The presence of the immune system as GO term in the downregulated genes and not in the upregulated genes is in line with what was observed during induced reproductive development in male eel [20]. In our study, 53 genes related to the immune system processes or development were downregulated. One of the gene included is *NCOR1* that in our study was present also in other biological processes such as response to growth hormone, formation of blood and cellular components (hemopoiesis), and plays a key role in reallocation of metabolic resources during fasting. In fact in mice, *NCOR1* negatively regulates many enzymes involved in fatty acid oxidation, desaturation, and elongation [68]–[70] and is involved in regulating mitochondrial and peroxisomal fatty acid oxidation [71]. During fasting, the activity of this gene is suppressed by autophagy, and this allow the Peroxisome Proliferator Activated Receptor Alpha (*PPARα*), to induce lipid oxidation [72]. PPARα is in fact

confirmed to be upregulated in our dataset. The interaction among NCOR1 and PPARα during fasting, extensively studied in mice, is still unknown in fish, but our analysis indicates that this regulation may occur also in fish.

Conclusion

Induced vitellogenesis of female European eel resulted in differential expression of genes involved in response to estrogen and reproduction, to allow gonad and oocyte development. At the same time, since eels do not feed during this process, several differentially expressed genes were linked with the requirement of energy and metabolite reallocation and several of which were differentially expressed during fasting in other species. Upregulated and downregulated genes showed different biological processes. Upregulated genes were involved in biological processes related to the biosynthesis of several molecules, energy production, mobilization and molecule transport. In contrast, downregulated genes were involved in the development and morphogenesis and to the immune system, as an indication of biological processes that may have reduced activity during vitellogenesis in this complex and distinctive fish species. Altogether, the results provide for the first time a holistic view (both at a phenotypical and a high-throughput molecular level) of the changes that occur in the liver of the European eel during unusual vitellogenesis, were oocyte maturation is accompanied by stopping the feeding regime. These findings represent the benchmark for further investigations of vitellogenesis processes.

Data availability

Raw RNA-Seq reads can be found in ENA (European Nucleotide Archive) under accession number PRJEB37006

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Tables

Table 1. Primers used for amplification of genes by qPCR. Overview of full gene name, accession number and target sequences.

Full name	Abbreviation	Accession no.	Primer (5′-3′) (F: Forward; R: Reverse)	Reference
Vitellogenin 1	*VTG1*	EU073127	F: GACAGTGTAGTGCAGATGAAG	[73]
			R: ATAGAGAGACAGCCCATCAC	
Vitellogenin 2	*VTG2*	EU073128.1	F: GATGCTCCCCTAAAGTTTGTG	[73]
			R: AGCGTCCAGAATCCAATGTC	
Beta-actin	*B-ACTIN*	DQ286836	F: AGCCTTCCTTCCTGGGTATG	[73]
			R: GTTGGCGTACAGGTCCTTAC	
Heat shock protein 90	*HSP90*	AZBK01838994	F: ACCATTGCCAAGTCAGGAAC	[15]
			R: ACTGCTCATCGTCATTGTGC	
Insulin like growth factor 1	*IGF1*	EU018410.1	F: TTCCTCTTAGCTGGGCTTTG	[15]
			R: AGCACCAGAGAGAGGGTGTG	
Insulin like growth factor 2	*IGF2*	AZBK01717674	F: ACAACGGATATGGAGGACCA	[15]
			R: GGAAGTGGGCATCTTTCTGA	
Elongation factor 1 alpha	*EF1A*	EU407824	F: CTGAAGCCTGGTATGGTGGT	[73]
			R: CATGGTGCATTTCCACAGAC	

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Table 2. Summary of Illumina sequencing data and mapped reads for the samples.

Week	Sample name	Total reads (n.)	Mapped reads (%)	Mapped reads unique (%)	Unmapped reads (%)
Week 0	Liver1	56,376,640	54.65	52.80	45.35
	Liver2	51,679,828	54.46	52.51	45.54
	Liver3	52,031,932	54.19	52.33	45.81
	Liver4	57,653,658	53.66	51.80	46.34
	Liver5	57,380,028	55.42	53.53	44.58
	Liver6	39,401,684	51.90	50.22	48.10
	Liver7	43,495,786	51.01	49.29	48.99
	Liver8	43,493,164	53.53	51.81	46.47
Week 9	Liver9	48,866,080	53.47	51.64	46.53
	Liver10	53,694,222	51.86	49.79	48.14
	Liver11	47,043,110	53.57	52.12	46.43
	Liver12	54,471,766	56.87	55.07	43.13
	Liver13	55,455,006	48.54	43.03	51.46
	Liver14	57,780,420	55.61	53.97	44.39
	Liver15	52,550,906	58.60	50.30	41.40
	Liver16	47,857,560	58.96	56.85	41.04

