Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Preclinical Sciences and Pathology

Philosophiae Doctor (PhD)
Thesis 2021:14

An Atlantic cod (*Gadus morhua* L.) pituitary model system for reproductive and reprotoxic testing *in vitro*

Et modellsystem for testing av reproduktive og reprotoksikologiske effekter på torskehypofysen *in vitro*

Kristine von Krogh

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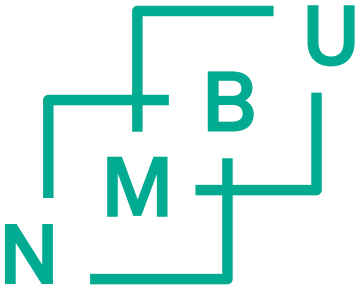
Oslo, 2021



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Det fins ei en eneste fisk

Som er bedre enn torsken, faktisk

Det er da sant og visst

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Oslo, 15.09.20, Kristine von Krogh

Summary

Due to factors such as overfishing, climate changes and pollution, most fish stocks are in decline. This is also the case for the commercially important marine species, Atlantic cod (*Gadus morhua*). As a result, the last few decades there has been growing interest to cultivate this species. However, a major set-back in cod aquaculture is precocious sexual maturity in farmed fish, leading to decreased flesh quality and quantity, higher susceptibility to diseases and spread of gametes from the net-pens to the surrounding environment. Sexual maturation in fish, as in other vertebrates, is regulated through the brain-pituitary-gonadal (BPG) axis. The brain produces gonadotropin releasing hormone (Gnrh) which stimulate synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) from gonadotrope cells in the pituitary. Fsh and Lh travel through the blood to the gonads, where they initiate gametogenesis and sex steroid production. The BPG axis is modulated through external factors such as temperature and light, and internal factors such as nutritional status. In recent years, endocrine disruptive (ED) properties have been demonstrated in many commercial compounds released into the environment, some of which have the capacity to affect fish development and reproduction. The exact mechanisms behind many of the above-mentioned regulating factors are largely unknown. Therefore, the primary aim of this thesis was to investigate further the reproductive-related regulation of the Atlantic cod pituitary. In order to do so, a primary culture model system using dispersed pituitary cells was developed. The system was optimized to mimic cod physiology and the natural environment, leading to stable, healthy, and physiologically relevant cultures. Once established, the culture system was subsequently used to investigate potential effects from sex steroids, the stress hormone cortisol, and the suspected ED contaminants, bisphenol A (BPA) and tetrabromobisphenol A (TBBPA), on pituitary gene expression and cell viability. By using cod donors at different stages of sexual maturity, potential maturity-dependent effects were also assessed. The results demonstrate that pituitary cultures are receptive to direct mechanisms from both endogenous and exogenous factors and that the Atlantic cod pituitary is a site for direct influence from sex steroids, stress, and pollutants. Dependent on dose and stage of sexual maturity, all tested substances were able to affect cell viability and gene expression and could potentially modulate cod reproductive function. For future work, this model system can be a useful tool for investigating physiological mechanisms in the pituitary and for screening of potential effects from environmental contaminants.

Sammendrag

Grunnet blant annet overfiske, klimaendringer og forurensing er mange av verdens fiskebestander synkende. Dette gjelder også torsk (*Gadus morhua*), en kommersielt viktig saltvannsart. De lave bestandene har initiert kultivering av torsk, men næringen har møtt flere store problemer, blant annet at oppdrettstorsken blir kjønnsmoden tidligere enn normalt. Tidlig kjønnsmodning kan føre til nedsatt kjøttkvalitet og - kvantitet, høyere mottagelighet for sykdom, samt genetisk forurensing til miljøet via spredning av gameter fra merdene. Hos fisk, som hos andre virveldyr, styres kjønnsmodningen gjennom hjerne-hypofyse-gonade-aksen (BPG-aksen). Hjernen produserer gonadotropin-frigjørende hormon (Gnrh) som stimulerer syntese og frigjøring av follikkelstimulerende hormon (Fsh) og luteiniserende hormon (Lh) fra gonadotrope celler i hypofysen. Fsh og Lh blir fraktet via blodbanen til gonadene, der de igangsetter gametogenese og produksjon av kjønnssteroider. BPG-aksen blir regulert via eksterne faktorer som temperatur og lys, samt interne faktorer som næringstilstand. De siste årene har det blitt påvist endokrinforstyrrende effekter fra flere kommersielle stoffer som slippes ut i miljøet. Noen av disse har også kapasitet til å påvirke fiskers reproduksjonsevne. De presise mekanismene bak flere av de overnevnte reguleringsfaktorene er ennå ikke kartlagte. Det primære målet for denne avhandlingen var å undersøke reproduksjons-relaterte reguleringer av torskehypofysen. For å kunne studere dette nærmere utviklet vi et modellsystem bestående av separerte hypofyseceller i primærkultur. Systemet ble optimalisert i henhold til torskens fysiologi og naturlige miljø, noe som førte til stabile, friske og fysiologisk relevante kulturer. De optimaliserte kulturene ble siden benyttet til å undersøke mulige effekter fra kjønnssteroider, stresshormonet kortisol og de antatt endokrinforstyrrende stoffene bisfenol A (BPA) og tetrabromobisfenol A (TBBPA) på genuttrykk og celleviabilitet i hypofysen. Ved å ta prøver fra torsk i ulike kjønnsmodningsstadier kunne vi også vurdere mulige stadiumseffekter. Resultatene viser at hypofysekulturer kan benyttes til å studere mekanismer forårsaket av både endogene og eksogene faktorer, og at torskehypofysen kan påvirkes direkte av kjønnssteroider, stress og forurensing. Avhengig av dose og modningsstadium påvirket alle substansene både celleviabiliteten og genuttrykket og kan dermed modulere torskens reproduktive funksjoner. For framtidig arbeid kan dette modellsystemet være et nyttig verktøy for å undersøke fysiologiske mekanismer i hypofysen og til å screene for mulige effekter fra miljøforurensende stoffer.

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Optimized conditions for primary culture of pituitary cells from the Atlantic cod (*Gadus morhua*). The importance of osmolality, pCO2, and pH

Kjetil Hodne, Kristine von Krogh, Finn-Arne Weltzien, Olav Sand, Trude Marie Haug

General and Comparative Endocrinology **2012,** *178* (2), 206-215

https://doi.org/10.1016/j.ygcen.2012.06.005

Paper II:

Sex steroids differentially regulate *fshb*, *lhb* and *gnrhr* expression in Atlantic cod (*Gadus morhua*) pituitary

Kristine von Krogh, Gunnveig Toft Bjørndal, Rasoul Nourizadeh-Lillabadi, Kjetil Hodne, Erik Ropstad, Trude Marie Haug, Finn-Arne Weltzien

Reproduction **2017,** *154* (5), 581-594

https://doi.org/10.1530/rep-17-0208

Paper III:

Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation

Kristine von Krogh, Gunnveig Toft Bjørndal, Rasoul Nourizadeh-Lillabadi, Erik Ropstad, Trude Marie Haug, Finn-Arne Weltzien

Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **2019,** 236, 110517

https://doi.org/10.1016/j.cbpa.2019.06.017

Paper IV:

***In vitro* effects of BPA and TBBPA on cell viability and reproduction-related gene expression in Atlantic cod pituitary**

Kristine von Krogh, Erik Ropstad, Rasoul Nourizadeh-Lillabadi, Trude Marie Haug, Finn-Arne Weltzien

Fishes **2019**, *4*(3), 48

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Abbreviations

Throughout this thesis, standard gene names and symbols specific for each species are applied. These can be found at community databases for particular organisms, e.g. human: www.genenames.org; and mouse: www.informatics.jax.org. For fish, the standard nomenclature for zebrafish (zfin.org) are applied to all species. For humans and non-human primates, gene symbols are generally in upper-case italicized characters (i.e. *GENE*), while protein symbols are upper-case non-italicized characters (i.e. PROTEIN). For mice and rats, protein symbols are the same as for humans, while gene symbols are italicized, first character in upper-case (i.e. *Gene*). For fish, gene symbols, as well as full gene names, are in lower-case italicized letters (i.e. *gene*), while protein symbols are non-italicized, with the first character in upper-case (i.e. Protein).

Abbreviation	**Full name**
11-KT	11 - ketotestosterone
1R	first whole genome duplication
20β -S	17α,20β,21-trihydroxy-4-pregnen-3-one
2R	second whole genome duplication
3R	third whole genome duplication
4R	Fourth whole genome duplication
AB	AlamarBlue
Acth	adrenocorticotropic hormone
Amh	anti-Müllerian hormone
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Ar	androgen receptor
arp2	*actin-related protein 2* (gene)
bactin	*beta actin* (gene)
BLAST	Basic Local Alignment Search Tool
BPA	bisphenol A
BPG	brain-pituitary-gonadal
BPI	brain-pituitary-interrenal
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
Cb	cerebellum
cDNA	complementary DNA
CF	carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate-actetoxymethyl ester
CG	chorionic gonadotropin
Cq	quantification cycle
Crh	corticotropin-releasing hormone

$CuSO_4$	copper sulphate
CV	coefficient of variation
D	diplotene
DAG	diacylglycerol
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNaseI	deoxyribonuclease I
DPH	17α,20β-dihydroxy-4-pregnen-3-one
E	efficiency
E2	17β-estradiol
ED	endocrine disruptor
EDCs	endocrine disruptive chemicals
ef1a	*elongation factor 1- alpha* (gene)
EPV	early previtellogenic follicle
Er	estrogen receptor
ERK	extracellular signal-related kinase
EtOH	ethanol
EU	European Union
EV	early vitellogenic follicle
F	cortisol
Fsh	follicle-stimulating hormone
Fshr	Fsh receptor
Fshβ	Fsh, beta subunit
fura-2AM	fura-2 acetoxymethyl ester
GABA	gamma-aminobutyric acid
GC	glucocorticoid
gDNA	genomic DNA
Gh	growth hormone
GH3	a rat pituitary tumour cell line
Gnih	gonadotropin inhibitory hormone
Gnrh	gonadotropin releasing hormone
Gnrhr	Gnrh receptor
Gnrhr1b	Gnrh receptor, type 1b
Gnrhr2a	Gnrh receptor, type 2a
Gnrhr2b	Gnrh receptor, type 2b
Gnrhr2c	Gnrh receptor, type 2c
GPα	glycoprotein hormone, alpha subunit
Gr	glucocorticoid receptor
GSI	gonadosomatic index
Gth	gonadotropin
hCG	Human chorionic gonadotropin
Hyp	hypothalamus

Igf-3	insulin-like growth factor 3
Im	immature
IP_3	inositoltriphosphate
ISRPF	International Symposium on the Reproductive Physiology of Fish
ITIS	Integrated Taxonomic Information System
IUCN	International Union for Conservation of Nature and Natural Resources
IUPAC	International Union of Pure and Applied Chemistry
L/Z	leptotene/zygotene
LC_{50}	lethal concentration, 50%
Lh	luteinizing hormone
Lhr	Lh receptor
Lhβ	Lh, beta subunit
LPV	late previtellogenic follicle
LV	late vitellogenic follicle
MCF-7	a human breast adenocarcinoma cell line
Me	mature
Med	medulla
MeOH	methanol
Mg	maturing
MIS	maturation inducing steroid
mOsm	milliosmole
Mr	mineralocorticoid receptor
mRNA	messenger RNA
Msh	melanocyte stimulating hormone
NCS	newborn calf serum
NE	norepinephrine
NMBU	Norwegian University of Life Sciences
NPY	neuropeptide Y
NTC	non-template negative control
O	oogonium
OB	olfactory bulb
Osm	osmolality
OT	olfactory tract
OTec	optic tectum
P	pachytene
P4	progesterone
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	phosphate-buffered saline
pCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PD	*pars distalis*
pen-strep	penicillin and streptomycin
PI	*pars intermedia*

Pit	pituitary
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PN	*pars nervosa*
PNEC	predicted no effect concentration
PPD	*proximal pars distalis*
Prl	prolactin
PV	postvitellogenic follicle
qPCR	quantitative PCR
qRT-PCR	quantitative RT-PCR
RA	retinoic acid
rcf	relative centrifugal force (G-force, *g*)
RIN	RNA Integrity Number
RNA	ribonucleic acid
RNase	ribonuclease
RPD	*rostal pars distalis*
rpm	revolutions per minute
RT-PCR	reverse transcription - PCR
S	sperm
SAGE	serial analysis of gene expression
SC	spermatocyte
SCo	spinal cord
SD	standard deviation
SEM	standard error of mean
SF	serum-free
Sl	somatolactin
Sp	spent
SPA	spermatogonia, type A
SPA_{diff}	SPA differentiated
SPA_{und}	SPA undifferentiated
SPB	spermatogonia, type B
SS	secondary spermatocyte
SSC	spermatogonial stem cell
SSR	synthetic serum replacement (serum substitute)
ST	spermatid
SZ	spermatozoa
T	testosterone
T3	triiodothyronine
T4	thyroxine
TBBPA	tetrabromobisphenol A
Tel	telencephalon
Tm	melting temperature

TNS	taxonomic serial number
Tsh	thyroid-stimulating hormone
ZFL	a zebrafish hepatocyte cell line

Species nomenclature

Upon first mention, the common name of a species will be followed by its scientific name. Subsequent descriptions of the species will be by common name only.

Common name:	**Scientific name:**
African catfish	*Clarias gariepinus*
Amazon molly	*Poecilia formosa*
Anglerfish	*Photocorynus spiniceps*
Atlantic cod	*Gadus morhua*
Atlantic croaker	*Micropogonias undulatus*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic pollock	*Pollachius pollachius*
Atlantic salmon	*Salmo salar*
Black seabream	*Acanthopagrus schlegeli*
Black-spotted pond frogs	*Rana nigromaculata*
Brown trout	*Salmo trutta*
Capelin	*Mallotus villosus*
Chinese grass carp	*Ctenopharyngodon idellus*
Chum salmon	*Oncorhynchus keta*
Cinnamon clownfish	*Amphiprion melanopus*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Deep sea ceratioid anglerfish	*Cryptopsaras couesi*
Eurasian perch	*Perca fluviatilis*
European eel	*Anguilla anguilla*
European hake	*Merluccius merluccius*
European sea bass	*Dicentrarchus labrax*
Fathead minnow	*Pimephales promelas*
Flying cod (family)	Exocoetidae
Frogfish (family)	Antennariidae
Giant oarfish	*Regalecus glesne*
Gilt-head seabream	*Sparus aurata*
Goldfish	*Carassius auratus*
Haddock	*Melanogrammus aeglefinus*
Harbour porpoise	*Phocoena phocoena*
Herring (family)	Clupeidae
Hybrid striped bass	*Morone saxatalis x chrysops*
Japanese eel	*Anguilla japonica*
Japanese quail	*Coturnix japonica*
Largemouth bass	*Micropterus salmoides*
Mangrove killifish	*Kryptolebias marmoratus*
Mariana snailfish	*Pseudoliparis swirei*

Marine medaka	*Oryzias melastigma*
Masu salmon	*Oncorhynchus masou*
Medaka	*Oryzias latipes*
Minke whale	*Balaenoptera acutorostrata*
Mozambique tilapia	*Oreochromis mossambicus*
Mudskippers (familiy)	Oxudercidae
New Zealand snapper	*Pagrus auratus*
Nile tilapia	*Oreochromis niloticus*
Ocean sunfish	*Mola mola*
Pacific salmon (genus)	*Oncorhynchus* spp.
Rainbow trout	*Oncorhynchus mykiss*
Red sea bream	*Pagrus major*
Ringed seal	*Phoca hispida*
Saithe	*Pollachius virens*
Scale-less carp	*Gymnocypris przewalskii*
Seahorses (genus)	*Hippocampus* spp.
Striped bass	*Morone saxatilis*
Tilapia hybrid	*Oreochromis niloticus* × *O. aureus*
Tongue sole	*Cynoglossus semilaevis*
Urohaze-goby	*Glossogobius olivaceus*
Zebrafish	*Danio rerio*

1

Introduction

1. Introduction

1.1 General background

Amongst vertebrates, teleost fish constitutes the largest and most diverse infraclass, with over 30 000 described species world-wide (FishBase, 2020; Eschmeyer's Catalog of Fishes, 2020). This number covers 96% of all extant fish species and just under half of all vertebrate species (IUCN, 2020). Teleosts belong to the ray-finned, bony fishes and appeared in the fossil records some 150 million years ago. However, phylogenetic estimates based on nuclear genes suggest the most recent common ancestor of living teleosts to be over 300 million years old (see Figure 1) (Near et al., 2012). Since, teleosts have spread to all waters of the world, both fresh, brackish and salt, and some, such as the mudskippers (Oxudercidae), can even live on land for long periods at the time (Polgar et al., 2010). The diversity in morphology is astonishing, ranging from just a few millimetres, such as the minute male anglerfish *Photocorynus spiniceps* (Pietsch, 2005), to the giant oarfish (*Regalecus glesne*), reaching over 8 meters length, and the ocean sunfish (*Mola mola*), reaching up to 2.3 metric tons weight (McClain et al., 2015). Similarly, there are large variations in physiology and behaviour between the teleost species. For instance, the Mariana snailfish (*Pseudoliparis swirei*) withstands the immense pressure living at over 8 000 meters depth (Gerringer et al., 2017), while the scale-less carp (*Gymnocypris przewalskii*) inhabits the alkaline waters of Lake Qinghai at 3 200 meters above sea-level (Matey et al., 2008). Frogfish (Antennariidae) walk at the sea bottom floor using their pelvic and pectoral fins (Arnold et al., 2014), while the flying cod family (Exocoetidae) use those same fins to glide above the sea surface for hundreds of meters (Fish, 1990). The basis for this remarkable diversity is probably provided by the third whole-genome duplication (3R) that occurred in the teleost fish lineage over 300 million years ago, following the two genome duplications (1R/2R) from earlier in vertebrate evolution (Steinke et al., 2006) (see Figure 1). Some teleosts, such as the salmonids, have even had an additional fourth duplication event (4R) (Lien et al., 2016). Because of these duplication events, a pool of duplicated genes now available to evolve new functions was generated, enhancing teleost capacity for rapid genomic evolution. The genetic diversity is also reflected in diverse reproductive strategies, as teleosts display every mode of reproduction found in vertebrates (Desjardins and Fernald, 2009). In terms of gender systems,

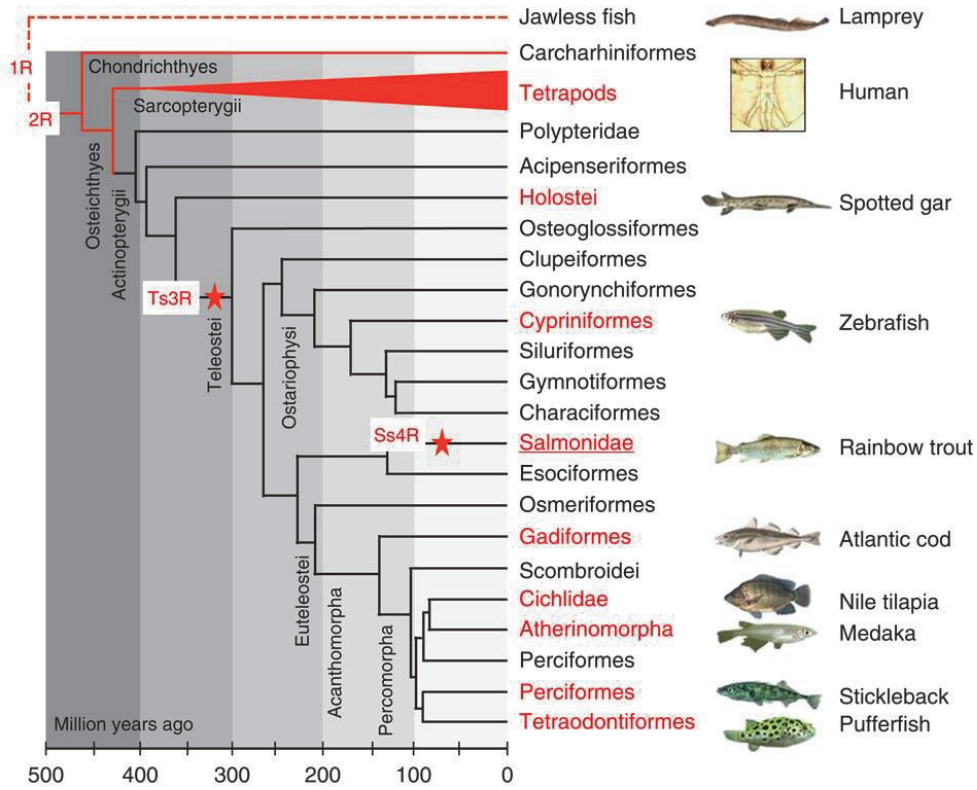

Figure 1.1 Phylogenetic description of vertebrate evolution, with the whole-genome duplication events indicated (1-4R). The species to the right represents groups in which a genome sequence is available (marked in red). **1R**; first whole genome duplication, **2R**; second whole genome duplication, **Ts3R**; teleost-specific third whole genome duplication, **Ss4R**; Salmon-specific fourth whole genome duplication.
Figure from Berthelot et al. (2014), CC BY-NC-SA 3.0.

some species, such as the mangrove killifish (*Kryptolebias marmoratus*), are hermaphrodites, possessing both male and female gonads simultaneously (Sakakura et al., 2006), and others, like the gilt-head seabream (*Sparus aurata*), are sequential hermaphrodites, changing sex during their lifetime (Pauletto et al., 2018). Most fish, however, are gonochoristic, and develop only as males or females and remain the same sex throughout life (Devlin and Nagahama, 2002; Heule et al., 2014; Smith and Wootton, 2016). In general, teleosts reproduce through sexual reproduction, but there are exceptions, such as the above-mentioned mangrove killifish, that can be self-fertilizing (Sakakura et al., 2006), or the Amazon molly (*Poecilia formosa*), that reproduce through parthenogenesis (Lampert and Schartl, 2008). Sexual reproduction takes several strategies and behaviours. Some species are diadromous, meaning they migrate between sea and fresh water to spawn, such as the anadromous Atlantic salmon (*Salmo salar*) (Jonsson and Jonsson, 2009) or the catadromous

European eel (*Anguilla anguilla*) (van Ginneken and Maes, 2005). Some are littoral spawners, while others spawn in streams. Some spawn in fall and some in spring. Some spawn several times per season (iteroparity) and others, like the Pacific salmon (*Oncorhynchus* spp.), spawn only once during their lifetime (semelparity) (Morbey et al., 2005). A few species fertilize internally, but the vast majority have external fertilization (Smith and Wootton, 2016). Some lay eggs on plants, land or simply release them into the water, while other bear live offspring, and some brood their young in their mouth or other body cavities (Desjardins and Fernald, 2009). Some species, like seahorses (*Hippocampus* spp.), display sex-role reversal, with the males incubating the eggs (Foster and Vincent, 2004). The fecundity, in terms of gamete production, can range from a few to tens of millions between species. Teleosts also display a wide array of mating behaviours, ranging from competing females to sneaky males (Desjardins and Fernald, 2009). Most fish are polygamous, but some form monogamous relationships (Whiteman and Côté, 2004). Some fish display rather extreme monogamy, like males of the deep sea ceratioid anglerfish (*Cryptopsaras couesi*), that attach and parasitically merge themselves to the females, existing entirely as a sack of sperm after maturity (Pietsch, 1975). While parental care is displayed in some species, with paternal care occurring more often than maternal, being non-parental is far more common (Smith and Wootton, 2016). In just 200-300 million years, teleost fish have evidently evolved to an extremely varied clade!

Historically, fish have always been important to humans as a food source, although fish uses in recent years have increased to include recreational benefits (angling), ornamental uses (aquaria fish), as well as a vertebrate model in research. Because of over-exploitation and other anthropogenic impacts such as habitat loss, climate change and pollution, fish stocks, as well as fish biodiversity, are declining globally (Clausen and York, 2008; FAO, 2018; Garcia and Rosenberg, 2010; Johnson et al., 2017; Jonsson and Jonsson, 2009; Mayer, 2019). According to the International Union for Conservation of Nature and Natural Resources (IUCN, 2020), there are now 2,721 threatened fish species. An increasing human population combined with declining fish stocks puts pressure on the food industry. As a result, there has been growing interest to cultivate fish species, both through stocking practices and aquaculture industries. As of 2016, there were 369 different fish species farmed commercially worldwide and about half of the human consumption of fish were derived from aquaculture, while this figure is expected to increase to 70% by 2030 (FAO, 2018). Fish aquaculture can represent several advantages. First, it provides a reliable source of affordable animal protein (food security). Second, it can protect wild stocks of vulnerable and/or over-

exploited species. Third, fish harvesting in culture is easier and can, compared to many other traditional harvesting methods, reduce certain types of environmental impact and increase some aspects of animal welfare. For instance, trawling and purse seining used for wild-catch frequently causes by-catch, which kills or wounds marine mammals, other fish species, sea turtles, sea birds and invertebrates, depleting wildlife animal stocks (Hall et al., 2000; Read et al., 2006). Additionally, demersal trawling and dredging may inflict devastating damage upon the seabed, affecting entire ecosystems (Allen and Clarke, 2007). Fourth, aquaculture can have socioeconomical advantages, increasing local jobs and drive scientific knowledge and technology. However, there are also several disadvantages to fish aquaculture. First, in contrast to wild fish, cultured species need to be cared for and nurtured until harvest, demanding much resources. Furthermore, providing food for carnivorous species, such as salmonids and cod, may put pressure on those wild fish stocks used to supply fishmeal and fish oil for aquafeed (Naylor et al., 2000; Primavera, 2006). Second, there are still several animal welfare problems associated with aquaculture, for instance handling, high stock densities, constraining conditions, and increased susceptibility to pathogens, parasites and diseases (Ashley, 2007; Huntingford et al., 2006). Third, pollution from chemical release, such as antimicrobials or trace amounts of agricultural pesticides from leftover feed pellets, and the high nutrient waste load, can negatively affect local ecosystems (Ackefors and Enell, 1994; Cabello et al., 2013; Olsvik et al., 2019; Primavera, 2006). Fourth, negative interactions and impacts between farmed and wild fish, through for example altered migration patterns of wild stocks, genetic introgression between wild and escaped farmed fish, as well as predators (marine mammals, birds) being attracted to fish cages (Callier et al., 2018; Ford and Myers, 2008; Uglem et al., 2014; Zhang et al., 2013). Finally, a major recurring production problem in fish aquaculture is the onset of precocious sexual maturity (Svåsand et al., 1996; Taranger et al., 2010; Zanuy et al., 2001). Precocious maturity leads to decreased filet quality, increased mortality, and risk of genetic pollution from escaped eggs/larvae, as well as economic loss for the farmer (Jørstad et al., 2008; McClure et al., 2007; Taranger et al., 2010). The early maturation is most likely a combined effect of the absence of predators and steady access to food in culture pens. Despite attempts to correct the issue of early puberty in fish farming through methods such as photoperiod control, induced triploidy, selective breeding and others, there are still limitations concerning the commercial use of these methods (Fraser et al., 2012; Taranger et al., 2010).

Based on the above-mentioned problems facing fish populations world-wide, it is necessary to preserve fish stocks. Cryopreservation of fish gametes for future stocking is a

promising method for preservation of fish biodiversity, but currently, the technology is available only for fish sperm and not eggs (Mayer, 2019). For some species, stocking practices, rearing fish in hatcheries and subsequently releasing them into the wild, have been used as a conservational tool (Aprahamian et al., 2003; Brown and Day, 2002), but also this practise have limitations. For instance, while hatchery-reared fish can contribute to increased fish biomass, they compete with wild species for limited natural resources (Amoroso et al., 2017). Furthermore, reduced genetic diversity and fitness of wild populations have been reported following stocking (Aprahamian et al., 2003; Bartley and Bell, 2008). While aquaculture will undoubtedly significantly contribute to future food security, as well as alleviating pressure of threatened wild stocks, several constraints must be addressed to improve sustainability of aquaculture practices. For wild stock rehabilitation, habitat restoration and fishing moratoria will probably be necessary for some species.

Figure 1.2 Fish aquaculture may offer a solution to the increasing demand for steady food sources but comes with many negative side-effects. Pictured is an Atlantic salmon (*Salmo salar*) fish farm at Vestmanna, Faroe Islands. Photo: © Erik Christensen. CC-BY-SA 3.0

1.2 Atlantic cod

Amongst the most popular commercial food fishes in the world is the Atlantic cod (*Gadus morhua*), a marine species belonging to the Teleostei infraclass and Gadiformes order (Figure 1.3) (FAO, 2018; Kurlansky, 1997). It is found in the North Atlantic and Arctic oceans; in the west, from Ungava Bay in Canada down to Cape Hatteras, North Carolina, and in the east, from the Barents Sea down to the Bay of Biscay. It is also distributed around the coasts of Greenland and Iceland (FishBase, 2020). Considered a demersal species, cod mainly resides over continental shelfs at 150 – 200 meters depth but have been found down to at least 850 meters (Cohen et al., 1990; Marteinsdottir et al., 2005). When feeding and spawning, they can exhibit more pelagic behaviour (Cohen et al., 1990). Cod form compact schools during the day, and scatter at night. While some groups of cod are relatively stationary, others, such as the Greenland cod, perform migrations up to 1000 km for feeding and spawning (Cohen et al., 1990). Oceanic fish can reach body lengths of up to 2 meters, while local fish tend to be smaller. They can live to be 20 years old. Being omnivorous predators, cod prey upon various species, such as capelin (*Mallotus villosus*), herrings (Clupeidae), crustaceans and amphipods (Blanchard et al., 2005; Johansen, 2003; Link et al., 2009). The famous barbel protruding from its lower jaws is used for finding prey buried in the sand. Cod are also cannibals, with the proportion of cod as feed positively correlated to body size (Bogstad et al., 1994; Link et al., 2009). In turn, many species prey on cod eggs, larvae and young juveniles. Larger specimens are mainly eaten by marine mammals, such as minke whale (*Balaenoptera acutorostrata*), harbour porpoise (*Phocoena phocoena*) and ringed seal (*Phoca hispida*) (Bogstad and Gjøsæter, 2001; Folkow et al., 2000; Link et al., 2009; Nilssen et al., 2004). Sexual maturity is reached from 2-7 years of age, dependent on population (Myers et al., 1997b). The spawning season varies geographically from January to May, with southern populations spawning earlier than northern, and lasts 60 to 90 days (Blanchard et al., 2005; Hall et al., 2004; Ottersen and Sundby, 2005; Otterå et al., 2006). The act of spawning occurs preferentially in the evening or at night, preceded by a mating ritual where females select their mate based on male courtship behaviour such as grunting, a drumming noise produced by muscles associated with the swim-bladder, and fin display (Brawn, 1961; Engen and Folstad, 1999; Hutchings et al., 1999; Kjesbu, 1989). The male holds the female using his pelvic fins and positions himself belly-to-belly to her, his vent just below hers, while they spawn (Brawn, 1961; Hutchings et al., 1999).

Taxonomy

Vernacular names:
Atlantic cod (English), torsk (Norwegian, Swedish and Danish), bacalao (Spanish), Kabeljau/Dorsch (German), kabeljauw (Dutch), merluzzo nordico (Italian), bacalhau-do-atlântico (Portuguese) and morue de l'Atlantique (French)

Scientific name: *Gadus morhua* Linnaeus, 1758 (TSN:164712)

Kingdom: Animalia
Phylum: Chordata
Subphylum: Vertebrata
Superclass: Osteichthyes
Class: Actinopterygii
Subclass: Neopterygii
Infraclass: Teleostei
Superorder: Paracanthopterygii
Order: Gadiformes
Family: Gadidae
Subfamily: Gadinae
Genus: *Gadus*
Species: *Gadus morhua*

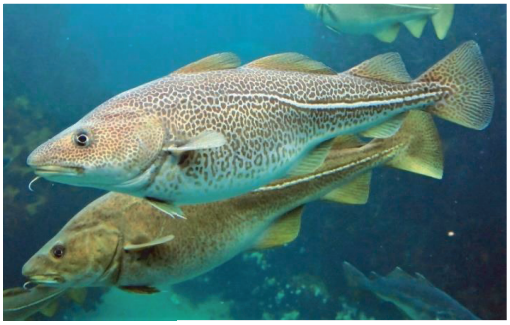

Photo: © Joachim S. Müller (CC BY-NC-SA 2.0)

Figure 1.3 Taxonomy of Atlantic cod. **TNS**; taxonomic serial number, issued by the Integrated Taxonomic Information System (ITIS), https://www.itis.gov/index.html

Atlantic cod are iteroparous batch spawners, releasing up to 21 egg batches at intervals of 45 to 70 hours (Kjesbu et al., 1996). Each batch can contain 300,000 eggs, making cod one of the most fecund fish in the world (Chambers and Waiwood, 1996; Cohen et al., 1990; Kjesbu, 1989). Cod display no parental care, the fertilized, buoyant eggs are released into the free water masses and hatch after approximately 1- 4 weeks dependent on temperature (Laurence and Rogers, 1976). Both eggs and larvae (see Figure 1.4) are pelagic, confined to the upper 50 meters of the water column (Ottersen and Sundby, 2005). The larvae, only a few millimetres long, drift motionlessly until they start feeding at around 3 days post hatching

(Brown et al., 2003; Hall et al., 2004). After a few months, juvenile cod seek out the bottom and spend the first few years of their life cycle following a benthic existence on the nursery grounds before joining the adult population (Cohen et al., 1990). Cod are generally gonochoristic, although hermaphroditic specimens have been reported (Cohen et al., 1990).

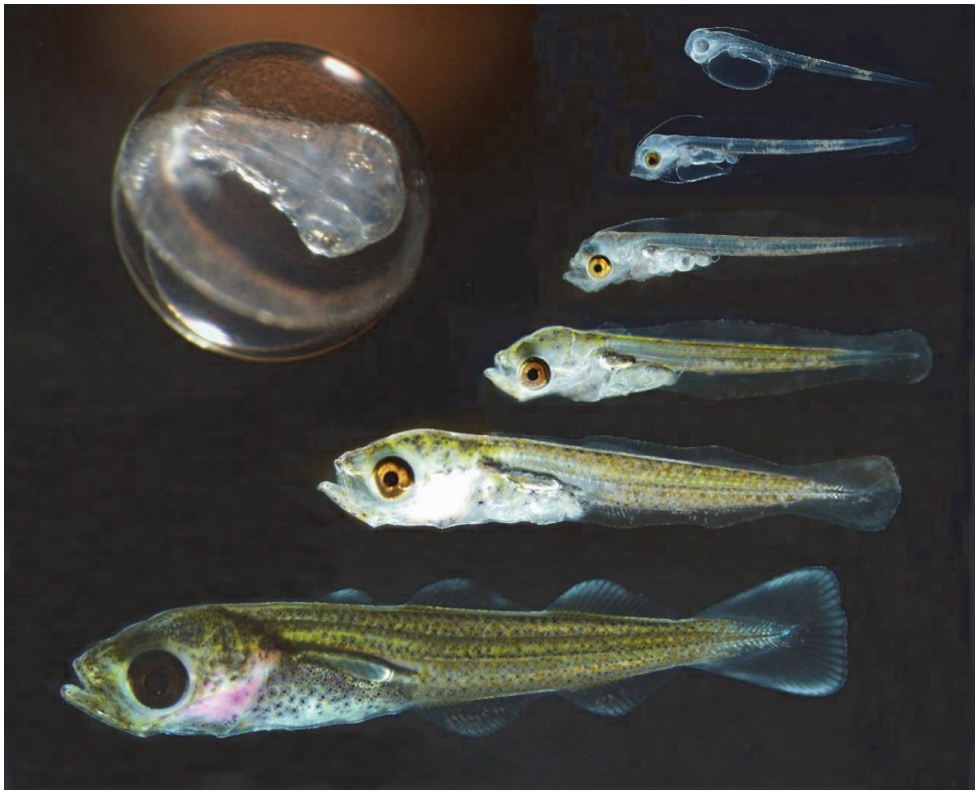

Figure 1.4 Atlantic cod development. The buoyant, transparent egg in the upper left corner, and newly hatched larvae to the upper right. Photo: © Terje van der Meeren and Ørjan Karlsen, Norwegian Institute of Marine Research. Reprinted with permission.

Historically, culturally and economically, the Atlantic cod has always been an important species. Wars have been fought (the so-called Cod Wars), cities have been founded, and whole populations kept alive because of this fish (Kurlansky, 1997; Steinsson, 2017). In Norway, the Vikings used dried cod as food on their boat journeys. In fact, the Norwegian name for cod, «torsk», is derived from the Old Norse word «turfiskr», meaning dried fish (Høberg, 2020). The white muscle of cod is lean and high in protein, making it very nutritious, and mildly flavoured, making it very versatile (Kurlansky, 1997; Lambert and Dutil, 1997). Cod is jokingly referred to as “the beef of the sea”, and the meat is prepared

salted, dried, smoked, in brine or fresh (Cohen et al., 1990). Dried cod is still part of the traditional Norwegian Christmas meal, weirdly, for many foreigners, prepared in lye. Almost all parts of the cod body are utilized; the tongue, cheek, liver and rye are considered delicacies, the skin is used for leather and collagen production, the swim-bladder for isinglass and the liver for crude oil. Most Norwegians eat this oil supplemented with D-vitamin during the winter months (rule of thumb; all months containing an "r"). Norway is a major exporter of cod and have been so for centuries, and as such, it is a very important fish species for Norwegians. Museums, art, music and poems have been created to honour this beloved fish (Figures 1.5 and1.6).

Figure 1.5 Cod is an important fish for Norwegians, and for many, the relationship is established from an early age. Here is my niece, Othilie, at 1-year old, admiring some pretty specimens at Atlanterhavsparken, The Norwegian Aquarium. Photo: © Ingvild von Krogh Strand. Reprinted with permission

There are two main Norwegian cod populations; Northeast Artic cod (called "skrei" in Norwegian, meaning "wanderer"), with long spawning migration, and coastal cod with short or no spawning migration. Coastal cod is often subdivided into two populations; coastal cod above 62° N and coastal cod below 62° N. However, recent genetic analysis suggests the existence of several gradient coastal cod subpopulations, and that the former division should be re-evaluated (Dahle et al., 2018). In general, *skrei* are larger and mature sexually at a later age than northern coastal cod, who again are larger and mature later than southern coastal cod

(Berg and Albert, 2003; Blanchard et al., 2005; Godø and Haug, 1999; Myers et al., 1997b; Ottersen and Sundby, 2005).

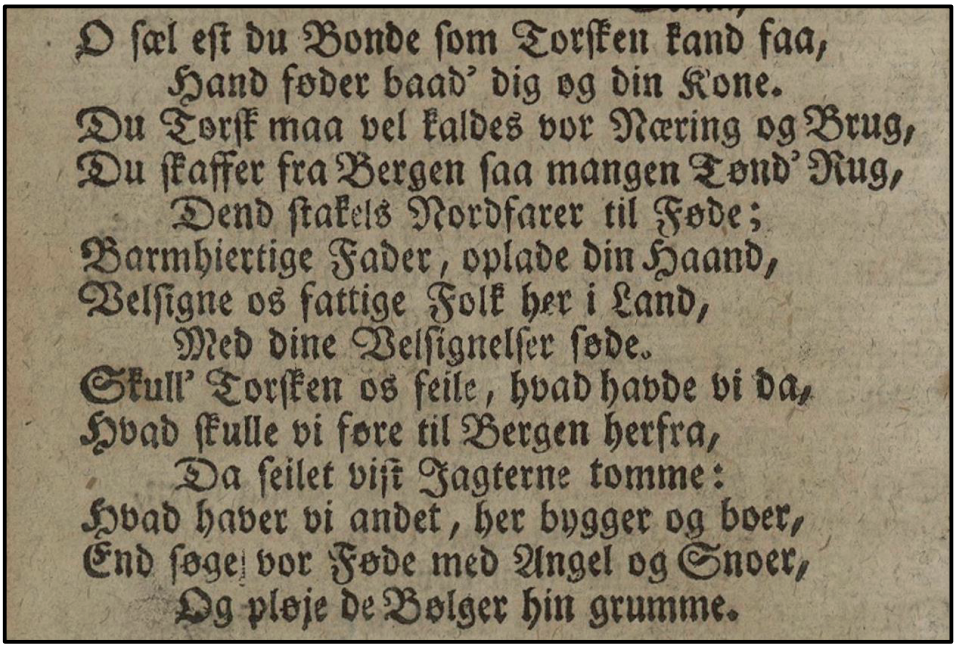
O sæl eſt du Bonde ſom Torſken kand faa,
Hand føder baad' dig og din Kone.
Du Torſk maa vel kaldes vor Næring og Brug,
Du ſkaffer fra Bergen ſaa mangen Tønd' Rug,
Dend ſtakels Nordfarer til Føde;
Barmhiertige Fader, oplade din Haand,
Velſigne os fattige Folk her i Land,
Med dine Velſignelſer ſøde.
Skull' Torſken os feile, hvad havde vi da,
Hvad ſkulle vi føre til Bergen herfra,
Da ſeilet viſt Jagterne tomme:
Hvad haver vi andet, her bygger og boer,
End ſøge vor Føde med Angel og Snoer,
Og pløje de Bølger hin grumme.

Figure 1.6 An ode to cod, describing its importance in 17th century Norway; "Should the cod fail us, what then would we hold?"
From *Nordlands Trompet* (*Trumpet of Nordland*), a poem praising nature, life and people in the North of Norway, by poet Petter Dass. Published posthumously in 1739 (in old Norwegian). Public domain.

Transcribed version:
O sæl est du Bonde, som Torsken kand faa,
Hand føder baad' dig og din Kone.
Du Torsk maa vel kaldes vor Næring og Brug,
Du skaffer fra Bergen saa mangen Tønd' Rug,
Dens stakels Nordfarer til Føde;
Barmhiertige Fader, oplade din Haand,
Velsigne os fattige Folk her i Land
Med dine Velsignelser søde.
Skuld' Torsken os feile, hvad havde vi da,
Hvad skulle vi føre til Bergen herfra,
Da seilet vist Jagterne tomme;
Hvad haver vi andet, her bygger og boer,
End søge vor Føde med Angel og Snoer,
Og pløie de Bølger hin grumme.

While once abundant in our fjords, Atlantic cod stocks, not only in Norway, but worldwide, have declined substantially the last few decades. Historically low levels were detected in many of the North Atlantic stocks in the early 1990s (Lilly et al., 2008; Myers et al., 1997a). The decline is generally contributed to extensive overfishing, but there is reason to believe that also climatic changes and recruitment failure are influencing factors (Drinkwater, 2002; Johannessen et al., 2011; Lilly et al., 2008; Shelton et al., 2006). As a result of the decline, fishing quotas have been regulated to allow stock recovery. Presently, Atlantic cod is classified by the IUCN as a vulnerable species (Sobel, 1996). It should be noted that this assessment has not been updated since the mid-1990s and needs re-evaluation. Some stocks, like that of *skrei*, have shown promising recovery, while others, like that of the Grand Banks off Newfoundland, have not, despite restricted quotas (Lilly et al., 2008; Marteinsdottir et al., 2005; Shelton et al., 2006). In the summer of 2019, a moratorium on cod fishing in the Oslofjord was imposed, not only commercially, but also for private persons, in hopes to preserve what is left of the stock (Norwegian Directorate of Fisheries, 2019).

The continued decline of many tradition cod stocks, together with the increasing market demand for cod, has resulted in a growing interest in the aquaculture of this species. While the basis for modern cod aquaculture was initiated already in the1880s by captain G.M. Dannevig in Arendal, Southern Norway, large scale hatching and raising of cod larvae were first achieved in 1983 (Danielssen et al., 2014; Øiestad et al., 1985). This achievement triggered some commercial aquaculture of cod, but profitability was low. From around 2000, technological advances led the industry into a new wave, increasing production to thousands of tonnes per year (Danielssen et al., 2014; Jørstad et al., 2008). However, as with many other species, cod aquaculture has faced several problems, such as high early life stage mortality, diseases, escapes, and precocious sexual maturity (Hansen et al., 2001; Kjesbu et al., 2006; Rise et al., 2014; Taranger et al., 2010). Gonadal development is energetically a costly process and is highly undesirable in aquaculture as sexual maturation results in both reduced somatic growth and reduced fillet quality. In addition, sexual maturation is associated with immunosuppression, rendering fish more susceptible to disease. Cod spawn spontaneously in captivity even when mated pairs of adults are maintained in isolation (Chambers and Waiwood, 1996), increasing the risk for genetic effects of escapees on wild stocks due to spawning in the net-pens (Jørstad et al., 2008). By combining modern technologies and refining management practices, it is possible that sexual maturation can be controlled. This would be of great benefit, not just to the industry in terms of increased meat harvest, and for the animals themselves in term of better animal welfare, but also for the environment in terms

of less waste product, less genetic pollution, and less resources per kilo of market ready fish. However, presently, the knowledge on the regulatory mechanisms controlling teleost pubertal development is still scarce, and there is need to gain more information. Puberty and the start of gametogenesis is initiated following the activation of the brain-pituitary-gonadal (BPG) axis, the endocrine system that controls and regulates sexual maturation in fish, as well as in other vertebrates (reviewed in Dufour and Rousseau, 2007; Okuzawa, 2002; Schulz and Goos, 1999; Weltzien et al., 2004). The BPG axis is affected by both internal and external factors, so to be able to control teleost reproduction, now and in the future, it is therefore essential to elucidate basic mechanisms, regulators, and influencing factors, including those of anthropogenic origin, such as climate change, pollution and endocrine disruptors, of this axis.

1.3 The brain – pituitary – gonadal (BPG) axis

The aim of sexual reproduction is to transfer recombined maternal and paternal genetic information to the next generation. Successful reproduction is dependent on mature adults being both healthy and having sufficient energy reserves to undertake the production of viable gametes (eggs and sperm), as well as other energetically demanding processes such as migration, courtship, and in some species parental care. Furthermore, to provide offspring with the best chance for survival, optimal timing of reproduction must be ensured. The endocrine connections between the brain, pituitary and the paired gonads constitute the axis that integrates physiological and environmental signals and controls sexual maturation in vertebrates. From hypothalamic neurons in the brain, gonadotropin-releasing hormone (Gnrh) is released onto cells in the pituitary, where binding of Gnrh to the Gnrh receptors in the gonadotrope membranes initiates intracellular pathways leading to the synthesis and subsequent release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) into the bloodstream. On reaching the gonads, Fsh and Lh bind to their respective receptors, activating gametogenesis and steroidogenesis. The sex steroids follow the blood back to the higher level of the axis, providing feedback of the steroid producing status of the animal, which, in turn, modulates the hormonal output by the brain and pituitary by feedback mechanisms (Figure 1.7).

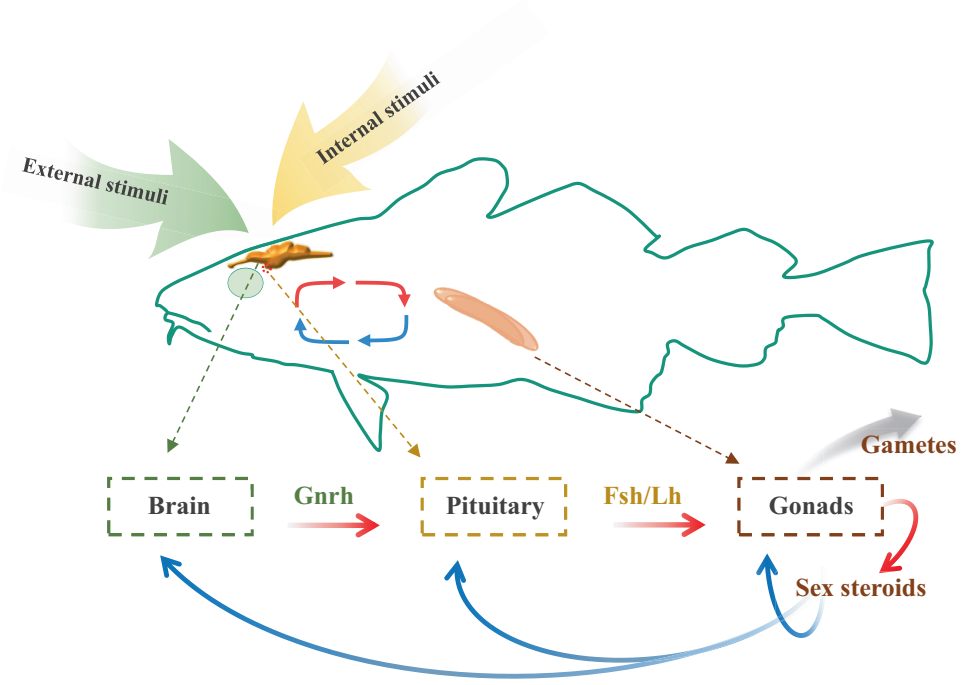

Figure 1.7 The BPG axis in teleost fish, see text for details

1.3.1 Brain

In the teleost brain (Figure 1.8), the main regulatory site for reproductive function is the pre-optic area of the hypothalamus. The brain receives information from external and internal sources, and the hypothalamus integrates this information into neurohormonal output relayed to the pituitary. External factors include photoperiod, temperature, population density, water quality, diet, pheromones, social clues and behaviour (Amano et al., 1999; Choi et al., 2010; Hildahl et al., 2013; Karlsen et al., 2006; Kovalev and Yaragina, 2009; Sorensen and Stacey, 2004; Taranger et al., 2010; White et al., 2002). Internal factors include numerous neurotransmitters such as kisspeptins, dopamine, serotonin, glutamate, γ-aminobutyric acid (GABA), neuropeptide Y (NPY), pituitary adenylate cyclase activating polypeptide (PACAP) and norepinephrine (NE), as well as hormones from the circulation, such as cortisol (F) and gonadal steroids (reviewed in Consten et al., 2001; Dufour et al., 2020; Gopurappilly et al., 2013; Levavi-Sivan et al., 2010; Peter and Yu, 1997; Taranger et al., 2010; Trudeau, 1997; Trudeau et al., 2000; Zohar et al., 2010). Because reproduction is an energy demanding process, input from the body regarding energy stores, through signals

such as insulin, growth factors, leptin and ghrelin, may also affect the hormonal output from the brain (Dufour and Rousseau, 2007; Levavi-Sivan et al., 2010; Taranger et al., 2010). Eventually, the integrated output is conveyed from the hypothalamus to the gonadotropes in form of neurohormones such as Gnrh, dopamine and gonadotropin inhibitory hormone (Gnih). The former is considered the most important hypothalamic factor and stimulates the release of the gonadotropins (Fsh and Lh; see chapter 1.3.2). Several isoforms of Gnrh exist, and at least 15 invertebrate and 15 vertebrate variants have been identified to date (Roch et al., 2011). These decapeptides generally branch into three paralogue groups; GnRH1, GnRH2 and GnRH3, with two or three forms usually expressed in most vertebrates (Dufour et al., 2020). In amphioxus (lancelets), a fourth paralogue, GnRH4, is found. However, GnRH4 appears exclusive to these genera (Roch et al., 2014). In teleosts, there are at least eight Gnrh variants (Kah et al., 2007), and in Atlantic cod, three have been described, with Gnrh1 likely being a pseudogene and Gnrh3 the probable hypophysiotropic form (Hildahl et al., 2011a).

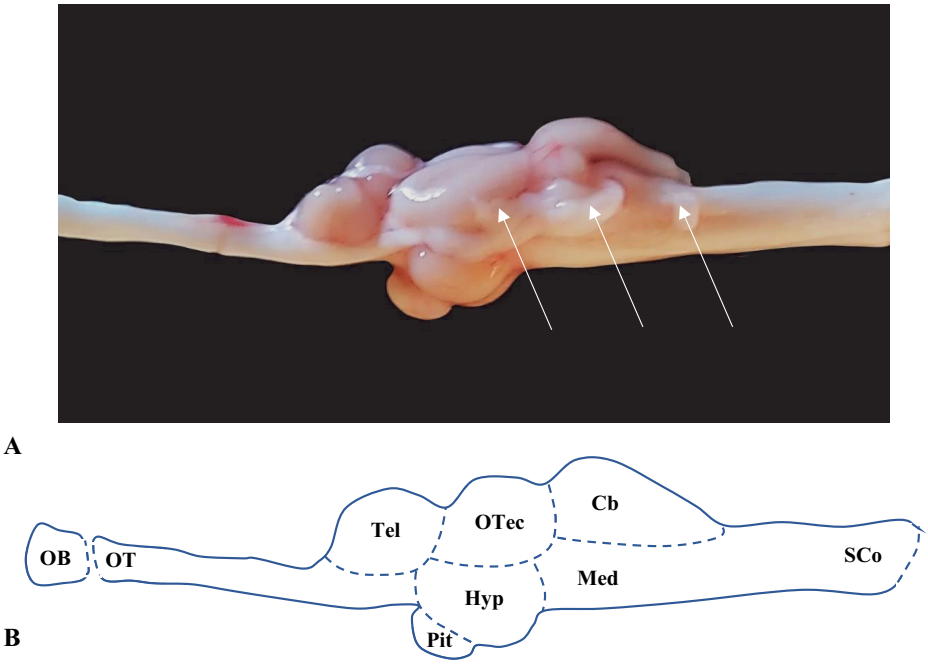

Figure 1.8 The teleost brain with attached pituitary. **A**) Dissected brain from Atlantic cod (*Gadus morhua*). The olfactory tract and spinal cord are cut and not shown in their entirety. White arrows point to cut nerves. **B**) Schematic sagittal view of the brain, with olfactory bulbs indicated. **OB**; olfactory bulb, **OT**; olfactory tract, **Tel**; telencephalon, **OTec**; optic tectum, **Cb**; cerebellum, **Hyp**; hypothalamus, **Med**; medulla, **Pit**; pituitary, **SCo**; Spinal cord. Photo: ©Kristine von Krogh, Weltzienlab, NMBU.

Dopamine is an inhibitory agent and works through depressing both Gnrh and gonadotropin release (Dufour et al., 2010; Zohar et al., 2010). The role of dopaminergic inhibition varies between species, leading to intense blocking in some species but with no effect in others (Dufour et al., 2010). Two decades ago, GnIH was discovered in the brain of the Japanese quail (*Coturnix japonica*) (Tsutsui et al., 2000) and has since been described also in several fish species (Amano et al., 2006; Sawada et al., 2002). Although still poorly investigated, and despite its name, Gnih has been shown to exert both a stimulatory and inhibitory effect on teleost gonadotrope activity, depending on factors such as season, species and mode of administration (Moussavi et al., 2013; Muñoz-Cueto et al., 2017). To my knowledge, there is no available information on the role of either dopamine or Gnih in the regulation of Atlantic cod reproduction.

1.3.2 Pituitary

The pituitary is often referred to as the "master gland" of the body. This title is earned through its involvement in almost every endocrine process, from growth and reproduction to metabolism and stress. In all vertebrates, the pituitary lies in a bony hollow (the *sella turcica*) immediately ventral to the forebrain and is anatomically connected to the hypothalamus through a short neural stalk, the infundibulum (de Beer, 1924; Schreibman et al., 1973). The pituitary itself is divided into the neurohypophysis and the adenohypophysis, also known as the posterior and anterior pituitary, respectively. The term "hypophysis" comes from Greek, meaning "lying under", referring to its position relative to the brain. The neurohypophysis originates from a neuroectodermal down-growth of the diencephalon and contains the *pars nervosa* (PN), consisting mainly of nerve terminals from the pre-optic hypothalamic area as well as glia-like supporting cells (pituicytes) (Pogoda and Hammerschmidt, 2007; Weltzien et al., 2014). In teleosts, the PN secretes vasotocin and isotocin, homologues to the mammalian vasopressin and oxytocin (Feng and Bass, 2017). In most mammals and birds, the neurohypophysis is located posterior to the adenohypophysis, whereas in teleosts, its it normally dorsally situated (Pogoda and Hammerschmidt, 2007). The adenohypophysis is embryologically derived from an ectodermal up-growth of the anterior roof of the oral cavity, known as Rathke's pouch, and contains several types of endocrine cells; gonadotropes, lactotropes, somatotropes, thyrotropes, corticotropes and melanotropes (see Figure 1.9) (de Beer, 1924; Pogoda and Hammerschmidt, 2007; Schreibman et al., 1973; Weltzien et al.,

2004), as well as non-secretory follicular and stellate cells (Golan et al., 2016). The endocrine cells produce gonadotropins (Gths, i.e. Fsh and Lh), prolactin (Prl), growth hormone (Gh), thyroid-stimulating hormone (Tsh), adrenocorticotropic hormone (Acth) and melanocyte stimulating hormone (Msh), respectively. Fish possess an additional type of pituitary endocrine cell, the somatolactotrope, which produces somatolactin (Sl), a hormone related to Prl and Gh, first isolated from the Atlantic cod pituitary (Kaneko, 1996; Rand-Weaver et al., 1991). Furthermore, it was recently demonstrated that Atlantic salmon possesses two distinct thyrotrope cell populations, each expressing different paralogues of *tshb* (Fleming et al., 2019). While tetrapods also may display two thyrotrope subpopulations, they express a single *TSH* gene. Additionally, whereas pituitary Gth in mammals are generally produced by one cell, teleost have two separate gonadotrope cells producing either Fsh or Lh (Kanda et al., 2011; Weltzien et al., 2014). However, in some teleost species, the gonadotropes can be bi-hormonal and even change phenotype (Fontaine et al., 2020). The adenohypophysis can be divided into the anteriorly situated *pars distalis* (PD), subdivided into the *rostral pars distalis* (RPD) and the *proximal pars distalis* (PPD), and the posteriorly situated *pars intermedia* (PI) (Weltzien et al., 2004). Unlike mammals, there is no strict morphological separation between the PD and the PI in fish (Pogoda and Hammerschmidt, 2007). The distribution of endocrine cells also differs between the classes. While the tetrapod pituitary cells are arranged in a mosaic pattern, teleost cells are separated into more discrete populations, similar to the embryonic compartmental organization (Figure 1.9) (Pogoda and Hammerschmidt, 2007; Weltzien et al., 2004). Lactotropes and corticotropes are located in the RPD, somatotropes, gonadotropes and thyrotropes in the PPD and somatolactotropes and melanotropes in the PI. In some teleosts, a band of Lh-producing gonadotropes can additionally be found at the periphery of the PI (Weltzien et al., 2003). A final difference between the mammalian and teleost pituitary is the anatomical connection between the hypothalamus and the adenohypophysis. In mammals, hypothalamic neurohormones are delivered to the endocrine cells through secretion at the median eminence and subsequent transport through the hypothalamo-hypophyseal portal system (Vázquez-Borrego et al., 2018). Teleosts lack such a portal system, and instead neurosecretory fibres of the hypothalamus project through the infundibulum and the PN into the adenohypophysis and either directly innervate the endocrine cells or secrete their products into the vascular spaces surrounding these cells (Ball, 1981; Hodne et al., 2019; Kah et al., 1983; Weltzien et al., 2004; Zohar et al., 2010).

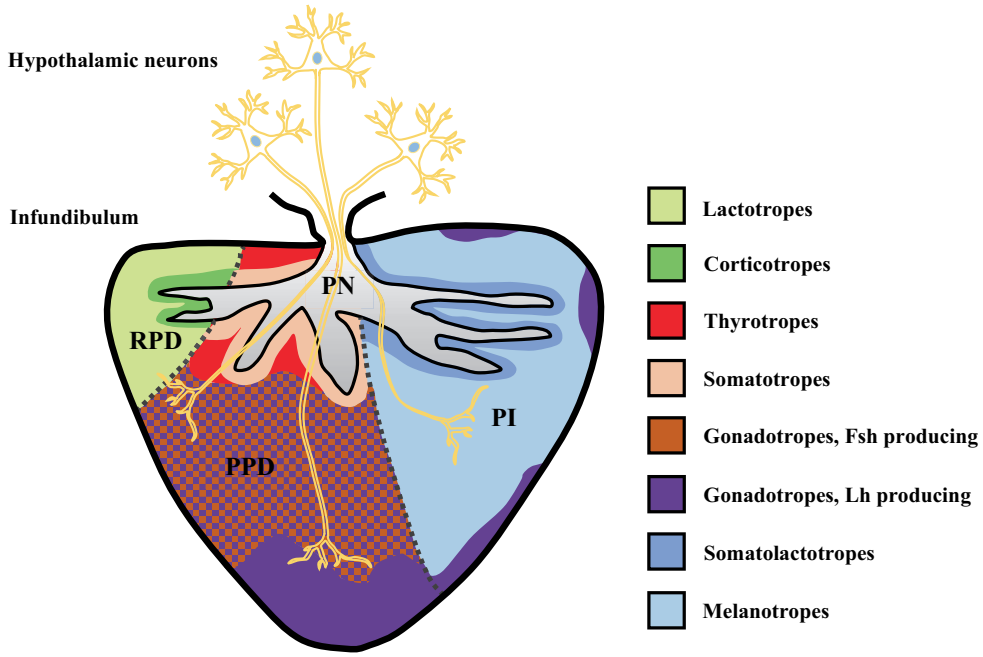

Figure 1.9 Organization of the teleost pituitary represented here by a schematic sagittal section through the Atlantic halibut (*Hippoglossus hippoglossus*) pituitary. The different compartments, cell types and the hypothalamic innervation are indicated. **RPD**; *rostral pars distalis,* **PPD**; *proximal pars distalis,* **PI**; *pars intermedia,* **PN**; *pars nervosa.* Adapted from Weltzien et al. (2003).

1.3.2.1 Gnrh receptors

Hypothalamic Gnrh exert its action by binding to Gnrh receptors (Gnrhrs) in the gonadotrope membranes, initiating intracellular pathways leading to the subsequent synthesis and release of gonadotropin (Yaron et al., 2003). Gnrhrs belong to the transmembrane rhodopsin β sub-family of G-protein coupled receptors, which upon binding of Gnrh activate G-proteins $Gq_{/11}$ or Gs. Activation of $Gq_{/11}$stimulates phospholipase C (PLC), generating inositoltriphosphate (IP_3), which release intracellular Ca^{2+}, and diacylglycerol (DAG). Ca^{2+} and DAG activate protein kinase C (PKC), increasing Gth synthesis. Ca^{2+} can also bind to calmodulin, increasing Gth release. Activation of Gs stimulates adenylyl cyclase, which elevates intracellular cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA) (reviewed in Levavi-Sivan and Avitan, 2005; Yaron et al., 2003). Gnrhrs exist in several isoforms which may be differentially expressed dependent on life stage, season, time of day, sex and species (Fontaine et al., 2020; Hildahl et al., 2013;

Lethimonier et al., 2004). The expression level may also be affected by other factors, such as Gnrh, dopamine, sex steroids and social status (Fontaine et al., 2020; Gopurappilly et al., 2013). Mammals normally express one or two isoforms (Hapgood et al., 2005), whereas teleosts, because of the 3R and 4R events described above, generally express multiple Gnrhr isoforms (Gopurappilly et al., 2013; Hildahl et al., 2011b; Sefideh et al., 2014). For instance, six *gnrhr* paralogues were recently identified in the Atlantic salmon genome, five of which were expressed in the pituitary (Ciani et al., 2020). While fish have two major groups of Gnrhr, each group containing several paralogues, there is currently no clear consensus regarding the nomenclature and classification system for the Gnrhr variants (Flanagan et al., 2007; Hildahl et al., 2011b; Ikemoto and Park, 2005; Lethimonier et al., 2004; Levavi-Sivan and Avitan, 2005; Sefideh et al., 2014). However, in this thesis, the nomenclature suggested by Hildahl and co-workers is used (2011b). In Atlantic cod, four gene variants of Gnrhr have been described, three of which are expressed in the pituitary; *gnrhr1b*, *gnrhr2a* and *gnrhr2c* (Hildahl et al., 2011b). Following the gene expression of these variants through the reproductive cycle, it was discovered that the pituitary transcript level of both *gnrhr1b* and *gnrhr2a* increased during sexual maturation, while *gnrhr2c* expression remained steady without seasonal variation. The relative expression of *gnrhr2a* was about a hundred-fold higher, and more closely correlated to the gonadal development, than that of *gnrhr1b*, indicating that Gnrhr2a is the likely mediator of the Gnrh response in cod (Hildahl et al., 2011b).

1.3.2.2. Gonadotropin and gonadotropin receptors

In all vertebrates, pituitary Gths (Fsh and Lh) stimulate gonadal growth, development and steroidogenesis. Together with Tsh and the placentally derived chorionic gonadotropin (CG), Fsh and Lh constitute a family of structurally related heterodimeric glycoproteins. The heterodimers consist of an α-subunit common to all four hormones, and a β-subunit specific for each hormone (Pierce and Parsons, 1981). Purification of both FSH and LH from mammals was achieved during the late 50's (Ellis, 1958; Squire and Li, 1959), and in most other vertebrate classes within the next two decades (Hartree and Cunningham, 1969; Licht and Papkoff, 1974; Papkoff et al., 1976). In teleosts, however, it was long believed that only a single subtype of gonadotropin existed, until a second subtype was discovered in chum salmon (*Oncorhynchus keta*) in the late 80's (Suzuki et al., 1988a; Suzuki et al., 1988b; Suzuki et al., 1988c). Initially named GthI and GthII, due to their uncertain homology to

mammalian gonadotropins, the formal change to Fsh and Lh, respectively, was decided during the 6th ISRPF in Bergen in 1999 (Jalabert, 2008).

The Gths are synthesized in the gonadotrope endoplasmic reticulum and transported through the Golgi apparatus before being stored in secretory vesicles. These vesicles are emptied through exocytosis into the extracellular space, from which the hormones diffuse into the blood circulation. The rate of secretion is regulated for Lh vesicles but can be both constitutive and regulated for Fsh vesicles (Takahashi et al., 2016). In addition to the hypothalamic signals described above, other neuropeptides are suggested to be directly involved in the Gth release, such as neurokinin B, glutamate, NPY and GABA (Biran et al., 2014; Trudeau et al., 2000; Zohar et al., 2010). In mammals, kisspeptin also stimulates direct secretion of LH (Gutiérrez-Pascual et al., 2007). Recent work suggests that this is also the case in teleost (reviewed in Gopurappilly et al., 2013; Somoza et al., 2020). Finally, hypophyseal paracrine factors, and interrenal and gonadal hormones, such as activin, follistatin, cortisol (F), and sex steroids, respectively, can regulate gonadotrope activity (Aroua et al., 2012; Levavi-Sivan et al., 2010; Yaron et al., 2003). The effects from F and sex steroids will be discussed in more detail in chapter 1.4.

Reaching the gonads, the Gths bind to Gth receptors in membranes of gonadal somatic cells. The Fsh receptor (Fshr) and the Lh receptor (Lhr), like the Gnrhrs, belong to the superfamily of transmembrane G-protein coupled receptors and activate the Gs/cAMP/PKA pathway (Hirai et al., 2002; Levavi-Sivan et al., 2010; Ogiwara et al., 2013). While mammalian FSH and LH interact with their respective receptors in a specific manner, and with little overlap in biological function (Moyle et al., 1994), the teleost Gth receptors are more promiscuous. In some species, both Gths can activate both receptors, while in others, either one or both receptors are ligand specific, with transactivation only occurring at supraphysiological levels (Burow et al., 2020; Levavi-Sivan et al., 2010; Li and Cheng, 2018; Schulz et al., 2010). In mammals, the FSHR is located on Sertoli and granulosa cells in the testis and ovary, respectively, and the LHR located on Leydig cells in the testis and both theca and granulosa cells of the ovary (Themmen and Huhtaniemi, 2000). In fish, it seems that both Gth receptors can be found in theca, granulosa, Sertoli and Leydig cells, but that the expression pattern might vary according to species and of stage of sexual maturity (Devlin and Nagahama, 2002; García-López et al., 2009; Garcia-Lopez et al., 2010; Ogiwara et al., 2013). For instance, in vitellogenic coho salmon (*Oncorhynchus kisutch*) follicles, Fshr was found in both theca and granulosa cells, whereas in the preovulatory follicle, Fshr was only

found in theca cells. In contrast, Lhr was detected only in the granulosa layer of the preovulatory follicle (Miwa et al., 1994).

The Gths are differentially secreted into the circulation during the reproductive cycle, suggesting that they play different roles regarding gonadal development also in teleosts. In general, Fsh is involved in steroid production and early gametogenesis (Devlin and Nagahama, 2002; Levavi-Sivan et al., 2010; Swanson et al., 2003). In females, Fsh recruits oocytes into vitellogenesis and is involved in vitellogenin uptake (Tyler et al., 1997; Tyler et al., 1991), while in males, Fsh stimulates spermatogonial and Sertoli cell proliferation (Schulz et al., 2015). The main role of Lh is in steroid production in the later stages of gametogenesis, leading to spermiation in males and ovulation in females (Devlin and Nagahama, 2002; Levavi-Sivan et al., 2010; Swanson et al., 2003).

Because of the variety in reproductive strategies and gonadal development profiles in teleosts, different species may require differential Gth regulation and steroid circulation profiles. Some species spawn daily (e.g. captive medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*)), some have an annual reproductive cycle, releasing one or several batches of gametes through the reproductive season (e.g. rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod), while others spawn once in their lifetime (e.g. chum salmon and European eel). Gonadal development can be roughly divided into three groups; synchronous, group-synchronous and asynchronous development (Nagahama, 1983). Generally, species that spawn once in a lifetime (semelparity/total spawners) have synchronously developing gonads, those that spawn once a year (iteroparity/single-batch or multi-batch spawners) have group-synchronous development, with at least two gamete populations at different developmental stages, and those that spawn daily have asynchronously developing gonads. In single-spawning species, Fsh levels generally increase during early gonadal maturation, while Lh levels peak during final maturation and ovulation/spermiation (Levavi-Sivan et al., 2010). In daily and multi-batch spawning species, the gonads have batches of gametes undergoing gametogenesis while simultaneously ovulating/spermiating (see Figure 1.10), which might necessitate a more complex regulation and interaction of Lh and Fsh. For instance, a study measuring plasma Gth between spawning rounds in the multi-batch spawning female Nile tilapia (*Oreochromis niloticus*), found that Fsh levels peaked both during vitellogenesis and prior to spawning, the latter peak probably to recruit the next generation of follicles, whereas Lh levels were relatively high during vitellogenesis in addition to peaking before spawning (Aizen et al., 2007). The high Lh levels during early phases indicate that also Lh may be involved in vitellogenesis in this species. To my knowledge, plasma Gth levels have not been

determined through the reproductive cycle of Atlantic cod, also a multi-batch spawner. However, pituitary Gth gene transcripts levels from female cod indicate an intermediate pattern, with both *fshb* and *lhb* being expressed throughout the reproduction cycle, increasing in correlation with the gonadosomatic index (GSI), but with a distinct additional peak in *fshb* expression during vitellogenesis (Mittelholzer et al., 2009). In males, gonadotropin expression followed a more similar pattern as that described above for single spawners (Almeida et al., 2011). Although gene transcript levels may be an indicator of protein synthesis levels, it says little of protein release, and future studies are needed to elucidate this relationship in cod.

1.3.3 Gonads

The gonads are paired organs located in the dorsal part of the body cavity, producing sex steroids and functional gametes. In females, the cellular reproductive unit for sexual reproduction is the ovum, produced in the ovary, whereas in males, it is the spermatozoon, produced in the testis. In most teleosts, the ovary is a hollow sac-like organ, connected posteriorly by the oviduct to the genital papilla. The ovary is compartmentalised by several extending ovigerous lamellae lined by germinal epithelium (Jalabert, 2005; Kagawa, 2013). The testis is an elongated organ consisting of thin-walled tubules or lobules housing the germinal epithelium, the lumen of which is connected to the main sperm duct, and an interstitial compartment containing the somatic cells (Billard et al., 1982; Grier, 1981). Somatic cells of both sexes comprise steroidogenic, nurturing and supportive cells. Steroid hormones are cholesterol-derived molecules, produced in the mitochondria of steroidogenic cells. There, cholesterol is processed into pregnenolone, which in turn, through a series of enzymatic reactions catalysed mainly by cytochrome p450 enzymes, can be transformed into all the other steroid hormones (reviewed in Rajakumar and Senthilkumaran, 2020; Tokarz et al., 2015; Young et al., 2005). Gonadal steroidogenesis occurs in the Leydig cells of the testis, and both granulosa and theca cells of the ovary (Devlin and Nagahama, 2002). Stimulated by the Gths, these cells produce sex steroids such as 17β-estradiol (E2), testosterone (T), dihydrotestosterone (DHT), progesterone (P4), 11-ketotestosterone (11-KT), and maturation inducing steroids (MISs), such as 17α,20β-dihydroxy-4-pregnen-3-one (DPH) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (Asahina et al., 1985; Margiotta-Casaluci et al., 2013; Margiotta-Casaluci and Sumpter, 2011; Nagahama, 1997; Tokarz et al., 2015). The granulosa cells are not strictly steroidogenic but receive precursors from the theca

cells that they process and release. For instance, T released from theca cells is aromatized to E2 in the granulosa cells, a process regulated through maturity-dependent levels of Cyp19a (aromatase) (Kagawa, 2013; Young et al., 2005). Steroids are lipophilic and therefore not stored in vesicles, as the Gths are, but released by diffusion upon synthesis, whereupon they bind to nuclear and membrane receptors in target cells (Young et al., 2005). The gonadal steroids are differentially produced through the reproductive cycle and besides their involvement in gametogenesis they control and regulate a number of associated reproductive processes including reproductive behaviour and the development of secondary sexual characters, as well as important steroid feedback to the higher levels of the BPG-axis (see section 1.4.1).

Gametogenesis, i.e. oogenesis and spermatogenesis, is the process in which germ stem cells develop into functional reproductive units. These processes are regulated by Gth and consist of germ cell proliferation and differentiation, as well as meiosis, turning the cells from diploid to haploid units. In females, oogonia proliferate mitotically until they enter meiosis. Meiosis proceeds until it is arrested at the first diplotene stage, resulting in a primary oocyte. Unlike mammals, that are born with a fixed number of primary oocytes, teleost oogonia can keep on proliferating in adults, generating new stocks of young oogonia and early follicles (Jalabert, 2005; Kagawa, 2013). During the previtellogenic phase of oogenesis, the oocyte grows, forms a chorion, and becomes surrounded by two follicular layers; the inner granulosa cell layer and outer theca cell layer. This phase is suggested to be under androgen control (Kortner et al., 2009; Kortner et al., 2008). In the following vitellogenic phase, oocytes undergo dramatic growth, primarily as a result of sequestration of the yolk protein precursor vitellogenin, which is produced mainly in the liver following Fsh-stimulated E2 release from the follicular layers, that will later serve as nutrition for the developing embryo. The post-vitellogenic phase is initiated by Lh-stimulated MIS release and leads to final oocyte maturation. This step includes the oocyte re-entering meiosis, completing the first miotic division and extruding the first polar body, before meiosis is re-arrested in metaphase II. Final maturation also includes hydration and ooplasm clearance (Iwamatsu et al., 1988; Kagawa, 2013; Parenti and Grier, 2004) (for oogonia developmental stages, see Figure 1.10). After ovulation, where the oocyte is extruded from the follicle layers and released, and fertilization, the oocyte finishes the last stages of meiosis, extruding the second polar body. Another Gth, hCG, though not produced in fish, is structurally so similar to Lh that it is able to induce teleost ovulation (and spermiation), which is a useful tool for the aqua-industry (Goo et al., 2015; Mollah and Tan, 1983).

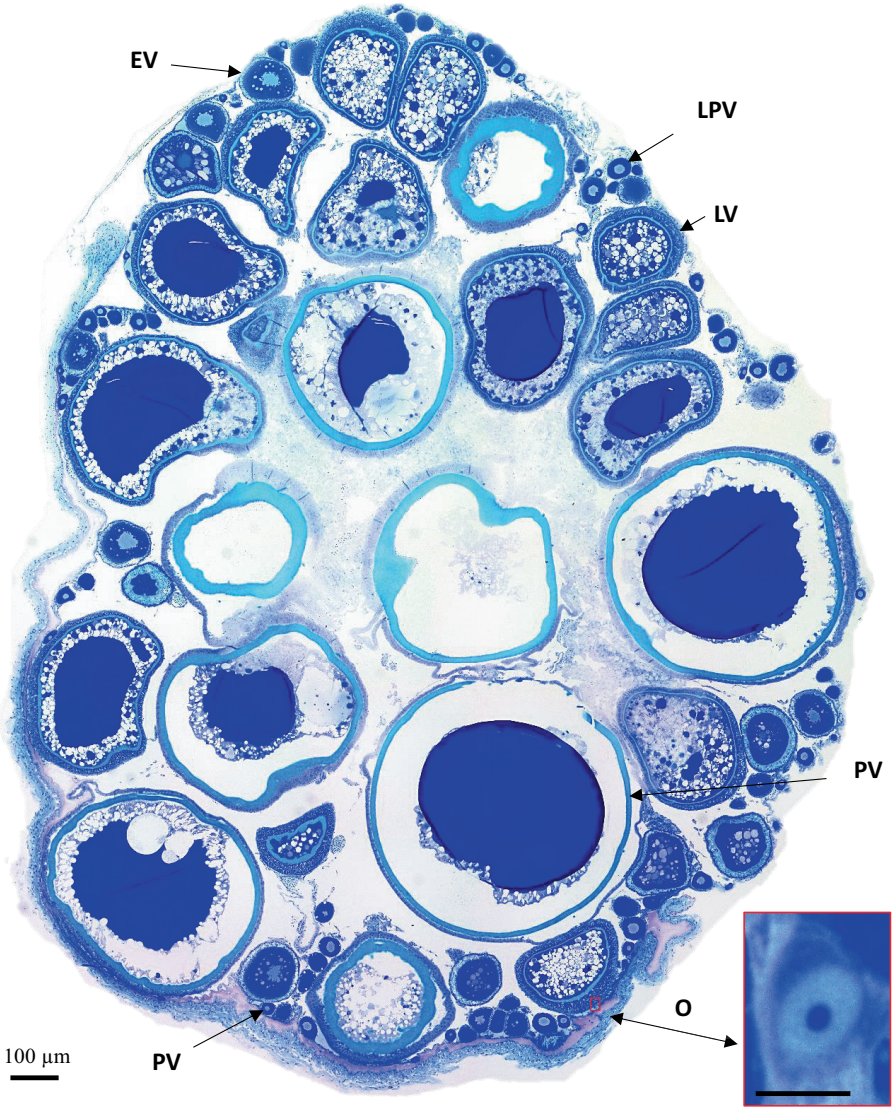

Figure 1.10 Sagittal section through medaka (*Oryzias latipes*) ovary. Medaka is an asynchronous spawner with simultaneous presence of all phases of oocyte development; oogonium (**O**), early previtellogenic follicle (**EPV**), late previtellogenic follicle (**LPV**), early vitellogenic follicle (**EV**), late vitellogenic follicle (**LV**) and postvitellogenic follicle (**PV**). Stages are based on Iwamatsu et al. (1988). The inset shows an enlargement of the red square containing an oogonium. Scale bar of the inset is 10 µm. The histological section is 3 µm thick and stained with Toluidine Blue O. Photo: ©Kristine von Krogh, Weltzienlab, NMBU

In males, the germinal epithelium of the testis contains spermatogonial stem cells, the undifferentiated spermatogonia type A (SPA_{und}), that self-renew through E2 stimulation (Miura et al., 1999). The spermatogenic process is initiated and maintained by androgens,

especially 11-KT, the main teleost androgen. Both Fsh and Lh can stimulate Leydig cell androgen release, which in turn stimulate Sertoli cell activity (Schulz and Miura, 2002; Young et al., 2005). During spermatogenesis, the germ cells are assisted by Sertoli cells which are co-located with the germ cells inside the seminiferous tubules (or lobules) and produce growth factors and provide structural support. Different from mammals, where the Sertoli cells support different stages of developing spermatogonial cells, the teleost Sertoli cells envelop the single SPA_{und} through cytoplasmic extensions, creating a cyst in which the germ cells subsequently develop synchronously (Schulz et al., 2015). The first resulting cells after such a division are the differentiated spermatogonia type A (SPA_{diff}), now connected by a cytosolic bridge, which enables synchronous development (Schulz et al., 2010). The Sertoli cells themselves cells proliferate along with the germ cells, adjusting to the growing volume of the developing cyst (Morais et al., 2013). Fsh stimulates Sertoli cell proliferation and release of growth factors, such as insulin-like growth factor 3 (Igf-3), that promotes spermatogenesis, while simultaneously supressing Sertoli cell release of inhibiting substances, such as anti-Müllerian hormone (Amh) (Morais et al., 2017; Nóbrega et al., 2015; Skaar et al., 2011). Through mitotic divisions, SPA_{diff}'s generate spermatogonia type B (SPB) cells, that will continue dividing mitotically for a few generation, the exact number being species specific (Schulz et al., 2010). Entering meiosis, the SPB give rise to primary spermatocytes. The first meiotic division gives rise to secondary spermatocytes, that immediately enters the second mitotic division, giving rise to haploid spermatids. These will no longer divide but differentiate into spermatozoa (spermiogenesis), after which the cyst opens to release sperm into the tubular lumen (spermiation) (for spermatogenic stages, see Figure 1.11). Spermiation is controlled by 11-KT in some species, whereas MIS is the main regulating factor in others (Schulz et al., 2010).

In Atlantic cod, the fluctuation of sex steroid plasma concentration through the reproduction cycle have been reported in several studies (Almeida et al., 2009; Dahle et al., 2003; Norberg et al., 2004). In females, the E2 plasma profile closely follows the GSI, peaking in spawning season, while T levels are stable through the season, except for a drop in the post-spawning stage. Vitellogenesis starts months before spawning and commences normally between September and November (Kjesbu, 1991; Skjæraasen et al., 2006). In males, T and 11-KT production increase until spawning season before dropping to almost zero afterwards (Almeida et al., 2009; Norberg et al., 2004). Spermatogonial proliferation starts around August, while meiosis is first observed from October (Almeida et al., 2008). For a gross description of cod gonadal development, please see chapter 2.1.1.

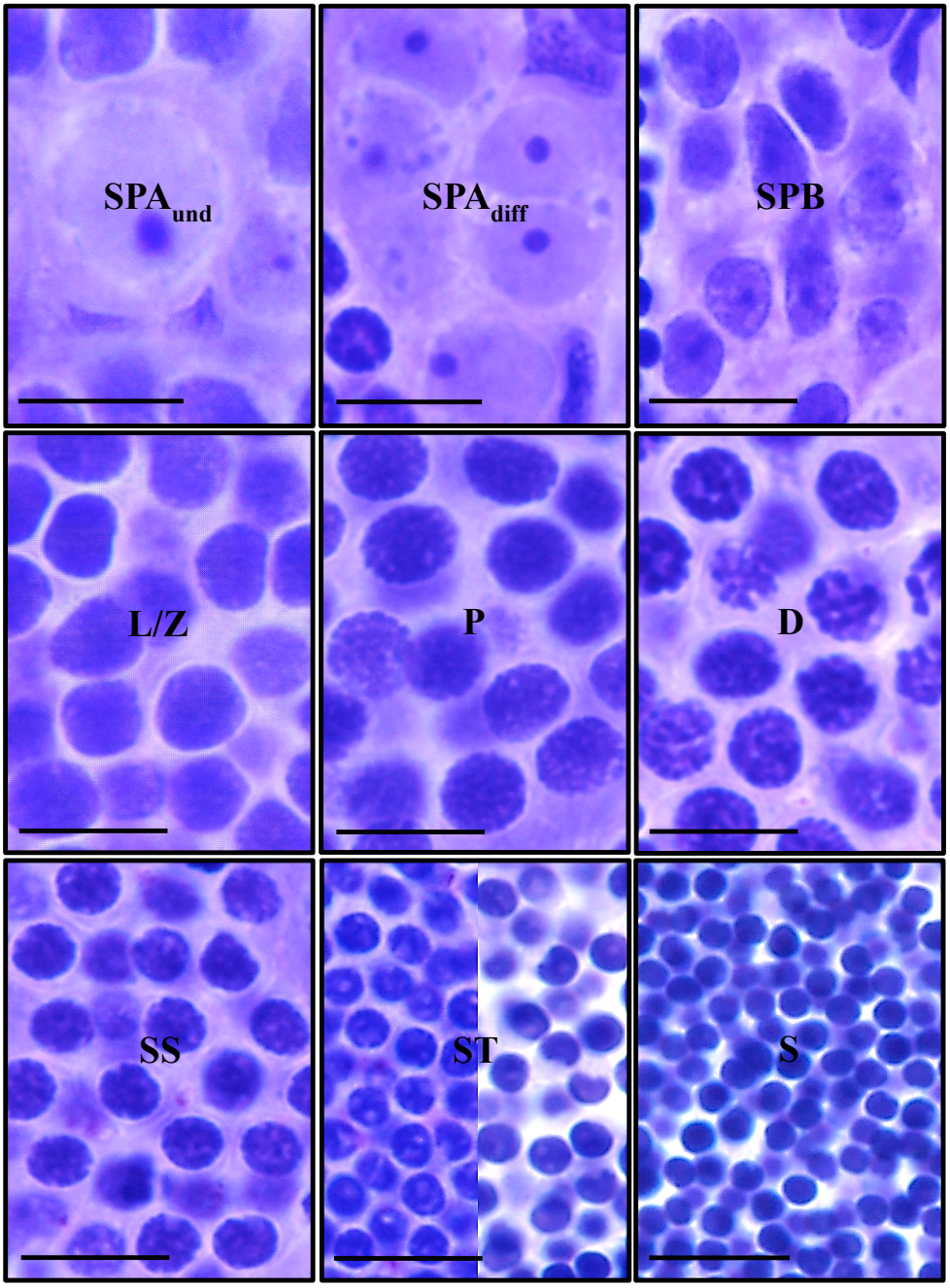

Figure 1.11 Histological sections of the spermatogenic cell stages in zebrafish (*Danio rerio*); undifferentiated spermatogonia A (**SPA$_{und}$**), differentiated spermatogonia A (**SPA$_{diff}$**), spermatogonia B (**SPB**), primary spermatocytes leptotene/zygotene (**L/Z**), pachytene (**P**) and diplotene (**D**), secondary spermatocytes (**SS**), spermatids (**ST**) and spermatozoa (**S**). Stages are based on the description by Leal et al. 2009. All bars are 10 µm. Sections are 3 µm thick and stained with Toluidine Blue O.
Photos: © Kristine von Krogh, Weltzienlab, NMBU

1.4 Regulation of the BPG axis

As described above, numerous factors can modulate BPG-axis output. The following section will focus on three subcategories of influencing factors most relevant to this thesis; sex steroid feedback, stress and endocrine disrupting chemicals (EDCs).

1.4.1 Sex steroid feedback

In all vertebrates, including teleosts, gonadal estrogens, androgens, and progesterons can exert positive or negative feedback on the higher levels of the BPG-axis, affecting their activity. There is also evidence for short-loop feedback onto the gonads themselves (Young et al., 2005). For instance, in the African catfish (*Clarias gariepinus*), T inhibits Leydig cell Cyp17 (cytochrome p450 enzyme) activity, thereby supressing androgen production (Cavaco et al., 1999). In the brain, hypothalamic neurons may express steroid receptors and thus be sensitive to steroid feedback, which can lead to changes such as altered gene expression or hormonal output (Alvarado et al., 2016; Amano et al., 1994; Vacher et al., 2002). This has for instance been demonstrated in both goldfish (*Carassius auratus*) and medaka, where double *in situ* hybridisation staining demonstrated the presence of estrogen receptors (Ers) in Kiss neurons, and furthermore, that ovariectomy and/or E2 treatment altered kisspeptin gene expression (Kanda et al., 2012; Mitani et al., 2010). At the pituitary level, modified gene transcription, synthesis and/or release of Gths after steroid replacement in intact or gonadectomized animals have been reported in several teleost species; e.g. rainbow trout (Billard, 1978), African catfish (Cavaco et al., 2001; de Leeuw et al., 1986), coho salmon (Larsen and Swanson, 1997), Atlantic salmon (Borg et al., 1998), striped bass (*Morone saxatalis*) (Holland et al., 1998), Atlantic Croaker (*Micropogonias undulatus*) (Khan et al., 1999), goldfish (Huggard et al., 1996; Kobayashi et al., 2000; Sohn et al., 1998), hybrid striped bass (*Morone saxatalis x chrysops*) (Klenke and Zohar, 2003), Nile tilapia (Levavi-Sivan et al., 2006), red sea bream (*Pagrus major*), and European eel (Aroua et al., 2007). The results reported from these studies can be consequences of both direct, e.g. through estrogen response elements in Gth promotor regions or regulation of dopamine and Gnrh receptor levels, and indirect, i.e. through modulation of hypothalamic neurons, effects on the pituitary (Levavi-Sivan et al., 2006). However, there are some issues using gonadectomized animals. First, not all sex steroids are necessarily removed, as these can be produced in other tissues, such as interrenal cells, adipose tissue and the central nervous system (Diotel et al., 2018;

Rajakumar and Senthilkumaran, 2020). Second, gonadectomy not only removes gonadal steroids, it also removes gonadal peptides, such as activin and follistatin. Both these substances are known to affect Gth output (Aroua et al., 2012). Finally, gonadectomy may cause discomfort and stress for the animal, conditions that are known to affect reproduction (see next subsection). It is therefore possible that the reported results from such studies are not caused by the absence of (gonadal) sex steroids alone. Using cultured organs, where the systemic input is removed, direct effects from treatments can be investigated. Indeed, several pituitary *in vitro* studies have confirmed that gonadal steroids have direct effects on pituitary Gth gene expression and synthesis (Ando et al., 2004; Aroua et al., 2007; Huggard-Nelson et al., 2002; Levavi-Sivan et al., 2006; Lin and Ge, 2009; Melamed et al., 1997; Rebers et al., 2000; Sohn et al., 2001; Tse et al., 2013).

In general, the feedback exerted by sex steroids is positive in immature fish and negative during late stages of gametogenesis. However, in addition to maturational stage, the nature of the feedback may vary due to the specific steroid, steroid concentration, administration, species, sex and other factors (Levavi-Sivan et al., 2010; Okuzawa, 2002; Saligaut et al., 1999), meaning that e.g. Lh and Fsh are not necessarily affected equally by the same hormone within one species at a certain stage. For instance, in sexually maturing female masu salmon (*Oncorhynchus masou*), Lh was upregulated, whereas Fsh was unaffected after 10 ng/ml E2 treatment (Ando et al., 2004). Prior to this thesis, there were, to my knowledge, no available studies on sex steroid feedback in Atlantic cod or other gadoids.

1.4.2 Stress

In both fish and mammals, stress, a state in which there is a real or imagined threat to an organism's homeostasis, can affect reproduction (reviewed in Geraghty and Kaufer, 2015; Milla et al., 2009; Schreck, 2010). Fish are exposed to several stressors during their lifetime, both under farmed and wild conditions. In aquaculture, this could be crowding, handling and diseases, while in the wild, it could be predators, starvation, climate changes or anthropogenic noise, amongst others (Ashley, 2007; Barton and Iwama, 1991; Huntingford et al., 2006; Mickle and Higgs, 2017; Pankhurst and Munday, 2011). The primary physiological stress response involves the activation of the brain-pituitary-interrenal (BPI)-axis, where corticotropin-releasing hormone (Crh) is released from hypothalamic neurons onto corticotropes in the adenohypophysis, stimulating adrenocorticotropic hormone (Acth) release into the circulation, which is turn activates the production and release of cortisol (F),

the main teleost glucocorticoid (GC), from the interrenal cells (Wendelaar Bonga, 1997). F is produced by steroidogenic cells in the interrenal tissue in much the same manner as the gonadal steroids described above and binds to glucocorticoid receptors (Grs) in target tissues upon release. The action of F has been demonstrated to affect all levels of the BPG axis, such as hypothalamic Gnih and Gnrh gene expression (Choi et al., 2017; Goos and Consten, 2002), pituitary Gth gene expression and plasma concentration (Carragher et al., 1989; Choi et al., 2017; Huang et al., 1999) and gonadal steroid production (Mandiki et al., 2017; Reddy et al., 1999). While chronic stress is generally considered negative for reproduction, some studies indicate that F may also have a positive effect during sexual maturation. For instance, a study using organ cultures of Japanese eel (*Anguilla japonica*) testes, found that F administration enhanced 11-KT induced spermatogonial proliferation (Ozaki et al., 2006). As with the gonadal steroids, the reproductive effects of F seem to be dependent on concentration, duration and manner of administration, species, stage of sexual maturity, sex and other factors (Carragher et al., 1989; Leatherland et al., 2010; Milla et al., 2009).

In Atlantic cod, stress has been shown to affect reproductive behaviour, by e.g. fewer and altered courtship sequences, irregular spawning, and abandoning of spawning grounds (Bogevik et al., 2012; Engås et al., 1996; Morgan et al., 1999). Stress is also associated with reduced fecundity, reduced embryo viability and increased number of abnormal larvae in this species (Kjesbu, 1989; Morgan et al., 1999; Sierra-Flores et al., 2015). Furthermore, high levels of F decreased T plasma levels in female cod and altered
gene expression in their eggs and embryos (Kleppe et al., 2013). Effects of stress or F on the higher levels of the BPG axis in cod were, to my knowledge, unstudied prior to this thesis.

1.4.3 Endocrine disruptive substances (EDCs)

The last few decades, there has been growing concern about industrial chemicals released to the environment and their impact on wild-life and human health (Colborn et al., 1993). An increasing number of these man-made chemicals have been found to negatively impact endocrine systems, including those of the BPG and BPI axes, and are collectively known as endocrine disruptive chemicals (EDCs) (Tokarz et al., 2015). There are also natural sources of EDCs, such as phytoestrogens, mycoestrogens and animal hormones (Goksøyr, 2006). EDCs act by either modifying the natural hormone production of an animal or mimicking or blocking natural hormones by binding to their receptors. Aquatic wildlife, including fish, are particularly vulnerable to pollutants as aquatic environments represent the

ultimate sink for most anthropogenic chemicals. Further, early life history stages, such as eggs and larvae, are more vulnerable compared to adults due to their high surface area:volume ratio, and their lack of avoidance behaviour. Finally, as most EDCs are lipophilic and prone to both bioconcentration and biomagnification, wildlife higher up the food chain, including carnivorous fish such as cod, are more vulnerable to EDC exposure. Deleterious effects of EDCs have been widely reported in fish, most noticeably the feminization of males following exposure to estrogenic pollutants (xenoestrogens). In addition to feminization effects in males (induction of vitellogenin and presence of ovotestes), the disruption in other reproductive traits have been reported, including neuroendocrine disruption, altered GC and sex steroid production, thyroid disruption, impaired gametogenesis, reduced fecundity and sperm quality, and impaired reproductive behaviour (Goksøyr, 2006; Jalabert et al., 2000; Jobling and Tyler, 2003; Lyche et al., 2010; Matthiessen, 2003; Page et al., 2011; Scholz and Mayer, 2008; Waye and Trudeau, 2011).

Two suspected EDCs are bisphenol A (BPA), and its halogenated derivative tetrabromobisphenol A (TBBPA) (European Union, 2008; WHO/ICPS, 1995). The former is widely used in the production of polycarbonate plastics and epoxy resins, while the latter is one of the most common flame-retardant compounds used in industry. Both are ubiquitously detected in the environment (Crain et al., 2007; Flint et al., 2012; Gong et al., 2017; Huang et al., 2012; Morris et al., 2004; Yang et al., 2012). While BPA has been demonstrated to act at all levels of the teleost BPG-axis (Chung et al., 2011; Faheem et al., 2019; Faheem et al., 2017; Hatef et al., 2012; Huang et al., 2017; Mandich et al., 2007; Molina et al., 2018; Qin et al., 2013; Rhee et al., 2010; Wang et al., 2019), there is currently little knowledge about TBBPA in this regard. A few available teleost studies indicate that TBBPA exposure can decrease egg production and hatching rate, as well as interfere with the thyroid hormone system (Chan and Chan, 2012; Huang et al., 2017; Kuiper et al., 2007a; Kuiper et al., 2007b).

Atlantic cod has been used a model to assess endocrine modulation from several potential EDCs (Martin-Skilton et al., 2006; Meier et al., 2007; Tollefsen et al., 2011; Yadetie et al., 2018), including BPA (Larsen et al., 2006). The latter study demonstrated that BPA was able to induce male vitellogenin production, a classical biomarker for estrogenic effects in teleosts (Scott et al., 2006). However, potential effects of BPA at the different levels of the BPG axis in this species, are currently unknown. For TBBPA exposure, potential direct effects on the higher levels of the BPG axis have only been assessed in two vertebrate species to date (van der Ven et al., 2008; Zhang et al., 2018a), and was prior to this thesis unstudied in cod and other teleosts.

1.5 Aims of the study

The overall aim of my thesis has been to gain better knowledge of how the Atlantic cod pituitary is regulated regarding reproductive function and how internal and external factors may directly affect this function. In order to explore this topic, I wanted to establish a physiologically relevant pituitary cell culture system, which subsequently could be used to study potential direct effects from different exposures. Furthermore, by collecting, culturing and exposing pituitaries throughout the reproductive cycle, potential significance of donor sexual maturity stage could be investigated.

Based on this, 4 sub-goals were formulated;

i) Develop and validate a physiologically relevant primary pituitary cell culture system for Atlantic cod (**paper I**)

ii) Use the pituitary culture system to investigate potential direct feedback mechanisms from physiologically relevant doses of sex steroids on cell viability and reproductive gene expression. Furthermore, investigate if these effects are dependent on the stage of sexual maturation (**paper II**)

iii) Use the pituitary culture system to investigate potential direct effects from basal and stress levels of cortisol on cell viability and reproductive gene expression. Furthermore, investigate if these effects are dependent on the stage of sexual maturation (**paper III**)

iv) Use the pituitary culture system to investigate potential direct effects from the suspected endocrine disruptive chemicals bisphenol A and tetrabromobisphenol A on cell viability and reproductive gene expression. To assess potential cytotoxic and reprotoxic levels for these chemicals in our system, expose the cells to a wide range of concentrations for each chemical (**paper IV**)

2

Methods

2. Methods

2.1 Animals

From 2010 to 2013, pituitaries from 257 coastal Atlantic cod (*Gadus morhua* L., 0.5 – 4.1 kg) of both sexes were dissected and utilized for a total of 42 primary cultures. The cod were obtained either from Austevoll (western coast of Norway) or the Oslo fjord, both sites at approx. 60° N. Fish from Austevoll were dissected on site and the pituitaries transported in cell media on ice by plane to Oslo, where cultures were immediately prepared. Fish caught in the Oslo fjord were either dissected on site or transported to the aquarium facilities at the University of Oslo, where they were kept for at least one week prior to sacrifice. While in captivity, the cod were fed shrimp daily. Aquaria were supplied with recirculating filtered seawater at a salinity of 28 ‰ and temperature of 7-12 °C. The light cycle was adjusted to fit the natural night/day rhythm in Oslo. A general permission to keep the animals in the facilities was given by the Norwegian animal research authority (S-2008/108215) and all animals were kept and handled in agreement with their requirements. The experiments described in this thesis were performed on pituitaries and not on the animals themselves, and as such, a specific ethical approval for this study was not needed (Norwegian legislation for use of animals in research, Chapter II, §6). Care was nonetheless taken so that stress and suffering of the fish were minimized. We found that covering the eyes of the cod immediately after netting calmed the fish and kept it from moving around, allowing the procedures to be carried out swiftly and precise. The fish were euthanized by rapid decapitation, severing the spinal cord and the dorsal aorta. To expose the pituitaries, the skulls were cut open, the main brain nerves cut, and the brains flipped over (Figure 2.1). Once exposed, the pituitaries were carefully transferred to ice-cold modified cell medium (for modifications, see chapter 2.2) and kept on ice until further culture preparation.