Figures

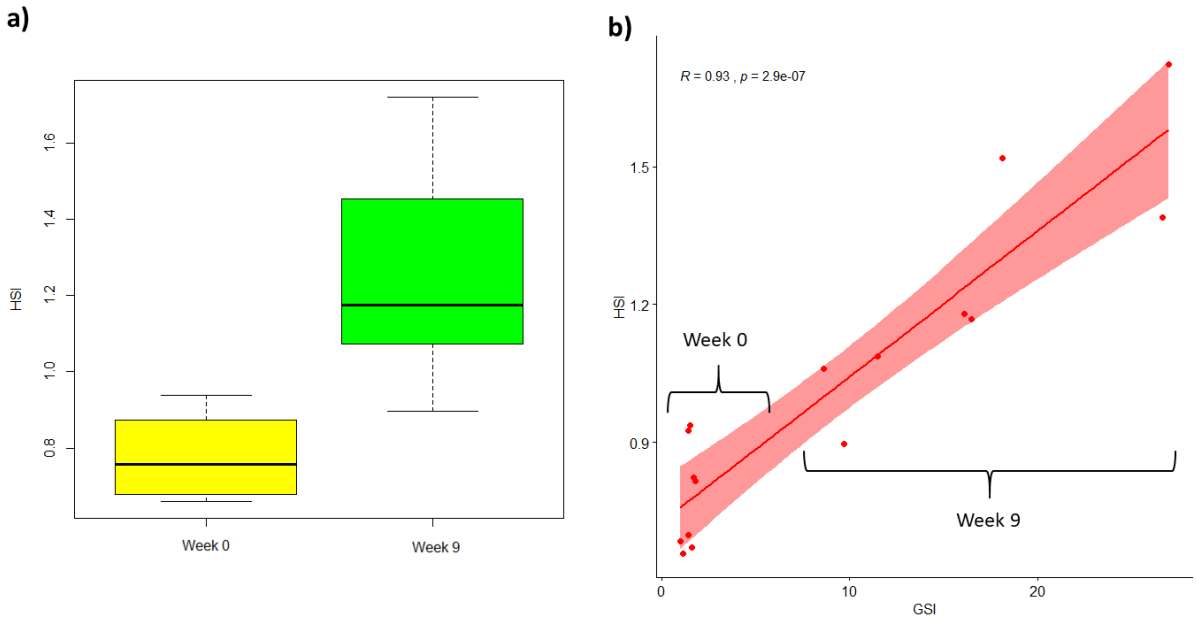

Fig 1. Statistical distribution of hepatosomatic index (HSI) of European eel before and during vitellogenesis (a) and correlation with gonadosomatic index (GSI) (b).

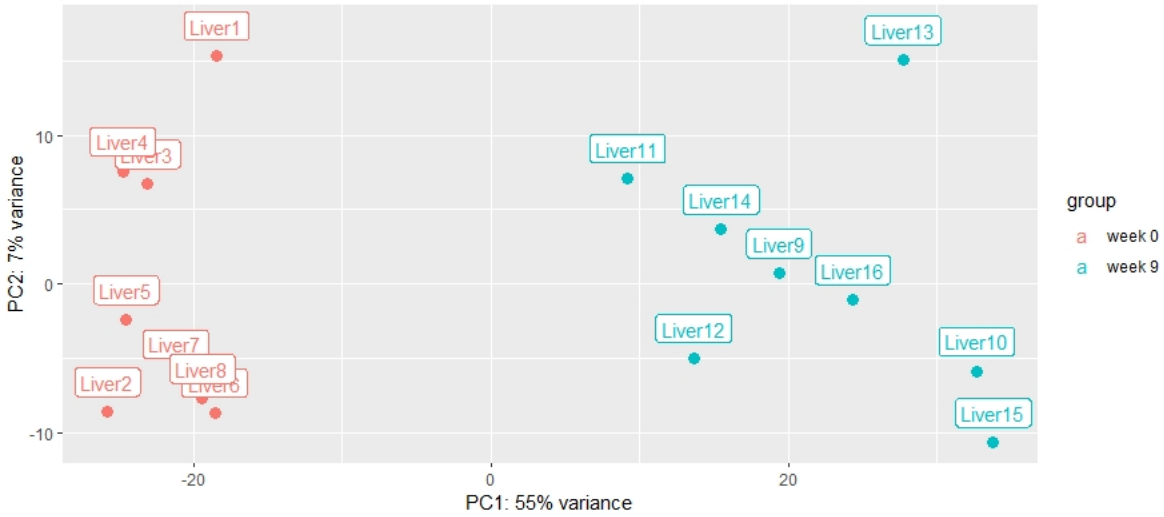

Fig 2. PCA plot of the differential expression analysis for European eel liver from previtellogenic (sampled in week 0) and late vitellogenic females (sampled after 9 weeks of treatment).