2.1.1 Gonadosomatic index (GSI)

After excising the pituitary, the gonads were removed from the body cavity, and both the body and gonads weighted. This allowed us to calculate the gonadosomatic index (GSI; (gonadal weight/total body weight) × 100). The GSI is a useful indicator of sexual maturity level, but as the weight of an e.g. maturing gonad can be identical to a newly spent one (see Figure 2.2) and the somatic weight decreases during the season by the energy expenditure of

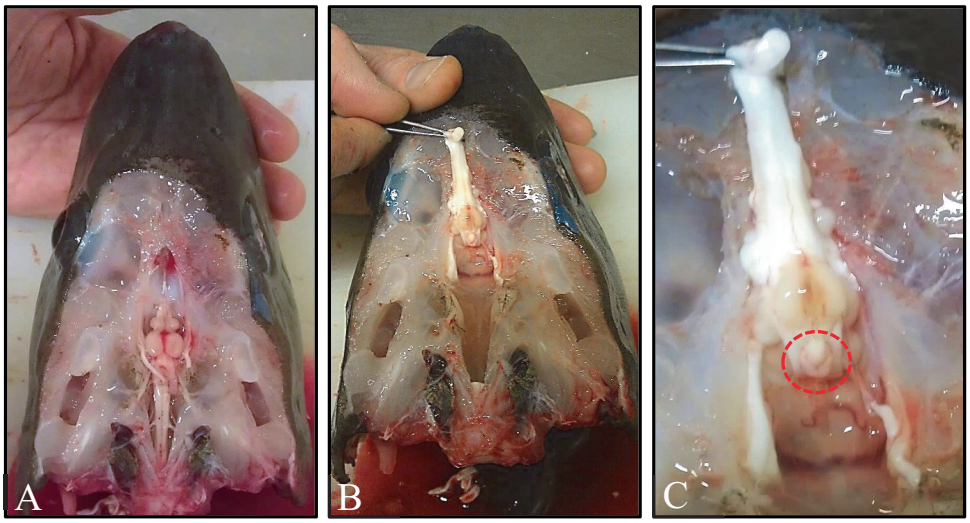

Figure 2.1 Dissection of the cod pituitary.
A) First, the skull roof is removed with a large, sharp knife. B) Second, the nerves are cut, and the brain flipped over to expose the pituitary. C) Enlargement of B, with the pituitary marked by a red circle.

gonadal growth (Karlsen et al., 1995), the GSI by itself is not a precise measurement and should be used in combination with a visual inspection of the gonads.

In cod, the immature ovary has small blood vessels, little tissue texture and transparent or pale orange colour. Note that the colour of the ovary itself is not a sure characteristic, as the ovarian colour may be influenced by diet (Powles, 1958). By making a small incision into the tissue, the status of the oocytes can be examined. In the immature ovary, only small, transparent oocytes are present. Ovaries from recovering specimens that spawned the previous season also contain only small, transparent oocytes and can be difficult to separate from immature specimens based on visual inspection alone. If such a separation is necessary, smaller fish (< 40 cm) are generally immature and recovering ovaries tends to be firm (Kjesbu, 1991; Powles, 1958). During maturation and vitellogenesis, the ovary tissue becomes more dense and pinker in colour, with semi-transparent and opaque oocytes clearly visible. In more mature individuals, less semi-transparent oocytes are present, and the amount of vascular tissue increases. In the fully mature gonad, large, translucent hydrated and/or ovulating oocytes are present and easy to identify. In spawning females, the eggs will freely release by applying a light pressure to the ovary. The newly spent ovary is flaccid, contains no hydrated eggs and have thickened ovarian walls. It may contain unshed eggs undergoing atresia, and the blood vessels are still large.

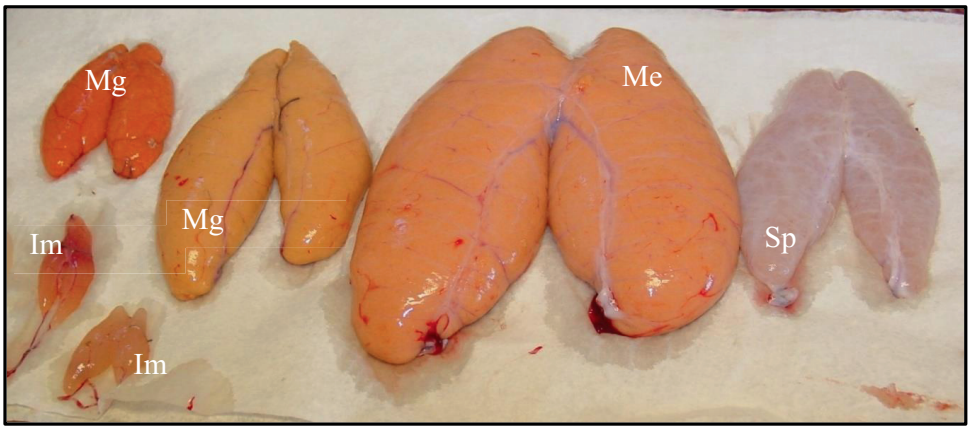

Figure 2.2 Atlantic cod ovaries
The Atlantic cod ovaries can differ quite a lot in size, still belonging to the same gross maturational stage. Similarly, ovaries from different stages can have an approximately equal size.
Im; immature, **Mg**; maturing, **Me**; mature, **Sp**; spent

The spermatogenic parenchyma of the Atlantic cod testis consists of lobes arranged around a central efferent duct (Almeida et al., 2008). Immature testes are slender, with small blood vessels, and small pinkish lobes containing mainly spermatogonia. In the maturing testes, the lobe size increases, still pink in the periphery where the spermatogonial cells are located, but with an off-white area increasing in size towards the sperm duct as the germ cells develop. This off-white area contains mostly spermatocytes and round spermatids. Closest to the sperm duct, a milky white area consisting of elongated spermatids and spermatozoa appears as the germ cells complete spermatogenesis. With further maturation of the testes, the pink zone in the periphery becomes progressively smaller until full maturation is reached, and the lobes are all white and filled with sperm. The GSI can increase >40-fold during this process (Almeida et al., 2008). In spawning individuals, the milt run freely at the touch (Powles, 1958). Spent testes are flaccid, have decreased lobe size with pinker colour, with larger blood vessels still present. Some residual sperm may be present in lobes and sperm duct. For pictures of the gross maturational stages of male and female cod, see **paper II**, supplementary data.

Cultures prepared for testing and development of the culture conditions, i.e. **paper I**, did not separate pituitaries based on sexual maturity stages of the donor fish. For the exposure studies (**papers II-IV**), however, the stage of sexual maturation was a key factor and pituitaries were separated in different cultures based on the identified maturational status of each donor. The division of maturity stages used in our studies was rough; immature, maturing, mature and (newly) spent. For a more nuanced and precise classification of sexual

maturity, i.e. distribution and developmental stages of germ cells (see chapter 1.3.3), histological analysis is required. This was not deemed necessary for our experimental design.

Since the fish themselves were not exposed to test chemicals or experimentally treated in any way, after dissection and weighting, the remains of the fish bodies were washed and utilized for food for the group members.

2.2 Cell cultures

2.2.1 Background

Studying organs *in vitro* can give insight to cell physiology and biochemistry, uncover regulatory mechanisms working directly at the organ level, and serve as a potential screening system for various chemicals. In primary cell cultures, tissues are removed from plants or animals, dissociated and grown in a physiologically ambient environment. Prior to our work, pituitary primary cell cultures had been established for several teleosts, including rainbow trout (Fåhræus-van Ree et al., 1982; Weil et al., 1986), common carp (*Cyprinus carpio*) (Ribeiro and Ahne, 1982; Ribeiro et al., 1983), African catfish (*Clarias gariepinus*) (de Leeuw et al., 1984), goldfish (Chang et al., 1990a; Chang et al., 1990b), tilapia hybrid (*Oreochromis niloticus* × *O. aureus*) (Levavi-Sivan and Yaron, 1992), European eel (Montero et al., 1996), Chinese grass carp (*Ctenopharyngodon idellus*) (Wong et al., 1998), coho salmon (Larsen et al., 1998), Mozambique tilapia (*O. mossambicus*) (Seale et al., 2003), black seabream (*Acanthopagrus schlegeli*) (Chan et al., 2004) and zebrafish (Lin and Ge, 2009). However, fish cell and organ cultures are traditionally based on mammalian protocols, with only minor adjustments to piscine physiology. Similarly, the first study on Atlantic cod pituitary primary cultures had only temperature (12 °C) adjusted to cod physiology (Haug et al., 2007). Realizing that these conditions were not ideal, the first objective was to develop and validate *in vitro* protocols optimal for the culture of Atlantic cod pituitary cells.

2.2.2 Adjusting culture conditions to Atlantic cod physiology

In culture, cells can no longer rely on systemic osmoregulation of the extracellular fluid through the kidneys or the chloride cells of the gills. Cell membranes are generally freely permeable to water and cells will shrink or swell depending on the extracellular fluid being hyper- or hypoosmotic, respectively. As rapid changes in volume can change cell properties, it is important that different solutions used for establishing and maintaining cell

cultures are comparable in osmolality (osmoles of solute per kilogram of water) and that this osmolality is adjusted to the physiology of the species at hand. Marine fish tends to have higher plasma osmolality than freshwater fish and mammals (Schmidt-Nielsen, 1997; Waymouth, 1970). Still, most commercial cell culture solutions are adjusted to mammalian physiology, around 300 milliosmoles (mOsm). For instance, the Leibovitz's L-15 cell medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, Massachusetts, U.S., catalogue no.: 11415049) and phosphate buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc., catalogue no.: 10010023) used in this study, have reported osmolality ranges of 280 - 315 mOsm and 300 - 340 mOsm, respectively. The wide range in osmolality for these common laboratory solutions increases the possibility to induce cellular osmotic stress when using them to culture fish cells. Furthermore, osmotic stress can be induced when switching between different solutions, which emphasises the importance of measuring each batch of new solutions and adjust them to protocol standard. The original protocol for Atlantic cod pituitary primary culture (Haug et al., 2007) used M199 cell culture media (Gibco; Thermo Fisher Scientific, Inc., catalogue no.: 12340030). For M199, the osmolality range was not given in the company product description but was measured in our lab to be approx. 280 mOsm. Atlantic cod has a plasma osmolality of ~340 mOsm, with concentrations negatively correlated to lowered temperature and salinity (Audet et al., 1993; Larsen et al., 1997; Magill and Sayer, 2004; Olsen et al., 2008). Therefore, for the new cod culture protocol, all working solutions, i.e. L -15 cell culture media, PBS and the extracellular solution, were adjusted to approximately 320 mOsm using 1M NaCl, ensuring no more than 5 mOsm difference between solutions. For cell media, osmolality was adjusted after adding glucose and, optionally, serum supplements.

Fish breathe through gills ventilated by large volumes of water. As CO_2 has high solubility in water and passes freely over biological membranes, in fish, metabolic CO_2 is efficiently cleared from the body, leading to low plasma partial pressure of CO_2 (pCO_2) compared with lung breathing animals. Low pCO_2 results in high blood pH (7.7-7.9) and low HCO_3^- concentrations (Claiborne et al., 2002; Marshall and Grosell, 2006). The M199 culture media used in the original cod pituitary culture protocol is supplemented with 26.2 mM HCO_3^-, which, when incubated at 5 % CO_2 atmosphere, gives a pH of approximately 7.4. This is not ideal for fish cell incubation, as a physiological pH is of great importance for the activity of enzymes and the stability of proteins. In addition to pCO2 and buffer capacity, temperature affects pH. Decreasing temperatures increases pH, leading to typical plasma pH of 7.8 at 20 °C and 7.95 at 10 °C (Cameron, 1989; Marshall and Grosell, 2006). Atlantic cod

is a cold-water species, with adults preferring temperatures between 0-12 °C (Sundby, 2000). At 12 °C, Atlantic cod has a plasma pH just shy of 7.9 (Larsen et al., 1997). Based on this, we adjusted the PBS to a pH of 7.85 at 12 °C using 1 M NaOH, and cultured the cells in L-15 media added 10 mM $NaHCO_3$, which, when incubated at a 0.5 % CO_2 atmosphere (pCO_2 of 3.8 mm Hg) at 12 °C, gave a medium pH of 7.85.

2.2.3 Adjusting other protocol parameters

In addition to adjusting incubation conditions to mimic that of cod physiology, we also refined other aspects of the protocol to increase cell yield, viability, stable gene expression and suitability for electrophysiological experiments. At the start of this thesis, most physiological parameters were already established. My work should determine optimal centrifugation speed (revolutions per minute; rpm) and duration during the dissociation procedures, culture well coating and cell seeding density, as well as evaluate potential effects of serum supplements in the culture media.

Both mechanical and chemical dissociation can potentially damage cells leaving them vulnerable to other laboratory procedures. We observed that, when centrifuging cell suspensions at high rpm in order to create a pellet, many cells burst in the process. Low rpm, on the other hand, left many viable cells in the supernatant. Testing a series of different centrifugation speeds, and different durations, we found the best compromise between a good pellet and intact cells to be 1200 rpm for 10 min (at 4 °C). In our centrifuge, the radius of the rotor was approximately 11.5 cm, giving a relative centrifugal force (rcf, or G-force) of around 185 *g*.

Due to suboptimal attachment of the cells, which was especially challenging for electrophysiological experiments, different coatings for culture cell wells and petri dishes were tested. We compared three different scenarios; precoated Corning CellBIND plates/dishes, and Corning Costar plates/dishes coated in the lab with either poly-L-lysine or poly-D-lysine (all plates, dishes and solutions from Sigma-Aldrich, Saint-Louis, MO, USA). The plates were prepared by adding a few drops of the solution to each surface and sterilizing them under UV-light for about 45 min. Prior to cell seeding, the wells/dishes were washed with Milli-Q water and dried. We found 0.1 mg/mL poly-L-lysine to be the best fit for our protocols.

Numbers of cells present in a culture well can affect cellular interactions, growth rate and attachment to the surface, in addition to physiological responses like, for instance, Gnrh

responsiveness (Weil et al., 1986). To obtain optimal seeding density of the pituitary cells, densities from 5×10^4 to 2×10^5 cells/cm^2 well surface were tested. The wells were scored based on cell attachment and survival, as well as suitability for patch clamp. The lowest and highest density had lower relative cell survival compared to intermittent densities. Furthermore, cells incubated at the highest concentration tended to form lumps, which lowered surface attachment and aptness for patch clamping. The best seeding density for the cod pituitary cells was deemed to be 1.5×10^5 cells/cm^2.

Serum supplements are commonly added to cell culture media to stimulate cell growth, aid cell adhesion and increase buffer capacity (Sigma-Aldrich and ECACC, 2001). However, serum is not always beneficial, dependent on cell type and experiment (Bowers and Dahm, 1993). Furthermore, sera are subject to batch-to-batch variation and introduce potential risk of contamination to the media. A synthetic serum replacement (SSR), designed without proteins or other substances of biological origin and therefore reducing contamination or interference risk (Bertheussen, 1993), maintained pig liver endothelial cells functional for over 20 days (Elvevold et al., 2005), thus providing an interesting substitute to traditional serum. To assess potential effects of adding serum supplement to the culture media, we compared electrophysical properties, cell viability, and the expression of reproductive-relevant genes, between cells cultured in serum-free media (SF) and cells cultured in media supplemented with either 5 % new-born calf serum (NCS) or 5 % SSR. The results of this assessment are described in **paper I**. The general observation was that serum supplements did not affect the resting membrane potential or the number of surviving cells but did increase metabolic activity and membrane integrity compared to SF cells. For gene expression, no differential effects were seen between SF and SSR cells for the first 7 days, whereas SSR cells had higher expression levels at day 14. Cells exposed to NCS had generally lower levels of expression than both other groups. Furthermore, we observed (not quantified) that cells subjected to NCS supplement aggregated more, formed more debris, and were more challenging to patch than cells subjected to SF or SSR. We therefore concluded SSR to be the better supplement for cod pituitary cells. Unfortunately, our SSR batch became contaminated, and we experienced unforeseen difficulties obtaining more. However, our design for the upcoming experiments (**papers II-IV**) included only cultures lasting 7 days, and as there were only minor differences between cells cultured with SF or SSR media within this timeframe, we decided to use SF media subsequently.

2.2.4 Final protocol

The final protocol was based on the previous work by Haug et al. (2007) and Montero et al. (1996), with the adjustments described above incorporated. The PBS was prepared by cooling the solution to 12°C, and then adjusting pH to 7.85 and osmolality to 320 mOsm. After sterile filtration, 2 mL pen-strep (Penicillin and Streptomycin, 20 U/ml, Lonza, Verviers, Belgium) was added. Cell culture media, L-15, was prepared by adding 10 mM $NaHCO_3$ and 1.8 mM D(+)-glucose (Sigma-Aldrich) prior to adjusting the osmolality. After sterile filtration, 1 mL of pen-strep was added.

Immediately after dissection, pituitaries were transferred to modified L-15 media and kept on ice until dissociation (see Figure 2.3). Returning to the lab, pituitaries were immediately washed in cold PBS media and visible remains of blood vessels removed. Using a scalpel, the pituitaries were cut into approximately 1 mm^3 pieces. The tissue fragments were transferred to an Erlenmeyer flask, 25 mL trypsin (type II-S, 1 mg/mL, Sigma-Aldrich) dissolved in PBS added, and placed in a shaking water bath at 18 °C, initiating the chemical dissociation of the tissue. After 45 min, the trypsin solution was replaced by 25 mL trypsin inhibitor (type I-S, 1 mg/mL, Sigma-Aldrich) and ~30 µg deoxyribonuclease I (DNaseI, Sigma-Aldrich) dissolved in PBS, after which the cells were incubated for another 20 min in the water shaker bath. Both trypsin and trypsin inhibitor solutions were filtered through a Millipore filter (Merck, Kenilworth, NJ, USA) before use. Following the chemical dissociation, mechanical dissociation was performed by adding a few mL of ice-cold PBS and gently sucking the fragments in repeated passages through a disposable pipette (see Figure 2.3 B). Dissociated cells were collected in a new tube and the process repeated until

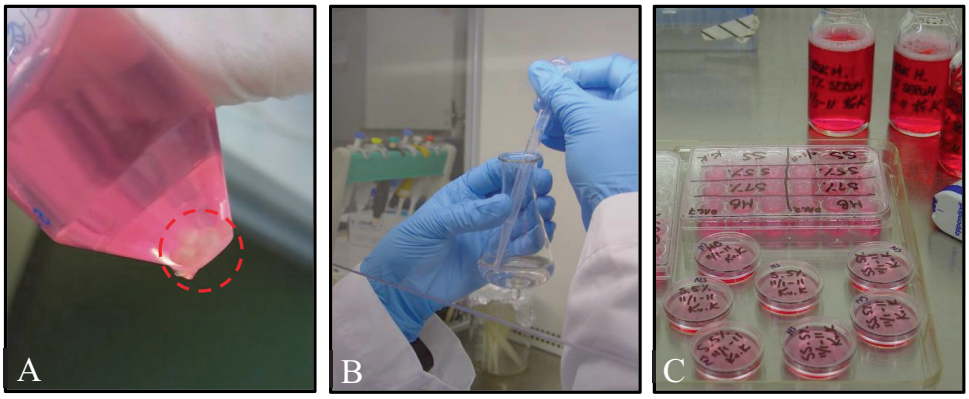

Figure 2.3 Cell culture preparations

A) A pool of dissected pituitaries in cell media. B) In ice-cold PBS, pituitary fragments are mechanically dissociated with a pipette. C) Plated cells added their respective media, w/wo specific treatments

most lumps were dissolved. Remaining tissue fragments were removed by filtering the cell suspension through a nylon mesh (70 µm). The filtrate was then centrifuged at 185 *g* for 10 min at 4 °C, before the supernatant was removed and the pellet resuspended in culture medium. Cell concentration was estimated using a Bürker counting chamber (haemocytometer). The cells were seeded in culture wells pre-coated with poly-L-lysine at a density of 1.5×10^5 cells/cm^2. For gene analysis samples, cells were seeded in 24-well plates (3×10^5 cells/well), and for viability assays, they were seeded in 96-well plates (5×10^4 cells/well). Cells for electrophysiological samples were seeded in plastic dishes (Corning), inside a central silicon ring of approx. 1 cm^2 (1.5×10^5 cells/dish) to improve cell placement for the subsequent patch-clamp analysis. The cultures were incubated at 12 °C in a humidified atmosphere with 0.5 % CO_2. After 24 hours, healthy cells were attached to the poly-L-lysine coated well surfaces, and by renewing the media, damaged and detached cells were removed. For the remainder of the incubation period, culture media were renewed every three days. The optimized culture conditions allowed the cells to remain viable in culture for over two weeks (For a visual impression of the cell development during culture, see Figure 2.4 for a 1-week series, or the supplementary data of **paper 2** for a 3-week series).

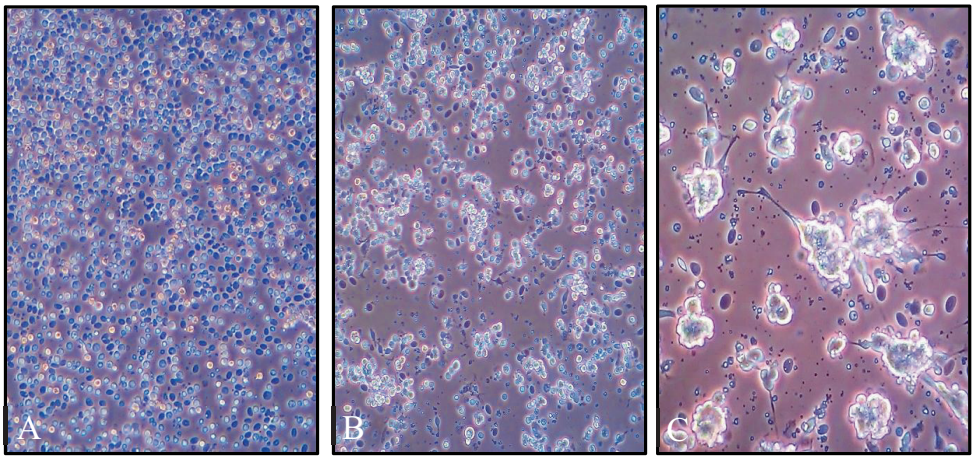

Figure 2.4 Cells in culture
A) Day 0, newly seeded cells. B) Day 1, after removal of dead or unattached cells.
C) Day 7 (picture taken at higher resolution), the cells start to aggregate and form small extensions between cell colonies. Left longer in culture, these aggregates and extensions would continue to grow. Note that some of the cells appear blue under the microscope.

The number of pituitaries pooled per culture varied between 2-6 in cultures used for testing the culture protocol, including cell density and centrifugation speed (**paper I**), and 4-21 in cultures used for exposure studies (**papers I-IV**), the final number dependent on pituitary size, fish availability, and scale of experiment(s) performed on the pool. The size of the cod pituitary is correlated both to maturational status of the fish, with maturing individuals having larger pituitaries, and to the size of the fish, with larger specimens having larger pituitaries (Woodhead, 1971). Pituitaries from both sexes were pooled in the cultures. Although it would have been preferable to pool pituitaries from the different sexes separately, the limited availability of fish necessitated the need to pool pituitaries from both sexes.

2.3 Exposures

At day 4 in culture, the cells were exposed to their respective treatments (see Table 1). This preincubation period was chosen to allow cells to recover from the stress of dissociation and to stabilize, and is in agreement with other studies in the field (Baker et al., 2000; Larsen et al., 1998; Levavi-Sivan et al., 2004; Weil et al., 1986). By day 4, the cells were firmly attached and had started to spurt outgrowths, which we consider a healthy sign. Prior to exposure, stock solutions of all compounds were prepared in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway) and stored at -20°C until use. The steroid hormones, 17β-estradiol (E2), testosterone (T), 5α-dihydrotestosterone (DHT) and cortisol (F), were kept in stock for up to 3 months, whereas the bisphenols, bisphenol A (BPA) and tetrabromobisphenol A (TBBPA), were kept for up to 1 week. All compounds were diluted in cell media prior to cell exposure, with working solutions having a final EtOH concentration of 0.2% (34.25 mM). To ensure accurate pipetting, as EtOH expands with increasing temperature, stocks were diluted at room temperature. To prevent possible reactions between the lipophilic compounds and plastic, all solutions were prepared in sterile glass tubes. In each experiment, controls w/wo EtOH (solvent control/control blank) were included.

Exposure doses of E2, T, and F (see Table 1) were based on low and high physiological levels (Almeida et al., 2009; Dahle et al., 2003; King and Berlinsky, 2006; Norberg et al., 2004; Olsen et al., 2008; Staurnes et al., 1994). While 11-ketotestosterone (11-KT) is considered the main androgen in teleosts, we chose to use DHT in our study. Ideally, 11-KT and DHT, which are both non-aromatizable androgens, should have been included; DHT for its known role in other vertebrates and potential role in teleosts (Asahina et al., 1985; Latz and Reinboth, 1993; Margiotta-Casaluci et al., 2013; Margiotta-Casaluci and

Sumpter, 2011; Martyniuk et al., 2013; Pasmanik and Callard, 1988), and 11-KT for its known physiological role in teleosts (Mayer et al., 1990; Schulz and Miura, 2002; Tokarz et al., 2015). However, since access to fish were limited and DHT has been shown to bind the androgen receptor with higher affinity than 11-KT (Jørgensen et al., 2007; Sperry and Thomas, 1999), we chose to use the former. Doses were based on cod plasma 11-KT values (Almeida et al., 2009). Exposure doses of BPA and TBBPA covered both environmentally relevant and potentially cytotoxic concentrations (Crain et al., 2007; Flint et al., 2012; Gong et al., 2017; Huang et al., 2012; Morris et al., 2004; Yang et al., 2012). Exposure lasted 72 h for all treatments. The duration of exposure was based on similar studies, with typical incubation lasting 12-168 h (Levavi-Sivan et al., 2006; Lin and Ge, 2009; Melamed et al., 1997; Rebers et al., 2000; Sohn et al., 2001; Tse et al., 2013).

Table 1. Experimental treatments

Paper	Compound	IUPAC ID	Abbreviation	Exposure doses
II	17β-estradiol	(17β)-estra-1,3,5(10)-triene-3,17-diol	E2	2.5 and 25 ng/mL
	Testosterone	Androst-4-en-17β-ol-3-one	T	2 and 20 ng/mL
	5α-Dihydrotestosterone	5α-Androstan-17β-ol-3-one	DHT	2 and 20 ng/mL
III	Cortisol	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	F	10 and 100 ng/mL
IV	Bisphenol A	4,4'-(propane-2,2-diyl)diphenol	BPA	10^{-9} to 10^{-3} M
	Tetrabromobisphenol A	4,4′-(propane-2,2-diyl)bis(2,6-dibromophenol)	TBBPA	10^{-9} to 10^{-4} M

All compounds were purchased from Sigma-Aldrich, dissolved in ethanol and diluted in culture media prior to cell exposure

2.4 Viability measurements

During culture, cells will inevitably eventually die, either due to apoptosis or necrosis. To assess the absolute number of surviving cells, each experimental well was visually inspected, and its cell content manually counted. The cell suspension for counting was prepared by removing the cell medium, wash with 1 mL PBS, then replacing the PBS with 200 µL 0.25 % Trypsin-EDTA (Sigma-Aldrich) and leaving it for 40s. After removing the enzyme solution, the remaining film was allowed to incubate for 3 min to complete cell detachment, before 1 mL PBS was added and pipetted over the surface several times. All solutions used for washing and detachment were transferred to a collecting tube and the suspension homogenized by gently flipping the tube. From the middle of the suspension, 50 µL was removed and mixed with 50 µL of the dye Trypan Blue (Sigma-Aldrich). Trypan

Blue is not absorbed by viable cells, but stains cells with a damaged cell membrane and can therefore be used as an indicator of non-viable cells. From the mixture, a sample was counted using a Bürker chamber. The volume of the Bürker chamber was 10^{-4} mL, so by multiplying the number of viable cells by the dilution factor and 10^4, the concentration of viable cells per mL suspension could be calculated. All samples were counted blindly, with one person preparing a sample in the Bürker chamber, and another counting it without knowing its identity.

As manual counting is quite time-consuming, and we had many wells to count, we tested the automated cell counter, CountessTM (Thermo Fisher Scientific), as an alternative. However, the machine repetitively overestimated the number of dead cells and was therefore not suited for our cells. The probable reason for this was that some pituitary cells appear blue under the microscope (see Figure 2.4). When counting manually, it is possible to distinguish between Trypan Blue stained cells and those appearing blue naturally, but the CountessTM was not able to do so. As a result, manual counting was performed for all estimations of absolute number of surviving cells.

However, not only cell death is of consequence when determining cell health. This became apparent during the optimization process of the protocol described above; mammalian culture conditions may have kept the cells alive, but optimized conditions produced more viable cells, as well as stabilized membrane potentials and increased action potential firing frequency. To assess more information about the cell conditions, we investigated the possibility of utilizing a combination of two fluorometric assays; AlamarBlue (AB) assay and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) assay (both from Sigma-Aldrich), established for fish hepatocyte primary culture by Schreer and colleagues (2005). The AB assay indicates cell metabolic activity and is based on the reduction of the blue, non-fluorescent dye resazurin to the pink, fluorescent resorufin by mitochondrial, cytosolic and microsomal enzymes (Bopp and Lettieri, 2008; Gonzalez and Tarloff, 2001). Resorufin can be detected spectrophotometrically or fluorometrically (Bopp and Lettieri, 2008). CFDA-AM is a nonpolar, non-fluorescent dye that diffuses rapidly into cells, but is converted by nonspecific cytoplasmic esterases of living cells into a polar fluorescent dye, carboxyfluorescein (CF), which has a low diffusion rate (Schreer et al., 2005). As an intact cell membrane helps maintaining a proper cytoplasmic milieu needed for the esterases to function properly, the CFDA-AM assay indicates plasma membrane integrity, and CF fluorescence reflects the viability of the cells in the culture. Neither AB nor CFDA-AM are cytotoxic, they require similar incubation times, and can be detected at different

wavelengths without interference (Bopp and Lettieri, 2008; Schreer et al., 2005). As such, AB and CFDA-AM can be applied in parallel on the same set of cells in each experiment, and the combined AB/CFDA-AM assay provide information to whether toxic chemicals act through disruption of cellular membrane permeability or cellular metabolism.

We performed the AB/CFDA-AM assay essentially as described by Schreer et al. (2005), using the modified fluorochrome concentrations introduced by Tollefsen et al. (2008). Prior to exposure, CFDA-AM was dissolved in 99.5 % dimethyl sulfoxide (DMSO) to a 4 mM stock solution and stored at -20°C until use, while AB was purchased as a ready-to-use solution and kept from light exposure at 4°C (all solutions from Sigma-Aldrich). Tris buffer (50 mM, pH 7.5) was prepared by dissolving 0.97 g/L trizma-base and 6.61 g/L trizma-HCl (both from Sigma-Aldrich) in distilled water. The buffer was filtered and stored at 4°C for up to six months. Cells were seeded as described above, with 5×10^4 cells/well in 96-well plates, incubated for 4 days prior to 72 h of exposure. At day 7, culture medium in all wells was replaced by 100 µL Tris buffer containing 5% AB and 4 µM CFDA-AM. The plate was covered in foil to avoid light exposure and incubated on a plate shaker for 30 min, after which the concentrations of fluorescent products were measured simultaneously for both probes using a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). For resorufin the excitation was 530 nm and emission 590 nm, while for CF, the excitation was 485 nm and emission 530 nm. Data was collected with the Gen5 Data Analysis Software (Bio-Tek Instruments Inc.). As a positive control for cell toxicity, and serving as intra/inter assay control, each plate included additional wells that were exposed to the known cytotoxic compound, copper sulphate ($CuSO_4$; 0.156, 0.625 or 2.5 mM, n = 6 per dose) (Tollefsen et al., 2008), for the last 24 h and analysed alongside the experimental wells at day 7. An interesting observation was that, under the microscope, cells exposed to the highest $CuSO_4$ concentration (2.5 mM) had normal appearances in the well but were revealed by the AB/CFDA-AM assay to be dead (see for instance **paper III**, supplementary material). This emphasizes the importance of adequate viability measurements, as visual inspection alone does not suffice. The stable results from the $CuSO_4$ controls between plates and cultures, indicated the AB/CFDA-AM assay a suitable and fast method to determine viability of the cod pituitary cells.

2.5 Gene expression analysis

Proteins are involved in essentially every aspect of cellular life, from organizing the DNA to catalysing chemical reactions, transporting materials across the plasma membrane, providing cell structure, cell movement and aiding cell division. Different cell types have different functions and produce proteins that reflect these functions. In response to internal and external stimuli, the protein synthesis profile may be altered both in level and in type. To synthesize a specific protein, cells transcribe a part of their DNA, i.e. the gene segment coding for that protein. Transcription creates a messenger RNA (mRNA) molecule, consisting of specific nucleotides complementary to the gene segment. After a series of post-transcriptional modifications, this mRNA molecule can be translated and folded into a functional protein. Though not all mRNAs are translated into functional proteins, and one mRNA molecule may be translated multiple times (Yan et al., 2016), analysing a cell's gene transcript levels provides useful indications of its protein synthesising state.

To measure gene expression levels, there are several techniques available, such as Northern blotting, RNase protection assay, microarrays, serial analysis of gene expression (SAGE), RNA sequencing and quantitative polymerase chain reaction (qPCR). In this study, we used the qPCR technique, where levels of mRNA are quantified by reverse transcription of the RNA to complementary DNA (cDNA) followed by a targeted amplification of the cDNA molecule that can be monitored in real-time. By measuring the increase in fluorescence signal from DNA-binding dyes or sequence-specific probes during the successive rounds of amplification, the amount of each specific target can be determined. The qPCR technique has been available since the early 1990's (Higuchi et al., 1992; Higuchi et al., 1993; Wittwer et al., 1997) and is established as a fast, accurate and sensitive method for nucleic acid quantification (Bustin et al., 2009).

2.5.1 Preparations for qPCR

In qPCR, it is possible to make an absolute quantification of transcript levels using either external standards or prepared standard curves for each amplicon (Bustin, 2000; Dhanasekaran et al., 2010). This was our original goal, and we wanted to use an external standard to normalize the gene expression data. In order to add a specific quantity of external RNA to each sample, the exact number of cells had to be determined upon harvesting. We decided to make aliquots of 10 000 cells from each sample, as this would allow us to make

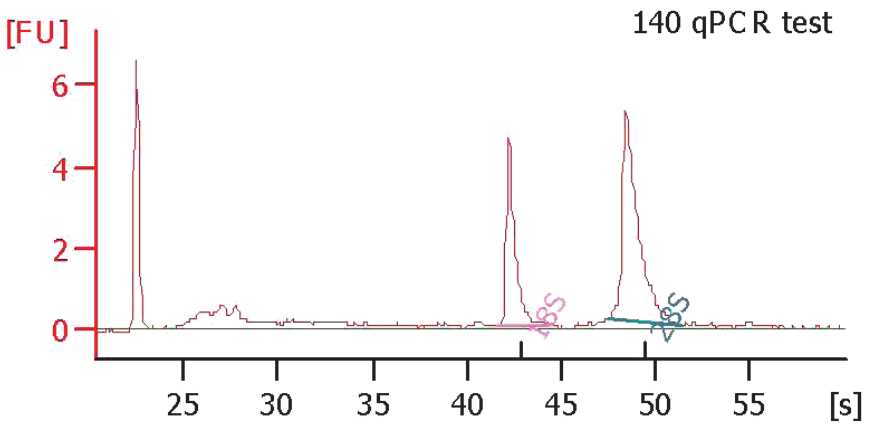

Figure 2.5 Example of Bioanalyzer electropherogram, showing the peaks of the 18S and 28S ribosomal subunits. The first peak is an internal marker. The RNA integrity number (RIN) for this sample was 9.2.

several replicates per well, and to make use of the CellsDirect qRT-PCR kit (Thermo Fisher Scientific), which removes the need for an RNA purification step when testing with ≤ 10,000 cells per reaction. To quantify the cells, each well was trypsinated as described above, the cell suspension centrifuged, and the pellet resuspended prior to manual counting in the Bürker chamber. However, the harvest caused many cells to burst and it proved difficult to produce equal replicates. Despite increasing the cell count by improving the trypsin protocol, we found the method too unstable and too time-consuming to continue using it. We therefore abandoned harvesting an exact number of cells, and hence, both CellsDirect and absolute quantification of expression. For the subsequent experiments, each sample was harvested as a whole, total RNA was isolated from the cells, and gene expression normalized relative to internal reference genes.

To access the mRNA, the cells must be lysed, and the RNA extracted. In our new approach, total RNA was isolated using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) applied directly into the culture wells. TRIzol® Reagent lyses the cells while maintaining the integrity of the RNA, due to effective inhibition of RNase activity. When performing the harvest, media was removed, and the wells washed with PBS before adding 300 µL TRIzol. Each well was stirred with a pipette, the TRIzol collected and snap frozen in liquid nitrogen before being stored at -80 °C until RNA isolation. Each plate had 24 wells, and each well took a few minutes to harvest. To minimize potential effects of the time lag from the first to the last sample of the plate, the plate was kept on ice, and one sample from

each experimental group harvested before moving on to the second sample from each group and so on.

The RNA isolation was performed using chloroform phase separation and isopropanol precipitation. During the first rounds of extraction, however, the RNA yield and quality were lower than expected. We found that adding an additional 700 µL of TRIzol prior to the phase separation, adding a small amount (1.5 µL) of GlycoBlue (Ambion; Thermo Fisher Scientific, Inc.), a blue dye covalently linked to glycogen that makes small pellets more visible, to the precipitation step and coprecipitate it with the RNA, as well as performing a second precipitation step using 0.3 M sodium acetate, increased the RNA yield and quality substantially. To remove possible traces of genomic DNA (gDNA), all samples were treated with DNase (TURBO DNA-free, Ambion; Thermo Fisher Scientific, Inc.). RNA quantity was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific). The NanoDrop also measures nucleic acid purity, by estimating the ratio of absorbance at 260/280 nm. Nucleic acids absorb at 260 nm, whereas proteins or phenol absorb around 280 nm. A ratio of ~ 2 is considered pure for RNA, and values from 1.7-2.1 were deemed acceptable. The RNA quality was further assessed using the Agilent 2100 Bioanalyzer and RNA LabChip® kit (Agilent Technologies, Santa Clara, CA, USA). The Bioanalyzer generates an electropherogram that provides detailed assessment of the RNA and determines the 18S to 28S ribosomal subunits ratio, i.e. the RNA integrity number (RIN), as an indication of RNA degradation (see Figure 2.5). RIN values are reported on a scale from 1-10, with 10 being the most intact. RNA with a RIN value above 8 was deemed acceptable for further analysis.

Since the qPCR technique is based on the amplification of DNA, using DNA polymerases, the RNA must to be converted into DNA prior to PCR. Using enzymatic reverse transcription, cDNA with the same nucleotide sequence as the initial gene segments, minus the introns, can be synthesized from the mRNA molecule. We used 500 ng RNA per sample, random primers and Superscript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) to synthesize the first strand cDNA. The cDNA was stored at -20 °C until qPCR analyses.

2.5.2 qPCR

The qPCR analyses were performed using the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland), with SYBR Green I Master (Roche) detection. SYBR Green is a fluorescent dye that binds to any double-stranded DNA and

generates a fluorescence signal proportional to the amount of DNA produced each cycle (Bustin, 2000). Since this dye only binds to double-stranded DNA, potential presence of single-stranded DNA or RNA will not contaminate the results.

In order to amplify our targets in the qPCR reaction, primers specific to each product were designed. The Atlantic cod genome sequence was published early during this work (Star et al., 2011), but parts of it was accessible from an earlier time point (http://www.ensembl.org/Gadus_morhua/Info/Index). The available scaffolds allowed us to make primers that uniquely selected our target regions of DNA, verified through BLAST searches. The primers were designed using the Primer3 shareware (Koressaar and Remm, 2007; Untergasser et al., 2012) (http://frodo.wi.mit.edu/primer3/input.htm). At least one primer from each pair spanned an exon-exon border to avoid amplification of potential traces of gDNA. To avoid incorrect hybridization or failed annealing, each primer pair had similar melting temperatures (Tm) close to the reaction annealing temperature. The primers were theoretically tested for possible hairpin loops and primer dimer formations using Vector NTI (Thermo Fisher Scientific). For this thesis, the genes of interest were *lhb* (GenBank ID: DQ402374), *fshb* (GenBank ID: DQ402373), *gnrhr1b* (GenBank ID: GU332297) and *gnrhr2a* (GenBank ID: GU332298.1), all related to pituitary reproductive function. As synthesis of the β-subunit genes of Lh and Fsh is considered the rate-limiting step in the overall production of these hormones (Kaiser et al., 1997; Pierce and Parsons, 1981), we only studied the gene expression levels of these subunits. Primers for four different reference genes, *arp2*, *bactin*, *ubiquitin* and *ef1a*, were also designed. All primers were purchased from MWG-Biotech AG (Ebersberg, Bayern, Germany). The efficiencies of the primers were verified through running a standard dilution curve on cDNA extracted from whole pituitaries. The qPCR reactions contained 5 µL of SYBR Green I Master, 1 µL (5 µM) forward primer, 1 µL (5 µM) reverse primer, and 3 µl diluted cDNA. To activate the *Taq* polymerase, the qPCR reactions were initiated by 10 min at 95 °C. This was followed by 42 cycles consisting of denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, and elongation for 6 s at 72 °C. After every elongation, the fluorescence was measured and used to determine the quantification cycle (Cq). Plotting the fluorescent signal from each sample against the cycle number creates an amplification curve. The rate at which the polymerase converts the reagents of the procedure to amplicon is a measure of the qPCR efficiency (E) (Taylor et al., 2010). Using the relationship between increasingly diluted cDNA and the corresponding Cq values, a standard curve can be calculated (see Figure 2.6 for an example). Evenly spaced amplification curves will produce a linear standard curve. The E of the assay is calculated

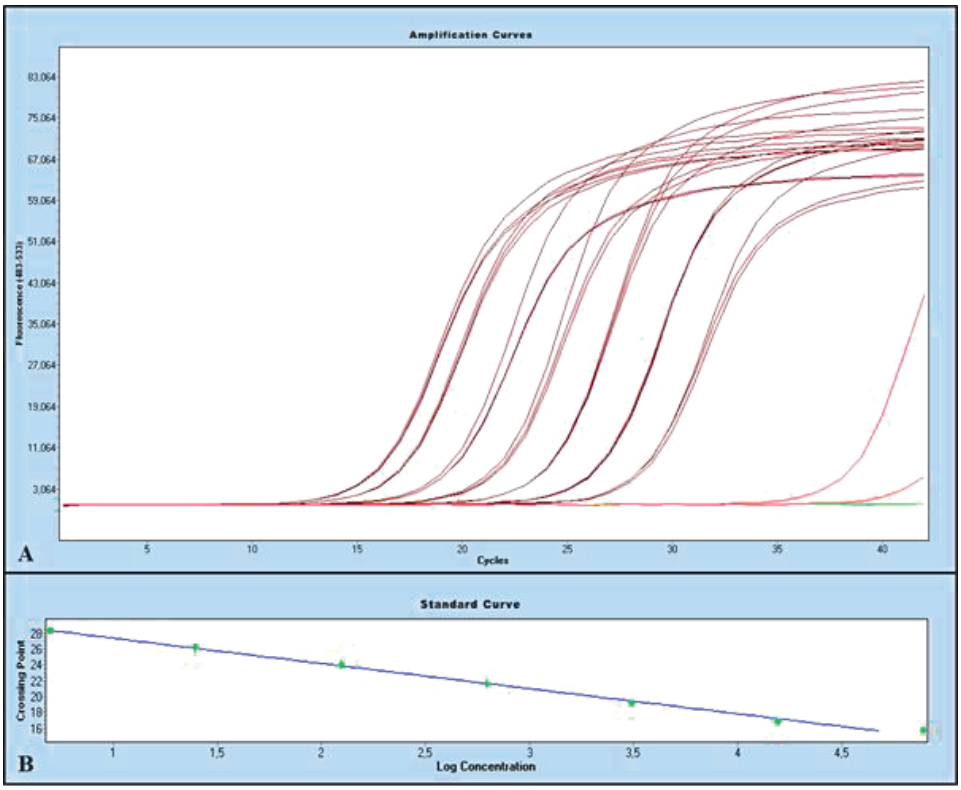

Figure 2.6 Example of the amplification curves created by qPCR. **A**) Ubiquitin primer testing on a series of cDNA dilutions, samples in triplicate, **B**) The corresponding calculated standard curve, E = 2.06. Screenshot from the LightCycler 480.

using the slope of the regression line, $E = 10^{-1/slope}$. A slope of -3.32 corresponds to E = 2, meaning that there is a doubling of product during each PCR cycle. Efficiencies between ~ 1.8 and 2.1 were accepted (see Table 2 for primer sequences, amplicon sizes and efficiencies). The specificity of the primers was verified by analyses of the amplified PCR products. Firstly, the melting curves, based on slowly heating the reaction mixture from 65 °C to 98 °C directly following each individual PCR reaction, were inspected, making sure that each curve displayed a single sharp peak. Because SYBRGreen binds to double-stranded DNA, a loss of fluorescence occurs when a sample is heated through the Tm of the product. The shape and position of a melting curve is dependent on the GC/AT content, length, and sequence of the product and can be used to distinguish between similar products (Ririe et al., 1997). Secondly, the samples were loaded on a 2.5 % agarose gel stained with SYBRsafe DNA gel stain (Invitrogen; Thermo Fisher Scientific, Inc.) and electrophoresis ran to verify the molecular weight of the product against a DNA ladder. A single band also verifies that a

Table 2. qPCR primers used in this thesis

Targets		Primer sequences	Amplicon sizes (nt)	Efficiencies*
lhb	F:	5`-GTGGAGAAGAAGGGCTGTCC-3`	81	1.97/1.93
	R:	5`-GGACGGGTCCATGGTG-3`		
fshb	F:	5`-GAACCGAGTCCATCAACACC-3`	63	2.02/1.84
	R:	5`-GGTCCATCGGGTCCTCCT-3`		
gnrhr1b	F:	5`-GCTACTCCCGAATCCTCCTC-3`	73	1,96
	R:	5`-CGCCTCAGGTATGACTCTCC-3`		
gnrhr2a	F:	5`-TTCACCTTCTGCTGCCTCTT-3`	113	1.90/1.99
	R:	5`-TCCGTGGAGGAAAGATTGTC-3`		
b-actin	F:	5`-TTCTACAACGAGCTGAGAGTGG-3	102	2.06/1.84
	R:	5`-CATGATCTGGGTCATCTTCTCC-3		
arp2	F:	5`-GGAGGTTAGAAGTAGCAAGGAGC-3`	107	2.11/1.94
	R:	5`-TGCTGACTCTCACGGAGTTG-3`		
ef1a	F:	5`-CCTTCAACGCCCAGGTCAT-3`	100	1,92
	R:	5`-AACTTGCAGGCGATGTGA-3`		
ubiquitin	F:	5`-TGTCAAAGCCAAGATTCAGG-3`	111	1.80/1.86
	R:	5`-TGGATGTTGTAATCCGAGAGG-3`		

***** Efficiencies were retested when primers were reordered. First value is from paper I, second from papers II-IV. **F**; Forward, **R**; Reverse

single product has been amplified, if not by chance there are several products of equal length. Thirdly, the samples were extracted from the gel, the DNA purified and inserted into a pGEM-T Easy vector (Promega, Madison, WI, USA) before being transformed into JM109 competent cells (Promega). From positive colonies, DNA was extracted and send to the ABI-lab at the University of Oslo for sequencing. The resulting sequences were verified by BLAST search. To determine which reference gene was best suited, we used the Excel-based software program, BestKeeper (Pfaffl et al., 2004). As the ideal reference gene is expressed at a constant level at all stages of development, and remains unaffected by the experimental treatment, we included Cq values obtained from samples representing both control and treated cells, from different stages of maturity. The BestKeeper programme evaluates whether the target genes are differentially expressed under an applied treatment. The evaluation was performed separately for primers used in **paper I** and primers used for subsequent papers. For **paper I**, we tested *ubiquitin*, *b-actin* and *arp2* primers. They had standard deviations (SDs) of 0.35, 0.53 and 0.42, respectively, and as ubiquitin proved most stable, this was chosen as the reference gene for this study. For **papers II-IV**, *arp2*, *b-actin*, *ubiquitin* and *ef1a* were tested. They had SDs of 0.39, 0.45, 0.43 and 0.39, respectively. While *arp2* and *ef1a* had identical SDs, the Cq value of *arp2* was much higher than that of *ef1a* (27.06 vs 20.40, respectively). Subsequently, *ef1a* (GenBank ID: DQ402371.1) was used to normalize the qPCR data. To calculate the change in expression of the genes of interest, an

efficiency-corrected relative expression method was used (Pfaffl, 2001; Weltzien et al., 2005):

$$\text{Relative expression} = E_{\text{target}}^{\Delta Cq(\text{calibrator-sample})} \times E_{\text{reference}}^{\Delta Cq(\text{sample-calibrator})}$$

The calibrator consisted of a mix of cDNA from all individual samples analysed at the same time and were used to adjust for assay-to-assay variation. The mixture was diluted and analysed in the same manner as the individual samples and added in duplicate to all plates in the run. In addition to the positive calibrator, two negative control reactions for every primer pair were included by substituting the cDNA template with nuclease-free water (Ambion; Thermo Fisher Scientific, Inc.). This was done to confirm that the reagents were not contaminated and to assess for primer–dimers.

In **paper II**, in order to analyse the gene expression patterns of Gnrh receptors in gonadotropes, experiments using single-cell qPCR were included. The pituitary primary cultures consist of all cell types of the pituitary, and this technique allowed us to identify individual cells, as well as investigate their gene expression levels. This method was optimized previously by members of our group (Hodne et al., 2010). To ensure that the mRNA content originated from a single cell, the cytosol was collected through a patch pipette following giga seal formation. To avoid RNA attaching to charged residues on the glass surface, the glass pipettes were silanized using Sigmacote (Sigma-Aldrich; Thermo Fisher Scientific, Inc.). As a perforating agent, giving low access resistance, and to stabilize the giga seal, b-escin was used (Sigma-Aldrich; Thermo Fisher Scientific, Inc.) (Fan and Palade, 1998). The cytosol content was transferred to RNase-free tubes for RNA amplification using the MessageBOOSTER cDNA Synthesis Kit (Epicenter Biotechnologies, Madison, WI, USA), which enables RNA amplification and cDNA synthesis directly from cell lysates, without the need to purify total RNA (Meis and Khanna, 2009). An oligo(dT) primer containing a T7 promoter and SuperScript III reverse transcriptase were used to synthesize first-strand cDNA. The kit includes an *in vitro* transcription reaction after second strand cDNA synthesis, which generates thousands of RNA molecules for every molecule of double-stranded cDNA template (Meis and Khanna, 2009). Using random primers, the amplified RNA was then reverse-transcribed into cDNA. qPCR was performed as described above.

2.6 Electrophysiology

In **paper I**, electrophysiological recordings of membrane potentials and action potentials were included to verify cell viability after optimizing the culture protocol. The plasma membrane surrounding animal cells consists mainly of phospholipids, sterols, and membrane proteins. Some of these proteins allow transport of ions from one side of the membrane to the other, either through active transport or by providing channels through which they can diffuse. Ion pumps actively move ions across the membrane and establish concentration gradients, while ion channels allow ions to move down those concentration gradients. The membrane potential is the resulting difference in voltage, or electric potential, between the interior and the exterior of the cell created by the differences in permeability of the different ions and of the uneven ion concentration across the membrane. Opening or closing ion channels in the membrane will cause transient local changes in the membrane potential. The changing electric field can affect other channels, causing them to open or close as well. In excitable cells, such as the endocrine cells in the teleost pituitary, a sufficiently large depolarization can induce an action potential (Halnes et al., 2019; Haug et al., 2007; Levavi-Sivan et al., 2005; Xu and Cooke, 2007; Yu et al., 2010). In mammalian pituitary cells, action potentials allow controlled influx of Ca^{2+} over the plasma membrane and are important for hormone secretion (Fontaine et al., 2020; Stojilkovic, 2006; Stojilkovic, 2012). While less investigated in teleosts, recent studies in medaka gonadotropes indicate that increased action potential firing frequency can facilitate Ca^{2+} influx sufficient to induce hormone release (Fontaine et al., 2018; Hodne et al., 2019).

To study changes in membrane voltage and currents of the pituitary cells, the patch-clamp technique, which isolates a small patch of membrane using a glass capillary, was used. The interaction between the glass and the membrane phospholipids creates a tight seal that prevents currents from flowing between the tip of the glass and the isolated membrane. The method has been available since the mid-1970s (Hamill et al., 1981; Neher and Sakmann, 1976; Sakmann and Neher, 1984) and is considered the golden standard for measuring cell electrophysiological properties. The experiments in **paper I** were performed using perforated patch configuration, which keeps the intracellular environment of the cell intact during the experiment, with b-escin as the perforating agent. Patch electrodes were made from borosilicate glass with filament, connected to an EPC-9 patch-clamp amplifier controlled by PULSE software (HEKA Elektronik, Lambrecht, Germany). Recordings were collected at 12 °C, using a Peltier element for temperature control (TC-10, Dagan Corporation, Minneapolis,

MN, USA). To prevent vibrations from the floor, the microscope used to observe the cells was placed on a vibration-free table, and to avoid inductive noise, the set-up was mounted inside a Faraday cage. Data analyses of the recorded signals were performed using PULSFIT (HEKA) and Origin (OriginLab Corporation, Wellesley, MA, USA).

2.7 Microfluorometry

Activation of Gnrh receptors in teleost gonadotropes is followed by elevated cytosolic Ca^{2+} influx from both internal and external sources (Fontaine et al., 2020; Hodne et al., 2019; Hodne et al., 2013; Levavi-Sivan and Yaron, 1993; Mollard and Kah, 1996; Strandabø et al., 2016; Yaron et al., 2003). This increased Ca^{2+} concentration ($[Ca^{2+}]_i$) facilitates the secretion of gonadotropin hormones (Chang et al., 2009; Fontaine et al., 2020; Yaron et al., 2003). In **paper I**, we used Ca^{2+}-imaging, a microfluorometric technique that visualizes the local concentration of Ca^{2+} in cells or tissues using fluorescent indicators (Hayashi and Miyata, 1994), to test whether the cultured cells responded to Gnrh. Cells used for Ca^{2+}- imaging were seeded in dishes with a central glass bottom, coated with poly-L-lysine. Prior to experiments, the cells were incubated with 5 µM fura-2 acetoxymethyl ester (fura-2AM) (Invitrogen; Thermo Fisher Scientific, Inc.), a lipid-soluble molecule able to pass through cell membranes. Once inside the cell, endogenous esterases removes the acetoxymethyl group, generating fura-2, a ratiometric (dual-wavelength) fluorescent dye which binds to free intracellular calcium. For imaging, the dish was mounted on an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). The cells were stimulated by a 10^{-7}M mixture of Gnrh 1, 2, and 3 (Bachem, Bubendorf, Switzerland). When Ca^{2+} binds to fura-2, it changes the optimum excitation wave-length. Ca^{2+}-free fura-2 has a peak excitation wavelength of 380 nm, whereas Ca^{2+}-bound fura-2 has a peak excitation wavelength of 340 nm (Bootman et al., 2013). By recording the ratio between emissions at excitation wavelengths 340 and 380 nm, cytosolic $[Ca^{2+}]_i$ can be calculated. Imaging Workbench 6 (INDEC BioSystems, Santa Clara, CA, USA) software was used for recording and analysis.

2.8 Statistics

Statistical analyses can reveal data irregularities or patterns, help interpret results, allow comparison between groups and treatments, and verify conclusions. Knowledge about statistical analysis can also help with correct experimental design. In biology, statistical

analysis often revolves around hypothesis testing. For each experiment in this thesis, a null hypothesis stating that the specific treatment had no effect, or that there was no difference between experimental groups and control, was formulated and the claim tested. The significance level for rejection of the null hypotheses was set to 0.05 for all experiments. For single cell qPCR and electrophysiological data, individual cells were treated as one sample. For the other experiments, technical replicates from individual experimental wells were averaged, and each experimental well treated as one sample. The fold change of each exposed sample was calculated relative to the mean of its respective solvent control and used in the subsequent analysis. To maintain the variance in control data, individual control samples were calculated in the same manner and included in the analysis. All data were tested for normality and equal variances by Shapiro–Wilk W test (Shapiro and Wilk, 1965) and Levene's test (Levene, 1960), respectively. If the criteria for parametric testing were not met, the data were either log-transformed to meet the criteria or analysed by non-parametric methods. Data that deviated markedly from other observations (having a z-score with an absolute value greater than 3), i.e. outliers, were removed from the dataset. For non-parametric testing, that are based on the group median and not the mean, outliers were generally kept, except when there were reasons to believe that the sample did not represent biological variation. The specific tests used to analyse individual experiments are described in each paper. Statistical analyses were performed using either Prism (GraphPad Software, San Diego, CA, USA) or JMP software (SAS Institute Inc, Cary, NC, USA).

3

Synopsis, results

Paper I

As most fish tissue cultures are based on mammalian protocols, in this study, we aimed at developing a more physiologically relevant primary cell culture of the Atlantic cod (*Gadus morhua*) pituitary. Imitating cod plasma parameters, we adjusted the working solutions to a pH of 7.85 and an osmolality of 320 mOsm, and the incubation atmosphere to 0.5% CO_2 at 12°C. By these simple alterations of culture conditions, the cells remained viable for over two weeks. Compared to cod pituitary cells cultured under standard conditions, cell viability was enhanced, resting membrane potential was stabilized, and the proportion of cells firing action potentials was increased seven-fold. In addition, the cells showed stable responses to Gnrh over the two-week period, measured by the relative increase in cytosolic Ca^{2+} concentration following activation of the Gnrh receptors. Addition of serum supplements to the media did not affect the resting membrane potential or the number of surviving cells, however, it did increase metabolic activity and membrane integrity compared to cells incubated serum-free. Measured over a 14-day period, serum substitute appeared a more beneficial supplement than new-born calf serum regarding gene expression. However, until day 7, no difference was seen between cells incubated in serum-free or serum substitute supplemented media.

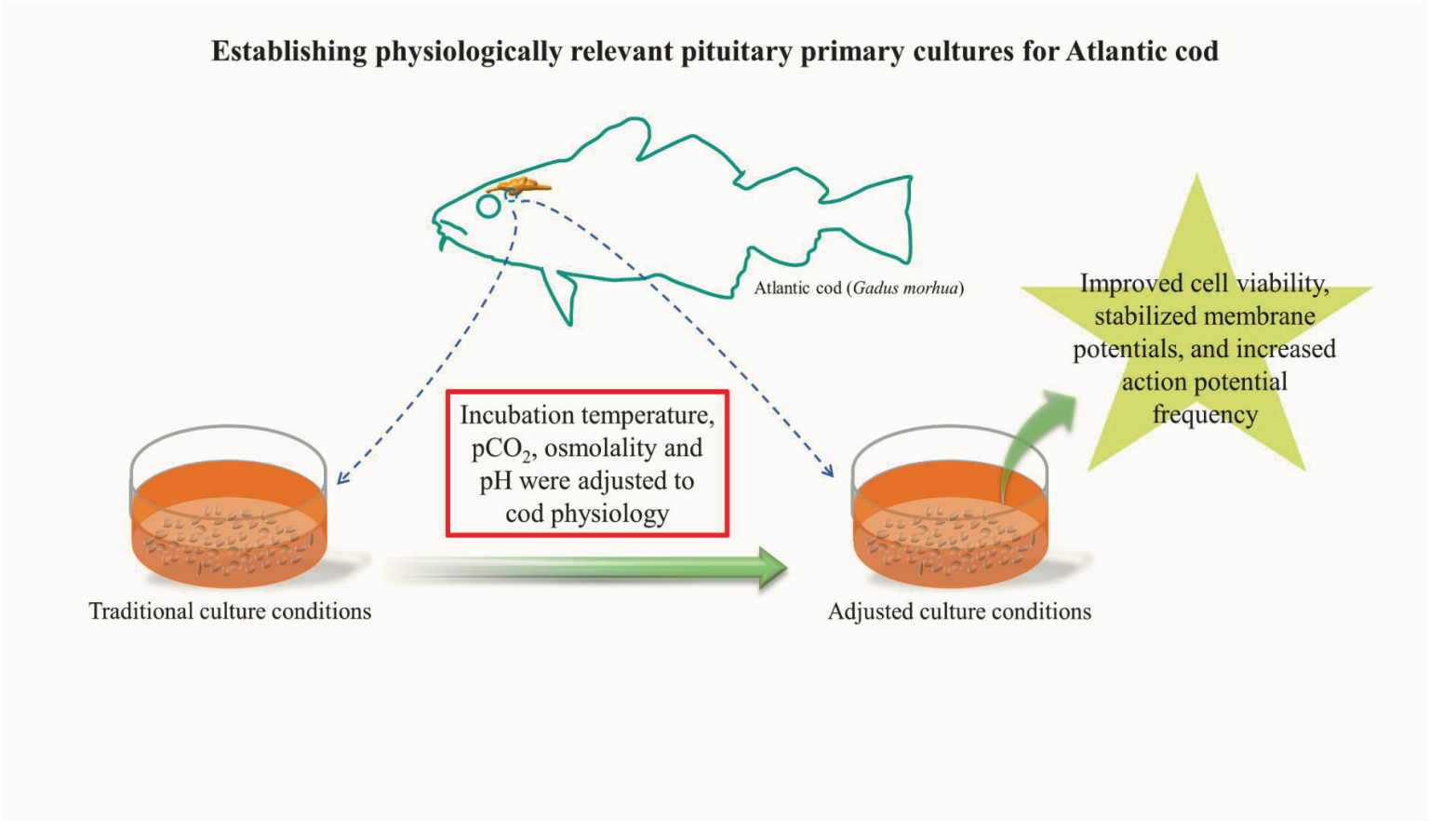

Figure 3.1 Graphical abstract, paper I

Paper II

Sex steroids exert feedback from the gonads to the higher levels of the BPG axis. The nature of the feedback depends on many factors, such as species, sexual maturity stage, concentrations and others. Since little is known about the steroid feedback in Gadiform fish, in this study, we aimed at investigating potential direct effects of two physiologically relevant concentrations of estradiol (E2), testosterone (T) and dihydrotestosterone (DHT) on cell viability and reproductive gene expression (*fshb*, *lhb*, *gnrhr1b and gnrhr2a*) in Atlantic cod pituitary, using the primary culture system from **paper I**. Cultures were prepared from fish at three different sexual maturity stages (maturing, mature and spent). The results demonstrated that all steroids stimulated cell viability throughout the reproductive season, except in cells from spent donors, where the androgens had no effect. Gene expression levels were differentially affected dependent on steroid treatment, gene in question and donor maturity stage. Only DHT affected *lhb* transcript levels, and only in cells from mature fish, while all steroids affected *fshb* levels at some point. This is the first time effects on *fshb* expression from a non-aromatizable androgen has been demonstrated in a multibatch spawner *in vitro*. Using single cell qPCR, both Gnrh receptors were found to be co-expressed in several gonadotropes. However, the results point to Gnrhr2a as the main gonadotropin modulator in cod, both because it was the dominating Gnrh receptor expressed in gonadotropes, and because its expression levels were regulated by all the sex steroids, while the expression levels of *gnrhr1b* remained unaffected by the same exposures.

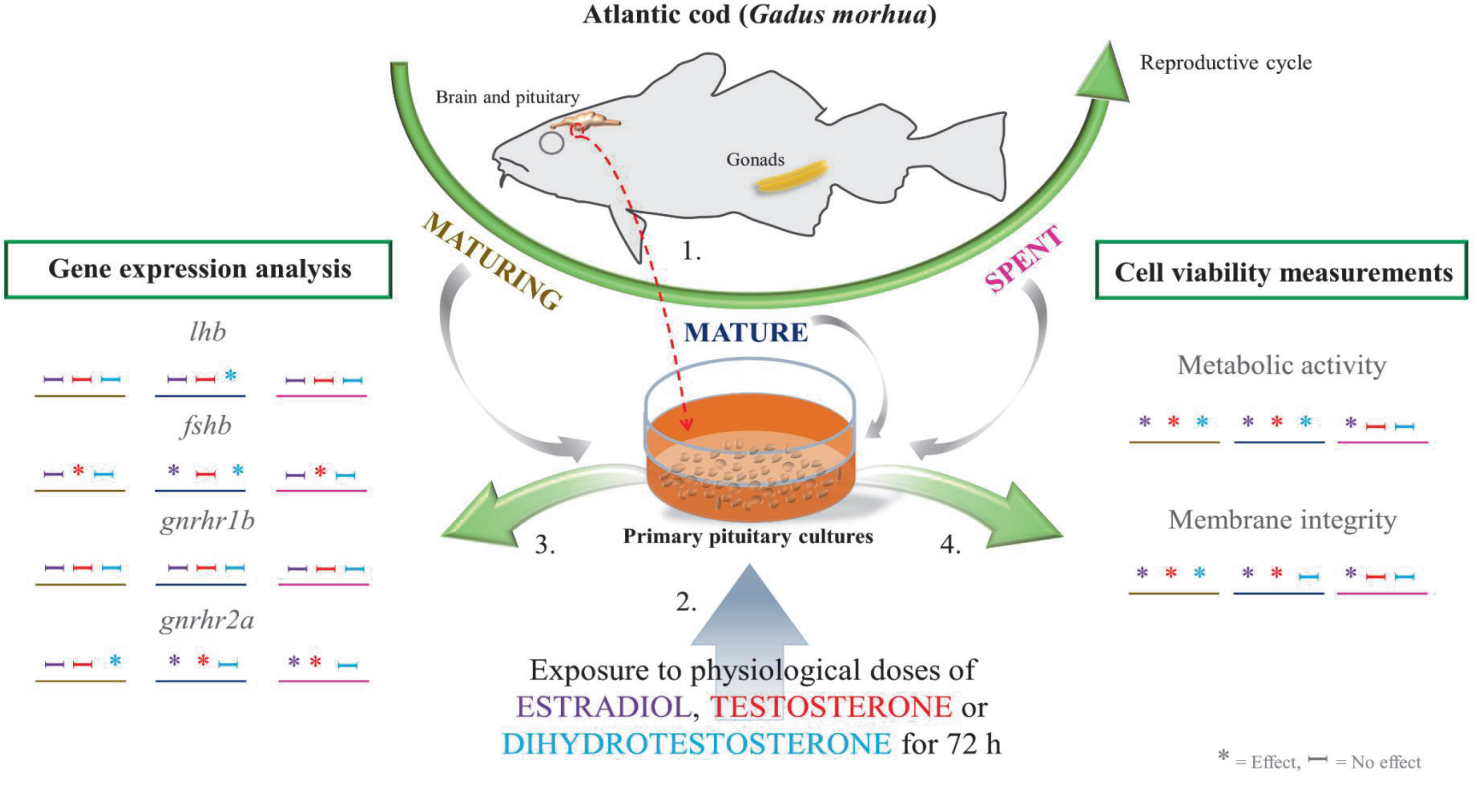

Figure 3.2 Graphical abstract, paper II

Paper III

During stress, plasma cortisol (F) levels rises. F is known to affect all levels of the BPG-axis but with differential effects reported based on species, duration, concentration and maturational stage, amongst others. In Atlantic cod, high F concentrations are associated with altered reproductive behaviour, lowered fertilization and increased number of abnormal larvae. As the effect of F on the higher levels of the BPA-axis is not known in this species, we aimed to investigate the potential direct effects of basal and stress F levels at the pituitary level. Using the pituitary system from **paper I**, we prepared cultures from donors at different stages of sexual maturity (maturing, mature and spent) and measured effects on cell viability and reproductive relevant gene expression (*fshb*, *lhb*, *gnrhr1b and gnrhr2a*).

Basal F levels did not affect cell viability, whereas elevated stress-induced levels stimulated both cellular metabolism and membrane integrity. Gene expression was dose-dependently activated and varied with the stage of donor sexual maturity for all genes except *lhb*, where no effects were detected. The effects of F treatment on *fshb* expression were dual; inhibiting in cells from maturing and spent donors but stimulating in cells from sexually mature fish. The transcript levels of the Gnrh receptors were affected only in cells from spent donors, with *gnrhr1b* being downregulated by basal F levels and *gnrhr2a* upregulated by stress F levels. This is the first teleost study to look at the expression of Gnrh receptors in the pituitary following F treatment.

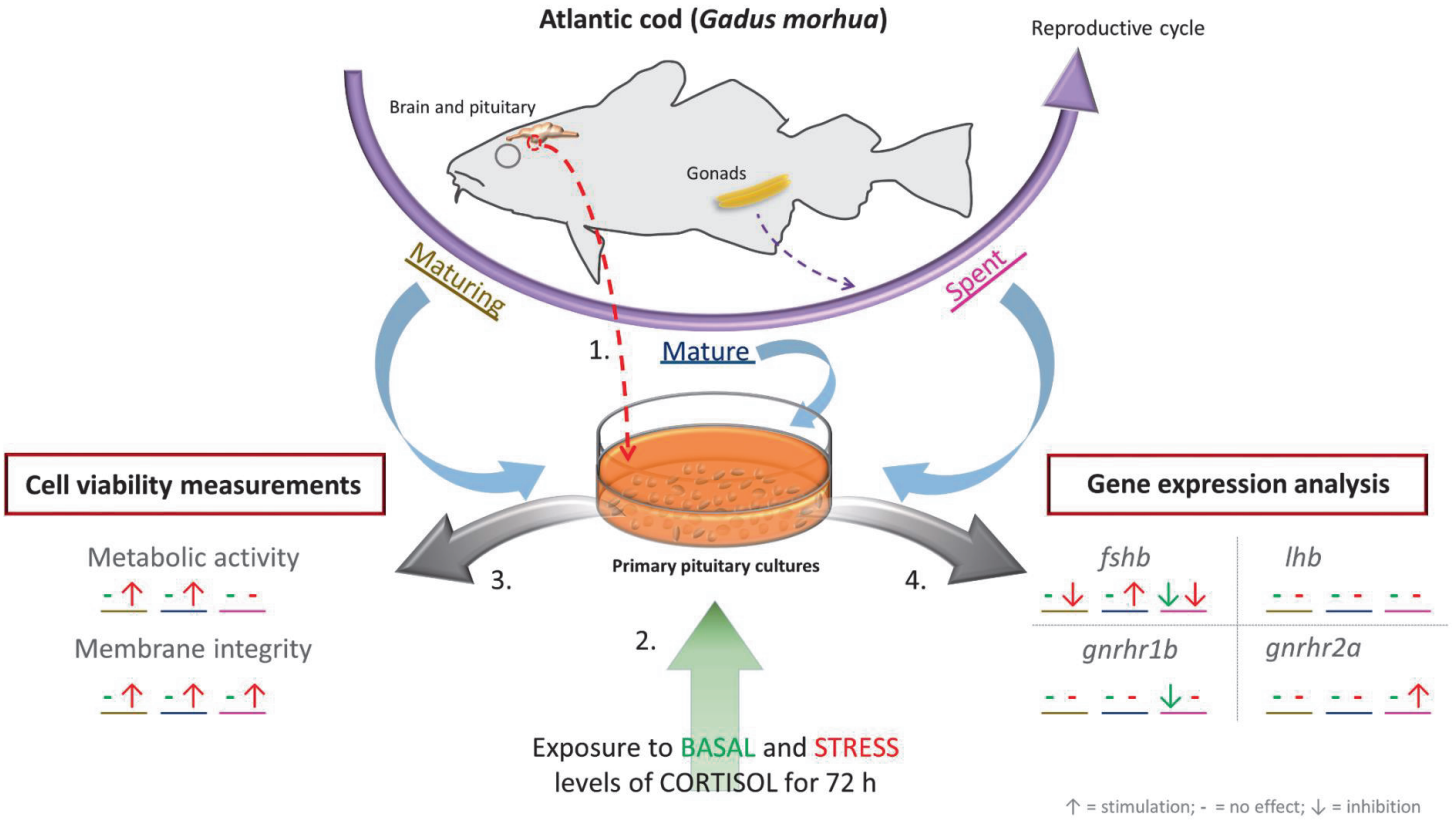

Figure 3.3 Graphical abstract, paper III

Paper IV

The last decades have witnessed increasing concern over the potential endocrine disruptive effects of industrial chemicals in our environment. The aim of this paper was to investigate if the widely used chemicals bisphenol A (BPA) and its brominated derivative, tetrabromobisphenol A (TBBPA), could potentially affect pituitary cell viability and reproductive gene expression (*fshb*, *lhb*, *gnrhr1b and gnrhr2a*) in Atlantic cod, using the pituitary system from **paper I**. While effects from BPA exposure on reproduction have been studied in several species, this is the first vertebrate study investigating its effects on the pituitary *in vitro*. For TBBPA, there are no previous studies in fish looking at the higher levels of the BPG-axis neither *in vivo* nor *in vitro*, and only two studies from other vertebrate taxa. This is the first vertebrate study to assess potential effects from TBBPA exposure on the pituitary *in vitro*, and the first to look at potential TBBPA effects on the expression of Gnrh receptors in general. At environmentally relevant concentrations, both bisphenols stimulated cell viability. Exposure at higher concentrations found an approximate LC_{50} at 10^{-4}M for BPA and 10^{-5}M for TBBPA, indicating increased cytotoxicity from bromination for these cells. In terms of gene expression, BPA and TBBPA had differential effects; BPA stimulated *gnrhr2a* expression, but had no effect on the other genes investigated, whereas TBBPA stimulated the transcript levels of both gonadotropins, but did not affect expression of the Gnrh receptor genes, except through a downregulation at cytotoxic levels.

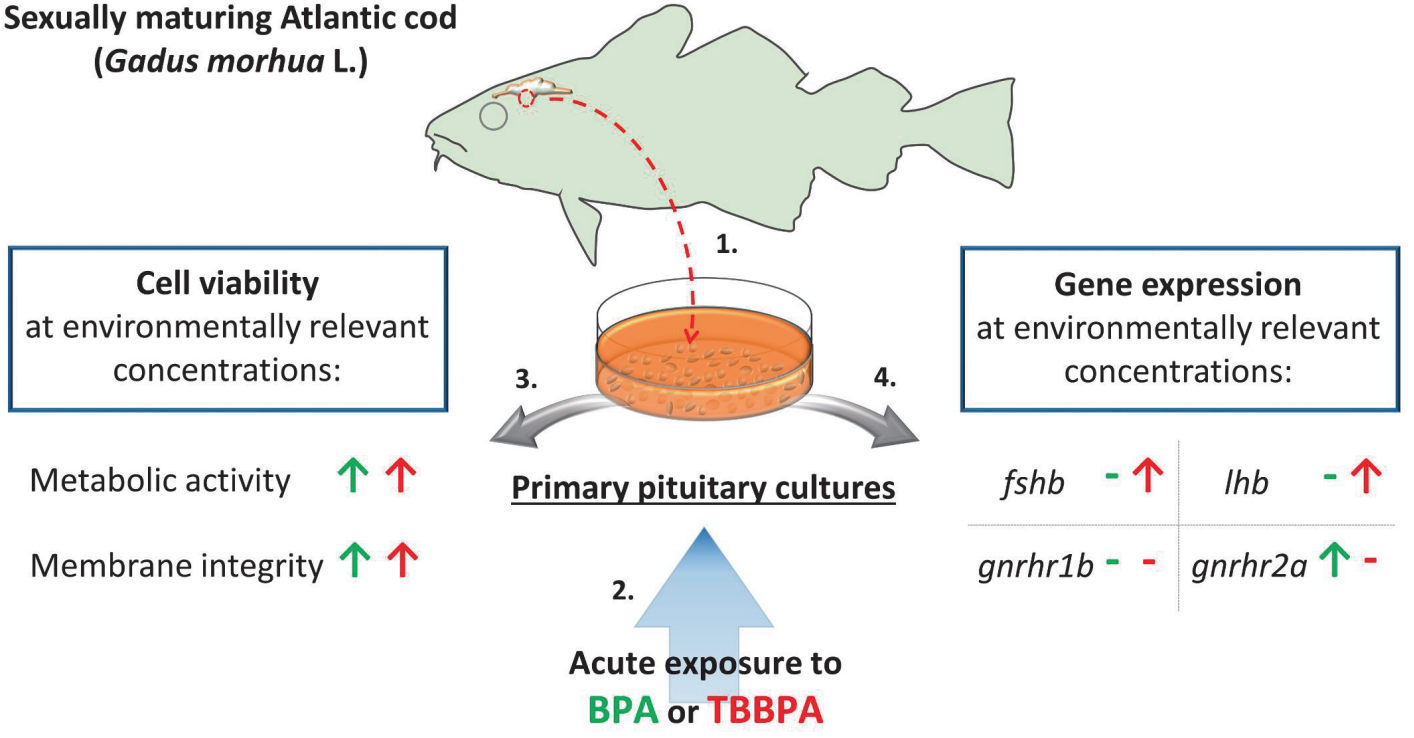

Figure 3.4 Graphical abstract, paper IV

4

Methodological considerations

4. Methodological considerations

While the general theory, modifications and advantages/disadvantages of the methodologies used in this thesis are described in chapter 2, some aspects of the experiments require a separate discussion, which will be covered in this section.

4.1 The utility of primary cultures

The term "*in vitro*" (Latin: "in glass") refers to the study of microorganisms, organs, tissues or cells outside their biological environment. These methods can be used to model various characteristics of the more complex *in vivo* (Latin: "within the living") condition. To study an organ, such as the pituitary, *in vitro*, it can be cultured either intact, as sliced or chopped tissue, or as dispersed cells. For this thesis, we chose the latter. There were several advantages to using primary cultures instead of living animals for our experiments. First, to reveal mechanisms within the BPG-axis, it is important to localize in which part(s) of the axis the regulation occurs. *In vitro*, without interference of multiple variable factors otherwise present in the intact animal, the experimental conditions can be controlled in a manner not possible *in vivo*, and direct mechanisms can be assessed (Baksi and Frazier, 1990). Second, cultures are cost effective and environmentally friendly, as small quantities of test chemicals are needed and quantities of toxic wastes are reduced. These two first points apply to all forms of primary culture, while the next concerns dispersed cells. Third, dissociating the pituitary yields millions of cells, offering multiple replicates from the same source, which allows for testing of several parameters simultaneously with low biological variation between experimental groups. While this is even more true for cell lines, where cells basically are clones, and most biological variation is removed, cell lines are often based on metastatic or genetically manipulated cells, which, after several generations and passages, may not be as physiologically relevant as their primary source (Kaur and Dufour, 2012). Fourth, the many cells generated by one pituitary reduces the number of animals sacrificed per experiment. According to the European Commission (2020), the number of vertebrate animals used for research and testing in the European Union (EU) exceeded 9 million annually from 2015 – 2017. This does not include animals utilized for organs or tissues, or animals bred and killed without being used in experiments. The 3R's (Replace, Reduce, Refine), first described by Russel and Burch (1959), are guidelines for an ethical approach to animal testing and part of the European Union's legislative framework for research animals (EU, 2010). Not only does

the use of primary cultures reduce number of killed animals, it also improves animal welfare by excluding potential pain, stress and discomfort from experimental treatments, such as toxicological assessment. Intact organ cultures also contribute to the latter, but do not reduce the number of animals needed for experiments, as each organ is one sample.

While primary cultures are a powerful tool, they have limitations. Compared to cell lines, which offer consistent samples and reproducible results, primary cultures have more biological variation, and are cost and labour intensive. Compared to *in vivo* experiments, primary cultures have several shortcomings and cannot replace animal testing entirely. The results achieved in culture can give mechanistic and physiological insight, but as systemic input and regulation are removed, the results do not necessarily reflect results from similar tests *in vivo*. The conditions for Atlantic cod pituitary cultures were improved by our work in **paper I**, and the cells in these experiments are cultured in a more physiologically relevant environment than most traditional fish cultures. This does not mean that the *in vivo* condition is mimicked. In addition to the lack of plasma nutrients, input from hypothalamic neurons, hormones and other factors while in culture, the dissociation process reduces cell-cell interactions and removes the connecting network of follicular stellate cells that aids intercellular communication (Golan et al., 2016) and gap junction connections. Dissociation can also disrupt membranes and may induce necrosis and apoptosis. Furthermore, exposure doses *in vitro*, even if adjusted to plasma levels, would likely differ from doses in the intact body, as other available molecules could interact with the compounds, and the liver, kidney or gills could metabolise and/or excrete them (Colombo et al., 1972; Evans et al., 2005; Tokarz et al., 2015; Zhang et al., 2018b).

Another issue associated with primary cell cultures is the sample size in statistical analysis. In our case, every experiment consisted of multiple wells created from a common pool of dispersed pituitaries and each well were considered a sample. Making separate cultures for each fish was not an option as one pituitary does not contain enough cells for all controls and treatments. Pooling pituitaries was therefore necessary. While this gives enough cells for several replicates, wells prepared from one pool are not considered statistically independent and may represent a form of pseudoreplication (Hurlbert, 1984). What constitutes pseudoreplication is a complicated issue and evaluating the strength of the experimental design is not straight forward. The classical example of pseudoreplication is treating technical replicates, i.e. repeated measurements from one sample, as individual samples. In our experiments, all technical replicates from individual wells were averaged into one observation. An experimental unit, i.e. one sample, is “the entity that is randomly and

independently assigned to experimental conditions" (Lazic et al., 2018). In cell cultures prepared from cell-lines, where all cells essentially are clones, one experiment is considered the experimental unit and each well a technical replicate. This can also be the case for experiments sampling individual animals, such as fish, if those fish are raised or experimentally treated in the same tank (Hurlbert, 1984). For this thesis, we used wild fish caught randomly from the sea. These fish have individual life histories, and likely have higher levels of biological variability than fish reared in fish facilities with low genetic heterogeneity and uniform living environments. Furthermore, since the pituitary consists of many cell types and each primary culture was prepared from pooled pituitaries from several fish, each culture well contained a unique mixture of cells, all randomly assigned to each well and treatment. As gene expression and cell viability occur at the level of the cell, the cells themselves can be considered the biological unit, and each well the experimental unit, for our experiments. In cell culture, there is a chance that the wells can be artificially homogenous. While this is more common for cell-lines, I assessed the biological variation in our cultures by calculating the variation in gene expression levels between technical replicates from individual wells and between different wells exposed to similar treatments. The technical replicates differed by a maximum of 4% (average 0.26% ± 0.69, n = 68), while average variation between wells belonging to the same treatment group was 17.1% (± 9.63) (not published). The Cq-levels of the reference genes were stable between wells, indicating an even number of cells per well. This suggests that the variation between wells was a result of biological variation. Even with the random distribution and the biological variation, for genuine replication, experimental treatments should be applied independently to each experimental unit, and those units should not influence each other (Lazic et al., 2018). It is unlikely that the wells could influence each other in our set-up, however, the cells within one well could, and "cell" is therefore not considered an experimental unit. But, while each well was exposed independently, every treatment was prepared from one solution, meaning that if something was wrong with that solution, this would affect all samples belonging to the corresponding treatment group. Furthermore, all plates and dishes from one experiment were incubated simulatiously, meaning that if something was wrong with the incubator parameters, this would affect all cells. Because of this potential correlation of treatment errors, "wells" do not meet all criteria for statistical independence. However, it might be that this is negligible, as treatment errors in many cases would be detected, as, for instance, the incubators have detectors and alarms. Nevertheless, incorrect use of experimental units is a huge source of type I errors, i.e. false positives, in literature, and caution should be taken when designing

experiments and performing statistical analysis (Hurlbert, 1984). In our first papers, we did not address these issues in the statistical analyses and performed the standard ANOVA (Analysis of variance), a test that requires independence, to assess differences among the group means. Later, after consulting with a statistician, we learned that we could correct for the non-independence by running the ANOVA weighted for the wells within each culture, which, in practice, is an ANCOVA (Analysis of covariance) with wells as the covariate. It is worth mentioning that I redid the analysis for previous papers and compared the statistical results from the ANOVA vs the ANCOVA, and only minor differences were detected. This is reassuring for the statistical results reported in the other papers. With that said, in biological experiments, there is growing concern that statistical significance and the p-value is given too much emphasis (Amrhein et al., 2017; Amrhein et al., 2019). Biological significance does not necessitate statistical significance, and statistical significance does not demonstrate biological significance. The desire to categorize effects as either significant or non-significant implies that biological responses are binary. This can lead to the wrong conclusion that treatments "have no effect" unless they reach statistical significance. Biological systems, however, can respond with gradual, nuanced and differential effects, and with vast variation. Using model systems with low biological variability, e.g. cell-lines, makes potential effects from treatment easier to detect, and increases the probability for achieving statistical significance, but does not reflect the true biological population. Primary cultures, with higher biological variability and more biological relevance, are in such cases better models.

In summary, primary cultures are a powerful tool for physiological and toxicological testing and can provide valuable mechanistic data for the design of further *in vivo* studies, but care must be taken in the design of the experiment to mimic the biology of the animal in question, assure biological variation and true replication, and to interpreted results in the right context.

4.2 Mixed sex cultures

All primary cultures were prepared from pools of pituitaries from both sexes. In culture, the gonadal steroids, as well as any other systemic input, are removed. It is not known if isolated Atlantic cod pituitary cells behave differently between the sexes while in culture. As there is no or little sexually dimorphic external anatomical traits in Atlantic cod, sex can usually not be determined (except in sexually mature specimens) without gonadal inspection, i.e. dissection. In these experiments, wild cod were used and access to fish was

limited. Both practically and ethically, a low number of sacrificed animals was desired, so we chose to separate cultures based on maturational stages only. At the start of our experiments (in 2010), there was little available information on sex differences of cultured teleost pituitary cells. In multibatch spawners, most studies investigating gonadal steroid feedback *in vitro*, were performed on mixed sex cultures (Huggard-Nelson et al., 2002; Huggard et al., 1996; Lin and Ge, 2009; Sohn et al., 2001). In studies looking at one sex only, information for the other sex was lacking, and comparison could not be made (see **paper II,** Tables 2 and 3). Regarding effects from F, BPA or TBBPA exposure, there were, to my knowledge, no teleost studies describing sex differences at the pituitary level *in vitro* either in multibatch species or other spawning types. There are, however, studies on rat pituitaries, which have demonstrated sex differences in gonadotropin secretion following F exposure *in vitro* (D' Agostino et al., 1990; Suter and Schwartz, 1985a; Suter and Schwartz, 1985b). The available information on sexual differences in gene expression of gonadotropin subunits or Gnrhr in culture is generally scarce, also in fish with other reproductive strategies. An exception is found in a study of the masu salmon, a single spawner, where such sex differences were investigated *in vitro* (Ando et al., 2004). The results demonstrated that, following E2 exposure, *fshb* levels were equal between the sexes, while *lhb* levels increased at lower E2 concentrations in males than females. For gonadotropin hormone release, there are more *in vitro* studies available that compare sex dependent effects (Ando et al., 2004; Baker et al., 2000; Levavi-Sivan and Yaron, 1993; Weil and Marcuzzi, 1990a; Weil and Marcuzzi, 1990b). These studies suggest that there are some effects of donor sex on pituitary cells *in vitro*, but the results seem dependent on species, endpoint, exposure and stage of sexual maturity.

To my knowledge, no new reports comparing teleost sex dependent *in vitro* effects on viability or reproduction-related gene expression have emerged since the publication of **papers II-IV**. In hindsight, it is therefore regrettable that we didn't separate the cultures based on donor sex, for several reasons; first, there is a general lack of information on sex differences in teleost pituitaries *in vitro*, especially for multibatch species. Second, no information about potential sex-dependent regulation in Atlantic cod pituitary could be provided. Third, potential sex differences might have masked effects in our studies. However, sex differences cannot create an effect where there is none. Therefore, the results from **papers II-IV** demonstrate that both steroids and environmental pollutants can act directly at the pituitary in Atlantic cod but cannot contribute knowledge as to whether these effects are sex dependent.

4.3. The use of ethanol (EtOH) as a solvent

To be able to expose the pituitary cells to non-polar compounds, we had to use an organic solvent. The water solubility at 25 °C for the compounds used in this thesis (**papers II-IV**), are listed in Table 3. The solubility of the compounds at 12 °C, at which our solutions were prepared, have not been reported, but solubility has been shown to decrease with decreasing temperatures (Corrales et al., 2015; European Union, 2006; European Union, 2008; Shareef et al., 2006; WHO/ICPS, 1995). Although the solubility for DHT and F are higher than that of E2 and T, and BPA higher than TBBPA, we wanted to use a common solvent control, with similar concentration, for all experiments, both to save cells when experiments were run in parallel, and to be able to compare effects between the different compounds. There were two main reasons to why we chose to use EtOH as the solvent. First, all the above-mentioned compounds readily dissolve in EtOH. Second, EtOH is the most commonly used solvent in these types of studies, with reported EtOH working concentrations ranging from 0.01 to 1% (see supplementary Table 1, **paper III**, for solvent use in cortisol exposure studies). Unfortunately, in our experiment, the working concentration of 0.2.% EtOH affected both cell viability parameters, and for two cultures in **paper III**, also the gene expression. We did not react to this during the experimental period, because the effects appeared moderate (see for instance supplementary Figure 1 and 2, **paper IV**). Furthermore, the effects were dependent on stage of sexual maturation. In maturing and mature fish (**papers II-IV**), no effects were seen on gene expression, while gonadotropin gene expression were elevated by the solvent in cells from spent donors in the cortisol experiment (**paper III**). In terms of cell viability, both metabolic activity and membrane integrity were negatively affected, regardless of maturational stage. For maturing fish, however, the effect seemed more pronounced (**papers II and III**). These cultures had a surplus of females, so the negative effect on viability could be a result of maturational state and/or that female cells are more sensitive to EtOH treatment. It is uncertain why negative effects were evident for viability, but not gene expression. The pituitary cultures consisted of all pituitary cell types, so one hypothetical explanation is that cells other than the gonadotropes and those that express *gnrhr* were more affected by the EtOH treatment, but this remains to be tested.

In the literature, there is a lack of thorough disclosure regarding solvent control use and effects. First, many studies do not state if a solvent is used (for instance; Levavi-Sivan et al., 2006; Lin et al., 2010; Lin and Ge, 2009). Of course, in some cases it is possible that the exposure compounds were dissolved without solvent use, which, depending on the dose and

polarity of the compound, is feasible. Other times, there would clearly be need for a solvent and the information is just omitted. Second, if the use of a solvent is reported, its working concentration is often lacking (for instance; Ando et al., 2004; Huggard-Nelson et al., 2002; Huggard et al., 1996; Iwakura et al., 2010). Third, it is often unclear whether the control has been adjusted to match the solvent content in the exposed groups. Forth, the effects of the solvent on the model system are not described, i.e. a medium blank control is not included. For my experiments, had the medium blank samples not been included in the experimental design, the negative effects from EtOH would not have been discovered. Furthermore, without the viability testing, the effects would probably have gone undetected. It is therefore a bit alarming that cell viability and the use, concentration and effects of solvents are both so underreported in exposure studies. We therefore emphasized these topics in methodological considerations of each paper and recommended a lower EtOH working concentration for future studies.

Table 3. Water solubility at 25 °C for the exposures used in this thesis (paper II-IV)

Compound	Solubility	Reference
E2	1.5 - 4 µg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/Estradiol#section=Solubility
T	23 µg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/Testosterone#section=Solubility
DHT	535 mg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/Androstanolone#section=Solubility
F	320 µg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/5754#section=Solubility
BPA	120 - 300 µg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/6623#section=Solubility
TBBPA	4.16 µg/mL	https://pubchem.ncbi.nlm.nih.gov/compound/6618#section=Solubility

Another issue with solvent use is that potential interaction effects between solvent and treatment are always possible, regardless of the concentration of the solvent. In order to test such a hypothesis in our system, an identical set-up without EtOH (or different concentrations of EtOH) would be required. For some of the exposures, it should be feasible to dissolve them at low concentrations without EtOH. Such an experiment would not only be mechanistically informative in regard to all the studies in which EtOH is used as a solvent, but additionally, as the combination of the compounds used in this thesis and EtOH is quite commonly present in humans *in vivo*, it could be of physiological importance as well.

5

Discussion

5. Discussion

The work presented in this thesis describes the development of a physiologically relevant model system of the Atlantic cod pituitary and the subsequent use of the established *in vitro* system to test potential effects from reproductive and reprotoxic agents.

5.1 Pituitary primary cultures

There are several reasons as to why we wanted to study the reproductive physiology of the Atlantic cod pituitary. First, to increase basal knowledge about reproductive regulation and mechanisms in the pituitary, which in turn can provide insight into the reproductive regulation of the entire organism. Second, because of the challenges regarding precocious sexual maturation in the aquaculture industry, to increase our understanding of the causal factors influencing sexual maturation. Third, as fish stocks are declining world-wide, to increase knowledge about potential impact of anthropogenic activities, such as pollution. Furthermore, while Atlantic cod is an economically and culturally important species, and worth investigating in its own right, due to the many physiological similarities between fish and other vertebrates regarding pituitary reproductive function, the cod pituitary can also serve as a model for other species. This is especially true for other Gadiformes fish. Despite including many other commercially important species, such as haddock (*Melanogrammus aeglefinus*), Atlantic pollock (*Pollachius pollachius*), saithe (*Pollachius virens*), and European hake (*Merluccius merluccius*), several of these listed as strong candidates for future cultivation (Kjesbu et al., 2006), the Gadiformes is a relatively little studied order.

In order to assess pituitary reproductive regulation, we started by developing an optimized protocol for the primary culture of cod pituitary cells. Adjusting the incubation parameters to mimic the natural environment of cod pituitary cells, especially in terms of pH, pCO_2, osmolality and temperature, we successfully established a healthy, stable and physiologically relevant primary culture system. The quality of the system was confirmed through physiologically reasonable gene expression patterns of *fshb*, *lhb* and *gnrhr2a*, stable resting membrane potentials and Gnrh responses, as well as enhanced cell viability and an increased proportion of action potential firing cells compared to cells cultured under traditional conditions. Moreover, the cultures remained viable for over two weeks (**paper I**). We are not the first to optimize pituitary culture condition based on physiology, e.g. Weil et al. (1986) adjusted the culture media osmolality and pH to match rainbow trout physiology. However, this study did not describe the incubation pCO_2 or whether the pH of the solution

was adjusted at incubation temperature, so the final pH is not certain. Nevertheless, while the protocol from Weil et al. was available for over two decades prior to our work, it has generally not been incorporated into the standard laboratory routines of the fish research community. Most commonly, in *in vitro* studies, only temperature is adjusted to match the physiology of fish. It is our hope that the work presented in **paper I** will establish a useful guideline and validated protocol for others studying piscine cells and tissues, leading to more physiologically relevant cultures and results. Furthermore, especially for widely used teleost model species like zebrafish, medaka and tilapia, optimizing culture protocols for each species, and standardizing these protocol parameters across labs and experiments, would likely reduce experimental variation and hence save resources and number of animals needed for experiments (addressing the 3Rs). Since the completion of **paper I**, our group has developed an optimized protocol for culturing the medaka pituitary (Ager-Wick et al., 2018).

5.2 Exposure studies

Direct effects of sex steroids, F and suspected EDCs at the pituitary level, have, to my knowledge, not previously been studied in Atlantic cod. This thesis also includes the first vertebrate study to describe reproductive effects at the pituitary level from BPA and TBBPA exposure *in vitro*. All treatments described in **papers II – IV** were able to affect both cell viability and gene expression, demonstrating that the cod pituitary can be a site for direct action of steroid feedback, stress and pollution, with the potential to modulate Atlantic cod reproductive function.

Cell viability measurement is often under reported in *in vitro* studies, hence we have emphasized its importance in our manuscripts. As mentioned in section 4.3, without such measurements, we would not have discovered the negative effects the solvent use had on our system. Given the potential harmful effects of solvent exposure on natural cell function, it is concerning that the impact of solvent use on cell health is omitted from many publications on fish *in vitro* studies. Regardless of solvent use, cell viability can be affected by many other factors, such as the method of cell dispersion, incubation conditions, and experimental treatment. Common for all experimental treatments in **papers II-IV** was that they generally reversed the negative effects on cell viability from the solvent. Negative effects on pituitary cell membrane integrity and metabolic activity from the experimental treatments were detected solely after exposure to high doses of BPA and TBBPA, 10^{-4} to 10^{-3} M and 10^{-5} to 10^{-4} M, respectively (**paper IV**). Bromination appeared to increase the cytotoxic effect, as the

lethal concentration at which approximately half the cells died (LC_{50}) was about ten-fold lower in TBBPA vs BPA exposed cells. However, comparable concentrations of such exposures are environmentally present only at extremely polluted sites, i.e. landfill leachates or similar (Crain et al., 2007; Morris et al., 2004). The cytotoxicity levels for cod pituitary cells were in concert with earlier reports, e.g. in a zebrafish hepatocyte cell line (ZFL), the 96 h LC_{50} was found to be ~3.58×10^{-4} M and 4.2×10^{-6} M, for BPA and TBBPA, respectively (Yang and Chan, 2015). At other doses tested, both BPA and TBBPA had a positive effect on cell viability. This was also the case for stress levels of F and both exposure doses of E2, T and DHT. Increased metabolic activity and membrane integrity can be measurements of enhanced health of the individual cell, but it can also reflect cell proliferation when measured upon a population of cells. While we did not test for proliferation in our set-up, there are previous findings that support this possibility. For instance, in prenatal mice, BPA stimulated gonadotrope proliferation (Brannick et al., 2012), and in chicken embryos, E2 increased pituitary cell numbers (Wu et al., 2009). In teleosts, the pituitary grows through life (Cavaco et al., 2001; Golan et al., 2014; Nozaki et al., 1990), and in the medaka, it has recently been demonstrated that 100 ng/ml E2 exposure stimulates Lh cell proliferation in both juvenile and adult fish (Fontaine et al., 2019).