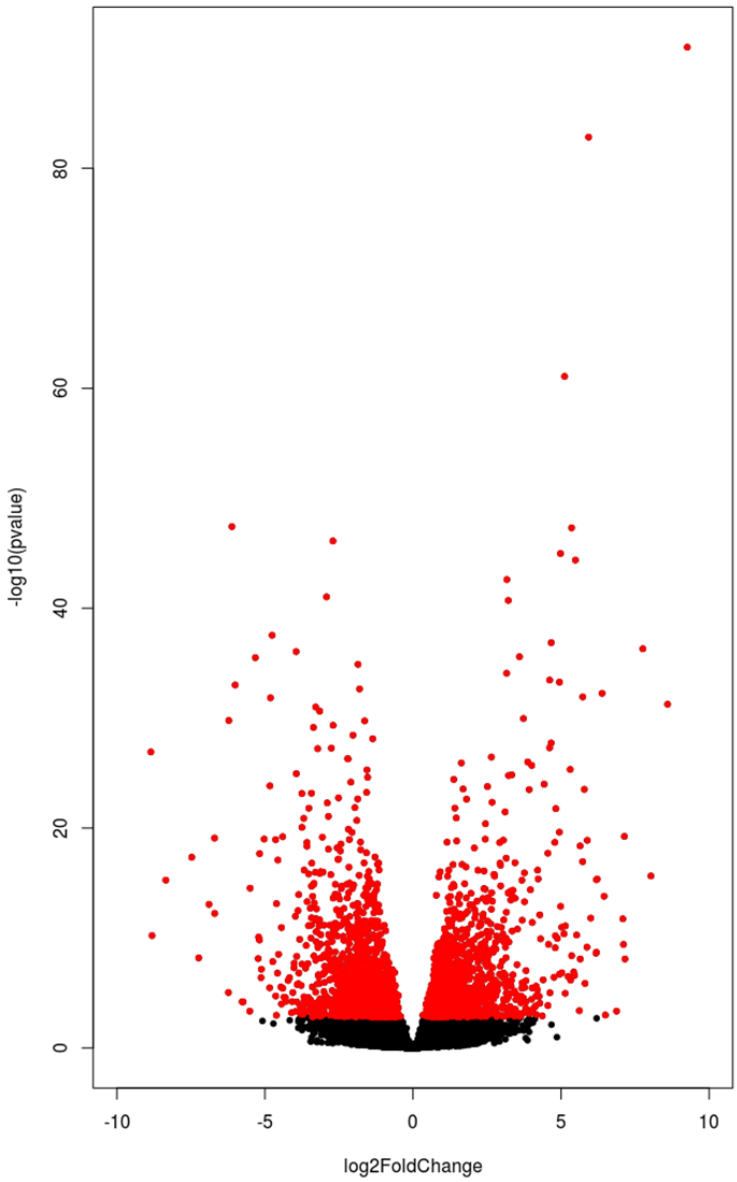

Fig 3. Volcano plot of the differential expression analysis. Each dot represents a gene. The x-axis reports the Log2fold change while the y-axis reports the –log10 of the adjusted P-value. All genes with adj. P < 0.01 were reported in red, with the left part (the negative part of the x-axis) representing the downregulated genes and the right part (the positive part of the x-axis) representing the upregulated genes.

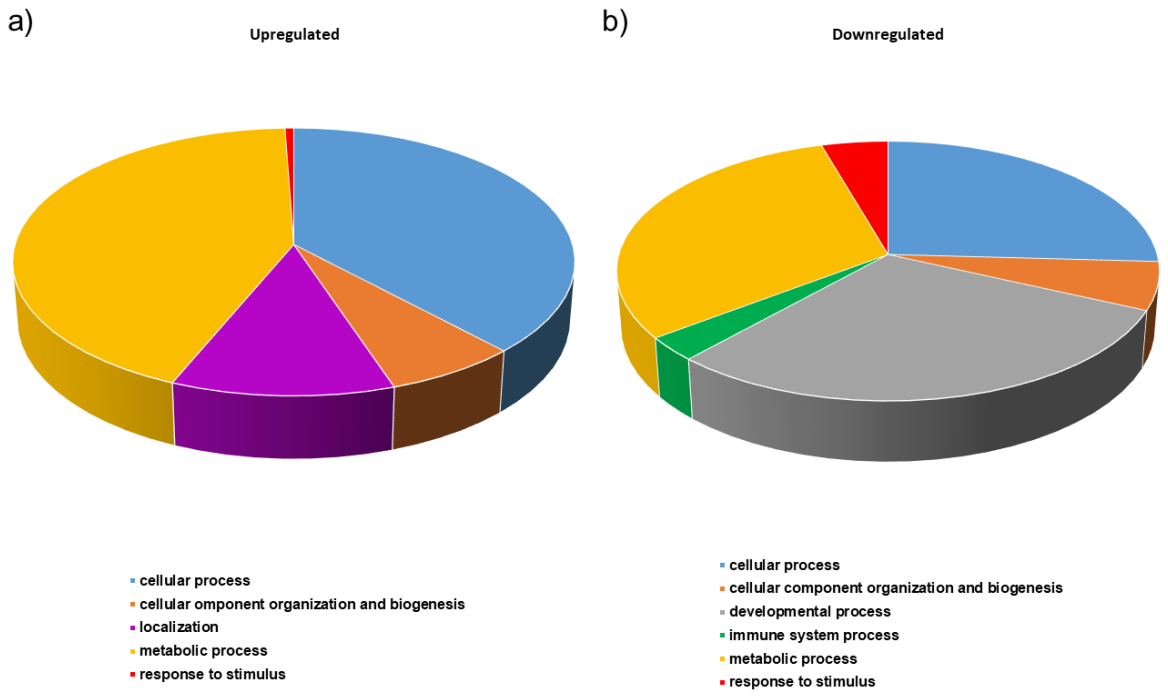

Fig 4. The proportions of significantly enriched terms belonging to the Biological Processes, for upregulated and downregulated genes with FDR less than 5%.

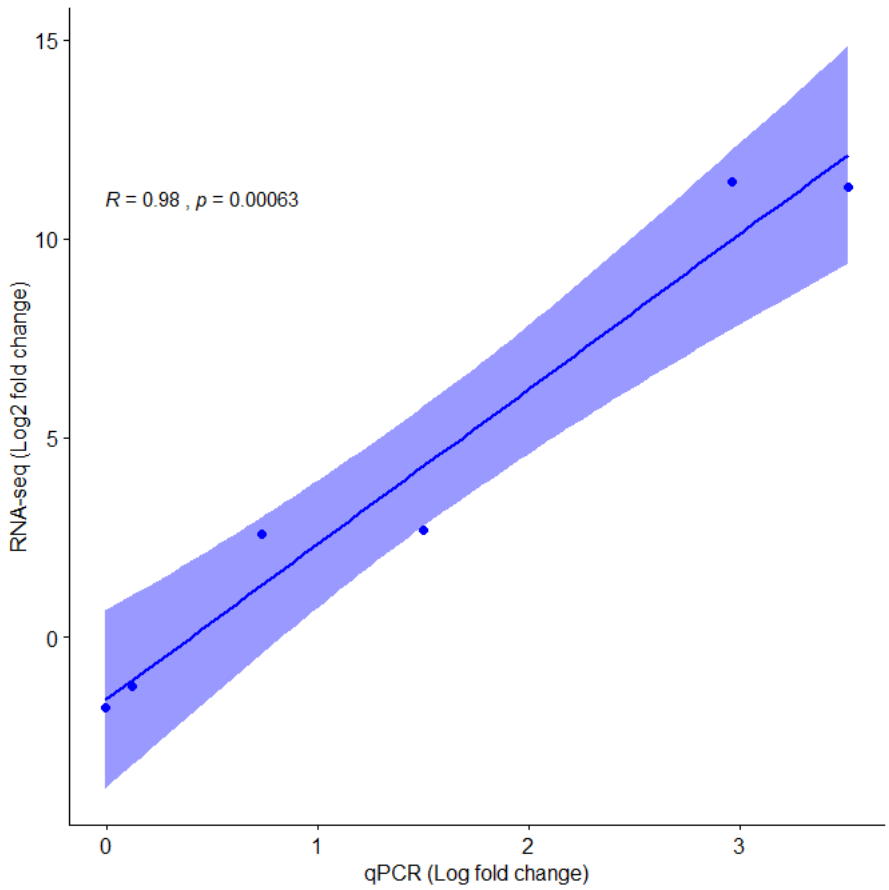

Fig 5. Correlation among the RNA-Seq (on the y-axis) and qPCR (on the x-axis) values for the same genes.

Supplementary captions

Supplementary data are available through the following link:
https://drive.google.com/drive/folders/1siXFtZXANAwS5Cwty58T6qJzF1uv3UpY?usp=sharing

Table S1: List of significantly differentially expressed genes, where annotation number based on the assembly, log2fold_change, P-value, homologous uniprot name (homologus_uniprot) and homologous gene symbol (gene_symbol) is reported.

Table S2: List of significantly enriched GO Biological processes from the overrepresentation analysis for the upregulated genes. Biological processes, subcategories of the biological process, GO-TERM number, Enrichment score and Genes detected in our analysis are reported.

Table S3: List of significantly enriched GO Biological processes from the overrepresentation analysis for the downregulated genes. Biological processes, subcategories of the biological process, GO-TERM number, Enrichment score and Genes detected in our analysis are reported.

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