Potential effects on cell viability from gonadal steroids and F were assessed over the reproductive cycle. Basal levels of F did not affect cell viability at any maturational stage, whereas stress levels stimulated both metabolic activity and membrane integrity at all stages. While E2 stimulated cell viability throughout the cycle, the androgen effect was seasonally dependent, with stimulating effects during the maturing and mature state, but with no effect in cells from spent fish. During maturation, the circulating levels of androgens in cod are high in both sexes (T in females, both 11-KT and T in males), while they drop close to zero after spawning (Almeida et al., 2009; Dahle et al., 2003; Norberg et al., 2004), in close concert with Gth subunit gene expression (Mittelholzer et al., 2009). In cultures made from spent donors, sex steroid and F treatment were not able to stimulate Gth gene expression (**papers II and III**). It is therefore tempting to speculate that an increase in cell viability is connected to increased synthesis and/or release of Gth. However, E2 and high F dose stimulated cell viability even in the spent stage. This could reflect cell proliferation, as described above, or that other cell types in the pituitary, for instance corticotropes, were affected by the treatment. It seems possible that viability-stimulated mechanisms are linked to factors that varies with the stage of sexual maturity, such as seasonally fluctuating expression levels of Ers or androgen receptors (Ars). For instance, in the largemouth bass (*Micropterus salmoides*), the

gene expression levels of two Er isoforms, Erα and Erβ, were seasonally dependent (Martyniuk et al., 2009), but in cod, this remains to be assessed. For F, it seems that such factors are also dose-dependently activated, as only stress, and not basal, levels were able to affect viability (**paper III**). In contrast, basal F levels were able to affect gene expression in cells from spent fish. F can bind to glucocorticoid receptors (Gr) or mineralocorticoid receptors (Mr) in their target cells, both of which are detected in teleost pituitary cells, including gonadotropes (Pepels et al., 2004; Stolte et al., 2008; Teitsma et al., 1999). In the common carp, one Mr and two Gr isoforms were expressed in the pituitary, all with different sensitivity to F (Stolte et al., 2008). This suggests that, depending on which receptor is activated, a differential cellular response could be initiated. While this is presently unstudied in Atlantic cod, it could potentially offer an explanation to the differential effects seen from F exposure. In addition to its dose-dependent effect of cell viability, stress levels of F were also able to induce both stimulating and inhibiting effects on *fshb* expression.

Because of its ability to act as an estrogen agonist and bind to Ers, BPA is classified as a xenoestrogen (Crain et al., 2007; European Union, 2008; Kloas et al., 2000). It is therefore interesting to compare the results from BPA exposure in **paper IV** to those from E2 exposure in **paper II**. BPA exposure ranged from 10^{-9} to 10^{-3} M, while E2 exposure was 2.5 and 25 ng/ml (~ 9.2×10^{-9} and 9.2×10^{-8} M, respectively). BPA exposure was assessed only in cultures prepared from maturing fish, so the comparison will be made to the equivalent cultures for the E2 exposures. In these cultures, E2 had no effect on gene expression but a stimulating effect on cell viability at 25 ng/ml. BPA on the other hand, stimulated *gnrhr2a* expression from 10^{-7} to 10^{-5} M and cell viability from 10^{-9} to 10^{-5} M. This suggests that BPA either binds to the Er in cod pituitaries with higher affinity than E2, or that it affects cell viability and gene expression through some other mechanism. TBBPA was also able to affect gene expression. Unlike BPA, TBBPA enhanced gene expression of both Gths, even at the lowest dose of 10^{-9} M. From the literature, there is only one available study on the effects of TBBPA exposure on Gth, an *in vivo* study on sexually mature male frogs (*Rana nigromaculata*) (Zhang et al., 2018a). This study described decreasing serum LH and FSH concentrations following TBBPA exposure, in opposite direction of our findings. However, there are many differences between the Zhang et al. study and ours, regarding species, *in vivo* vs *in vitro*, protein vs gene, and mature vs maturing models, making direct comparison difficult. As both BPA and TBBPA were able to affect pituitary cellular responses at environmentally relevant concentrations in **paper IV**, the effects and mechanisms of these chemicals should be further assessed in future studies.

While TBBPA was able to affect *lhb* expression, this was generally not achieved by the hormonal treatments in **papers II** and **III**, regardless of exposure dose and pituitary donor maturational stage. An exception was DHT, that stimulated *lhb* levels at both doses, but only in cells from mature donors. DHT, like 11-KT, is a non-aromatizable androgen, so it is likely that this action was facilitated by the Ar. As the effect was seasonally dependent, this suggests a seasonally dependent regulation of the Ar or other facilitating factors, as well. Neither E2, T nor F affected *lhb* levels in a statistically significant manner. However, a small (not statistically significant) stimulatory effect was observed in cells from mature fish after high dose E2 and F exposure. From the literature on steroid feedback in multibatch spawning species, the effects from E2 and T *in vitro* seem dependent on species, dose, maturational stage and length of incubation, as both positive, negative, and no effects have been detected after exposure regimes similar to ours (see Tables 2 and 3, **paper II**). In fish, there is little information regarding the effects from F on *lhb* expression. To my knowledge, there is only a single study available, performed on juvenile European eel pituitary cultures (Huang et al., 1999). That study found a positive effect on *lhb* transcripts after 12 d 10^{-6} M F incubation, but as the eels were juvenile, the incubation time longer and the exposure was 10-fold higher than our highest dose, a direct comparison to our study cannot be made. For Lh synthesis and release after stress or F exposure, on the other hand, there are several studies available (Carragher et al., 1989; Huang et al., 1999; Pankhurst and Van der Kraak, 2000; Pickering et al., 1987). However, the results between these studies are conflicting, and seem, like the results from gonadal steroid exposure, to depend on dose, species, and stage of sexual maturity. Interestingly, a study by Consten et al. (2001) demonstrated that Lh release from dissected pituitaries was unaffected by F treatment, but when treated with a combination of F and Gnrh, the Lh release decreased compared to Gnrh alone. Similar results on LH release, and *LHβ* transcription, are reported in mammalian pituitary cultures (Breen et al., 2012; Suter et al., 1988). Therefore, a possible explanation to the low hormone sensitivity of *lhb* observed in the present pituitary cultures, could be that *lhb* regulation is mainly Gnrh dependent in Atlantic cod. In contrast, *fshb* expression was affected by most experimental treatments at some point during the cycle, indicating the possibility of a more direct, Gnrh independent regulation of this gene. The only treatment not able to affect *fshb* transcription was BPA, but as most other treatments had a seasonally dependent effect, it cannot be excluded that such an effect exists for BPA as well.

Regulation of the Gnrh response can be at the brain level, modulating Gnrh synthesis and release from hypothalamic neurons, or it can be at the pituitary level, modulating the

number of available Gnrh receptors in the cells. In **paper II**, we found that both *gnrhr1b* and *gnrhr2a* were expressed in Atlantic cod gonadotropes. The expression of *gnrhr2a* was more prominent than that of *gnrhr1b*, which, combined with earlier findings that *gnrhr2a* expression levels follow the GSI (Hildahl et al., 2011b), and present findings that *gnrhr2a* transcripts levels increase following sex steroid treatment, points to Gnrhr2a being the main Gth modulator in this species. *gnrhr2a* levels were also stimulated by stress levels of F and high, but environmentally relevant, doses of BPA. If translated into protein, BPA and F exposure can enhance the Gnrh response, possibly increasing Gth output and disrupting normal gonadal development. In contrast, neither the sex steroids nor BPA were able to affect *gnrhr1b* expression. In fact, the only effect seen on *gnrhr1b* was in cells from spent fish, where F treatment decreased *gnrhr1b* transcript levels. While both receptors where expressed in gonadotropes, we did not explore if the other pituitary cell types express them as well. In other teleosts, Gnrh receptor gene expression or protein have been located in all the endocrine cell types of the pituitary (Flanagan et al., 2007; Illing et al., 1999; Parhar et al., 2005; Parhar et al., 2002), indicating that the Gnrh receptors are involved in many different processes. The cell specific expression of the receptors could also be dependent on the stage of sexual maturity. In a study by Parhar et al. (2005) in the Nile tilapia, transcripts of receptors phylogenetically close to *gnrhr1b* were detected in all cell types except thyrotropes. However, in immature fish *gnrhr1b* was absent from corticotropes, and in mature fish *gnrhr1b* was absent from somatolactotropes. Similarly, transcripts of receptors closely related to *gnrhr2a* was detected in all cell types in mature fish but were absent from thyrotropes and corticotropes in immature fish. The same study also demonstrated concurrent expression of several Gnrhr isoforms in individual cells, and, similar to our findings, that both *fshb* and *lhb*–producing gonadotropes co-expressed *gnrhr1b* and *gnrhr2a*. While the distribution and function of the different Gnrh receptor isoforms remains to be investigated in Atlantic cod, it is possible that the observed regulation of *gnrhr1b* and *gnrhr2a* expression from the experimental treatments occurred in cells other than gonadotropes.

5.3 Conclusions

The obtained results in this thesis present an optimized protocol for preparations of piscine *in vitro* cultures, a tool that may be useful for other researchers, and new knowledge about the regulation of reproduction-related parameters in the Atlantic cod pituitary, which can contribute to a better understanding of vertebrate reproduction in general. With the work in **paper I**, we achieved our first sub-goal, the successful establishment of a healthy, stable and physiological relevant primary culture system for the Atlantic cod pituitary. Through a series of exposure studies, using the established culture system to explore the potential direct effects from E2, DHT, T, F, BPA, and TBBPA on reproduction-related endpoints, the remaining sub-goals were achieved (**papers II-IV**). The results from these studies demonstrate that the Atlantic cod pituitary is a site for direct effects from sex steroid feedback, as well as the effects of stress and pollution. The reproductive cycle in iteroparous species is marked by seasonally dependent physiological events. This was reflected in the effects from almost all experimental treatments. While all substances were able to affect cell viability and gene expression, the effects were highly dependent on the stage of sexual maturity.

The expression of both *gnrhr1b* and *gnrhr2a* was confirmed to both *fshb* and *lhb* expressing gonadotropes. The expression of the latter receptor was more pronounced, and affected by sex steroids, suggesting this form to be the main gonadotropin regulator in cod. In general, the transcript levels *fshb* and *gnrhr2a* showed greater steroid sensitivity than that of *lhb* and *gnrhr1b*, indicating that for Lh and Gnrhr1b gene expression, the regulation is more dependent on other factors. BPA and TBBPA had differential effects on gene expression in the Atlantic cod pituitary but were both able to affect cellular responses at environmentally relevant concentrations and have the potential to modulate Atlantic cod reproductive function.

5.4 Future perspectives

The pituitary model system described in this thesis can be used to investigate physiological mechanisms and for screening of potential effects from environmental contaminants. However, before using the culture system for such experiments, the methodological uncertainties discussed in chapter 4 should be assessed and sorted out for physiological implications. This means i) preparing cultures separated on sex and assess potential sex differences under both basal and treated conditions, and ii) performing exposure studies comparing a range of solvents and solvent concentrations to assess potential cytotoxicity and potential interaction with the experimental treatment. Investigating these uncertainties are important for validating both the present work and many other published *in vitro* studies from this field. If the physiological consequences of mixed sex and solvent use are not significant, this can be reassuring for the work that has already been completed. If they are, however, then the investigations can bring new mechanistic insight and contribute to further optimization of culture conditions and exposure protocols.

Once a new protocol is established, I would use the system to test for seasonal effects of BPA and TBBPA exposure. Furthermore, as both bisphenols induced cellular responses at the lowest dose tested, I would include exposure at even lower concentrations. In addition to the genes already tested, I would test for potential effects on steroid hormone receptors, i.e. Ar, Er, Mr and Gr, as well as aromatase. To remove the possibility of potential pseudoreplication, I would prepare several smaller cultures, each containing only a few pituitaries, with fewer replicates per culture unit to compensate for the lowered number of cells and run these cultures in parallel with individually prepared exposure solutions.

The stress dose of F used in **paper III** was a mean value of measured stress levels from different studies (Herbert and Steffensen, 2005; King and Berlinsky, 2006; Morgan et al., 1999). However, some studies report several-fold higher levels of F following exposure to an acute stressor (Perez-Casanova et al., 2008). Because of the detrimental effects reported from stress on reproduction, and the fact that teleosts, both in the wild and under farmed conditions, can be exposed to substantial amounts of stress, I would have liked to repeat the experiment with higher doses of F. In addition, I would like to include immature fish in the experimental set-up, to see if stress affects normal reproductive development. As we did not perform histology on the cod gonads, and separated the cultures based on rough gonadal characteristics, it could be interesting to repeat the experiments separated by more fine-tuned reproductive stages, in order to link stages of gonadal development to when the pituitary is

potentially more sensitive to exposure. Furthermore, I would like to measure the circulating levels of F in individual fish prior to dissection. Plasma hormone levels are a pre-treatment to the cells, and wild caught fish might have very different background in terms of stress (and other exposure), which might leave the cells with differential sensitivity when in culture.

To gain more mechanistic insight to the present results, it would be interesting to investigate which cells express the different Gnrhr isoforms and steroid hormone receptors in the cod pituitary. This can be done for instance by double-staining immunohistochemistry or *in situ* hybridisation. Furthermore, it would be interesting to assess if the presence and level of expression in those cells vary with the stage of sexual maturity. To test whether the regulation of Gth and Gnrhr mRNA levels are Gnrh dependent, I would redo the steroid exposure studies w/wo co-exposure to Gnrh. By developing antibodies to cod Gths for the development and validation of specific ELISAs, Gth synthesis and release could be measured, which is likely more physiologically relevant than the measurement of gene expression. Moreover, measuring Gth levels and comparing it to gene expression levels could reveal new insight into their inter-relationship. Another aspect that would be interesting to investigate further, is the duration of exposure. It cannot be excluded that, had the exposure lasted shorter/longer or started at an earlier/later timepoint after seeding, the results could have differed. Finally, as the experimental treatments stimulated cell viability at almost all stages, I would test the possibility that this is a result of cell proliferation by co-incubating the cells with bromodeoxyuridine (BrdU), a proliferation marker.

The knowledge obtained from developing an improved cod pituitary culture system in this thesis work can be used to establish more physiologically relevant cultures for other species and for the other levels of the BPG-axis. Having an *in vitro* system for all levels of the BPG axis within one species could, in addition to being a powerful tool for investigating direct mechanisms at the individual axis levels, be useful for comparing *in vivo* vs *in vitro* results.

Advances in *in vitro* models enables relevant toxicity assessment in culture, which can replace animal testing, at least in early stage screening. Having stable, physiologically relevant models and combining the exposure results from such models with *in silico* methods, can likely help predict the outcome of chemical exposure and aid safety assessments in the future.

6

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6. References

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7

Appendix: Paper I-IV

I

Optimized conditions for primary culture of pituitary cells from the Atlantic cod (*Gadus morhua*). The importance of osmolality, pCO2, and pH

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Optimized conditions for primary culture of pituitary cells from the Atlantic cod (*Gadus morhua*). The importance of osmolality, pCO_2, and pH

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ABSTRACT

Protocols for primary cultures of teleost cells are commonly only moderately adjusted from similar protocols for mammalian cells, the main adjustment often being of temperature. Because aquatic habitats are in general colder than mammalian body temperatures and teleosts have gills in direct contact with water, pH and buffer capacity of blood and extracellular fluid are different in fish and mammals. Plasma osmolality is generally higher in marine teleosts than in mammals. Using Atlantic cod (*Gadus morhua*) as a model, we have optimized these physiological parameters to maintain primary pituitary cells in culture for an extended period without loosing key properties. L-15 medium with adjusted osmolality, adapted to low pCO_2 (3.8 mm Hg) and temperature (12 °C), and with pH 7.85, maintained the cells in a physiologically sounder state than traditional culture medium, significantly improving cell viability compared to the initial protocol. In the optimized culture medium, resting membrane potential and response to releasing hormone were stable for at least two weeks, and the proportion of cells firing action potentials during spawning season was about seven times higher than in the original culture medium. The cells were moderately more viable when the modified medium was supplemented with newborn calf serum or artificial serum substitute. Compared to serum-free L-15 medium, expression of key genes (*lhb*, *fshb*, and *gnrhr2a*) was better maintained in medium containing SSR, whereas NCS tended to decrease the expression level. Although serum-free medium is adequate for many applications, serum supplement may be preferable for experiments dependent on membrane integrity.

1. Introduction

In many *in vitro* studies of animal cells in primary culture, it is tacitly assumed that cultured, viable cells have properties similar to those of intact cells *in vivo*. However, important functional properties of the cells may be lost during dispersion of the cells and their subsequent maintenance in culture, although the cells may stay viable for an extended period. For example, the contractility of dissociated smooth muscle cells [7] and cardiomyocytes [23,43] kept under standard culture conditions may decay within a couple of days, which may be only a fraction of the time the cells stay "healthy" according to common viability tests. However, moderate adjustments of standard culture procedures and media are often sufficient to greatly improve the culture conditions for a specific cell type. Accordingly, a vast literature describes customized culture protocols for a large number of mammalian cell types (for reviews and protocols, see [2,15]). In comparison, specialized methods for culturing cells from exothermic vertebrates, i.e., fish, amphibians and reptiles, are rather few.

When we recently commenced an investigation of the functional properties of pituitary cells from the teleost Atlantic cod (*Gadus morhua*), we realized that the most commonly used procedures for establishing primary cultures of teleost cells were moderately adjusted protocols for mammalian cells. The main difference between most culture procedures for teleosts and mammals is the temperature, whereas important parameters like osmolality, pCO_2, pH, and serum supplement are usually similar. Still, scientists employing such methods for studying teleost cells in primary culture seem to achieve reasonable results. Therefore, in our initial work on cod pituitary cells [16], we used a slightly adjusted version of an established protocol for dissociation and incubation of pituitary cells from the European eel (*Anguilla anguilla*) [24]. The main modification of the culture procedure used for Atlantic cod, which is usually adapted to an ambient temperature below about 12 °C, was a reduction in temperature both during dissociation and incubation of the cells.

However, using these primary cultures in electrophysiological experiments, we realized that the culture conditions were suboptimal, and did thus start systematic trials in an attempt to optimize

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the different elements of the culture procedure. We aimed at designing a primary cell culture protocol that fulfilled the following criteria:

- For at least two weeks, the cells should be viable and in addition show all the properties listed below.
- The resting cell membrane potential should stay within the range for pituitary cells reported in other teleost species, for the whole time period.
- At least a subgroup of the cells should be able to generate action potentials, either spontaneously or triggered by current injections.
- The cells should continue to express key genes essential for the endocrine function of the pituitary.

It was an obvious criterion that the cells should stay viable for the required period. Furthermore, a sound negative resting membrane potential and action potential firing require high membrane integrity and consume energy, and do thus indicate healthy cells. Action potentials are important for regulation of secretion in mammalian pituitary cells, for review see [37,38]. Less is known about the role of action potentials in teleost pituitary cells [16,19,42,48], but voltage-activated Ca^{2+} channels are shown to be involved in regulation of secretion in several species, for reviews see [10,11,20,47]. Finally, genes linked to the functional properties under investigation must of course be expressed under the selected culture conditions. We are currently studying the regulation of sexual maturation and spawning in the Atlantic cod, and are focusing on the gonadotropin releasing hormone (GnRH)-induced production and secretion of gonadotropins, i.e., follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the pituitary gonadotrope cells. Consequently, the last criterion was that cultured cells should continue to express genes for the FSH ß-subunit (*fshb*), the LH ß-subunit (*lhb*), and GnRH receptor 2a (*gnrhr2a*), and respond to GnRH by a transient elevation of cytosolic free Ca^{2+} similar to in mammals [38] and goldfish [10].

In our effort to optimize the culture procedures, we focused on the following parameters, which are likely to be rather different in a cod pituitary *in vivo* compared to the standard mammalian culture conditions: temperature, osmolality, CO_2 partial pressure (pCO_2) and pH. By optimizing these parameters, we have designed a primary cell culture protocol yielding cod pituitary cells that allow studies of essential, functional properties for at least two weeks.

2. Materials and methods

2.1. Animals

Atlantic cod (1–3 kg) were captured in the Oslo fjord and kept in the aquarium facilities at the University of Oslo for a maximum of 2 weeks before being sacrificed. They were fed shrimps while in captivity. The aquaria were continuously perfused with seawater with salinity of 28% and a temperature of 7–8 °C. The light cycle was adjusted to fit the normal night/day cycle in Oslo. Both male and female fish were used in the study.

2.2. Initial procedure for primary culture of pituitary cells from the Atlantic cod

The initial procedure for preparing primary pituitary cultures from the cod [16] was based on established methods for mammalian cells, and similar to a procedure used successfully for the European eel [24]. Pituitaries were removed immediately after decapitation and placed in M199 medium (Invitrogen, Carlsbad, USA) on ice. The pituitaries were then washed with ice-cold phosphate-buffered saline (PBS; Invitrogen), chopped in approximately 1 mm^3 pieces with a scalpel and washed again. The tissue fragments were treated with trypsin (type II-S, 1 mg/ml PBS) for 45 min in a shaker water bath at 18 °C. The trypsin solution was then replaced with PBS containing trypsin inhibitor (type I-S, 1 mg/ml) supplemented with approximately 1 μg/ml DNaseI, before the tissue was incubated for another 20 min in the water bath. Subsequently, the tissue fragments were mechanically dissociated in ice-cold PBS by using a plastic pipette. The cell suspension was filtered through a nylon mesh and centrifuged for 10 min at 100*g* (4 °C). Cells were then resuspended in M199 medium supplemented with newborn calf serum (NCS; 5%) and seeded at a density of 1.5×10^5 cells/cm^2 in 35 mm plastic dishes or 24/96-well plates (Corning, Amsterdam, Netherland) coated with poly-L-lysine. The cells were kept at 12 °C in a humidified atmosphere of 5% CO_2 in air (pCO_2; 38 mm Hg) until start of experiments. The culture medium M199 contains 26.2 mM HCO_3^-, which provides a pH of approximately 7.4 in an atmosphere with 5% CO_2, and the pH of the PBS used in the first steps of the procedure, was also adjusted to 7.4. The osmolality of the PBS and the M199 medium was 270–280 mOsm. Apart from the culture medium and PBS, all chemicals were purchased from Sigma (St. Louis, MO, USA).

2.3. Modifications of the protocol for primary culture of pituitary cells from the Atlantic cod

In our attempt to develop an improved procedure for making primary culture of pituitary cells from the Atlantic cod, we tested the effects of the following adjustments of the initial protocol:

The culture medium M199, which contains 26.2 mM HCO_3^-, was replaced with the medium L-15 (Life Technologies (Invitrogen), Paisley, UK) added 1.8 mM D(+)-glucose. This medium does not include a pH buffer, and was supplemented with 10 mM $NaHCO_3$. When using the L-15 medium modified in this manner, the CO_2 content of the incubator atmosphere was lowered to 0.5%, corresponding to a pCO_2 of 3.8 mm Hg, which resulted in a pH of 7.85 in the medium at 12 °C.

The different L-15-based media tested in the present study were further supplemented with either NCS (5%), the artificial serum substitute SSR (5%) [4], or the medium was completely serum-free. Subsequent to these additions, the osmolality was adjusted to 320 mOsm with NaCl. Finally, the medium was Millipore-filtered (0.2 μm) before added Pen-Strep (20 U/ml, Lonza, Verviers, Belgium). The pH of the PBS was adjusted to 7.85 at 12 °C with NaOH, and the osmolality was increased to 320 mOsm with NaCl. The saline was then Millipore-filtered (0.2 μm) before added Pen-Strep (40 U/ml).

2.4. Solutions

The electrophysiological recordings were performed in the following standard extracellular solution (mM): 150 NaCl, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 1.8 glucose, 10 HEPES/NaOH, pH 7.85, 320 mOsm. The patch electrodes were filled with the following standard intracellular solution (mM): 120 CH_3SO_3K, 20 KCl, 10 HEPES/NaOH, 20 sucrose, pH 7.2, 290 mOsm.

2.5. Electrophysiology

The patch-clamp recordings were carried out at 12 °C, using a peltier element for temperature control (TC-10, Dagan Corporation, Minneapolis, MN, USA), as described by Haug et al. [16] and Hodne et al. [17]. All experiments were performed with the perforated patch configuration, which prevents loss of organic molecules from the cytosol. Perforation was achieved using β-escin, following the

procedure described by Sarantopoulos et al. [33]. In brief, β-escin from a stock solution (25 mM) was added to the pipette solution (50 μM), which was then vortexed for 1 min. The stock solution was kept at −20 °C for up to two weeks, whereas the pipette solution was prepared daily. Both the stock solution and the final pipette solution were protected from direct light by aluminum foil. The patch electrodes were made from borosilicate glass with filament, and the electrode resistance was 2–5 MΩ. The electrodes were connected to an EPC-9 patch-clamp amplifier controlled by the software PULSE (HEKA Elektronik, Lambrecht, Germany). The recorded signals were digitized at 4–10 kHz, filtered at 1/3 of the sampling rate, and stored on a computer. Data analysis was performed using PULSFIT (HEKA) and Origin (OriginLab, Massachusetts, USA).

2.6. Number of surviving cells and cell viability

The cells were seeded at a density of 1.5×10^5 cells/cm^2 in 96-well plates, corresponding to approximately 50,000 cells per well, and incubated in either serum-free L-15 medium, L-15 medium supplemented with 5% NCS, or L-15 medium with 5% SSR. The absolute number of remaining cells per well at a given time was assessed by counting the cells in a haemocytometer. For each well, the cell suspension for counting was prepared by removing the medium and washing the well with 1 ml PBS, before replacing the PBS with 200 μl 0.25% Trypsin- EDTA (Gibco, Invitrogen). The enzyme solution was then removed after 40 s, followed by 3 min incubation to allow the remaining trypsin to complete detachment of the cells. Finally, 1 ml PBS was added and the cell suspension was collected after stirring with a pipette.

To gain information about the condition of the cells, viability tests using two fluorescent indicator dyes, i.e., AlamarBlue (AB) (Invitrogen) and 5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) (Invitrogen), were performed. These tests indicate metabolic activity and membrane integrity, respectively [6]. The AB assay measures the conversion of the non-fluorescent dye resazurin into the fluorescent resorufin by the action of mitochondrial and other enzymes. In living cells able to maintain a cytoplasmic milieu that allows esterase function, non-fluorescent CFDA-AM is converted by nonspecific esterases to the fluorescent carboxyfluorescein (CF). The test procedures were based on the original report by Scheer et al. [35] and in accordance with the modifications described by Tollefsen et al. [41]. Cells were seeded in 96-well plates and incubated as described above. At day 7, the culture medium in the wells was replaced with 100 μl Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 μM CFDA-AM (from 4 mM stock in DMSO). The plates were then incubated in the dark on a shaker (85 rpm) for 30 min at room temperature, before the concentration of the fluorescence products was measured simultaneously for both probes with a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., USA). The software Gen5 was used for data collection (Gen5 Data Analysis Software, Bio-Tek Instruments Inc., USA). The employed excitation–emission wavelength pair was 530–590 nm for AB and 485–530 nm for CFDA. The fluorescence signal from each well was corrected for background fluorescence by subtracting the signal from wells without cells, and the adjusted values were expressed as percentage of the values for serum-free medium. As a positive control for cell toxicity, cells were incubated in serum-free medium with 2.6 mM $CuSO_4$ for 24 h from day 6 in culture. At this concentration, Cu^{2+} is lethal to the cells [41]. All experimental groups were in replicates of five wells and the whole experiment was repeated three times.

2.7. RNA extraction and qPCR

Cod pituitary cells seeded in 24 well plates at a density of approximately 300,000 cells per well were lysed in the wells by removing the culture medium, washing the wells with 300 μl ice-cold PBS, before adding 300 μl of Trizol (Invitrogen). After stirring with a pipette, the cell lysate was instantly frozen on liquid N_2 and stored at −80 °C. In order to extract total RNA, 700 μl Trizol was added to the lysate from each well, and total RNA was extracted following standard procedures and resuspended in 10 μl water (Ambion, Life Technologies, Carlsbad, USA). To remove genomic DNA, the RNA was DNase-treated using TURBO DNase-free (Ambion) according to the manufacturer's protocol. The quantity of the extracted RNA was determined spectrophotometrically (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the quality was assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies, USA) of the RNA Integrity Number (RIN). Only RNA samples with RIN number above eight were analyzed further.

qPCR analyses were carried out using the LightCycler 480 platform (Roche, Switzerland), as previously described [17,45]. First strand cDNA synthesis was performed on 1 μg total RNA using random hexamers, dNTP mix, and Super Script III reverse transcriptase (all from Invitrogen). The cDNA was stored at −20 °C until qPCR. Each PCR reaction mixture contained *Taq* DNA polymerase, SYBR green I detection dye, buffer, gene specific primers, and diluted (1:10) cDNA. The qPCR primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Potential primers were further analyzed using Vector NTI (Invitrogen) to test for possible self-annealing and primer dimer formations (see Table 1 for sequence details). In each pair, one primer was targeted to an exon-exon border to avoid amplification of genomic DNA. The primers were synthesized by MWG-Biotech AG (Ebersberg, Germany), diluted to 1 mM with nuclease-free water (Ambion) upon arrival, and stored at −20 °C. From the stock solution, working dilutions of 5 μM were prepared. All samples were run in duplicate, and in every round two non-template negative control (NTC) reactions were conducted for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). In addition, to account for plate-to-plate variation, a standard positive calibrator control in duplicate was included on every plate. The qPCR reactions were carried out using an initial step for 10 min at 95 °C to activate *Taq* polymerase, followed by 45 cycles consisting of 10 s at 95 °C, 10 s at 60 °C, and elongation at 72 °C for 6 s. The fluorescence was measured at the end of each cycle (after each elongation) and used for determining the quantification cycle values (Cq). A melting curve analysis was performed directly following the PCR by continuously reading the fluorescence while slowly heating the reaction mixture from 65 to 98 °C.

The relation between increasingly diluted cDNA starting material and the corresponding Cq was used for making cDNA dilution curves. After running the qPCR, the Cq was plotted against the logarithm of the relative concentration of the cDNA starting material. The efficiency (E) of the qPCR assay is described by the slope of the regression line $E = 10^{-1/\text{slope}}$. If the slope of the dilution curve is −3.32, the efficiency equals two, meaning that for each PCR cycle there is a doubling of product. The LC480 software calculates the efficiency directly, which was employed together with the dilution curve and melting curve analyses for optimizing the conditions for the various qPCR assays with regards to primers, elongation time, and annealing temperature.

In the present study, we investigated the expression of three different genes specifically related to pituitary function, i.e., *fshb* (GenBank ID: DQ402373), *lhb* (GenBank ID: DQ402374), and *gnrhr2a* (GenBank ID: GU332298.1). To allow accurate normalization of the qPCR, we also tested the stability of three reference genes, *bactin*, *arp2/3*, and *ubiquitin* using Bestkeeper Software [29]. The sequences of these genes were obtained by search in the recently sequenced Atlantic cod genome (http://www.ensembl.org/Gadus_morhua/Info/Index). All reference genes were stably expressed with a low standard deviation SD. However, ubiquitin

Table 1
qPCR primers used in the present study.

Target	Reference	Primer sequence	Amplicon size (nt)	Efficiency
lhb	Hodne et al. (2010)	5′-GTGGAGAAGAAGGGCTGTCC-3′ 5′-GGACGGGTCCATGGTG-3‘	81	1.97
fshb	Hodne et al. (2010)	5′-GAACCGAGTCCATCAACACC-3′ 5′-GGTCCATCGGGTCCTCCT-3′	63	2.02
gnrhr2a	Hildahl et al. (2011)	5′-TTCACCTTCTGCTGCCTCTT-3′ 5′-TCCGTGGAGGAAAGATTGTC-3′	113	1.90
bactin	Genome browser	5′-TTCTACAACGAGCTGAGAGTGG-3′ 5′-CATGATCTGGGTCATCTTCTCC-3′	102	2.06
arp2/3	Genome browser	5′-GGAGGTTAGAAGTAGCAAGGAGC-3′ 5′-TGCTGACTCTCACGGAGTTG-3′	107	2.11
ubiquitin	Genome browser	5′-TGTCAAAGCCAAGATTCAGG-3′ 5′-TGGATGTTGTAATCCGAGAGG-3′	111	1.80

proved most stable (SD = 0.35), while the SD of bactin and arp2/3 were 0.53 and 0.42, respectively. Thus, ubiquitin was used for normalizing the qPCR data. The relative expression levels were determined using an efficiency-corrected method [28]:

$$\text{Relative expression} = E_{\text{target}}^{\Delta Cq(\text{calibrator}-\text{sample})} \times E_{\text{references}}^{\Delta Cq(\text{sample}-\text{calibrator})} \quad (1)$$

2.8. Ca^{2+} imaging

For Ca^{2+} imaging, the cells were plated in dishes fitted with a central glass bottom and coated with poly-L-lysine. For improved attachment of the cells on the glass bottom, 5% NCS was added to the culture medium. Prior to experiments, the cells were incubated with 5 μM fura-2 AM (Life Technologies (Invitrogen, Carlsbad, CA, USA)) in standard extracellular solution for 60 min at 12 °C, followed by washout of the fura-2 ester, and further 30 min incubation. The dish was then mounted on an Olympus IX71 inverted microscope with objectives of high UV light transmittance (Olympus, Tokyo, Japan) for imaging. A Lambda 10-2 filter wheel (Sutter, CA, USA) switched the excitation light from a Lambda LS Xenon Arc Lamp (Sutter). Exposure times at the wavelengths were between 250 and 350 ms for 340 nm, and between 50 and 150 ms for 380 nm, according to the degree of fura-uptake in the cells. The ratio between emissions at these excitation wavelengths (F340/F380) reflects the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Emission of fluorescence at 510 nm was recorded using a Hamamatsu ORCA ER camera (Hamamatsu Photonics, Hamamatsu, Japan). The software Imaging Workbench 6 (INDEC BioSystems, Santa Clara, CA, USA) was used for recording and analysis. The digitized data were background-subtracted. In the present study, the relative increase in $[Ca^{2+}]_i$ is used as a measure of response to the GnRH. Therefore, calibration in order to determine the absolute Ca^{2+} concentrations was not performed. Responses were calculated from baseline to peak of the response, and presented as the ratio peak/baseline. In order to stimulate the cells, a mix of 10^{-7} M GnRH 1, 2 and 3 (Bachem, Bubendorf, Switzerland) in extracellular solution was prepared from three individual stock solutions of 10^{-3} M in DMSO. The GnRH solution was applied via a pressure ejection pipette (about 1 kPa) positioned about three cell diameters from the cell. No ejection artifacts were observed from this distance.

2.9. Statistics

Numerical data are presented as mean ± SEM if not otherwise stated. The significance of the observed effects was assessed using linear correlation analysis, *t*-test and one- or two-way ANOVA. A Tukey's post test was conducted subsequent to one-way ANOVA tests while a Bonferroni multiple comparisons test used following two-way ANOVA. Analysis of the cell count data was performed using Tukey's test, and the number of remaining cells were tested both as a continuous and a discrete variable with time. The observed values were tested for normality by applying the Shapiro–Wilk test. The differences between the mean fluorescence values obtained in media supplemented with either NCS or SSR and the mean value for serum-free medium were analyzed using the Student's t-test. In all cases, *p*-values <0.05 were regarded as statistically significant. All statistics except for cell viability tests were performed using GraphPad Prism version 5.0d for Mac (GraphPad Software, San Diego, CA, USA). Cell viability data were analyzed using JMP 7 software (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Condition of cells cultured in traditional medium

The initial protocol for making primary pituitary cell culture from the Atlantic cod was based on the M199 medium and was quite similar to standard procedures for dispersing and maintaining mammalian cells, except for the lower temperature (12 °C) during incubation. As an estimate of the general health of the cells, the resting membrane potential was recorded after 2 and 5 days in culture. The average membrane potential was −37.4 ± 2.2 mV ($n = 7$) after 2 days and most of the cells were firmly attached to the bottom of the dish. However, after 5 days in culture, the cell density was markedly reduced and the remaining cells were poorly attached to the bottom. The membrane potential of the few cells still possible to record from was −22.4 ± 1.0 mV ($n = 5$). After 7 days in culture, almost all cells were detached, and inaccessible for electrophysiological experiments.

The osmolality of the M199 medium is approximately 280 mOsm, whereas the osmolality of the cod plasma is closer to 320 mOsm. The quality of the cultures was greatly improved by increasing the osmolality of the incubation medium and all working solutions to 320 mOsm. In the osmolality-adjusted M199 medium, the resting membrane potential of the cells was recorded at day 1, 2, 4, 5, 7, and 8 in culture (Fig. 1). Compared to the values in the unadjusted medium, the average resting membrane potentials on day 2 and 5 were significantly increased to −43.4 ± 3.1 mV ($n = 21$) and −41.0 ± 5.1 mV ($n = 5$), respectively. Furthermore, more cells remained after 5 days in culture. The slope of the regression line showing resting membrane potential as a function of time was significantly different from zero, indicating that resting membrane potential (V_m) still declined with time (X) in culture ($F_{15.75,4}$, $V_m = -51.76 + 3.38X$, $P = 0.0165$). In fact, 80% of the variation in resting membrane potential can be explained by the time in culture. These recordings were performed in April, i.e., during the spawning season, and 12% of the recorded cells were able to generate action potentials, either spontaneously or triggered by current injections.

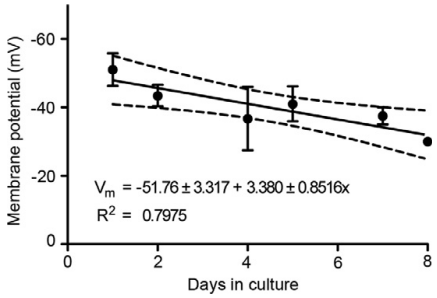

Fig. 1. Resting membrane potential of dispersed Atlantic cod pituitary cells in a culture medium developed for mammalian cells. The membrane potential was measured using a perforated whole cell patch-clamp configuration. The cells were maintained in the M199 culture medium, which contains 26.2 mM HCO_3^-, at a pCO_2 of 38 mm Hg and a pH of 7.4. The osmolality was adjusted to 320 mOsm and the temperature was kept at 12 °C. The data are presented as mean ± SEM (day 1; n = 10, day 2; n = 21, day 4; n = 3 day 5; n = 5, day 7; n = 2, day 8; n = 3). Linear correlation analysis shows that the slope of the regression line is significantly different from zero ($F_{15.75,4}$, P = 0.0165).

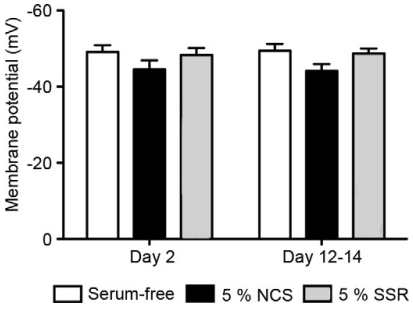

Fig. 2. Resting membrane potential of dispersed Atlantic cod pituitary cells in culture medium optimized for this species. The membrane potential was measured using a perforated whole cell patch-clamp configuration. The cells were maintained in L-15 culture medium with 10 mM HCO_3^-, at a pCO_2 of 3.8 mm Hg and a pH of 7.85. The osmolality was adjusted to 320 mOsm and the temperature was kept at 12 °C. The membrane potential at day 2 and day 12–14 were compared between serum-free optimized L-15 medium and optimized L-15 medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum substitute (SSR). The membrane potential of cells in serum free environment was −49.1 ± 1.7 mV at day 2 (n = 35) and −49.4 ± 1.8 at day 12–14 (n = 33). Cells in L-15 medium supplemented with 5% NCS had a membrane potential of −44.5 ± 2.4 mV (n = 23) at day 2 and −44.1 ± 1.8 at day 12–14 n = 41). Cells in L-15 medium supplemented with 5% SSR had a membrane potential of −48.3 ± 1.9 mV (n = 15) at day 2 and −48.7 ± 1.3 (n = 47) at day 12–14. The data are presented as mean ± SEM. There were no statistical differences in membrane potential between days or treatments.

3.2. Condition of cells cultured in modified L15 media

Because the CO_2 and the HCO_3^- concentration in blood plasma are very different in mammals and fish, the next obvious step in the attempt to optimize the culture medium was to adjust these parameters to appropriate values for the Atlantic cod. Therefore, the M199 medium, which contains 26.2 mM HCO_3^-, was replaced with the HCO_3^--free medium L-15. Based on the physiological values measured in cod, the L-15 medium was adjusted by adding 10 mM HCO_3^- and the pCO_2 in the incubator was reduced to 0.5% (3.80 mm Hg), resulting in a pH of 7.85 in the medium at 12 °C. Finally, 1.8 mM glucose was added and the osmolality adjusted to 320 mOsm. The condition of cells cultured in this serum-free, adjusted L-15 medium was then compared to the properties of cells incubated in the adjusted L-15 medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum (SSR).

3.2.1. Membrane potential

After two days incubation in the serum-free L-15 medium, the average resting membrane potential of the cells was −49.1 ± 1.7 mV (n = 35). The corresponding values for cells incubated in L-15 medium supplemented with NCS or SSR were −44.5 ± 2.4 mV (n = 23) and −48.3 ± 1.9 mV (n = 25), respectively (Fig. 2). The differences between theses values are not significant, and none of the values are significantly different from the resting membrane potential of cells incubated for 2 days in the osmolarity-adjusted M199 medium. However, whereas the linear correlation analysis shows that the slope of the regression line is significantly different from zero in the M199 medium (Fig. 1), the resting membrane potential was stable for at least two weeks when the cells were incubated in any of the three versions of the L-15 medium (Fig. 2). After 12–14 days in culture, the resting potential was −49.4 ± 1.8 mV (n = 33), −44.1 ± 1.8 mV (n = 41), and −48.7 ± 1.3 mV (n = 47) for cells incubated in serum-free L-15, L-15 supplemented with NCS, and L-15 supplemented with SSR, respectively. Furthermore, whereas only 12% of the cells cultured in osmolality-adjusted M199 medium during the spawning season in April were excitable, action potentials (either spontaneous or triggered) were recorded from 82% of the cells incubated in the L-15 media during the same season. No obvious difference in excitability was observed between cells incubated in the three versions of the L-15 medium. During the post-spawning season (May), none of the cells cultured in M199 medium were able to generate action potentials, whereas 32% of the cells incubated in the L-15 media were electrically excitable. The observation that pituitary cells are more excitable during the spawning period than after cessation of spawning has interesting implications for our ongoing studies of the signaling pathways involved in GnRH-induced gonadotropin release and production, and further indicates that the improved culture conditions are more physiologically relevant than the initial protocols.

An indirect measure of the condition of a primary culture is how long the cells remain sufficiently attached to the bottom of the dish to allow electrophysiological experiments. Under the initial culture conditions, electrophysiological experiments were possible for 3.8 ± 2.3 days (n = 5). After adjustment of the osmolality of all solutions, the number of experimental days was 5.4 ± 2.0 (n = 8), which is an insignificant increase. On the other hand, after changing to L-15 medium and adjusting the HCO_3^- concentration, pCO_2, and pH, the average number of experimental days increased significantly to 14.0 ± 4.2 (n = 9) (p < 0.001).

3.2.2. Total cell count and cell viability

The total number of cells remaining after 2, 7, and 14 days in culture was used as a coarse estimate of the stability of cultures incubated in the three versions of the L-15 medium (serum-free, 5% NCS, 5% SSR) (Fig. 3A). Double blind counting of the cells was performed to eliminate bias. At day 2 in culture, approximately 50% of the seeded cells were lost, independently of the version of incubation medium. From day 2 until the end of experiment, the total cell number was negatively correlated with time for all media (Fig. 3A; p < 0.001), and at day 14, only around 30% of the cells were remaining. Thus, serum or SSR supplement alone do not reduce the decline of the number of remaining cells with incubation time.

To obtain more information about the condition of the cultured cells, assays based on the fluorescent indicators AB and CFDA-AM (Fig. 3B) were performed on cells after 7 days in culture. These fluorophores give an indication of metabolic activity and membrane integrity, respectively. The AB assay revealed a significantly

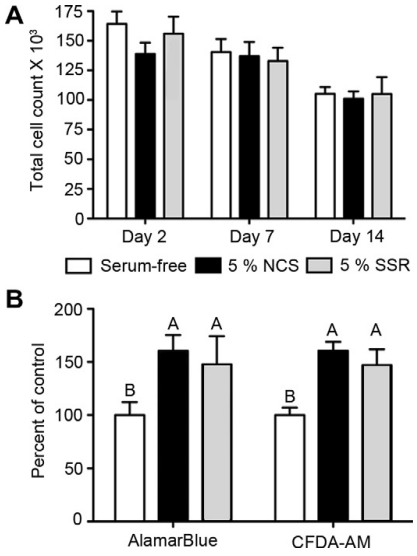

Fig. 3. Viability of dispersed Atlantic cod pituitary cells cultured in optimized medium with and without serum supplement. The cells were maintained in optimized L-15 culture medium (10 mM HCO_3^-, pCO_2 = 3.8 mm Hg, pH = 7.85), and cell viability was compared between cells in serum-free medium and medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum substitute (SSR). (A) Total number of cells in single wells at different times after dispersion. Approximately 300 000 cells were seeded in each well at day 0. It was a negative correlation between total cell number and time in culture for all the tested growth conditions ($p < 0.001$), but there was no significant difference between the different treatments. The data are given as mean ± SEM ($n = 9$). (B) Metabolic activity measured by the AlamarBlue (right graph) and CFDA-AM (left graph) assays. Data are represented as percentage of the mean fluorescence signal in serum-free medium ($n = 15$). The AlamarBlue fluorescence signal was significantly higher in medium supplemented with either 5% NCS or 5% SSR than in serum-free medium ($p < 0.01$). The CFDA-AM fluorescence signal was also significantly higher in L-15 medium supplemented with either 5% NCS or 5% SSR compared to serum-free medium ($p < 0.001$).

higher metabolic activity of cells incubated in L-15 medium supplemented with either serum ($p < 0.0001$) or SSR ($p < 0.01$) compared to cells cultured in serum-free L-15 medium. Membrane integrity measured by the CDFA-AM assay was also significantly higher for cells incubated in medium supplemented with serum or SSR ($p < 0.0001$) compared to cells cultured in serum-free medium. No difference between media supplemented with serum or SSR was detected with either assay.

$CuSO_4$ (2.6 mM) is lethal to the cells, and was used as a positive control for cell death. After 24 h exposure to $CuSO_4$-containing media, the measured metabolic activity and membrane integrity was 0 ± 7% and 0 ± 0% ($n = 15$), respectively, of that of cells incubated in serum-free media only. Interestingly, the $CuSO_4$-exposed cells appeared normal by visual inspection prior to analyses.

3.2.3. Gene expression

The effects of the three versions of the L-15 medium on the condition of the cultured cells were also compared by determining the expression of the genes *fshb*, *lhb*, and a gonadotropin releasing hormone specific receptor, *gnrhr2a*, which are important in the regulation of cod sexual maturation and spawning (Fig. 4). The expression of these three genes was measured using qPCR and normalized relative to the expression of *ubiquitin*. By employing two-way ANOVA, the following aspects were analyzed: (1) stability of gene expression with time in culture, (2) possible effects of serum or SSR in the incubation medium on the mean expression of *fshb*, *lhb*, and *gnrhr2a* at any given time in culture, (3) and statistical interactions describing possible differences in the time dependence of gene expression in the various versions of the incubation medium. The qPCR was performed on cell cultures prepared during the spawning season (March and April).

When pituitary cells are removed from the influence of the brain, GnRH will no longer stimulate synthesis and release of FSH and LH. Thus, as expected, a significant decline of mRNA expression with time was observed for *fshb* (time depended variation, $F2,45 = 55.81$, $P < 0.0001$). However, the mRNA expression of lhb does not show the same time-dependent decline. Still, variation with time is seen, about 12.5% of the variation in *lhb* expression can be explained by time in culture ($F2,45 = 3.93$, $P < 0.0267$). Interestingly, while *fshb* mRNA levels decreased with time in culture, there was an up regulation of the mean mRNA expression of gnrhr2a between day 2 and day 7 (time dependent variation, $F2,45 = 81.47$, $P < 0.0001$).

The culture medium had a significant impact on the mean expression of both *fshb* ($F2,45 = 14.96$, $P < 0.0001$) and *gnrhr2a* ($F2,45 = 55.81$, $P < 0.0001$), while no significant effects of culture medium were observed for *lhb*. The *fshb* mRNA levels were stabilized from day 2 until day 7 in serum-free medium and medium containing 5% compared to cells cultured in medium with 5% NCS. At day 14, the mean *fshb* mRNA levels in cells incubated in medium with 5% SSR was significantly higher than in cells incubated both in serum-free medium and medium with 5% NCS. Furthermore, the addition of 5% SSR prevented a further decrease in the mRNA levels of *fshb* from day 7 until day 14. The mean expression of *gnrhr2a* was suppressed at all time points in cells incubated in medium with 5% NCS compared to cells incubated in medium containing 5% SSR. Although serum-free medium led to increased expressional levels of *gnrhr2a* from day 2 until day 7 a significant drop in expression was observed from day 7 until day14. In contrast, the elevated *gnrhr2a* levels observed after 7 days persisted until at least 14 days in culture when the incubation medium included 5% NCS or SSR. The statistical interaction between the different media regarding the time dependence of both *fshb* expression ($F4,45 = 4.01$, $P = 0.0073$) and *gnrhr2a* expression ($F4,45 = 9.591$, $P = 0.0016$) were significant.

3.2.4. Response to GnRH

Finally, we tested whether the cultured cells also responded to GnRH during the full culture period. Normally, activation of GnRH receptors is followed by elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). This rise in $[Ca^{2+}]_i$ is dependent on the signaling cascade that activates PLC followed by increased levels of inositol 1,4,5-trisphosphate (IP_3) and ultimately release of Ca^{2+} from internal stores [10]. Thus, the increased levels of $[Ca^{2+}]_i$ serve as a robust indication that the cells maintain the capacity to respond to GnRH. To investigate changes in $[Ca^{2+}]_i$ we used microfluorometry based on the Ca^{2+}-sensitive fluorophore fura-2. $[Ca^{2+}]_i$ was monitored by recording the ratio between emissions at excitation wavelengths of 340 and 380 nm (F340/F380). We tested the cultures at day 3, 7, and 14 during late maturational and spawning season (November until March).

Fig. 5a illustrates a typical response following GnRH stimulation. An immediate elevation in $[Ca^{2+}]_i$ was observed followed by a slow return to baseline over several minutes. All recorded GnRH responses showed a similar initial elevation in $[Ca^{2+}]_i$ with a peak before a slow decline. Therefore, we used the ratio between the peak and the basal F340/F380 value as a measure of stability of the GnRH response (Fig. 5b). The GnRH response estimated as peak/baseline was for day 3; 1.71 ± 0.12 ($n = 5$), day 7; 1.63 ± 0.06 ($n = 10$), and day 14; 1.70 ± 0.06 ($n = 8$). There were no significant differences with time in culture during this period.

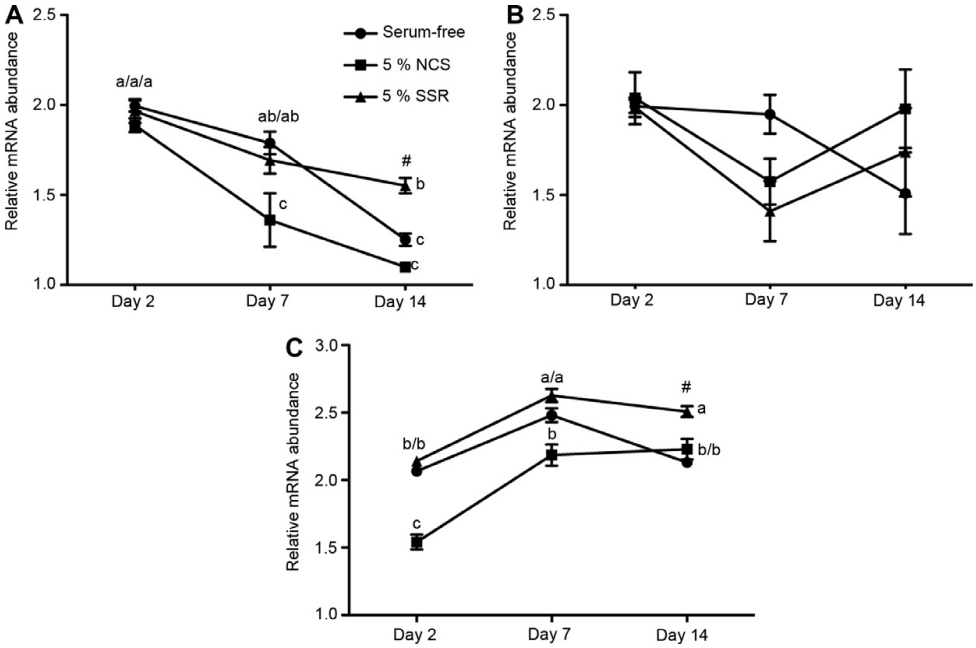

Fig. 4. Relative gene expression of dispersed Atlantic cod pituitary cells cultured in optimized medium with and without serum supplement. QPCR was used to measure relative expression of the genes *fshb*, *lhb*, and *gnrhr2a*. The cells were maintained in optimized L-15 culture medium (10 mM HCO_3^-, pCO_2 = 3.8 mm Hg, pH = 7.85), and gene expression was compared between cells in serum-free medium and medium supplemented with either 5% newborn calf serum (NCS) or 5% artificial serum substitute (SSR). The samples were collected from primary cell cultures after 2, 7 and 14 days in culture during the spawning season (March and April). The data are presented as mean ± SEM (n = 6). Lower case letters indicate statistical differences between individual mean values. (A) The *fshb* mRNA levels were dependent on both time ($F_{2,45}$ = 67.86, P < 0.0001) and culture medium conditions ($F_{2,45}$ = 14.96, P < 0.0001). A general decrease in *fshb* mRNA levels where observed, affected by culture conditions with a statistical significant interaction ($F_{4,45}$ = 4.01, P = 0.0073). (B) The *lhb* mRNA expression levels was only time dependent ($F_{2,45}$ = 3.93, P = 0.0267), with no significant differences between the three culture medium conditions. (C) The *gnrhr2a* mRNA expression levels were time dependent ($F_{2,45}$ = 81.47, P < 0.0001), with increased mRNA levels during the first 7 days in culture for all treatments. The expression of *gnrhr2a* was also affected by culture medium conditions ($F_{2,45}$ = 55.81, P < 0.0001). Furthermore, there was a statistically significant interaction of the time dependence observed in the three different culture media ($F_{4,45}$ = 9.591, P = 0.0016).

4. Discussion

4.1. Primary pituitary cultures from teleosts

Prior to our studies on the Atlantic cod, primary pituitary cultures have been made from several teleost species including: carp [31,32]; goldfish [9,48]; rainbow trout [44], European eel [24]; tilapia [46]; and zebrafish [21]. Trypsin was used for tissue digestion in the cell dispersion step in all these cases except for rainbow trout and zebrafish, where collagenase was used. It is a general feature of the culture protocols that the cells were incubated at a pH between 7.2 and 7.5 in an atmosphere containing 5% CO_2. The osmolality of the incubation media varied between 300 and 355 mOsm, and in some protocols it is unclear if all the solutions employed during the culture procedure were adjusted to exactly the same osmolality. Regarding serum supplements, most incubation media contained 1–10% mammalian serum, either horse serum or NCS. Only one protocol comprised serum-free incubation medium [46], whereas an artificial serum substitute was used in one case [44].

Although most of the previous studies on teleost pituitary cells in primary culture have yielded reasonable results, it is uncertain to which degree the cells were in a physiologically normal state. In our initial work on primary pituitary cells from the Atlantic cod, we adopted the established culture protocol for the European eel [24], which we considered to be the most promising. However, the shallow and rapidly decaying resting membrane potential of the dispersed cells strongly indicated that the culture conditions were suboptimal for Atlantic cod.

We did not systematically test different temperature regimes, but simply selected 12 °C, which is moderately above the ambient temperature experienced by the cod before being sacrificed, as the temperature during both incubation and the electrophysiological experiments. The enzymatic digestion of the tissue was performed at 18 °C, as a compromise between the low adaptation temperature of the cod and the much higher temperature required for maximum enzyme activity. For mammals, the most common enzyme for dissociation of pituitary cells is trypsin [15]. For teleost pituitary cultures, mammalian trypsin is also the most commonly used enzyme for tissue digestion (goldfish, *Carassius auratus*: [9,48]; tilapia, *Oreochromis mossambicus*: [46]; carp, *Cyprinus carpio*: [31,32], European eel[24]), but mammalian collagenase has also been used successfully (rainbow trout, *Oncorhynchus mykiss*: [44]; zebrafish, *Danio rerio*: [21]). The mammalian isoforms of these enzymes have relative low activity below 20 °C. Trypsin is the most efficient alternative and provides the shortest digestion time, which is a significant benefit when dissociating temperature-sensitive cells from animals acclimated to low temperatures. Therefore, we did not compare the various enzyme treatments, but decided to use trypsin for tissue digestion. In addition to these parameters, the enzymatic treatment for tissue degradation varies considerably between culture protocols.

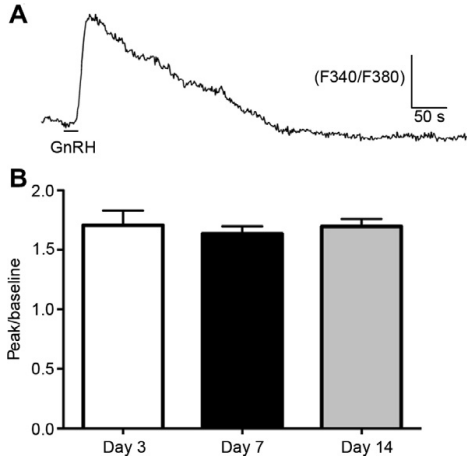

Fig. 5. Cytosolic Ca^{2+} response to GnRH1–3 in dispersed Atlantic cod pituitary cells during the culture period. Microfluorometric recordings using fura-2 loaded cells were used to monitor changes in cytosolic Ca^{2+} levels $[Ca^{2+}]_i$ following gonadotropin releasing hormone (GnRH) stimulation. The ratio between emissions at the excitation wavelengths 340 and 380 nm (F340/F380) reflects $[Ca^{2+}]_i$. (A) Represents a typical response to a mixture (10^{-7} M) of the three different GnRH forms for 10 s (black line under trace). An immediate elevation of $[Ca^{2+}]_i$ was followed by steady decline lasting for one to several minutes before returning to resting levels. (B) The ratio between the peak and basal F340/F380 levels was compared for GnRH responses in cells grown for different time in culture. Average peak/baseline ratio was for day 3: 1.71 ± 0.12 (n = 5), day 7: 1.63 ± 0.06 (n = 10), and day 14: 1.70 ± 0.06 (n = 8). The data are presented as mean ± SEM. No statistical differences were observed between the time points.

Although some of our tests of the culture quality are targeting gonadotropes in particular, i.e. gene transcription levels and GnRH response, most results are based on all cells in the culture dish and do not distinguish between the different pituitary cell types present. Still, we have no reason to believe that the culture conditions presented should not be equally optimized for all the different endocrine pituitary cell types, as they are closely related and exposed to the same microenvironment within the pituitary *in vivo*. Thus, we consider it likely that the results presented in this study apply to all pituitary cell types.

4.2. Improved procedures for establishing primary cultures of cod pituitary cells

4.2.1. The importance of osmolality

Most commercial culture media and salt solutions for mammalian cell cultures have an osmolality far below the plasma osmolality of the Atlantic cod at the temperature (7–8 °C) and salinity (28‰) of the seawater tanks at our animal facility [22,26]. It is a reasonable assumption that osmotic stress during the culture procedures might be deleterious for the cells. The condition of cells incubated in the initial M199 medium was markedly improved by adjusting the osmolality of all solutions to 320 mOsm, which is close to the normal osmolality of cod plasma [26]. The resting cell membrane potential increased significantly and reached values in the range reported for both goldfish and mammalian pituitary cells [30,39,42]. The resting membrane potential was also more stable after adjustment of the osmolality, although it still decayed with time in culture. It is possible that the improved condition of the cells is less dependent on the absolute value of osmolality than on keeping the osmolality constant during the whole process of cell dissociation, incubation, and electrophysiological recordings. A rapid change in osmolality is stressful to cells, in particular when being particularly fragile due to the dissociation procedure. It is thus important to measure and adjust the osmolality of all solutions used for establishing and maintaining the cell cultures.

4.2.2. The importance of pCO_2 and pH

Because CO_2 is easily exchanged over the gills, the pCO_2 in fish is only a small fraction of the pCO_2 in mammalian blood (40–46 mm Hg or 5.3–6.1 kPa, arterial-venous pCO_2) [34]. In teleosts, the arterial pCO_2 range from 1.7 to 3.4 mm Hg (0.23–0.45 kPa), while the venous pCO_2 range from 3.2 to 5.7 mm Hg (0.42–0.79 kPa), depending on the species [1]. Due to the low pCO_2 and the correspondingly low HCO_3^- concentration, the buffer capacity of the blood and extracellular fluids is lower in fish than in mammals and culture media adapted for mammalian cells. Mammalian culture media usually include buffer systems that result in pH 7.4 when the medium is equilibrated to the standard atmosphere (5% CO_2 in humidified air) and temperature (37 °C) in the incubator. Consequently, changing any of these parameters (temperature, pCO_2, $[HCO_3^-]$) in an attempt to adjust to the internal conditions of a teleost will alter the pH of the medium. Furthermore, the value for neutral pH increases with decreasing temperature [8]. Consequently, in cold-water fish, the blood plasma may have a pH about 0.5 units above normal values in mammals, which represents approximately the same alkalinity of the plasma in these animal groups. In teleosts, plasma pH ranges from 7.7 to 7.9, depending on temperature [12]. In the Atlantic cod, the plasma pH is ~7.9 at 12 °C [18]. Thus, it is to be expected that neither the pCO_2 nor the pH recommended for incubation of mammalian cells are optimal for teleost cells. Therefore, culture media customized for teleost cells should contain modified buffer systems compared to those used in most media developed for mammalian cells. By adjusting not only the osmolality, but also the interrelated parameters pCO_2, $[HCO_3^-]$, and pH of the incubation medium to physiological values for teleosts [12,34], the resting cell membrane potential became stable for at least two weeks in culture. In order to achieve this, the M199 medium, which contains 26.2 mM HCO_3^-, was replaced with the HCO_3^- free medium L-15 supplemented with 10 mM HCO_3^-. The CO_2 content of the incubation atmosphere was set to 0.5% (3.80 mm Hg), resulting in a physiological pH of 7.85 in the medium at 12 °C.

In addition to a deeper and more stable membrane potential, the adjustments of pCO_2, $[HCO_3^-]$, and pH significantly increased the electrical excitability of the cells. The cells also stayed attached to the bottom of the dish for about two weeks in the fully adjusted incubation medium, whereas most cells were detached after less than one week when the culture protocol only included osmolality adjustments.

Thus, by simple adjustments of the osmolality of all applied solutions and the pCO_2, $[HCO_3^-]$, and pH of the incubation medium, the physiological condition of the cultured cod pituitary cells was dramatically improved compared to the result following a traditional culture protocol adapted for mammalian cells.

4.2.3. The importance of serum supplement

Addition of bovine serum to the culture medium is standard procedure in most protocols for mammalian cell culture. The arguments for adding serum to the culture media range from improved cell adherence to the bottom of the culture vessel, to stronger plasma membranes and a generally improved condition of the cells [15]. However, except for blood cells and the endothelial cells lining the blood vessels, cells are not in direct contact with serum *in vivo*. Although serum supplement is usually beneficial for cells in culture, serum may also be detrimental for at least some cell types. Several reports have demonstrated that even mammalian primary cell cultures may benefit from serum-free media. For

example, Bowers and Dahm [7] showed that amniotic smooth muscle cells in culture maintained their contractility if serum was omitted from the culture medium. By growing pig liver sinusoidal endothelial cells in a serum-free medium supplemented with an artificial serum substitute, Elvevold et al. [14] managed to maintain functional cells for 20 days, compared to maximum 4 days in traditional serum-containing medium. The serum substitute contained no proteins or peptides that might interfere with the cell function, but did include transferrin, surfactant, adherence promoters, low molecular weight lipids, and replacements for metal ion buffers [4]. In order to keep total control of the experimental conditions, artificial serum supplement has previously also been used in the culture of head kidney macrophages from the Atlantic cod [36]. Thus, it is evident that serum-free media and media supplemented with serum or artificial serum substitutes should be compared when the aim is to develop optimized culture conditions for a specific cell type.

The improvements of the culture conditions discussed in the two previous sections were achieved using serum-free L-15 medium. Therefore, addition of serum (NCS) to the incubation medium is not required in order to maintain seemingly healthy cod pituitary cells in primary culture for at least two weeks. Nevertheless, in order to investigate if supplementing the incubation medium with NCS or the artificial serum substitute SSR is beneficial or not, electrophysiological properties, viability, and gene expression were compared in cells cultivated in serum-free medium and medium supplemented with either NCS or SSR.

Addition of neither NCS nor SSR to the incubation medium had any significant effect on the resting cell membrane potential, although it tended to be somewhat shallower when NCS or SSR were present in the medium. The number of surviving cells with time in culture was also unaffected by these supplements. In contrast to this lack of evident effects on electrophysiological properties and survival, both the metabolic activity and the membrane integrity of cells incubated in L-15 medium supplemented with NCS or SSR were significantly higher than in cells cultured in serum-free L-15 medium. The effects of NCS and SSR on these parameters were similar.

The physiological relevance of NCS and SSR supplements was further examined by measuring the expression of selected genes. The expected decrease in the expression of *fshb* with time in culture, due to absence of GnRH influence, was observed in all medium versions. At day 14 in culture, the expression of *fshb* was significantly higher in medium supplemented with SSR than in the other media. We did not observe a similar decrease in *lhb* expression. The differential GnRH-regulation of *fshb* and *lhb* will be the topic of further studies.

Transcription of the GnRH receptor gene *gnrhr2a* is essential regarding GnRH responses in pituitary cells. It is common that reduced ligand concentration induces upregulation of the related receptors. Because the tested media were lacking GnRH, it is not surprising that the expression of *gnrhr2a* was higher at day 7 than at day 2 in culture for all medium versions. Also for this receptor gene, the expression level was lowest in medium supplemented with serum, and highest in medium containing SSR. Furthermore, both SSR and NCS supplements caused the elevated *gnrhr2a* expression to remain also at day 14. Thus, for primary culture of cod pituitary cells, the gene expression data indicate that SSR is a preferable medium supplement compared to NCS if the aim is to study the GnRH response.

It is well documented that NCS promotes viability and substrate attachment in primary cultures of many types of teleost cells [5]. Also, we experienced that using NCS was necessary for proper attachment when cells where plated on glass bottom. Fish serum, on the other hand, may be beneficial or toxic, depending on cell type (reviewed in [27]). Barlian et al. [3] showed that it might be favorable to replace the poorly defined serum with calf serum albumin (BSA). However, the success was dependent on the type of BSA. Other similar proteins, like ovalbumin, did not have the same positive effect.

An important concern regarding total removal of serum from incubation media customized for pituitary cells is the fact that gonadotropin releasing hormone (GnRH) receptors, like many G protein-coupled receptors, are dependent on lipid rafts in order to be localized close to their cognate signaling molecules. Navratil et al. [25] showed that the GnRH receptors are localized to the low density membrane fraction together with c-raf kinase, and that depletion of cholesterol disrupts the association between the receptors and the lipid rafts. Cholesterol depletion also reduced the GnRH activation of ERK (extracellular signal-related kinase) and c-fos-regulated gene transcription. There is increasing evidence that also many ion channels are localized to lipid rafts, which can affect channel function [13]. For instance, the channel gating kinetics may be regulated by interactions between channel proteins and both lipids and other associated proteins in the raft.

Tocher et al. [40] showed that the lipid composition of the plasma membrane of fish cells in long-term cultures was affected by the NCS added to the medium, reflecting the lipid components of the serum more than those of the original fish tissue. Therefore, an optimal serum substitute should contain the essential factors for keeping the structure and the constituents of the plasma membrane intact, while omitting unknown growth factors and other signaling molecules that can interfere with the physiology of the cell.

5. Conclusion

By adjusting the osmolality of all solutions included in the culture protocol, and the temperature, pCO_2, [HCO_3^-], and pH of the incubation medium, to physiological values for the Atlantic cod, it was possible to maintain viable cod pituitary cells in primary culture for at least two weeks. During this period, the resting membrane potential was completely stable, and the expression pattern of key genes was physiologically reasonable. In addition, the cells responded similarly to GnRH during the whole culture period. In contrast, cells dispersed and maintained following a traditional culture procedure for teleosts displayed rapidly decaying resting membrane potentials and succumbed within one week. The cells were moderately more viable in medium supplemented with either NCS or an artificial serum substitute (SSR). Compared to serum-free medium, the gene expression was better maintained in medium containing SSR, whereas NCS tended to depress the expression level. Although serum-free medium is adequate for many applications, serum supplement may be preferable for experiments dependent on maximizing the membrane integrity and receptor function. Using a well-defined, artificial serum substitute, thus avoiding introduction of undesired signaling molecules, may be the ultimate solution in many experimental situations.

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II

Sex steroids differentially regulate *fshb*, *lhb* and *gnrhr* expression in Atlantic cod (*Gadus morhua*) pituitary

REPRODUCTION
RESEARCH

Sex steroids differentially regulate *fshb*, *lhb* and *gnrhr* expression in Atlantic cod (*Gadus morhua*) pituitary

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Abstract

Depending on the stage of gonad maturation, as well as other factors, gonadal steroids can exert either a positive or negative feedback at the brain and pituitary level. While this has been demonstrated in many teleost species, little is known about the nature of steroid feedback in Gadiform fish. Using an optimized *in vitro* model system of the Atlantic cod pituitary, the present study investigated the potential effects of two physiologically relevant doses of estradiol, testosterone (TS) or dihydrotestosterone (DHTS) on cell viability and gene expression of gonadotropin subunits (*fshb*/*lhb*) and two suggested reproduction-relevant gonadotropin-releasing hormone receptors (*gnrhr1b*/*gnrhr2a*) during three stages of sexual maturity. In general, all steroids stimulated cell viability in terms of metabolic activity and membrane integrity. Furthermore, all steroids affected *fshb* expression, with the effect depending on both the specific steroid, dose and maturity status. Conversely, only DHTS exposure affected *lhb* levels, and this occurred only during the spawning season. Using single-cell qPCR, co-transcription of *gnrhr1b* and *gnrhr2a* was confirmed to both *fshb*- and *lhb*- expressing gonadotropes, with *gnrhr2a* being the most prominently expressed isoform. While steroid exposure had no effect on *gnrhr1b* expression, all steroids affected *gnrhr2a* transcript levels in at least one maturity stage. These and previous results from our group point to Gnrhr2a as the main modulator of gonadotropin regulation in cod and that regulation of its gene expression level might function as a direct mechanism for steroid feedback at the pituitary level.

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Introduction

Sexual maturation in fish, as in other vertebrates, is controlled by the physiological connection between the brain–pituitary–gonad (BPG) axis compartments; gonadotropin-releasing hormone (Gnrh) secreted from hypothalamic neurons onto the pituitary stimulates the synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which in turn stimulate gonadal steroidogenesis and gametogenesis. Although the exact mechanisms are not known, activation of the Gnrh system seems to be the key event in the onset of teleost puberty (Weltzien *et al.* 2004). Gnrh exerts its action via specific Gnrh receptors (Gnrhr) located in target cell membranes. Several isoforms of Gnrhr exist in teleosts, and these may be differentially expressed during maturation and spawning. In Atlantic cod (*Gadus morhua*), four Gnrhr gene orthologues (*gnrhr1b*, *gnrhr2a*, *gnrhr2b* and *gnrhr2c*) have been identified (Hildahl *et al.* 2011), all of which were expressed in the brain, and three of which (*gnrhr1b*, *gnrhr2a* and *gnrhr2c*) were expressed in the pituitary. The expression of *gnrhr2c* was low and not seasonally regulated, while *gnrhr1b* expression showed increasing trends in expression during the mature and spent stages. *gnrhr2a* expression appeared to be closely correlated to gonadal maturation, indicating this forms the most likely mediator of the gonadotrope Gnrh response. Unlike mammals, teleost fish have two types of gonadotropes, individually producing either Fsh or Lh. Gnrh has been shown to stimulate both *fshb*- and *lhb*-expressing cells in cod (Hodne *et al.* 2013), but the Gnrh receptor type expressed in individual gonadotropes is yet to be determined.

The activation of the BPG axis and pubertal development is under the control of both external and internal factors, as well as feedback mechanisms within the axis itself (Dufour *et al.* 2010, Levavi-Sivan *et al.* 2010, Taranger *et al.* 2010, Zohar *et al.* 2010) and ensures that reproduction occurs at a time of optimal survival of the offspring. The synthesis and/or release of gonadotropins

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due to sex steroid feedback has been confirmed in several teleost species by measuring protein or transcript levels after steroid supplementation/replacement *in vivo* or after steroid exposure *in vitro* (Billard 1978, Kobayashi *et al.* 1989, Larsen & Swanson 1997, Borg *et al.* 1998, Holland *et al.* 1998, Khan *et al.* 1999, Cavaco *et al.* 2001, Huggard-Nelson *et al.* 2002, Aroua *et al.* 2007). The nature of the feedback can differ depending on maturational stage, hormone concentration, species, sex and other factors (Saligaut *et al.* 1999, Okuzawa 2002, Yamaguchi *et al.* 2006, and see Levavi-Sivan *et al.* 2010 and references therein).

Atlantic cod is a marine multibatch spawner with group-synchronous gonadal development, and spawns multiple times from February until May (Brander 1994). Reports describing the gonadotropic interplay in multibatch spawners are scarce compared to single batch spawners such as salmonids and eel, and little is known on the role of steroid feedback at this level in Atlantic cod or in Gadiformes in general. In contrast to single or total spawners, in which gametes develop synchronously, both multibatch and daily spawners display gametes at different stages of development at any one time. These two contrasting spawning strategies probably display differences in the gonadotropin regulation of gametogenesis, including different responses to circulating steroids at the higher levels of the BPG axis.

The objective of the present study was to investigate direct effects of sex steroid exposure on pituitary gonadotropin and Gnrhr gene expression during different stages of sexual maturity in Atlantic cod, using dispersed pituitary primary cultures as the model system. To acquire a greater understanding of the regulatory elements in gonadotropes, a secondary objective was to identify the expression profiles of *gnrhr* isoforms in individual *fshb*- and *lhb*-producing pituitary cells.

Materials and methods

Animals

Wild Atlantic cod (0.51–4.10 kg body weight) of both sexes were caught by trawling on the south coast of Norway. Following capture, fish were immediately transported to the University of Oslo and kept in recirculating aquaria supplied with 28% seawater. Fish were kept under ambient conditions of temperature (8–12°C) and photoperiod (60°N), and fed daily with frozen shrimp. All animals were kept and handled in agreement with the provisions enforced by the Norwegian Animal Research Authority. A specific approval for this study was not needed, as the animals themselves were not experimentally treated (Norwegian legislation for use of animals in research, Chapter II, §6). All procedures were approved by the Ethical Committee for Animal Experiments at the University of Oslo.

Dispersed pituitary cell cultures

Dispersed primary cultures of mixed sexes were prepared from pituitaries according to Hodne and coworkers (2012), with culture conditions optimized for Atlantic cod parameters in respect to osmolality, temperature, pCO_2 and pH. The optimized conditions allowed the pituitary cultures to be physiologically stable and viable for at least two weeks (see Supplementary data and Hodne *et al.* 2012, see section on Supplementary Data given at the end of this article). The pituitaries were sampled throughout the year to reflect different reproductive stages of cod, from maturation to the spent stage. Maturational status was determined based on macroscopic inspection of the gonads (see Supplementary data) and gonadosomatic index (GSI; (gonad weight/total body weight) × 100). Six cultures were included in the experiment, two from each of the following maturity stages; maturing, mature and spent.

In short, dissected pituitaries were pooled ($n = 5$–15), washed with modified phosphate-buffered saline (PBS; Life Technologies) and chopped into approximately 1 mm^3 pieces, before being treated with trypsin (type II-S, Sigma; 1 mg/mL PBS). The trypsin digestion was ended by replacing the solution with PBS containing trypsin inhibitor (type I-S, Sigma; 1 mg/mL) supplemented with approximately 1 µg/mL DNaseI (Sigma). Next, the tissue fragments were mechanically dissociated in ice-cold PBS, before the solution was filtered and centrifuged. Subsequently, the pellet was resuspended in modified L-15 and the dispersed cells seeded at a density of 1.5×10^5 cells/cm^2 in 24/96-well plates (Corning) precoated with poly-L-lysine (0.1 mg/mL, Sigma). The cells were incubated at 12°C in a humidified atmosphere of 0.5% CO_2 in air (pCO_2; 3.8 mmHg, which resulted in a medium pH of 7.85). After 24 h, medium was replaced to remove damaged and detached cells.

Steroid exposure

Stock solutions of 17β-estradiol (E2), testosterone (TS) and the non-aromatizable androgen dihydrotestosterone (DHTS) (all from Sigma) were prepared by dissolving the steroids in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway). Stock solutions were stored at −20°C for no longer than 3 months and diluted in modified L-15 medium before cell exposure. To prevent possible reactions between steroids and plastic, all solutions were prepared in sterile glass tubes. The working solutions for exposure had an EtOH concentration of 0.2%. For each experiment, two controls, either with or without EtOH were included. At day 4 of culture, the cells were exposed to either a low or high physiologically relevant dose of steroid (E2; 2.5 and 25 ng/mL (~9.2×10^{-9} and 9.2×10^{-8} M), TS and DHTS; 2 and 20 ng/mL (~6.9×10^{-9} and 6.9×10^{-8} M)) (Dahle *et al.* 2003, Norberg *et al.* 2004, Almeida *et al.* 2009) and incubated for an additional 72 h. Solvent control cells were exposed to 0.2% EtOH in the same manner. For gene expression analysis, six replicate wells in 24-well plates per treatment were prepared, whereas for viability tests, six replicate wells in 96-well plates were used.

Viability testing

A classical approach to measure cell viability is using the vital stain Trypan blue. However, in cod pituitary cultures, a proportion of the cells appear naturally blue under the microscope, making this approach suboptimal (see Supplementary data). So to gain information about the condition of the cells after 7 days in culture (w/wo exposure), viability tests were performed using two non-toxic fluorescent indicator dyes, AlamarBlue (AB) and 5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) (both from Life Technologies). Both assays measure the conversion of a non-fluorescent dye into a fluorescent dye by enzymes present in intact and viable cells and indicate metabolic activity and plasma membrane integrity respectively (Bopp & Lettieri 2008). Test procedures were carried out as described in Hodne and coworkers (2012). In short, cells were seeded, incubated and exposed to steroids as described previously. As a positive control for cell toxicity, an additional set of wells were prepared and incubated in modified L-15 medium. At day 6, the medium of these cells was replaced with L-15 supplemented with 2.5, 0.625 or 0.156 mM $CuSO_4$ ($n=6$). At the highest concentration, Cu^{2+} is lethal to the cells. The $CuSO_4$ wells were included on every plate used in the viability assays and served as both intra- and inter–assay-positive controls.

At day 7, the culture medium in all wells was replaced with 100 µL Tris buffer (50 mM, pH 7.5) containing both 5 % AB and 4 µM CFDA-AM (from 4 mM stock in DMSO). After 30 min of incubation, the concentration of the fluorescent products was measured simultaneously for both probes with a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) and the software Gen5 was used for data collection (Gen5 Data Analysis Software, Bio-Tek Instruments Inc.).

Quantification of gene expression

RNA extraction and cDNA synthesis

Cells used for gene expression analysis were harvested at day 7 of culture. A cell lysate was produced by removing the culture medium, washing with ice-cold PBS, adding 1 mL TRIzol (Life Technologies) and stirring the well content with a pipette. The lysate was transferred to an Eppendorf tube and instantly frozen in liquid N_2 and stored at −80°C. Total RNA was extracted in TRIzol following standard procedures, with the exception of adding 1.5 µL GlycoBlue (Ambion, Life Technologies) to the isopropanol step for visualization of the RNA pellet. The RNA was resuspended in 10 µL RNase-free water (Ambion). DNase-treated RNA (TURBO DNase-free (Ambion)) was quantified spectrophotometrically (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the quality was assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies) of the RNA integrity number (RIN). Only RNA samples with RIN number above 8 were analyzed further. First-strand cDNA synthesis was performed on 500 ng total RNA using random hexamer primers and Super Script III (Life Technologies), according to standard procedures, and stored at −20°C until qPCR.

Primers and reference genes

The qPCR primers were designed using Primer3 shareware (http://frodo.wi.mit.edu/primer3/input.htm). Potential primers were further analyzed using Vector NTI (Life Technologies) to test for possible hairpin loops and primer dimer formations (Table 1 for sequence details). In each pair, one primer was targeted to an exon–exon border to avoid amplification of genomic DNA. The expression of four different genes specifically related to pituitary function was investigated, i.e., *fshb* (GenBank ID: DQ402373), *lhb* (GenBank ID: DQ402374), *gnrhr1b* (GenBank ID: GU332297) and *gnrhr2a* (GenBank ID: GU332298.1). To allow accurate normalization of the qPCR, the stability of four reference genes, *arp2*, *bactin*, *ubiquitin* and *ef1a* was tested using Bestkeeper Software (Pfaffl *et al.* 2004), giving Cq geometric means and standard deviations (±Cq) of 27.06 (±0.39), 21.96 (±0.45), 22.19 (±0.43) and 20.40 (±0.39) respectively. For subsequent experiments, *ef1a* was used for normalization of the qPCR data.

Table 1 qPCR primers used in the present study.

Target	Reference	Primer sequence	Amplicon size (nt)	Efficiency
lhb	Hodne *et al.* (2010)	Forward: 5′-GTGGAGAAGAAGGGCTGTCC-3′ Reverse: 5′-GGACGGGTCCATGGTG-3′	81	1.93
fshb	Hodne *et al.* (2010)	Forward: 5′-GAACCGAGTCCATCAACACC-3′ Reverse: 5′-GGTCCATCGGGTCCTCCT-3′	63	1.84
gnrhr1b	This study	Forward: 5′-GCTACTCCCGAATCCTCCTC-3′ Reverse: 5′-CGCCTCAGGTATGACTCTCC-3′	73	1.96
gnrhr2a	This study	Forward: 5′-TTCACCTTCTGCTGCCTCTT-3′ Reverse: 5′-TCCGTGGAGGAAAGATTGTC-3′	113	1.99
bactin	Hodne *et al.* (2012)	Forward: 5′-TTCTACAACGAGCTGAGAGTGG-3 Reverse: 5′-CATGATCTGGGTCATCTTCTCC-3	102	1.84
arp2	Hodne *et al.* (2012)	Forward: 5′-GGAGGTTAGAAGTAGCAAGGAGC-3′ Reverse: 5′-TGCTGACTCTCACGGAGTTG-3′	107	1.94
ef1a	Hodne *et al.* (2010)	Forward: 5′-CCTTCAACGCCCAGGTCAT-3′ Reverse: 5′-AACTTGCAGGCGATGTGA-3′	100	1.92
ubiquitin	Hodne *et al.* (2012)	Forward: 5′-TGTCAAAGCCAAGATTCAGG-3′ Reverse: 5′-TGGATGTTGTAATCCGAGAGG-3′	111	1.86

qPCR analysis

qPCR analyses were carried out using the LightCycler 480 platform (Roche), as described previously (Weltzien *et al.* 2005, Hodne *et al.* 2012). All samples were run in duplicate, and in every round, three non-template negative control (NTC) reactions were conducted for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). In addition, to account for plate-to-plate variation, a standard positive calibrator control in triplicate was included on every plate. Each PCR reaction (10 µL) mixture contained 5 µL of SYBR Green I master mix (Roche), 1 µL (5 µM) of forward primer, 1 µL (5 µM) of reverse primer and 3 µL of diluted (1:10) cDNA. The qPCR reactions were carried out using an initial step for 10 min at 95°C to activate the *Taq* polymerase, followed by 42 cycles consisting of 10 s at 95°C (denaturation), 10 s at 60°C (annealing) and elongation at 72°C for 6 s. The fluorescence was measured after each elongation and used for determining the quantification cycle values (Cq). A melting curve analysis was performed directly following the PCR by continuously reading the fluorescence while slowly heating the reaction mixture from 65°C to 98°C. qPCR efficiencies (E) were determined based on cDNA dilution curves, which, together with the Cq values, were used to calculate the relative expression (Pfaffl 2001, Roche 2001):

$$\text{Relative expression } n = E_{\text{target}}^{\Delta Cq(\text{calibrator-sample})} * E_{\text{reference}}^{\Delta Cq(\text{sample-calibrator})}$$

The qPCR assay specificity was confirmed by agarose gel electrophoresis and sequencing.

Single-cell qPCR

In order to analyze *gnrhr* expression patterns in individual gonadotropes, qPCR was performed on transcripts harvested from single cells, as described by Hodne and coworkers (2010). To secure single-cell transcripts, the patch-clamp technique in whole cell configuration was used. During these experiments, cells were maintained in artificial extracellular solution (EC) comprising (mM): 150 NaCl, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 1.8 glucose and 10 HEPES/NaOH (pH 7.85) at 12°C, 320 mosmol. Patch pipettes were made from borosilicate glass with filament and had a resistance between 2 and 5 MΩ. Before fire polishing, the patch pipettes were silanized (Sigmacote, Sigma) to prevent extracellular contamination from attaching to the glass surface. The patch pipettes were filled with ribonuclease-free intracellular solution (mM): 120 CH_3SO_3K, 20 KCl, 10 HEPES/NaOH, 20 sucrose (pH 7.2), 290 mosmol. Following whole cell configuration, the cytosol was harvested into the pipette by applying gentle suction through tubing connected directly to the pipette holder. The cell content was transferred to 0.5 mL RNase-free tubes immediately after harvesting for RNA linear pre-amplification using MessageBOOSTER cDNA Synthesis Kit (Epicenter Biotechnologies, Madison, WI, USA). Transferred cytosol from individual cells was eluted into 3 µL of Quick Extract solution from the MessageBOOSTER cDNA Synthesis Kit together with Oligo(dT) primer containing a T7 promoter and SuperScript III (Life Technologies). Reverse transcriptase was used to synthesize first-strand cDNA from poly(A) RNA. After the second-strand cDNA synthesis, a high-yield *in vitro* transcription reaction was used to amplify the poly(A) RNA (mRNA). All single-cell qPCR was performed on cells from sexually mature cod, sampled during the spawning season.

Statistical analysis

Statistical analysis was performed using the JMP Pro12 software (SAS Institute Inc, Cary, NC, USA). Fold-changes of exposed samples relative to their respective solvent control mean were calculated and used in the subsequent analysis for both gene expression and viability data. To maintain control variance in the data set, each control sample was calculated in the same manner and included in the analysis. All data were tested for normality using the Shapiro–Wilk *W* test. In case of non-normality, log-transformed data gave a satisfactory fit to the normal distribution. Potential differences between control cells w/wo EtOH were tested by a one-way ANOVA. Differences in gene expression or viability between treatments were investigated with the Tukey–Kramer HSD test. Significance level was set to 0.05.

Results

Solvent control

For every primary cell culture, control wells with and without 0.2% EtOH (solvent) in the media were prepared. Viability assays performed on these cells revealed a significant negative effect from EtOH on both metabolic (mitochondrial) activity and membrane integrity (data not shown). However, qPCR analysis did not detect any influence on gene expression from the solvent (data not shown). Note that data from steroid exposed cells in the following sections are compared to effects seen in solvent control cells.

Cod pituitary cell viability after 7 days of primary culture

In general, 72 h of sex steroid exposure increased both metabolic activity (AB assay) and membrane integrity (CFDA-AM assay) (Figs 1, 2 and 3), with strong correlation between effects from each assay. No negative effects from the steroids were detected.

E2 exposure significantly increased viability compared to solvent control during all three reproductive stages (Fig. 1), with the exception of cells from maturing fish exposed to low (2.5 ng/mL) dose, which showed no effect from the treatment.

Androgen treatment significantly increased metabolic activity in cells from maturing and mature fish. Interestingly, in cells from mature fish the low dose (2 ng/mL) of DHTS gave a significantly stronger effect on metabolic activity than the high dose (Fig. 3). TS exposure stimulated membrane integrity in maturing and mature stages (Fig. 2), whereas effects from DHTS

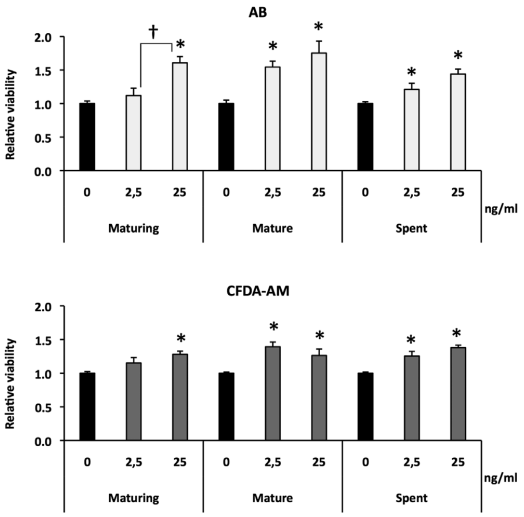

Figure 1 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of estradiol exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold-change relative to solvent control ± S.E.M. ($n = 8–12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P < 0.05$).

was seen only during the former with this parameter. No effect on viability was detected from androgen treatment on cells from spent fish.

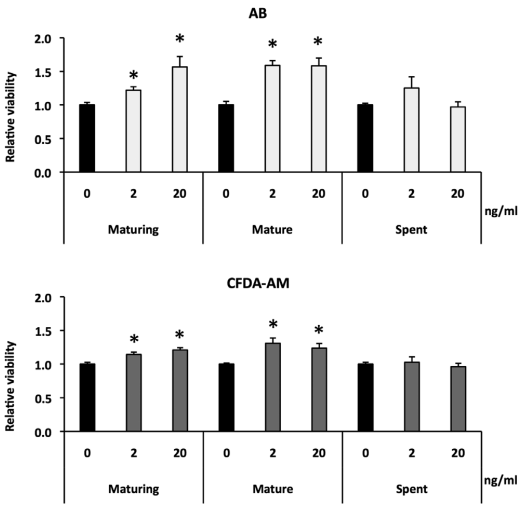

Figure 2 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of TS exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold-change relative to solvent control ± S.E.M. ($n = 8–12$). Asterisk indicates statistical difference from control ($P < 0.05$).

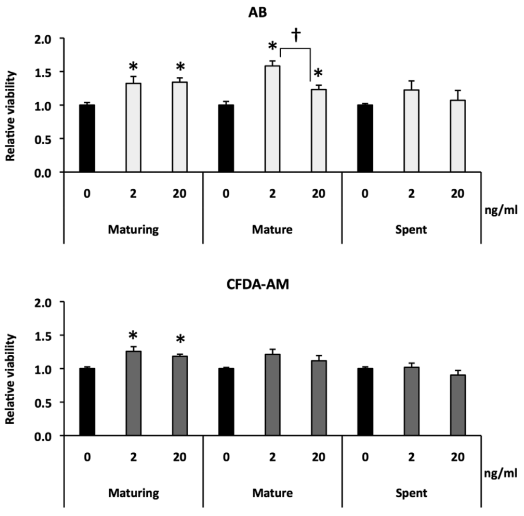

Figure 3 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of DHTS exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold change relative to solvent control ± S.E.M. ($n = 8–12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P < 0.05$).

Cod pituitary gene expression after 7 days of primary culture

Gene expression in individual gonadotropes

In order to identify which Gnrh receptors (Gnrhr) are expressed in cod gonadotropes, cytosol from individual non-exposed *fshb*- or *lhb*-expressing cells from mature fish was analyzed by single-cell qPCR. Ten *fshb*-expressing and 10 *lhb*-expressing cells were assessed, and 7 of each cell type expressed *gnrhr2a*. In the remaining cells, no *gnrhr* expression was detected. Of the 7 *gnrhr2a*-positive cells, three also expressed *gnrhr1b*, both in *fshb*- and *lhb*-expressing cells (Fig. 4), meaning both isoforms are expressed in individual gonadotropes. However, *gnrhr1b* expression was low and close to the detection limit of the qPCR assay (Cq values from 35 to 38). Thus, *gnrhr2a* seems to be the predominant receptor in both gonadotrope cell types, at least during spawning season (Cq values from 32 to 34).

Gene expression in steroid-exposed cells

Following 72 h of steroid exposure, gene expression of gonadotropin β-subunits and two Gnrhr's in the pituitary cells was determined by qPCR (Figs 5, 6 and 7). In general, steroid treatment had differential effects on gene expression, dependent on the type of steroid, target gene and time of year. Common for all three steroids was that

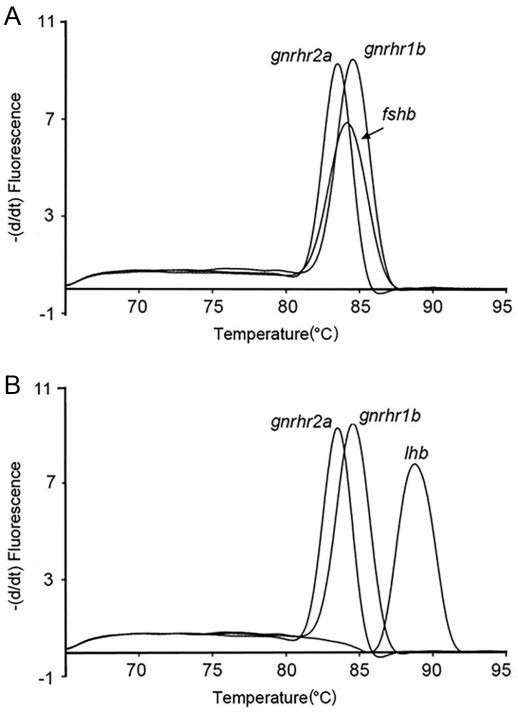

Figure 4 Transcripts of *gnrhr1b* and *gnrhr2a* in *fshb*- and *lhb*-expressing cells in Atlantic cod. Melting curve analysis of qPCR products from a single-cell was performed directly after the qPCR and plotted as the negative change in fluorescence per time as a function of temperature. Each qPCR product is reflected in a specific melting peak in the melting curve analysis. Expression of both *gnrhr1b* and *gnrhr2a* was detected in (A) *fshb*-expressing cells and (B) *lhb*-expressing cells.

gnrhr2a transcript levels showed the strongest treatment response, while, regardless of season, no response on *gnrhr1b* expression was observed.

In more detail, a significant increase in *gnrhr2a* levels was seen after high-dose E2 (25 ng/mL) exposure in cells from mature fish, while both E2 doses increased *gnrhr2a* expression during the spent stage (Fig. 5). Gonadotropin transcript levels were mainly unaffected by E2, with the exception of a significant increase in *fshb* expression following high-dose treatment in mature fish. A similar trend was observed with the expression of *lhb* ($P=0.08$).

The effects from TS exposure on *gnrhr2a* expression were seasonally dependent (Fig. 6). During maturation, no effects were seen, whereas the high dose (20 ng/mL) stimulated expression during the mature stage. Interestingly, in cells from spent fish, the effect of the low TS dose (2 ng/mL) on *gnrhr2a* expression was significantly different from both control and the high TS dose. A similar pattern was seen with *fshb*, where the low dose stimulated expression during maturation, while the high dose had no effect. During the mature stage, there were no observed effects on *fshb* expression, while transcript levels decreased following high-dose TS treatment in cells from spent fish. TS exposure had no statistically significant effects on *lhb* expression, although a decreasing trend was observed during the spent stage ($P=0.13$).

While E2 and TS stimulated *gnrhr2a* expression during mature and spent stages, DHTS affected *gnrhr2a* only in cells from maturing fish (Fig. 7), with the high-dose (20 ng/mL) exposure increasing expression. For gonadotropin gene expression, an effect from DHTS treatment was seen in cells from maturing fish. While the low DHTS dose (2 ng/mL) resulted in increased expression levels of both fshb and lhb, the high DHTS dose only significantly increased lhb expression levels, although fshb expression levels were also increased, but not significantly ($P=0.067$).

Discussion

Gonadotropes are important regulators of reproduction in vertebrates and are themselves subject to regulation from both hypothalamic input and systemic feedback. Using primary pituitary cell cultures as model system, this study describes the effects of sex steroid (E2, TS and DHTS) exposure (72 h) on cell viability and gene expression of gonadotropin β-subunits (*fshb* and *lhb*) and two Gnrh receptors (*gnrhr1b* and *gnrhr2a*) during three stages of sexual maturity in Atlantic cod. In addition, it demonstrates that both *fshb*- and *lhb*-producing gonadotropes can co-express two Gnrh receptor genes, *gnrhr1b* and *gnrhr2a*.

Cell viability

Very few studies have investigated pituitary cell viability after steroid exposure in fish, which is somewhat surprising as in most *in vitro* studies steroids are generally solubilized using solvents such as ethanol (EtOH) or dimethyl sulfoxide (DMSO), chemicals known to elicit deleterious effects on the health and properties of cultured cells (Baker & Kramer 1999, Santos *et al.* 2002). In this study, steroids were dissolved in EtOH to a final exposure concentration of 0.2%. This concentration did not affect gene expression of any gene analyzed, but did negatively affect the viability of the cells as compared to those exposed to EtOH-free media, both in terms of metabolic activity and membrane integrity. Consequently, all steroid exposed cells were compared to that of the solvent control. The negative effects on cell viability seen from EtOH were generally reversed with the addition of steroids. One proposed mechanism for the non-genomic effects of steroids is intercalation into the phospholipid bilayer,

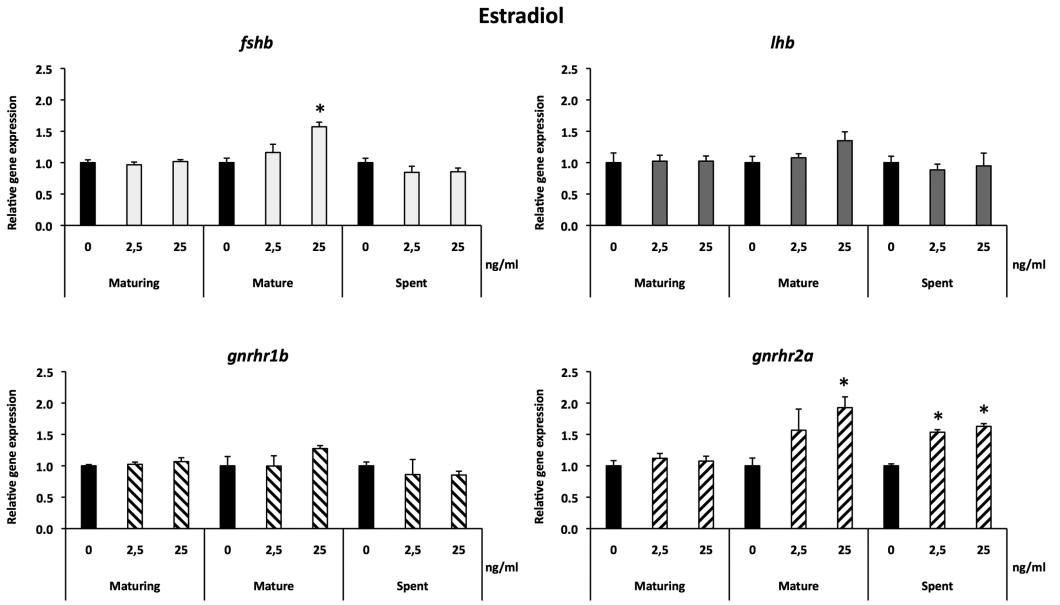

Figure 5 Gene expression in Atlantic cod pituitary cells after 72 h of estradiol exposure *in vitro*, presented as mean fold-change relative to solvent control ± S.E.M. ($n = 6–12$). Asterisks indicate statistical significant difference from control ($P < 0.05$).

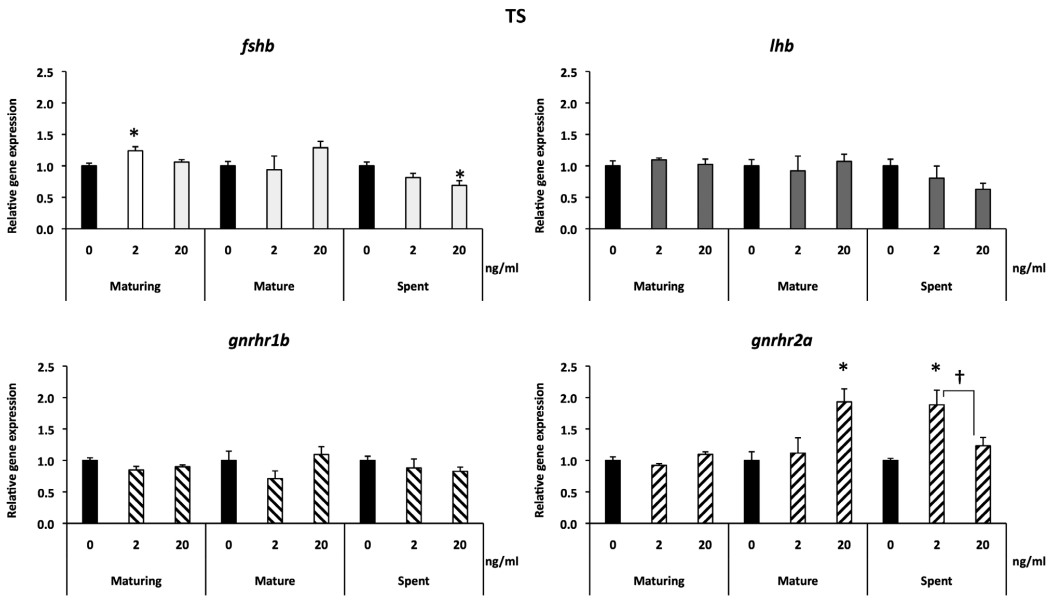

Figure 6 Gene expression in Atlantic cod pituitary cells after 72 h of TS exposure *in vitro*, presented as mean fold-change relative to solvent control ± S.E.M. ($n = 6–12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P < 0.05$).

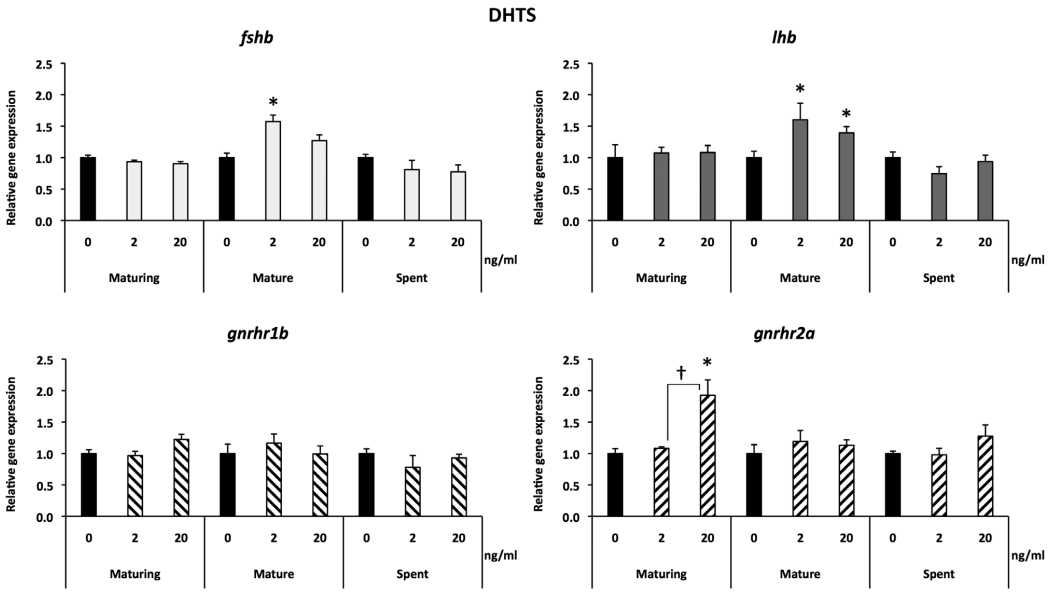

Figure 7 Gene expression in Atlantic cod pituitary cells after 72 h of DHTS exposure *in vitro*, presented as mean fold change relative to solvent control ± S.E.M. ($n = 6–12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P < 0.05$).

introducing structural and functional alterations of the cell membrane (Golden *et al.* 1998, Falkenstein *et al.* 2000, Whiting *et al.* 2000). In the present study, using physiological sex steroid concentrations, no negative effects on membrane integrity in cod pituitary cells was observed. This was not surprising, as membrane perturbations such as fluidity changes and leakage occur mainly at high, non-physiological, concentrations (Clarke *et al.* 1990, Shivaji & Jagannadham 1992). However, some differential effects were seen. While all steroids generally stimulated membrane integrity, E2 did so regardless of maturational state. While TS had no effect on pituitary cells from spent fish, DHTS had no effect on cells from both spent and mature fish. A similar pattern was evident from the metabolic activity assay. E2 stimulated cell metabolism at all three reproductive stages, while the androgens stopped being stimulatory in cells from the spent stage. This strongly implies that for androgens, there is a seasonal effect in terms of both membrane integrity and cellular metabolism. In spent fish, the endocrine activity of gonadotropes is low, with *fshb* and *lhb* synthesis being at its lowest level in the reproductive cycle (Mittelholzer *et al.* 2009). Although the present study was performed on all pituitary cell types and not just gonadotropes, it is tempting to speculate that there might be an association between gonadotropic endocrine activity and the androgen interplay with the plasma membrane and cellular metabolism in spent fish.

Gonadotropin expression

With the exception of the goldfish (*Carassius auratus*), that has been thoroughly studied (Kobayashi *et al.* 1989, 2000, Huggard *et al.* 1996, Sohn *et al.* 1998, 2001, Huggard-Nelson *et al.* 2002), most studies on teleost steroid feedback have been on single or total spawners. Tables 2, 3, 4 and 5 summarize studies on steroid effects on gonadotropin β-subunit mRNA synthesis in multibatch and asynchronous spawners (Tables 2, 3, 4 and 5: Hermaphrodite species are omitted; Tables 4 and 5: Studies using gonadectomized fish are omitted due to possible pain/stress interaction with reproduction). Common for the *in vitro* studies is that steroid exposure leads to no or stimulating effects on gonadotropin transcript levels, indicating that positive feedback can occur through mechanisms directly at the pituitary level. Inhibiting effects from steroids on gonadotropin transcripts have rarely been observed *in vitro*, but in the male African catfish (*Clarias gariepinus*) 24-h sex steroid (11-ketotestosterone (11-KTS), TS or E2) exposure inhibited *lhb* expression (Rebers *et al.* 2000). In the present study, TS inhibited *fshb* expression in spent cod. There are therefore likely, at least in some species, mechanisms that facilitate negative feedback directly at the pituitary level also.

Due to high levels of aromatase, the enzyme complex that converts TS to E2, in the brain and pituitary of teleosts

Table 2 *In vitro* studies of sex steroid effects on gonadotropin subunit gene expression in immature multibatch spawning fish[a].

	DHTS[1]/11-KTS[2]		E2		TS					
Species	*fshb*	*lhb*	*fshb*	*lhb*	*fshb*	*lhb*	**Sex**	**Dose (s)**	**Time**	**References**
Goldfish (*Carassius auratus*)	NM	NM	NM	NM	NM	↑	M+F	2–5000 ng/mL	15 h	Huggard *et al.* (1996)
Goldfish	NM	NM	2 ng/mL: (↑) 20 ng/mL: — 100–200 ng/mL: ↑	↑	NM	NM	M+F	2–200 ng/mL	15–16 h	Huggard-Nelson *et al.* (2002)
Goldfish	NM	NM	NM	NM	—	1/100 nM: (↑) 10 nM: ↑	M+F	1–100 nM	168 h	Sohn *et al.* (2001)
Nile tilapia (*Oreochromis niloticus*)	NM	NM	—	↑	NM	NM	F	10 nM–150 µM	48 h	Levavi-Sivan *et al.* (2006)
Tilapia hybrid (*O. niloticus* x *O.aureus*)	NM	NM	NM	NM	12/24/72 h: — 36/48 h: ↑	—	M	10 nM	12–72 h	Melamed *et al.* (1997)

[a]Immature fish are defined here as both those that have not yet reached puberty and those that have spawned previously, but are now in early gonadal recrudescence. Hermaphrodite species are omitted.
F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; —, No effect.

Table 3 *In vitro* studies of sex steroid effects on gonadotropin subunit gene expression in mature multibatch spawning fish[a].

	DHTS[1]/11-KTS[2]		E2		TS					
Species	*fshb*	*lhb*	*fshb*	*lhb*	*fshb*	*lhb*	**Sex**	**Dose** (s)	**Time**	**References**
Goldfish (*Carassius auratus*)	NM	NM	NM	NM	NM	2–20 ng/mL: — 2000 ng/mL: ↑	M+F	2–2000 ng/mL	15 h	Huggard *et al.* (1996)
Goldfish	NM	NM	NM	NM	10 nM: — 100 nM: (↑)	—	M+F	10–100 nM	48 h	Sohn *et al.* (2001)
Zebrafish (*Danio rerio*)	NM	NM	↑	↑	1 nM: — 10–100 nM: ↑	1 nM: — 10–100 nM: ↑	M+F	1–100 nM	72 h	Lin and Ge (2009)
Marine medaka (*Oryzias melastigma*)	NM	NM	↑	↑	NM	NM	M+F	1 µM	24 h	Tse *et al.* (2013)
Tilapia hybrid (*O. niloticus* x *O.aureus*)	NM	NM	NM	NM	0.01–0.1 nM: (↑) 1–100 nM: —	(↑)	M	0.1–100 nM	48 h	Melamed *et al.* (1997)
African catfish (*Clarias gariepinus*)	NM	0.1–2 ng/mL: —[2] 10 ng/mL: ↑[2] 50 ng/mL: (↑)[2]	NM	0.001–0.02 ng/mL: — 0.1–10 ng/mL: ↑ 50 ng/mL: (↑)	NM	NM	M	0.001–50 ng/mL	48 h	Rebers *et al.* (2000)
African catfish	NM	24 h: NM [1]/↓[2] 48 h: — [1]/(↑)[2]	NM	24 h: ↓ 48 h: (↑)	NM	24 h: ↓ 48 h: ↑	M	50 ng/mL	24/48 h	Rebers *et al.* (2000)

[a]Hermaphrodite species are omitted.
F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; – , No effect; ↓, significant decrease; [1], DHTS; [2], 11-KTS.

Table 4 *In vivo* studies of sex steroid effects on gonadotropin subunit gene expression in immature multibatch spawning fish[a].

	DHTS[1]/11-KTS[2]		E2		TS					
Species	*fshb*	*lhb*	*fshb*	*lhb*	*fshb*	*lhb*	**Sex**	**Dose** (s)	**Time**	**References**
Goldfish (*Carassius auratus*)	↓[2]	↓[2]	↓	↑	↓	(↑)	M	200 µg/fish	14 days	Sohn *et al.* (1998)
Goldfish	↓[2]	↓[2]	↓	↑	↓	↑	F	200 µg/fish	14 days	Sohn *et al.* (1998)
Goldfish	NM	NM	NM	NM	NM	12–48 h: (↓) 72–96 h:↑	M+F	20 µg/fish	12–96 h	Huggard *et al.* (1996)
Goldfish	NM	NM	NM	NM	NM	0.2–2 µg: ↑ 20 µg: –	M+F	0.2–20 µg/fish	24 h	Huggard *et al.* (1996)
Goldfish	NM	NM	0.02 µg: – 0.2–2 µg: ↑	↑	NM	NM	M+F	0.02–2 µg/fish	24 h	Huggard-Nelson *et al.* (2002)
Goldfish	5 µg[2]: ↓ 50 µg[2]: ↓	5 µg[2]: – 50 µg[2]: –	5 µg: ↓[2] 50 µg: ↓	5 µg: ↑ 50 µg: ↑	5 µg: ↓ 50 µg: ↓	5 µg: ↑ 50 µg: ↑	M+F	5/50 µg/g pellet 2% BW pellet/d	14 days	Kobayashi *et al.* (2000)
European sea bass (*Dicentrarchus labrax*)	↓[1]	(↑)[1]	↓	(↑)	↓	↑	M+F	20 mg/kg BW	12 days	Mateos *et al.* (2002)
Atlantic croaker (*Micropogonias undulatus*)	NM	NM	1 µg/g: ↓	1 µg/g: –	NM	NM	M+F	1/5 µg/g BW	120 h	Banerjee and Khan (2008)
Nile tilapia (*Oreochromis niloticus*)	NM	NM	5 µg/g: ↓ 250 µg/kg: (↓) 500 µg/kg: ↓	5 µg/g: ↑ 250 µg/kg: (↓) 500 µg/kg: ↓	NM	NM	F	250/500 µg/kg BW	6 days	Levavi-Sivan *et al.* (2006)
Red sea bream (*Pagrus major*)	–[2]	↑[2]	–	–	–	–	M	0.68 mg steroid/kg BW	30 days	Yamaguchi *et al.* (2006)
African Catfish (*Clarias gariepinus*)	NM	–[1]/↓[2]	NM	↑	NM	↑	M	30 µg/g BW	14 days	Cavaco *et al.* (2001)

[a]Immature fish are defined here as both those that have not yet reached puberty and those that have spawned previously, but are now in early gonadal recrudescence. Gonadectomized fish and/or hermaphrodite species are omitted.
BW, body weight; F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; –, No effect; (↓), nonsignificant decrease; ↓, significant decrease;
[1], DHTS; [2], 11-KTS.

(Diotel *et al.* 2010), effects from TS exposure might occur through both androgenic and estrogenic mechanisms. The level of brain aromatase gene expression and enzyme activity is itself modulated by steroids and has been shown to fluctuate with the reproductive stage and season in several species (Andersson *et al.* 1988, Pasmanik & Callard 1988*a*, Gonzalez & Piferrer 2003, Diotel *et al.* 2010). Hence, the amount of TS aromatization might vary and many studies on potential sex steroid feedback therefore include a non-aromatizable androgen to distinguish between actions mediated through estrogen receptors (Er) and androgen receptors (Ar). Despite 11-KTS being non-aromatizable and generally the most important teleost androgen, DHTS was chosen as the non-aromatizable androgen in this study, in part because this androgen has a high binding affinity for the Ar (Sperry & Thomas 1999, Jørgensen *et al.* 2007), and partly because it is often used in studies to demonstrate androgen effects (Martyniuk *et al.* 2013). Although the presence, let alone the biological activity, of DHTS has not been demonstrated in most teleosts, its synthesis and plasma levels has been reported in both male and female fathead minnow (*Pimephales promelas*) (Margiotta-Casaluci *et al.* 2013). In another species, the urohaze-goby (*Glossogobius olivaceus*), DHTS was the main product of *in vitro* steroidogenesis in testes tissue (Asahina *et al.* 1985). In addition, enzymatic activity of 5-α reductase, the enzyme that converts TS to DHTS, has been demonstrated in different tissues, including the brain and pituitary, in several teleost species (Pasmanik & Callard 1988*a*, Latz & Reinboth 1993, Margiotta-Casaluci *et al.* 2013), so it is likely that DHTS plays a biological role not yet described in teleosts. In the present study, DHTS stimulated *fshb* expression in cells from mature cod, which, to our knowledge, is the first time effects on *fshb* expression from a non-aromatizable androgen have been demonstrated *in vitro* in a multibatch spawner. Furthermore, DHTS, but not T, affected *lhb* expression. This result is in contrast to a study performed on mature male African catfish, where a relatively high dose (50 ng/mL) of DHTS had no effect on *lhb* expression *in vitro*, whereas a similar dose of TS did, presumably following aromatization to E2 (Rebers *et al.* 2000). In Atlantic cod, high E2 dose (20 ng/mL) had a stimulatory trend on *lhb* transcription in mature fish, but this result was not statistically significant. While TS had no effect on *lhb* levels, all steroids affected *fshb* expression at some point during the reproductive cycle, indicating that both estrogenic and androgenic mechanisms are involved in its regulation at the pituitary level in the cod. In a recent study on Atlantic cod, Nagasawa and coworkers (2014) demonstrated a seasonal variation in gonadal Er and Ar gene expression levels. To our knowledge, there is no available data regarding Er/Ar seasonal variation at the pituitary level in cod, although an earlier study reported changes in pituitary Er transcript levels over the reproductive cycle in the largemouth

Table 5 *In vivo* studies of sex steroid effects on gonadotropin subunit gene expression in mature multibatch spawning fish[a].

	DHTS[1]/11-KTS[2]		E2		TS					
Species	*fshb*	*lhb*	*fshb*	*lhb*	*fshb*	*lhb*	**Sex**	**Dose** (s)	**Time**	**References**
Goldfish (*Carassius auratus*)	—[2]	(↑)[2]	—	↑	—	(↑)	M	200 µg/fish	14 days	Sohn *et al.* (1998)
Goldfish	(↑)[2]	—[2]	(↓)	—	—	—	F	200 µg/fish	14 days	Sohn *et al.* (1998)
Goldfish	NM	NM	NM	NM	NM	↑	M + F	0.02–20 µg/fish	24 h	Huggard *et al.* (1996)
African Catfish (*Clarias gariepinus*)	NM	↑[2]	NM	NM	NM	NM	M	10, 20 and 100 µg/kg BW	5 days	Rebers *et al.* (1997)

[a]Gonadectomized fish and/or hermaphrodite species are omitted.
BW, body weight; F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; – , No effect; (↓), nonsignificant decrease; [1], DHTS; [2], 11-KTS.

bass (*Micropterus salmoides*) (Martyniuk *et al.* 2009). In the current study, the steroids had differential effects on gonadotropin subunits expression at the different reproductive stages. Fluctuations in Er/Ar levels could change tissue sensitivity or responsiveness to estrogens/androgens and might serve as a possible explanation to this observation. Furthermore, as mixed sex cultures were used in these experiments, no information about potential sex-dependent regulation can be provided. It is possible that results from one sex could mask results from the other. Potential male vs female differences regarding both gonadotropin steroid sensitivity and Er/Ar levels through the reproductive cycle would be interesting and relevant topics for future studies in Atlantic cod.

Gonadotropin-releasing hormone receptor expression

In cell cultures prepared from intact pituitaries, all pituitary cell types, not just the gonadotropes, are present. Previously, Gnrhr gene expression or protein has been demonstrated in gonadotropes, somatotropes (ST), lactotropes, thyrotropes (TT), melanotropes, corticotropes (CT) and somatolactotropes (SLT) (Illing *et al.* 1999, Parhar *et al.* 2002, 2005, Flanagan *et al.* 2007). In Nile tilapia (*Oreochromis niloticus*), Parhar and coworkers (2005) conducted a study examining receptor expression in individual pituitary cells. They showed that mRNA of receptors phylogenetically close to *gnrhr1b* was expressed in all cells types except TT, SLT (mature fish) and CT (immature fish). Similarly, mRNA of receptors closely related to *gnrhr2a* was detected in all cell types except immature fish TT and CT cells. The same study also showed that individual cells could concurrently express several isoforms of Gnrhr and that both *fshb* and *lhb*–producing gonadotropes expressed both *gnrhr1b* and *gnrhr2a*. In the present study, it was demonstrated that this also is true in Atlantic cod, with co-expression of both receptors detected in 30% of the gonadotropes investigated. While this points toward a role for both receptors in gonadotropin regulation, in both *fshb*- and *lhb*-expressing cells, *gnrhr2a* was clearly the predominant receptor. In a study on Gnrhr gene expression over the reproductive cycle in female Atlantic cod, Hildahl and coworkers (2011) showed that, in whole pituitary samples, only *gnrhr2a* isoform expression levels followed the same profile as the GSI, and was significantly upregulated during periods of reproductive activity. Even though the observed expression pattern could not be confined to only gonadotropes, these results indicate that *gnrhr2a* is the main receptor mediating gonadotropin expression in cod.

In teleosts, as well as mammals, steroid treatment has been shown to modify pituitary responsiveness to Gnrh and regulate Gnrhr mRNA abundance (Quinones-Jenab *et al.* 1996, Cowley *et al.* 1998, Yen *et al.* 2002, Levavi-Sivan *et al.* 2006, Lin *et al.* 2010). In the current study, *gnrhr1b* showed no, whereas *gnrhr2a* showed strong, sensitivity to steroid exposure. All sex steroids tested were able to stimulate the expression of *gnrhr2a*, which would, assuming its translation into functional protein, increases the sensitivity of gonadotropes to Gnrh stimulation and acts as a direct route for a positive feedback at the pituitary level. However, the mechanisms through which steroids affect *gnrhr2a* expression is not clear, as the stimulation occurred at different times of the year dependent on the steroid. During maturation, *gnrhr2a* transcripts were upregulated by DHTS exposure, while at later stages, DHTS did not affect its expression. Conversely, no effect was seen from E2 or TS during maturation, but both hormones stimulated *gnrhr2a* transcription in cells from mature and spent fish. In general, a close correlation exists between circulating steroid levels and GSI in Atlantic cod, with low plasma levels during early gonadal recrudescence, increasing during maturation, peaking at spawning, before declining rapidly in spent fish (Dahle *et al.* 2003). The steroid sensitivity of *gnrhr2a* does thus not correlate with circulating steroid levels. As with the gonadotropin regulation, seasonal and differential regulation or location of Ar/Er and their respective isoforms might be one explanation to this (Pasmanik & Callard 1988*b*, Harbott *et al.* 2007).

In the pituitary primary cultures used in the present study, control levels of *gnrhr1b* expression (data not shown) were quite stable over the reproductive cycle except for a small decrease during spawning season, and no effect on its transcription was seen following steroid exposure. In individual cells from mature fish, single-cell qPCR demonstrated that while *gnrhr1b* was co-expressed with *gnrhr2a* in the gonadotropes, the transcript levels

were low and sometimes undetectable. Taken together, these results indicate that, in Atlantic cod, Gnrhr1b is not a part of the sex steroid feedback mechanism at the pituitary level and its gene expression is more abundant in other pituitary cell types than gonadotropes. In cod, as in many other teleosts, somatic growth rate is low during times of gonadal growth and spawning (Hansen *et al.* 2001). The growth rate is mediated mainly through the somatotropic axis (Dai *et al.* 2015), where growth hormone (Gh) production may be stimulated by Gnrh (Marchant *et al.* 1989). Since *gnrhr1b* transcript levels decreased during spawning season in control cells and have been demonstrated in STs in other species (Flanagan *et al.* 2007), a tempting hypothesis for future studies is that this receptor is involved in Gh regulation.

Conclusions

In the present study, it was demonstrated that sex steroids (E2, TS and DHTS) stimulate cell viability and have the ability to directly affect transcript levels of gonadotropins and two Gnrhr orthologues in the Atlantic cod pituitary. Cell viability was not correlated to gene expression, as steroids induced overall stimulating effects on metabolic activity and membrane integrity, while transcript levels had more seasonally dependent patterns. Co-transcription of *gnrhr1b* and *gnrhr2a* was confirmed to both *fshb*- and *lhb*-expressing gonadotropes using single-cell qPCR, with *gnrhr2a* clearly being most prominently expressed. While steroid exposure had no effect on *gnrhr1b* expression, all steroids stimulated *gnrhr2a* levels at some stage in the reproductive cycle. These results point to Gnrhr2a being the main modulator of gonadotropin regulation in Atlantic cod, and that regulation of its gene expression level might function as a direct mechanism for positive steroid feedback at the pituitary level. The expression of *fshb* showed greater steroid sensitivity than that of *lhb* and was affected by all steroids at some reproductive stage, while only DHTS was able to affect *lhb* expression. The mechanisms behind the differential steroid sensitivity is not clear, but might be due to different hormonal regulatory elements in the promoter region of the genes, the level/existence of Er/Ar in cell types that express the individual genes or that these levels vary with season.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0208.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Sex steroids differentially regulate *fshb*, *lhb* and *gnrhr* expression in Atlantic cod (*Gadus morhua*) pituitary

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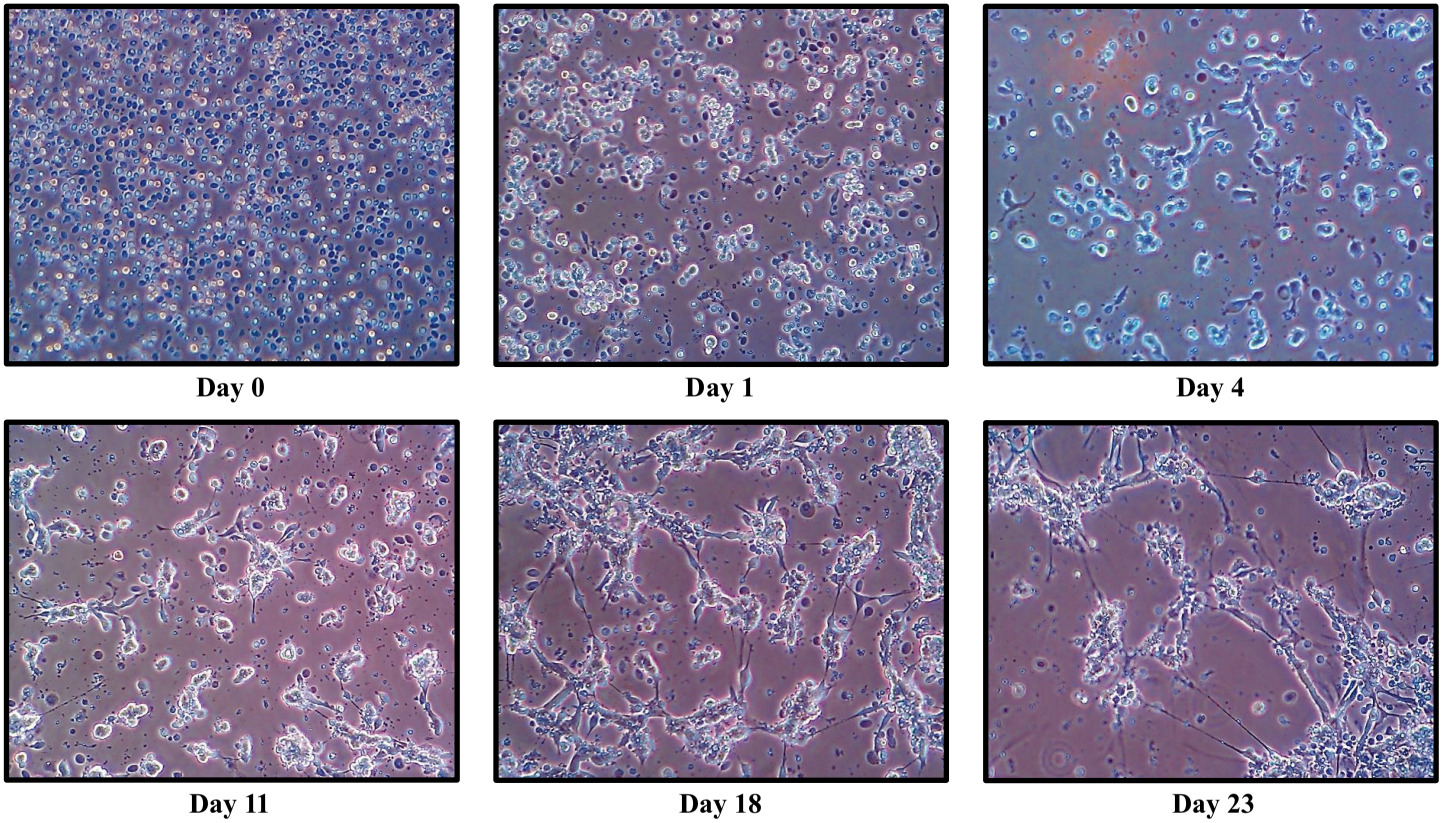
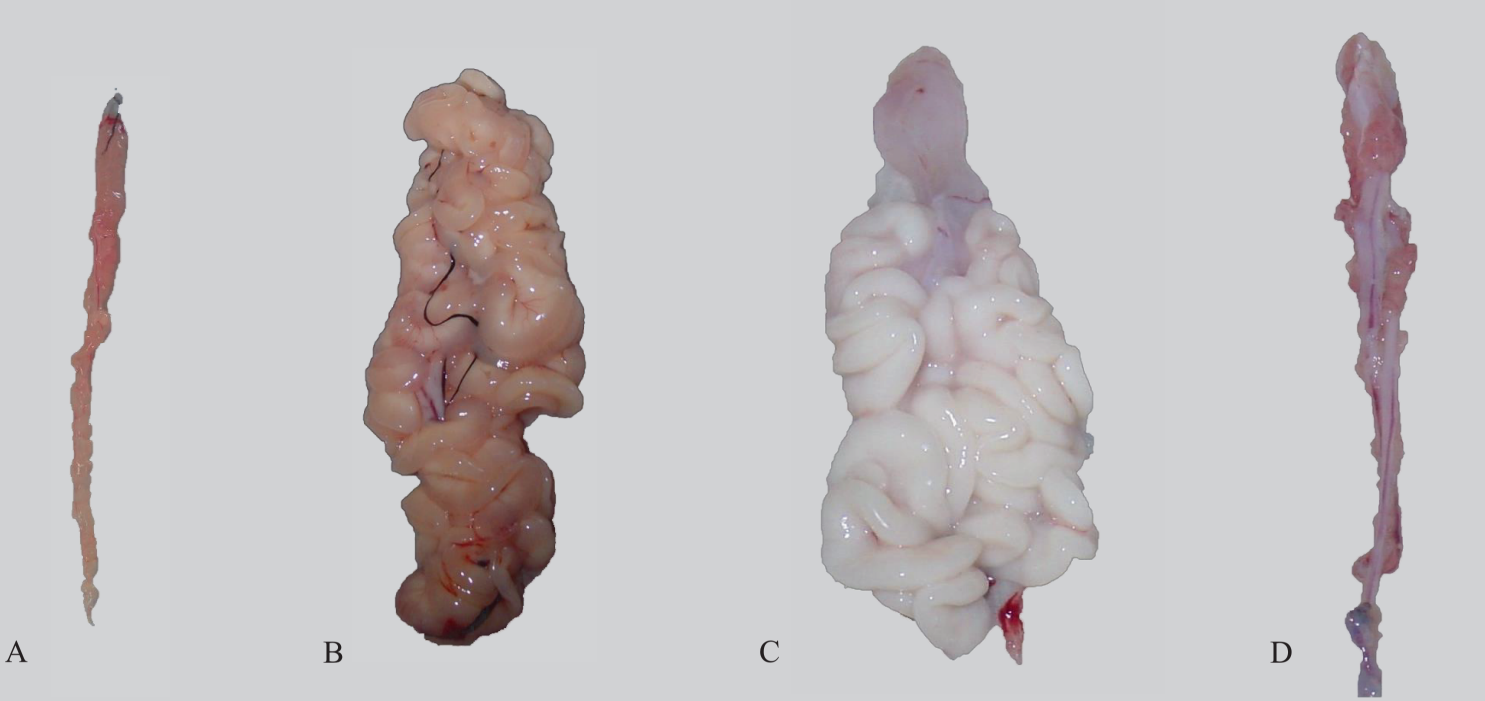
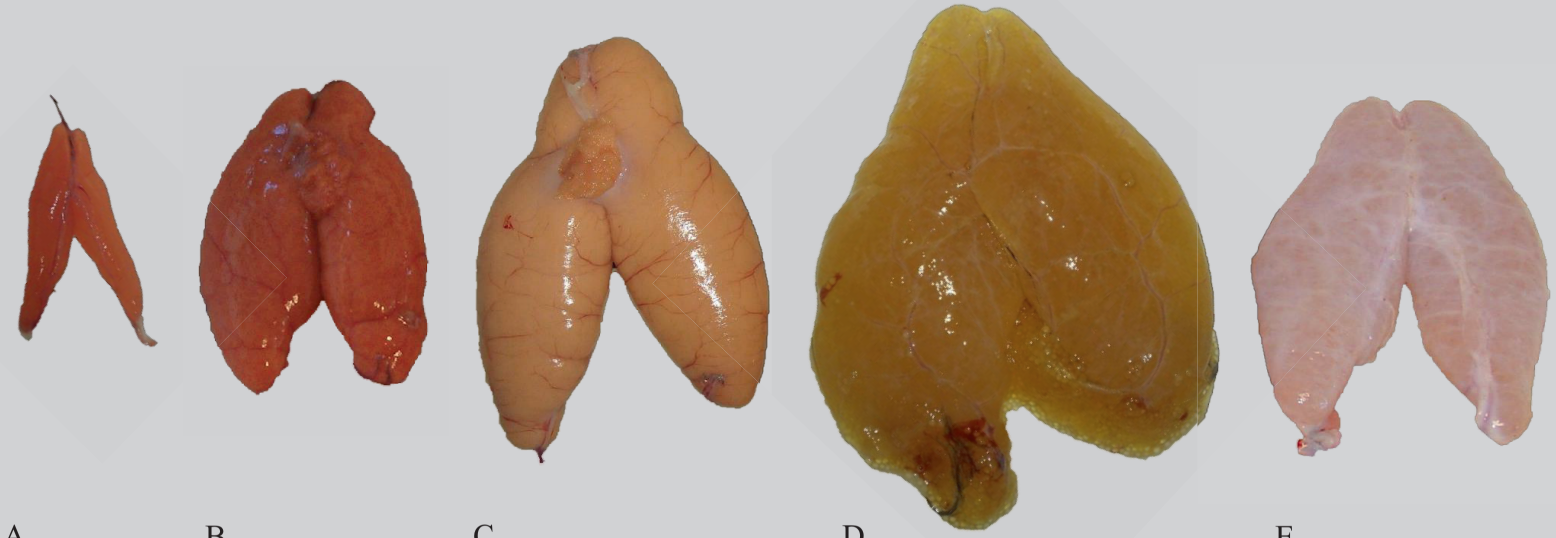
Supporting Figures 1-3



Supplementary Figure 1 Timeline of Atlantic cod (*Gadus morhua*) pituitary primary culture. Day 0; making of the culture. Day 1; medium change and removal of unattached cells. Small extensions are starting to form in a few blue colored cells. Day 4-18; cells starts to aggregate and form extensions between cell colonies. Day 23; about half of the cell aggregates are unattached to the culture well bottom and float in the medium. Note that some cells appear naturally blue under the microscope, making Trypan Blue staining a suboptimal viability measurement for these cells.



Supplementary Figure 2 Atlantic cod (*Gadus morhua*) testes at different stages of sexual maturity; Immature (**A**), Maturing (**B**), Mature (**C**) and Spent (**D**). Immature testes have small pinkish lobes and small blood vessels. In the maturing testes, the lobe size increase, still pink in the periphery where the spermatogonial cells are, but with an off-white area toward the sperm duct increasing in size as the germ cells develop. With further maturation of the testes, the pink zone in the periphery will become progressively smaller until full maturation is reached, and the lobes are all white and filled with sperm. Spent testes are flaccid, have decreased lobe size with more pink color, and large blood vessels still present. Some residual sperm may be present in lobes and sperm duct.



Supplementary Figure 3 Atlantic cod (*Gadus morhua*) ovaries at different stages of sexual maturity; Immature (**A**), Maturing (early; **B**, late; **C**), Mature (**D**) and Spent (**E**). The immature ovary has small blood vessels, little tissue texture and transparent or pale orange color. During maturation and vitellogenesis, the tissue become more dense and pinker in color, with opaque oocytes clearly visible. Vascular tissue increases. In the fully mature gonad, translucent hydrated and/or ovulating oocytes are present. The spent ovary is flaccid, contains no hydrated eggs and have thickened ovarian walls. The blood vessels are still large. As the size of maturing and spent ovaries can be similar, staging should not be based on the gonadosomatic index alone. For detailed staging of maturing oocytes, histological examination is the most accurate.

III

Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation

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Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation

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ABSTRACT

Through the action of cortisol, stress can affect reproductive biology with behavioural and physiological alterations. Using mixed sex primary pituitary cultures from Atlantic cod (*Gadus morhua*), the present study aimed to investigate potential direct effects of basal and stress level cortisol on the pituitary in terms of cell viability and reproduction-related gene expression at different stages of sexual maturity. Stress level of cortisol stimulated cell viability in cells derived from sexually maturing and mature fish. In cells from spent fish, high cortisol levels did not affect cell viability in terms of metabolic activity, but did stimulate viability in terms of membrane integrity. Basal cortisol levels did not affect cell viability. Ethanol, used as solvent for cortisol, decreased cell viability at all maturity stages, but did generally not affect gene expression. Genes investigated were *fshb*, *lhb* and two Gnrh receptors expressed in cod gonadotropes (*gnrhr1b* and *gnrhr2a*). Cortisol had dual effects on *fshb* expression; stimulating expression in cells from mature fish at stress dose, while inhibiting expression in cells from spent fish at both doses. In contrast, cortisol had no direct effect on *lhb* expression. While *gnrhr2a* transcript levels largely increased following cortisol treatment, *gnrhr1b* expression decreased in cells from spent fish and was unaffected at other maturity stages. These findings demonstrate that cortisol can act directly and differentially at the pituitary level in Atlantic cod and that factors facilitating these actions are dose-dependently activated and vary with level of sexual maturity.

1. Introduction

Sexual maturation and reproduction in teleosts are regulated through the brain-pituitary-gonadal (BPG) axis. Hypothalamic neurons secrete gonadotropin-releasing hormone (Gnrh) onto gonadotrope cells in the pituitary, leading to the synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which in turn stimulate gonadal development, gametogenesis and steroid production. The axis itself is regulated through an array of environmental factors, such as photoperiod, temperature, population density and food availability, as well as internal modulators such as the levels of sex steroids, dopamine, kisspeptins and others (reviewed by Golan et al., 2014; Levavi-Sivan et al., 2010). In addition, stress has been shown to interfere with the BPG-axis and affect fish reproduction.

Stress can be defined as a state in which there is a perceived threat to an organism's homeostasis, real or imagined. The factor causing this threat is the stressor, and the physiological and behavioural responses attempting to re-establish homeostasis is the stress-response (Charmandari et al., 2005; McEwen, 2000; Wendelaar Bonga, 1997). In fish, the stress response is characterized by the activation of the brain-pituitary-interrenal (BPI) axis and of sympathetic fibres leading to release of catecholamines from chromaffin cells in the head kidney (Wendelaar Bonga, 1997). Activation of the BPI-axis enhances corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoid (GC) release (Flik et al., 2006). The main teleost GC is cortisol, produced by steroidogenic interrenal cells functionally equivalent to the mammalian adrenal cortex. In fish, as in mammals, high levels of cortisol have been demonstrated to act on all levels of the BPG-axis, affecting Gnrh and gonadotropin levels, gonadal development, gamete quality, vitellogenin production, sex steroid

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levels and sexual behaviour (reviewed in Goos and Consten, 2002; Leatherland et al., 2010; Milla et al., 2009; Schreck, 2010; Schreck et al., 2001). The results, however, are inconsistent between studies. For instance, in cortisol-implanted sexually maturing brown trout (*Salmo trutta*), pituitary gonadotropin levels decreased in both males and females, while in juvenile female eel (*Anguilla Anguilla*) pituitary Lh levels increased after cortisol exposure, both *in vivo* and *in vitro* (Carragher et al., 1989; Huang et al., 1999). Moreover, cortisol administration to vitellogenic ovarian follicles decreased 17β-estradiol and testosterone production in rainbow trout (*Oncorhynchus mykiss*) and Eurasian perch (*Perca fluviatilis*) (Mandiki et al., 2017; Reddy et al., 1999), but had no effect in goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*) or the New Zealand snapper (*Pagrus auratus*) (Pankhurst et al., 1995). The differential responses seem dependent on species-specific regulations, age, sex and maturational stage, as well as dose, manner and duration of the treatment.

In Atlantic cod (*Gadus morhua*), an important commercial saltwater species belonging to the relatively little studied order of Gadiformes fish, stress has been shown to induce irregular spawning intervals and lowered fertilization rates (Bogevik et al., 2012; Kjesbu, 1989; Kjesbu et al., 1990). Moreover, high cortisol levels altered reproductive behaviour and lead to increased number of abnormal larvae (Morgan et al., 1999), as well as differential expression of cytogenesis-linked genes inn eggs and embryo (Kleppe et al., 2013) in this species. However, stress or cortisol effects at the higher levels of the BPG-axis in Atlantic cod are not known. The objective of the present study was therefore to investigate potential direct effects of cortisol at the cod pituitary level, using primary cell cultures as the model system. After reaching puberty at 2–3 years of age in the wild, Atlantic cod spawn annually. Gonadal maturation commence during late autumn, with full maturation and multiple spawning occurring from January until May (Dahle et al., 2003; Kjesbu, 1989). The pituitary cultures were prepared from fish at different stages of sexual maturity (maturing, mature and spent), and exposed to cortisol in doses corresponding to basal and stress plasma levels. Impact on cell viability and gene expression of gonadotropin (*lhb* and *fshb*) and two cod gonadotrope Gnrh receptor isoforms (*gnrhr1b* and *gnrhr2a*) (von Krogh et al., 2017) were recorded.

2. Materials and methods

2.1. Animals

Atlantic cod (0.5–4.1 kg body weight) from the southern Norwegian coast were captured and kept for at least one week in the aquarium facilities at the University of Oslo before being sacrificed. While in captivity, they were fed shrimp daily. Aquaria were continuously perfused with seawater with a salinity of 28‰ and a temperature of 8–12 °C. The light cycle was adjusted to fit the natural night/day rhythm in Oslo (60°N). Both male and female cod were used in the study. A general permission to keep the animals in the facilities was given by the Norwegian animal research authority (S-2008/108215) and all animals were kept and handled in agreement with their requirements. A specific approval for this study was not needed, as the animals themselves were not experimentally treated (Norwegian legislation for use of animals in research, Chapter II, §6).

2.2. Pituitary primary cell cultures

Dispersed pituitary primary cultures of mixed sexes were prepared using culture conditions optimized to the physiology of Atlantic cod (Hodne et al., 2012). Due to difficulties obtaining fish and the lack of external dimorphic sexual phenotypes in cod, in order to save fish, donors were not discriminated based on sex, but only on state of sexual maturity. Maturational status was determined based on visual inspection of the gonads (see von Krogh et al., 2017, supplementary data) and gonadosomatic index (GSI; [Gonadal weight / Total body weight] x 100). Two cultures were prepared from each stage (maturing, mature and spent) over a two-year period. The GSI (mean ± SD, M = males, F = females) for each stage was; maturing (M; 1.47 ± 1.78, F; 1.74 ± 0.67), mature (M; 3.60 ± 4.46, F; 4.71 ± 5.71) and spent (M; 0.11 ± 0.17, F; 0.62 ± 0.33). The sex ratios within cultures were; maturing stage (8F/7M and 7F/7M), mature stage (6F/2M, and 7F/2M) and spent stage (4F/5M and 3F/2M).

In short, dissected pituitaries were pooled (n = 5–15) and dissociated. Dispersed cells were seeded at a density of 1.5×10^5 cells/cm^2 in modified L-15 medium (Life Technologies, Carlsbad, CA, USA) and incubated at 12 °C in a humidified atmosphere of 0.5% CO_2 in air (pCO_2; 3.8 mmHg, which resulted in a medium pH of 7.85). After 24 h, culture media were replaced to remove damaged and detached cells.

2.3. Steroid exposure

At day 4 in culture, cells were exposed to cortisol. Stock solutions of cortisol (Sigma, St. Louis, MO, USA) were prepared by dissolving the steroid in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway). Before cell exposure, stocks were diluted to desired concentration in modified L-15 medium, with working solutions having a final EtOH concentration of 0.2% (34.25 mM). For each experiment, controls w/wo EtOH (solvent control/control blank) were included.

Two physiologically relevant doses of 10 ng/ml (2.76×10^{-8} M) and 100 ng/ml (2.76×10^{-7} M), corresponding roughly to Atlantic cod basal and stress plasma cortisol levels, respectively (King and Berlinsky, 2006; Staurnes et al., 1994), were applied. Six replicate wells in 24-well plates per treatment were prepared for gene expression analysis, whereas for viability tests, a minimum of six replicate wells in 96-well plates were used. Exposure lasted 72 h.

2.4. Viability testing

After seven days of culture (w/wo exposure), viability tests were performed using two non-toxic fluorescent indicator dyes, AlamarBlue (AB) and 5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) (both from Life Technologies). These assays indicate metabolic activity and plasma membrane integrity, respectively, and measure the conversion of a non-fluorescent dye into a fluorescent dye by enzymes present in intact and viable cells (Bopp and Lettieri, 2008). The test procedures were carried out as described by Hodne et al. (2012).

In short, cells were seeded, incubated and exposed to cortisol as described above. At day 7, culture medium in all wells was replaced with 100 µl Tris buffer (50 mM, pH 7.5) containing both 5% AB and 4 µM CFDA-AM (from 4 mM stock in DMSO). After 30 min of incubation, the concentration of fluorescent products was measured simultaneously for both probes using a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Data was collected with the Gen5 (Gen5 Data Analysis Software, Bio-Tek Instruments Inc.) software.

As a positive control for cell toxicity, serving as intra/inter assay control, each plate included additional wells exposed to copper sulphate ($CuSO_4$) (0.156–2.5 mM) the last 24 h of culture (n = 6 per concentration). The effects from $CuSO_4$ exposure were comparable between individual plates and cultures, indicating the AB/CFDA-AM assays a stable system for cytotoxicity measurement in these cultures (see supporting information, Fig. S1).

2.5. Quantification of gene expression

2.5.1. RNA extraction and cDNA synthesis

Cells used for gene expression analysis were harvested at day 7. Total RNA was extracted in Trizol (Life Technologies) and resuspended in 10 µl RNase-free water (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). DNase treated RNA (TURBO DNase-free (Ambion)) was quantified spectrophotometrically (NanoDrop, Thermo Fisher

Table 1
qPCR primers used in the present study.

Target	Reference		Primer sequence	Amplicon size (nt)	Efficiency
lhb	Hodne et al., 2010	Forward	5′-GTGGAGAAGAAGGGCTGTCC-3`	81	1.93
		Reverse	5′-GGACGGGTCCATGGTG-3`		
fshb	Hodne et al., 2010	Forward	5′-GAACCGAGTCCATCAACACC-3`	63	1.84
		Reverse	5′-GGTCCATCGGGTCCTCCT-3`		
gnrhr1b	von Krogh et al., 2017	Forward	5′-GCTACTCCCGAATCCTCCTC-3`	73	1.96
		Reverse	5′-CGCCTCAGGTATGACTCTCC-3`		
gnrhr2a	von Krogh et al., 2017	Forward	5′-TTCACCTTCTGCTGCCTCTT-3`	113	1.99
		Reverse	5′-TCCGTGGAGGAAAGATTGTC-3`		
bactin	Hodne et al., 2012	Forward	5′-TTCTACAACGAGCTGAGAGTGG-3	102	1.84
		Reverse	5′-CATGATCTGGGTCATCTTCTCC-3		
arp2	Hodne et al., 2012	Forward	5′-GGAGGTTAGAAGTAGCAAGGAGC-3′	107	1.94
		Reverse	5′-TGCTGACTCTCACGGAGTTG-3`		
ef1a	Hodne et al., 2010	Forward	5′-CCTTCAACGCCCAGGTCAT-3`	100	1.92
		Reverse	5′-AACTTGCAGGCGATGTGA-3`		
ubiquitin	Hodne et al., 2012	Forward	5′-TGTCAAAGCCAAGATTCAGG-3`	111	1.86
		Reverse	5′-TGGATGTTGTAATCCGAGAGG-3′		

Scientific), and the quality assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) of the RNA Integrity Number (RIN). Only RNA samples with RIN number above 8 were analysed further. First strand cDNA synthesis was performed on 500 ng total RNA using random hexamer primers and Super Script III (Life Technologies) according to standard procedures, and stored at −20 °C until qPCR.

2.5.2. Primers and reference genes

All qPCR primers (Table 1) were designed using Primer3 shareware (http://frodo.wi.mit.edu/primer3/input.htm). To test for possible hairpin loops and primer dimer formations, potential primers were further analysed using Vector NTI (Life Technologies). In each pair, one primer was targeted to an exon-exon border to avoid amplification of genomic DNA. The expression of four different genes specifically related to pituitary reproductive function, *i.e.*, *lhb* (GenBank ID: DQ402374), *fshb* (GenBank ID: DQ402373), *gnrhr1b* (GenBank ID: GU332297) and *gnrhr2a* (GenBank ID: GU332298.1) was investigated. To allow accurate normalization of the qPCR, the stability of four reference genes, *arp2*, *bactin*, *ubiquitin* and *ef1a* was tested using Bestkeeper Software (Pfaffl et al., 2004), giving quantification cycle value (Cq) geometric means and standard deviations (± Cq) of 27.06 (± 0.39), 21.96 (± 0.45), 22.19 (± 0.43) and 20.40 (± 0.39), respectively. In order to save sample, only one reference gene, *ef1a* (GenBank ID: DQ402371.1) was used for normalization of the qPCR data in the subsequent experiments.

2.5.3. qPCR analysis

qPCR analyses were carried out as previously described (Hodne et al., 2012; Weltzien et al., 2005), using the LightCycler 480 platform (Roche, Basel, Switzerland). All samples were run in duplicate, and in every round, three non-template negative control (NTC) reactions were conducted for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). To account for plate-to-plate variation, a standard positive calibrator control in triplicate was also included on every plate. The positive calibrator was prepared by mixing cDNA from all individual samples analysed at the same time. The mixture was diluted and analysed in the same manner as the individual samples. Each PCR reaction (10 µl) mixture contained 5 µl of SYBR Green I master mix (Roche), 1 µl (5 µM) of forward primer, 1 µl (5 µM) of reverse primer, and 3 µl of diluted (1:10) cDNA. The qPCR reactions were carried out using an initial step for 10 min at 95 °C to activate the *Taq* polymerase, followed by 42 cycles consisting of 10 s at 95 °C (denaturation), 10 s at 60 °C (annealing), and elongation at 72 °C for 6 s. The fluorescence was measured after each elongation and used for determining the Cq. Directly following the PCR a melting curve analysis was performed by continuously reading the fluorescence while slowly heating the reaction mixture from 65 °C to 98 °C. qPCR efficiencies (E) were determined based on cDNA dilution curves, which, together with the Cq values, were used to calculate the relative expression (Pfaffl, 2001; Roche, 2001):

$$\text{Relative expression} = E_{\text{target}}{}^{\Delta Cq(\text{calibrator}-\text{sample})} * E_{\text{reference}}{}^{\Delta Cq(\text{sample}-\text{calibrator})}$$

The qPCR assay specificity was confirmed by agarose gel electrophoresis and sequencing.

2.6. Statistics

Statistical analysis was performed using the JMP Pro12 software (SAS Institute Inc., Cary, NC, USA). Fold changes of exposed samples relative to their respective solvent control mean were calculated and used in the subsequent analysis for both gene expression and viability data. To maintain control variance in the data set, control samples were calculated in the same manner and included in the analysis. All data were tested for normality by the Shapiro-Wilk W test. In case of non-normality, log-transformed data gave a satisfactory fit to the normal distribution. To account for the non-independence of the data within each culture when comparing solvent control wells (w/0.2% EtOH) with control blank wells (wo/EtOH), a one-way analysis of covariance (ANCOVA) corrected for well replicates within each culture as a concomitant variable was run. The same model was used to compare solvent control cells with exposed cells within each maturational stage. Here, to elucidate differences between groups that showed a significant treatment effect, the Tukey–Kramer honestly significant difference (HSD) post-hoc test was conducted on the adjusted means. Significance level was set to 0.05.

3. Results

3.1. Effects of ethanol as a solvent

Control wells with and without 0.2% EtOH (solvent control and control blank, respectively) in the media were prepared for every primary cell culture. The AB and CFDA-AM viability assays performed on these cells revealed a statistically significant negative effect from EtOH on both the metabolic activity (Fig. 1A) and membrane integrity (Fig. 1B), respectively. Although consistent between maturational stages, the negative effects on cell viability appeared more pronounced in cells from sexually mature fish. No inhibition of gene expression was detected from the solvent, regardless of maturational state (Figs. 2 and 3). In contrast, the solvent increased gene expression of *fshb* and *lhb* in the spent stage (Fig. 2A and B). No solvent effects were detected on *gnrhr1b* and *gnrhr2a* expression (Fig. 3A and B). Note that data from

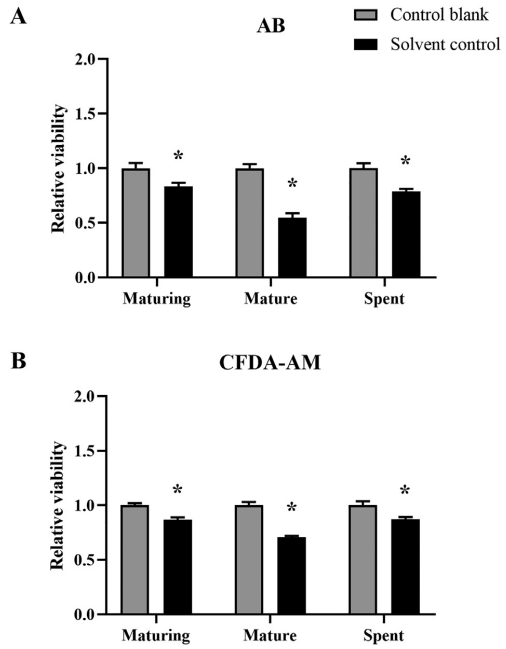

Fig. 1. Viability in terms of mitochondrial activity (AB assay; A) and membrane integrity (CFDA-AM assay; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 17–24). Statistical significance (*; $p < .05$) was assessed by ANCOVA.

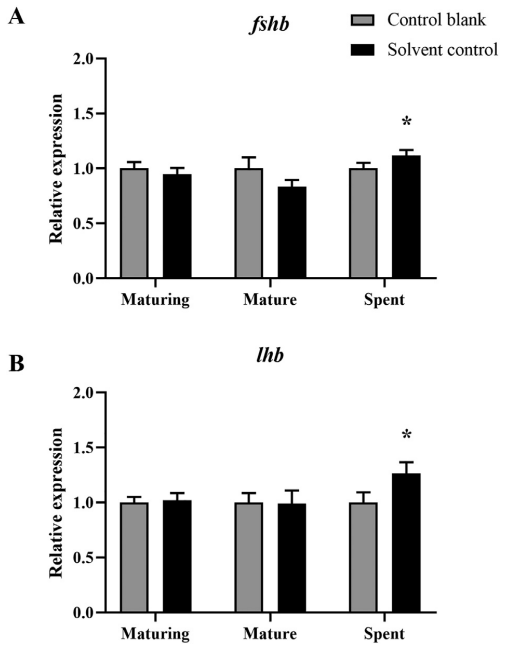

Fig. 2. Gene expression of gonadotropin subunits (*fshb*; A, *lhb*; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 12). Statistical significance (*; p < .05) was assessed by ANCOVA.

cortisol-exposed cells in the following sections are compared to effects seen in solvent control cells.

3.2. Cortisol exposure

3.2.1. Cell viability after cortisol exposure

Cell viability in terms of metabolic activity or membrane integrity (Fig. 4A and B, respectively) was unaffected by 72 h of basal cortisol level (10 ng/ml) exposure. On the other hand, stress levels of cortisol (100 ng/ml) stimulated both viability parameters in cells derived from maturing and mature fish. In spent fish, only the membrane integrity was significantly improved, although a similar tendency (p = .096) was seen also for the metabolic activity.

3.2.2. Gene expression after cortisol exposure

Depending on the target gene and time of year, cortisol treatment led to differential effects on gene expression, regarding both gonadotropin subunits (Fig. 5) and Gnrh receptors (Fig. 6).

Cortisol exposure had seasonally dependent effects on *fshb* expression (Fig. 5A). Basal cortisol levels had no effect on cells from mature and maturing fish, but decreased *fshb* transcription in cells from spent donors. In contrast, stress levels increased *fshb* expression at the mature stage, while decreasing expression in cells from maturing and spent fish, the decrease being more pronounced in the latter.

No significant changes in *lhb* transcript levels were seen following cortisol treatment (Fig. 5B), regardless of the maturational stage of the donor fish, although an increasing trend (p = .057) was observed in cells from mature fish exposed to stress level concentrations.

Similar to *fshb* expression, cortisol inhibited *gnrhr1b* expression in pituitary cells derived from spent fish (Fig. 6A). However, only basal levels of cortisol led to significantly reduced *gnrhr1b* transcript levels, although a similar trend was seen from stress level exposure (p = .095). In exposed cells derived from maturing and mature fish, *gnrhr1b* transcription levels followed those of solvent control. Regardless of season, a general increase in *gnrhr2a* expression was seen after stress level cortisol treatment (Fig. 6B), though statistically significant from solvent control only in cells derived from spent fish. Basal cortisol level did not affect *gnrhr2a* expression at any maturational stage.

4. Discussion

It is generally accepted that stress and high levels of cortisol can affect different aspects of reproduction, both in mammals and teleosts. The present study aimed to investigate direct effects of cortisol at the pituitary level in Atlantic cod and provides evidence that cortisol stimulates pituitary cell viability at stress-level concentrations and affects reproductive gene expression at both basal and stress levels in this species.

4.1. Methodical considerations

In Atlantic cod, peaking plasma cortisol levels during stress vary between studies and the severity of the stressor. Morgan et al. (1999) measured cortisol peaks of ~28 ng/ml in cod subjected to capture/confinement and ~127 ng/ml after otter trawl capture. Similar results to the latter value have been reported after exposure to a net stressor (~95 ng/ml; King and Berlinsky, 2006) and progressive hypoxia (~110 ng/ml; Herbert and Steffensen, 2005), while Perez-Casanova et al. (2008) measured ~450 ng/ml after acute temperature increase in juveniles. The stress dose of 100 ng/ml administered in this study must therefore be regarded to represent a mean of physiologically relevant

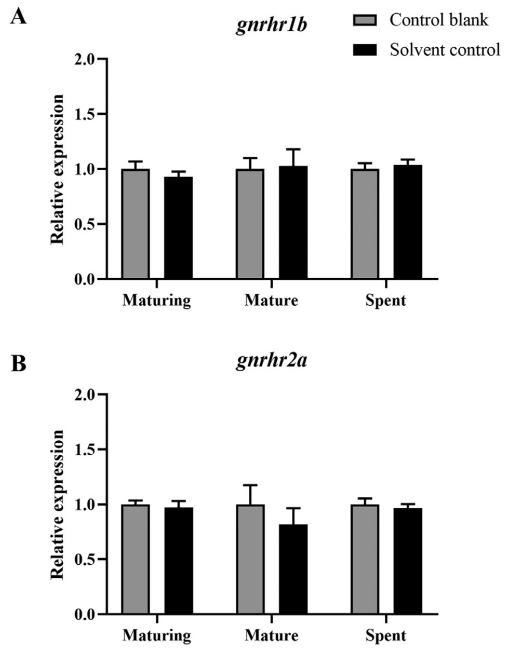

Fig. 3. Gene expression of two gonadotropin-releasing hormone receptors (*gnrhr1b*; A, *gnrhr2a*; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 12). Statistical significance (*; $p < .05$) was assessed by ANCOVA.

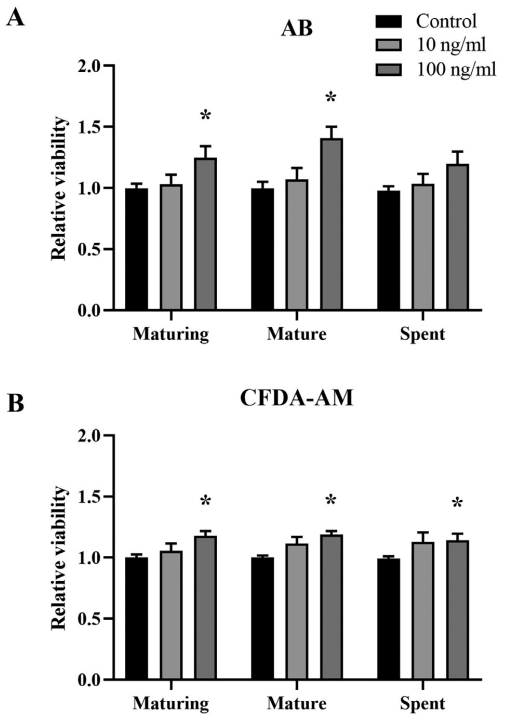

Fig. 4. Viability in terms of mitochondrial activity (AB assay; A) and membrane integrity (CFDA-AM assay; B) in Atlantic cod pituitary cells after 7 days of primary culture w/wo 72 h of cortisol exposure. Data is presented as mean fold change relative to mean solvent control ± SEM ($n = 9–12$). Statistical significance (*; $p < .05$) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

stress levels, and it cannot be excluded that the present results could have been different had the exposure dose resembled more extreme values. Glucocorticoids affect both sexes and do so in a dual manner, *i.e.* the effects can be both positive and negative, depending on the species in question, tissue investigated and maturational stage (Milla et al., 2009). However, there is reason to speculate that at least chronic stress could affect female reproduction harder than male reproduction as the nutritious investment is higher for eggs than sperm and because part of the allostatic load is reallocation of available metabolic resources (Leatherland et al., 2010). At the pituitary level, there are studies in rats showing differential effects between the sexes, both *in vivo* and *in vitro* (D' Agostino et al., 1990; Ringstrom et al., 1992; Suter and Schwartz, 1985a, 1985b). For instance, using perfused fragments of rat anterior pituitary, D'Agostino et al. (1990) found increased gonadotropin secretion by cortisol treatment in female donors, but not in males. In contrast, cortisol implantation of sexually maturing brown trout reduced pituitary gonadotropin levels, while maintaining plasma gonadotropin levels, in both sexes equally (Carragher et al., 1989). The present study pooled pituitary cells from both sexes in each culture prepared, and can therefore not contribute information regarding potential sex dependent mechanisms in Atlantic cod. The cultures prepared at the mature stage had seemingly a more pronounced decrease in viability following EtOH treatment than cultures from the other maturational stages. Since these cultures had a surplus of female donors, it is uncertain if the enhanced effect is a result of sex, *i.e.* that cells from females are more sensitive to EtOH, or of reproductive state, *i.e.* that during this period the cells are in such a state that they are more vulnerable.

The pituitary consists of many cell types, which may respond differentially to experimental treatment. Gene expression of Fsh and Lh subunits are probably limited to gonadotropes, whereas Gnrh receptors have been identified in several pituitary cell types, including corticotropes (Parhar et al., 2005). The cultures used in the present study were prepared from *in toto* pituitaries. Consequently, results presented here cannot be attributed to individual cell types within the pituitary. Furthermore, effects may be underestimated or undetected, as potential opposite effects from individual cells, either because of its type or because of the sex or history of its donor, would mask each other.

4.2. Cell viability

For relevant results in functional *in vitro* studies, it is crucial that the cells are physiologically healthy and stable while in culture. To ensure this, culture conditions should be adjusted to mimic the physiology of the animal in question. However, most fish *in vitro* studies are performed using mammalian protocols with only the temperature being adjusted to fish physiology. The present study used protocols optimized for Atlantic cod plasma osmolality, pCO2 and pH, as well as temperature (Hodne et al., 2012). These optimized conditions allow cultured Atlantic cod pituitary cells to maintain stable membrane potentials and steady Gnrh responses, along with the ability to fire action potentials for at least two weeks (Hodne et al., 2012). Moreover, cell metabolic activity and membrane integrity are significantly improved compared to traditional cell culture conditions.

Cortisol is a lipophilic steroid hormone and prior to cell media dilution, stock solutions are often prepared using a solvent, like EtOH. As many organic solvents, EtOH can be cytotoxic, and the working

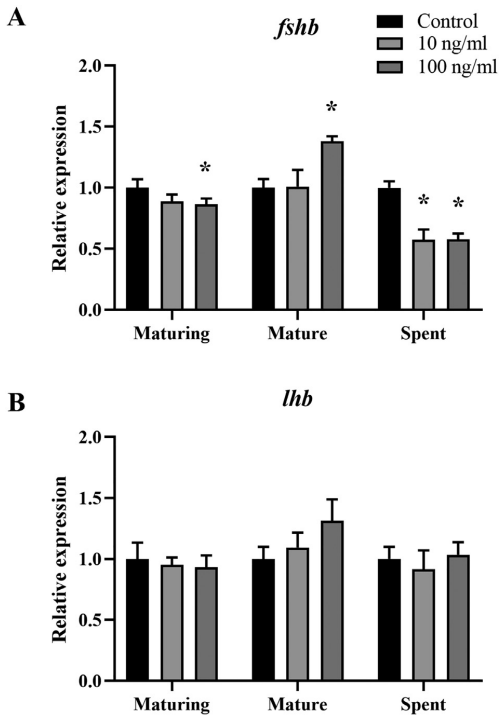

Fig. 5. Gene expression of gonadotropin subunits (*fshb*; A, *lhb*; B) in Atlantic cod pituitary cells after 72 h of cortisol exposure *in vitro*, presented as mean fold change relative to mean solvent control ± SEM (n = 6–12). Statistical significance (*; $p < .05$) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

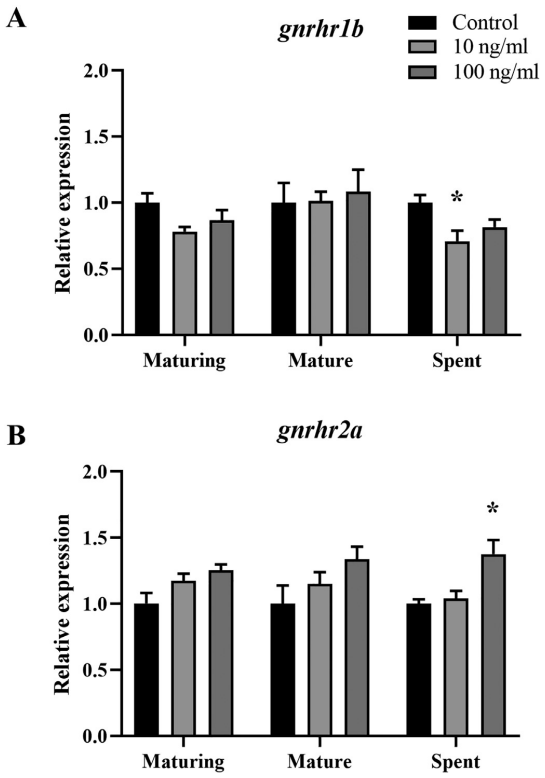

Fig. 6. Gene expression of two gonadotropin-releasing hormone receptors (*gnrhr1b*; A, *gnrhr2a*; B) in Atlantic cod pituitary cells after 72 h of cortisol exposure *in vitro*, presented as mean fold change relative to mean solvent control ± SEM (n = 6–12). Statistical significance (*; $p < .05$) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

concentration of 0.2% EtOH (34.2 mM) used in the present study caused negative effects on pituitary cell viability, both in terms of metabolic activity and membrane integrity, but did not negatively affect gene expression. Due to its small hydrophobic hydrocarbon chain, EtOH can physically insert into the lipid bilayer of membranes and alter their function and integrity (McKarns et al., 1997). Furthermore, EtOH is linked to mitochondrial dysfunction and increased levels of reactive oxygen metabolites (Baker and Kramer, 1999; Manzo-Avalos and Saavedra-Molina, 2010; McKarns et al., 1997), so the negative effects on cell viability seen here was not surprising. The addition of cortisol to the culture medium seemed to leave the cells more viable than solvent control cells. Positive effects on cell viability from exogenous steroid hormones have previously been observed using this pituitary cell model, where physiological concentrations of sex steroids increased viability at different maturity stages (von Krogh et al., 2017). The cortisol-induced increase in viability seen here was evident in cells at all reproductive stages, but in cells derived from spent fish, cortisol exposure had no significant effect on metabolic activity, indicating that the viability-stimulating mechanisms here are linked to factors that varies with sexual maturity. Furthermore, only stress-level dose of cortisol increased cell viability, which suggest that these factors are also dose-dependent in their activation. Cortisol exerts its action by binding to corticoid receptors (Cr), *i.e.* glucocorticoid receptors (Gr) or mineralocorticoid receptors (Mr), in the membrane or cytosol of its target cells. The level of available receptors, as well as the receptors' sensitivity to cortisol, regulate the strength of impact. Crs have been demonstrated in the pituitary and in gonadotropes of both mammals and teleosts (Breen et al., 2012; Kitahashi et al., 2007; Kononen et al., 1993; Pepels et al., 2004; Teitsma et al., 1999; Thackray et al., 2006), but are not yet investigated in cod, so their affinity for cortisol and potential pituitary regulation through the reproductive cycle in cod is presently not known. However, in the common carp (*Cyprinus carpio*), Stolte et al. (2008) demonstrated that one Mr. and two Gr isoforms were expressed in the pituitary and that all had differential sensitivity to cortisol, meaning that basal and elevated levels of cortisol could initiate differential cellular responses dependent on which receptor is activated. One such possible cellular response is mitosis, meaning that the increased viability observed presently could be a reflection of cell proliferation in cortisol treated wells. Though generally being considered inhibitory to cell proliferation, this is not always the case, and cortisol treatment did *e.g.* increase cell numbers at early stage embryogenesis in rainbow trout (Li et al., 2012). Still, as the viability scores of the cortisol treated cells in the current study never exceeded those of the control blank cells, with no EtOH added to the media, proliferation seems an unlikely explanation. It appears more probable then, that high-level cortisol somehow counteracts the cytotoxicity induced by EtOH. In addition to the well-known genomic effects, cortisol may act through non-genomic mediated mechanisms as well (Borski, 2000; Mommsen et al., 1999). One such proposed mechanism for steroids is intercalation into the phospholipid bilayer of the cell membrane, introducing structural and functional alterations (Falkenstein et al., 2000; Golden et al., 1998; Whiting et al., 2000). This might be a way for cortisol to reduce the

negative effects from EtOH on the membrane activity, but at the present stage, this remains a speculation and needs further elucidation. Nevertheless, for future studies it seems reasonable to recommend an even lower working concentration of EtOH.

4.3. Gene expression

In mammals, it has been suggested that some of the negative effects seen from stress and cortisol on reproduction is due to a reduction of Gnrh responsiveness in the pituitary (Breen and Karsch, 2004; Breen et al., 2012; Suter et al., 1988). This is mainly based on the observation that synthesis and release of LH, which normally increase following GnRH stimulation, decrease when coupled with glucocorticoids. For instance, rats receiving cortisol implantation and GnRH injection had lower plasma LH levels than rats receiving GnRH only (Suter et al., 1988). Similarly, restrained stress by itself did not affect mice *LHβ*-expression, but led to a significant reduction in *LHβ*-expression compared to unstressed mice when coupled with exogenous GnRH (Breen et al., 2012). The present study indicate that, similar to mammals, cortisol does not affect *lhb* synthesis directly in Atlantic cod. This is in contrast to data from juvenile eel, where *lhb* mRNA and Lh protein levels increased after cortisol exposure *in vitro*, demonstrating that in this species, cortisol effects on Lh are not necessarily Gnrh dependent (Huang et al., 1999). However, teleost data at the pituitary level are inconsistent and seem dependent on several factors, such as species and stage of sexual maturity. For instance, in sexually mature brown trout, acute stress was followed by high levels of both circulating cortisol and gonadotropin (Pickering et al., 1987), whereas neither confinement stress nor cortisol injections had any effect on plasma Lh in maturing rainbow trout (Pankhurst and Van Der Kraak, 2000). Furthermore, cortisol implantation decreased pituitary Lh levels in sexually maturing brown trout (Carragher et al., 1989), but increased pituitary Lh contents in juvenile eel (Huang et al., 1999). A study on male common carp fed cortisol-containing food during first sexual maturation reported unchanged pituitary Lh levels during pubertal development, but decreased Lh contents in the mature fish (Consten et al., 2001). The same study also investigated Lh release from dissected pituitaries, and found that basal Lh release was unaffected by cortisol treatment, whereas concurrent treatment with Gnrh and cortisol decreased Lh release compared to Gnrh alone in pituitaries from mature fish. Collectively, it seems clear that there are considerable differences between species regarding Lh synthesis and release, both in terms of direct and Gnrh-coupled effects. While no direct effects of cortisol on *lhb* mRNA was detected in the present study, except a stimulatory tendency in cells derived from mature donors, potential effect on Atlantic cod Lh regulation if cortisol is co-administered with Gnrh cannot be excluded and would be an natural next subject for future mechanistic studies. An additional cause to the observed differential effects between studies, especially regarding *in vitro* experiments, might be the solvent used to administer cortisol to the cells. Most studies described here has used EtOH as the solvent, with concentrations ranging from 0.01 to 1% (Summarized in Table S1, supplementary data). As very few studies include control blanks, it is hard to deduce the potential effects of EtOH on their experiments. To the best of our knowledge, no study has compared the effect of cortisol treatment with and without EtOH as a solvent. Future work investigating the potential interactions between EtOH and cortisol on pituitary cells might elucidate the observed differences between *in vitro* studies.

In addition to mechanisms downstream of the Gnrh receptor, such as reduced Lh synthesis or release, reduced Gnrh responsiveness might be caused by a down-regulation of available binding sites for the Gnrh ligand, *i.e.* the Gnrh receptors (Gnrhr). While *gnrhr1b* expression indeed was down-regulated in cells from spent cod, previous evidence points to Gnrhr2a as the main gonadotropin modulator in this species. This is based on the fact that *gnrhr2a* expression increases in concert with the gonadosomatic index and, while being co-expressed with *gnrhr1b* in both *lhb*- and *fshb*- expressing cells, *gnrhr2a* expression is more prominent than that of *gnrhr1b* and regulated by sex steroids (Hildahl et al., 2011; von Krogh et al., 2017). Here, basal cortisol levels had no effect on *grnhr2a* transcript levels, while stress levels generally increased *gnrhr2a* expression. This agrees with results from the mouse gonadotrope LβT2 cell line where dexamethasone, a synthetic glucocorticoid, increased *GnRHR* transcripts (Kotitschke et al., 2009; Turgeon et al., 1996). However, GnRHR numbers and GnRH affinity were unaffected by glucocorticoid treatment in rats *in vivo* (Suter et al., 1988). To our knowledge, this is the first teleost study to look at Gnrhr expression in the pituitary following cortisol treatment. Assuming translation of the mRNA into functional protein, the present result suggests that stress levels of cortisol enhances, rather than reduces, Gnrh responsiveness in terms of gonadotropin modulation in Atlantic cod.

As mentioned above, expression of *gnrhr2a* and *gnrhr1b* are evident in both gonadotropes in Atlantic cod (von Krogh et al., 2017). The distribution of these receptors in the other cell types of the cod pituitary is presently not known. However, Flanagan et al. (2007) showed that *Astatotilapia burtoni* somatotropes express *gnrh-r2-*^{PEY}, which is phylogenetically related to *Gadus morhua gnrhr1b*. It is therefore possible that Gnrhr1b is involved in growth hormone (Gh) regulation (Leatherland et al., 2010). Crs have been demonstrated in teleost Gh-cells (Kitahashi et al., 2007; Stolte et al., 2008), and both stress and high cortisol levels have been demonstrated to correlate with decreased somatic growth and plasma Gh levels (Farbridge and Leatherland, 1992; Small, 2004). Yet, whether *gnrhr1b* is expressed in cod somatotropes and Gnrhr1b involved in Gh regulation in this species, remains to be determined.

Unlike that of *lhb*, direct effects from cortisol on *fshb* expression was observed in the present study. However, the outcome seemed highly dependent on the stage of sexual maturation of the donor fish. In mature cod, cortisol at stress level enhanced *fshb* transcription. This is in concert with several studies on cortisol exposure performed on rats of both sexes, demonstrating both *in vivo* and *in vitro* that cortisol increases pituitary content of FSH and *FSHβ* mRNA, with and without exogenous GnRH (D' Agostino et al., 1990; Leal et al., 2003; Ringstrom et al., 1991; Suter and Schwartz, 1985b; Suter et al., 1988). Suter and Schwartz (1985a) suggested that the increase in FSH synthesis during a stressful situation might be a way for the animal to rapidly resume reproductive function after a period of stress has ended. However, in the present study, it cannot be ruled out that the increased *fshb* expression is a female cod phenomenon only, due to the skewed sex ratio in the cultures derived from mature fish. Studies from other teleosts indicate a negative effect on Fsh regulation from cortisol. In sexually maturing male common carps, *fshb* transcript levels decreased following cortisol treatment (Goos and Consten, 2002), whereas in immature cinnamon clownfish (*Amphiprion melanopus*), cortisol injection reduced plasma Fsh levels after both short- and long-term treatment (Choi et al., 2017). The present study found negative effects on *fshb* levels in cells derived from maturing and spent fish, the effect appearing more marked in the latter stage. During this spent stage, there is a quiescent period in Atlantic cod gonadal growth, with somatic growth and increasing energy reserves being prioritized (Pedersen and Jobling, 1989). The findings here suggest that in spent cod the recruitment of new gametes into proliferation is prohibited by cortisol through the down-regulation of *fshb*. As this occurred also at basal cortisol levels, it is possible that cortisol at this stage of the reproductive cycle is part of the homeostasis, making sure that the body does not prioritize gonadal recrudescence in times when offspring survival would be harder. The mechanisms at which similar concentrations of cortisol may act as an inhibitor of *fshb* during one stage and a stimulator during another is not established. It seems likely that either additional transcription factors interplay with the Crs at the binding sites of the *fshb* promotor causing activation or repression of the transcript, or that Cr receptors in Fsh cells are differentially regulated through sexual maturation, either in abundance or isoform expressed. The latter is indeed the case in tilapia, where single cell RT-PCR on Fsh cells revealed that while immature males expressed

both *gr1* and *gr2*, mature males expressed only *gr1*, but did so in significantly higher absolute number of *gr1* transcripts than the immature fish (Kitahashi et al., 2007). As the presence, let alone the regulation, of Crs is unknown in the cod pituitary, this would be an interesting topic for future studies.

5. Conclusions

The present study demonstrates that cortisol can exert direct and differential effects on pituitary cell viability and reproduction associated gene expression in an Atlantic cod primary culture system. Basal cortisol levels had no impact on cell viability and affected gene expression only in pituitaries from spent donor fish, while stress cortisol levels stimulated cell viability and affected gene expression at all maturational stages. The negative reproductive effects associated with high-level cortisol in cod is probably not a result of direct interaction with the *lhb* promotor, nor with a downregulation of *gnrhr* expression. However, cortisol can affect reproduction directly at the pituitary level through Fsh synthesis. As this effect was both positive and negative dependent on the reproductive stage of the donor fish, it is likely that some unmeasured elements, such as transcription factors, vary with sexual maturity in the cod gonadotropes and facilitate the action of cortisol.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.06.017.

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Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary dependent on stage of sexual maturation

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Supporting information, figure S1 and table S1

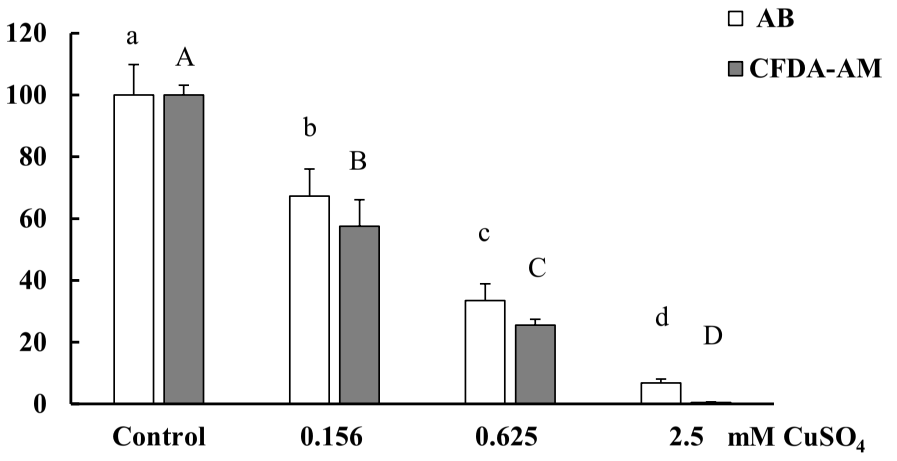

Figure 1. Intra- and inter-assay controls for cell viability testing

When measuring Atlantic cod (*Gadus morhua*) pituitary cell viability in terms of metabolic activity and membrane integrity (AlamarBlue (AB) and 5-carboxyfluorescein diacetate-actetoxymethyl ester (CFDA-AM) assays, respectively), different concentrations of the cytotoxic substance copper sulphate ($CuSO_4$) served as a positive intra/inter assay control.

The effects from $CuSO_4$ exposure were comparable between individual plates and cultures, indicating a stable system. The data presented represents a typical fluorescent reading and is the mean percent of control ± SEM (n=6). Different letters (a-d, A-D) indicate statistical difference ($p<0.05$; tested with ANOVA followed by Tukey HSD).

Note that, all though 24 h of 2.5 mM $CuSO_4$ exposure was lethal to almost 100 % of the pituitary cells, many cells would still appear viable under the microscope, confirming the necessity of adequate viability measurements.

Table S1. Solvent vehicle used for cortisol exposure in in vitro studies referenced in this manuscript

Reference	Solvent	Solvent concentration	Control blank included
Breen et al., 2012	EtOH	0.1 %	No
Consten et al., 2001	Not declared/used	N/A	N/A
D'Agostino et al., 1990	EtOH	0.05 %	No
Huang et al., 1999	EtOH	0.2 %	No
Kotitschke et al., 2009	EtOH	0.1 %	No
Li et al., 2012	EtOH	0.1%	No
Leal et al., 2003	EtOH	Not declared	No
Mandiki et al., 2017	EtOH	Not declared	Yes **
Pankhurst et al., 1995	EtOH	1 %	No
Reddy et al. 1999	EtOH	1 %	No
Sathiyaa & Vijayan, 2003	EtOH	0.01 %	No
Stolte et al., 2008	EtOH	0.1 %	No
Suter & Schwartz, 1985a	EtOH	0.01%	No
Suter & Schwartz, 1985b	Not declared/used*	N/A	N/A
Thackray et al., 2006	EtOH	Not declared	No
Turgeon et al., 1996	Not declared/used	N/A	N/A

* Not declared, but presumed to be the same as in the parallell study (Suter & Schwartz, 1985a)

** EtOH had a negative effect on aromatase activity compared to control blank cells, but no effect on steroid production, except a small negativ effect on E2 production in some cases. EtOH = ethanol, N/A = not applicable

IV

***In vitro* effects of BPA and TBBPA on cell viability and reproduction-related gene expression in Atlantic cod pituitary**

fishes

MDPI

Article

In Vitro Effects of Bisphenol A and Tetrabromobisphenol A on Cell Viability and Reproduction-Related Gene Expression in Pituitaries from Sexually Maturing Atlantic Cod (*Gadus morhua* L.)

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Abstract: Bisphenol A (BPA) and tetrabromobisphenol A (TBBPA) are widely used industrial chemicals, ubiquitously present in the environment. While BPA is a well-known endocrine disruptor and able to affect all levels of the teleost reproductive axis, information regarding TBBPA on this subject is very limited. Using primary cultures from Atlantic cod (*Gadus morhua*), the present study was aimed at investigating potential direct effects of acute (72 h) BPA and TBBPA exposure on cell viability and the expression of reproductive-relevant genes in the pituitary. The results revealed that both bisphenols stimulate cell viability in terms of metabolic activity and membrane integrity at environmentally relevant concentrations. BPA had no direct effects on gonadotropin gene expression, but enhanced the expression of gonadotropin-releasing hormone (GnRH) receptor 2a, the main gonadotropin modulator in Atlantic cod. In contrast, TBBPA increased gonadotropin transcript levels but had no effect on GnRH receptor mRNA. In conclusion, both anthropogenic compounds display endocrine disruptive properties and are able to directly interfere with gene expression related to reproductive function in cod pituitary cells at environmentally relevant concentrations in vitro.

Keywords: gonadotropin; GnRH receptor; endocrine disruption; reproduction; teleost

1. Introduction

During the last few decades, attention towards the potentially harmful effects of industrial chemicals released into the environment has increased dramatically. Many of these chemicals are endocrine disruptive compounds (EDCs), capable of interfering with normal endocrine function in animals, including fish. One important and susceptible endocrine system is that of reproduction. The physiological compartments of vertebrate reproduction comprise of the brain—pituitary—gonadal (BPG) axis, where neuroendocrine and endocrine substances relay communication between the different axis levels. In teleosts, gonadotropin-releasing hormone (GnRH), released through neuron fibers from the preoptic area and binding to its receptors in the pituitary, stimulates gonadotropin synthesis and release [1]. The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in turn, bind to receptors in the gonads, stimulating gametogenesis and steroidogenesis. Once in the bloodstream, sex steroids will provide feedback to the higher levels of the BPG axis [2–4].

Bisphenol A (BPA) and its halogenated derivative tetrabromobisphenol A (TBBPA) are widely used industrial and commercial chemicals, the former mainly used in the production of polycarbonate plastics and epoxy resins, and the latter used as a flame retardant in combustible products [5,6]. Both bisphenols are ubiquitously detected in the environment, although at highly variable concentrations dependent on sample matrix and location [7–12]. Peak measurements of BPA have been 21 µg/L in surface waters, 56 µg/kg dry weight (DW) in suspended solids and 17.2 mg/L in landfill leachates [7–9]. Seawater levels are usually below 1 µg/L, but BPA could potentially be leaching at marine sites of accumulated plastic waste. Further, BPA leaches faster and may withstand degradation longer in saltwater than in freshwater [7,8,13–16]. Measured peak levels of TBBPA have been 4.87 µg/L in lake waters, 600 µg/kg DW in sewage sludge, 9.8 mg/kg DW in freshwater sediment and 1.8 µg/L in seawater [10–12].

BPA is a well-known endocrine disruptor and has been demonstrated to act on all levels of the teleost BPG-axis; brain [17–21], pituitary [22–25], and gonads [26–29]. Considerably less information is currently available for TBBPA in this regard, with only a few teleost reproductive studies conducted. These studies indicate that TBBPA can affect teleost reproductive success through decreased egg production, delayed hatching, and decreased hatching rate [28,30,31]. Estrogenic activity from TBBPA exposure has been demonstrated in mammalian in vitro systems [32–34], while in teleosts, estrogenic activity has, to our knowledge, not been studied in vitro, and results in vivo are conflicting [30,35–39]. However, the capacity of TBBPA to act as a thyroid-disrupting compound has clearly been demonstrated in both mammalian and teleost assays, e.g., TBBPA can bind transthyretin with higher potency than the natural ligand thyroxine [30,33,40–42].

For both BPA and TBBPA, the vast majority of reproductive studies have been on gonads, with fewer studies focusing on the higher BPG-axis levels. For TBBPA, no such studies exist in teleosts, while to our knowledge only two studies exist for other vertebrate taxa [43,44]. While BPA is rather well-studied, most teleost reports are from small, freshwater species. The aim of the present study was therefore to investigate potential direct effects of BPA and TBBPA at the pituitary level in a marine teleost, the Atlantic cod (*Gadus morhua*). Using pituitary primary cultures from sexually maturing individuals, cell viability and expression of reproductive related genes, i.e., the gonadotropins (*FSHb* and *LHb*) and two gonadotropic GnRH receptors (*GnRHr1b* and *GnRHr2a*), following acute exposure to a range of concentrations were assessed.

2. Materials and Methods

2.1. Animals

Atlantic cod of both sexes were captured from the southern Norwegian coast (approx. 60° N) on four separate occasions. Specific ethical approval for this study was not needed as the animals themselves were not experimentally treated (Norwegian legislation for use of animals in research, Chapter II, §6). Care was nonetheless taken so that stress and suffering of the animals were minimized. All fish were euthanized by swift decapitation at location prior to immediate dissection of pituitary and gonads. Dissected pituitaries were kept in modified L-15 medium (Life Technologies, Carlsbad, CA, USA, see below for modifications) on ice until culture preparation. After dissection, body and gonads were weighted and the gonadosomatic index (GSI; (Gonadal weight/Total body weight) × 100) calculated. As GSI by itself is not a precise indicator of sexual maturity, gonads were also visually inspected and staged according to von Krogh et al. [3] (Supplementary data). In total, four cultures were prepared using pooled pituitaries from sexually maturing donor fish (n = 48; body weight: 1.48 ± SD 0.56 kg; GSI: 6.35 ± SD 3.28). Sex ratios of donors within cultures were 3M/3F, 3M/1F, 11M/6F, and 10 M/11F (M: males; F: females).

2.2. Dispersed Pituitary Primary Cell Cultures

The present study followed a previously described, optimized protocol for primary cultures of Atlantic cod pituitaries [45]. The optimized conditions included cell density, pCO_2, and incubation

temperature, as well as pH and osmolality for working solutions. In short, pooled pituitaries were chemically and mechanically dissociated and seeded in culture wells pre-coated with poly-L-lysine (0.1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) at a density of 1.5×10^5 cells/cm^2. Cells were incubated in modified L-15 medium, adjusted to 320 mOsm, at 12 °C in a humidified atmosphere of 0.5% CO_2 (pCO_2; 3.8 mmHg, which resulted in a medium pH of 7.85). After 24 h, culture medium was replaced to remove damaged and detached cells.

2.3. BPA and TBBPA Exposure

Stock solutions of bisphenol A (BPA; 4,4′-(propane-2,2-diyl)diphenol, Sigma-Aldrich, CAS: 80-05-7, ≥99%) and tetrabromobisphenol A (TBBPA; 4,4′-(propane-2,2-diyl)bis(2,6-dibromophenol), Sigma-Aldrich, CAS: 79-94-7, 97%) were prepared in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway) and stored at −20 °C for up to one week. Prior to cell exposure, stocks were diluted to the desired concentration in modified L-15 medium, with working solutions having a final EtOH concentration of 0.2% (34.25 mM). For each experiment, controls w/wo EtOH (solvent control/control blank) were included. For viability tests, exposure doses were 10^{-9} to 10^{-3} M (0.22 to 228,290 µg/L) for BPA and 10^{-9} to 10^{-4} M (0.54 to 54,390 µg/L) for TBBPA (n = 6–8 per dose). For gene expression studies, exposure doses were 10^{-9} to 10^{-5} M (n = 6 per dose) for both compounds. Note that, due to the limited number of pituitaries, not all doses were included in all four cultures, leading the number of replicates to vary between treatments. Cultures were allowed to settle until day 4, by then the cell count was stable and the cells had started to spurt outgrowths, before being exposed to either BPA or TBBPA. Exposure was given as a single dose and incubation lasted 72 h.

2.4. Viability Assays

Viability testing was carried out using two non-toxic fluorescent indicator dyes, AlamarBlue (AB) and 5-carboxyfluorescein diacetate-actetoxymethyl ester (CFDA-AM) (both from Life Technologies). These assays indicate metabolic activity and plasma membrane integrity, respectively, and measure the conversion of a non-fluorescent dye into a fluorescent dye by enzymes present in intact and viable cells [46]. The test procedures followed the description by Hodne et al. [45]. In short, cells were seeded in 96-well plates (Corning, Amsterdam, The Netherlands) and incubated for 4 days before being exposed to either BPA or TBBPA for 72 h as described above. At day 7, culture medium in all wells was replaced with 100 µL Tris buffer (50 mM, pH 7.5) containing both 5% AB and 4 µM CFDA-AM (from 4 mM stock in dimethyl sulfoxide (DMSO)). After 30 min of incubation, the concentration of fluorescent products was measured simultaneously for both probes using a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Data was collected with Gen5 Data Analysis Software (Bio-Tek Instruments Inc.). As a positive control for cell toxicity, serving as intra/inter assay control, each plate included additional wells that were exposed to copper sulfate ($CuSO_4$; 0.156–2.5 mM, n = 6 per dose) for the last 24 h and analyzed alongside the experimental wells at day 7. The effects from $CuSO_4$ exposure were comparable between individual plates and cultures (data not shown) and similar to previously published data from this cell model (see von Krogh et al. [47], Supporting information), indicating stable cultures.

2.5. Quantification of Gene Expression

2.5.1. RNA Extraction and cDNA Synthesis

Cells used for gene expression analysis were seeded in 24-well plates (Corning), exposed to either BPA or TBBPA at day 4 as described above, and harvested at day 7. Cells were lysed and homogenized by pipetting in Trizol (Life Technologies), from where total RNA was extracted, before being re-suspended in 10 µL RNase-free water (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). DNase treated RNA (TURBO DNase-free (Ambion)) was quantified spectrophotometrically (NanoDrop, Thermo Fisher Scientific), and the quality assessed by electrophoretic validation (Bioanalyzer, Agilent

Technologies, Santa Clara, CA, USA) of the RNA Integrity Number (RIN). RNA samples with RIN numbers above 8 were allowed further analysis. Using 500 ng total RNA, first strand cDNA synthesis was performed using random hexamer primers and Super Script III (Life Technologies) according to standard procedures. cDNA was stored at −20 °C until qPCR.

2.5.2. Primers and Reference Genes

qPCR primers (Table 1) were designed using Primer3 shareware (http://frodo.wi.mit.edu/primer3/input.htm) and theoretically tested for possible hairpin loops and primer dimer formations using Vector NTI (Life Technologies). In each primer pair, one primer targeted an exon-exon border to avoid amplification of potential traces of genomic DNA. The genes of interest were *LHb* (GenBank ID: DQ402374), *FSHb* (GenBank ID: DQ402373), *GnRHr1b* (GenBank ID: GU332297) and *GnRHr2a* (GenBank ID: GU332298.1), all related to pituitary reproductive function. Four reference genes, *arp2*, *bactin*, *ubiquitin*, and *ef1a*, were tested using Bestkeeper Software [48], giving quantification cycle value (Cq), geometric means, and standard deviations of 27.06 (±0.39), 21.96 (± 0.45), 22.19 (±0.43), and 20.40 (±0.39), respectively. *ef1a* (GenBank ID: DQ402371.1) was considered most stably expressed and was subsequently used to normalize the qPCR data.

Table 1. qPCR primers used in the present study.

Target	Reference		Primer Sequence	Amplicon Size (nt)	Efficiency
lhb	[49]	Forward Reverse	5′-GTGGAGAAGAAGGGCTGTCC-3′ 5′-GGACGGGTCCATGGTG-3′	81	1.93
fshb	[49]	Forward Reverse	5′-GAACCGAGTCCATCAACACC-3′ 5′-GGTCCATCGGGTCCTCCT-3′	63	1.84
gnrhr1b	[3]	Forward Reverse	5′-GCTACTCCCGAATCCTCCTC-3′ 5′-CGCCTCAGGTATGACTCTCC-3′	73	1.96
gnrhr2a	[3]	Forward Reverse	5′-TTCACCTTCTGCTGCCTCTT-3′ 5′-TCCGTGGAGGAAAGATTGTC-3′	113	1.99
bactin	[45]	Forward Reverse	5′-TTCTACAACGAGCTGAGAGTGG-3′ 5′-CATGATCTGGGTCATCTTCTCC-3′	102	1.84
arp2	[45]	Forward Reverse	5′-GGAGGTTAGAAGTAGCAAGGAGC-3′ 5′-TGCTGACTCTCACGGAGTTG-3′	107	1.94
ef1a	[49]	Forward Reverse	5′-CCTTCAACGCCCAGGTCAT-3′ 5′-AACTTGCAGGCGATGTGA-3′	100	1.92
ubiquitin	[45]	Forward Reverse	5′-TGTCAAAGCCAAGATTCAGG-3′ 5′-TGGATGTTGTAATCCGAGAGG-3′	111	1.86

2.5.3. qPCR Analysis

Using the LightCycler 480 platform (Roche, Basel, Switzerland), qPCR analyses were carried out as previously described [45,50]. Three non-template negative control (NTC) reactions were included for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). To account for plate-to-plate variation, a standard positive calibrator control, prepared by mixing cDNA from all individual samples, was also included on every plate in triplicate. All samples were run in duplicate. Each PCR reaction (10 µL) mixture contained 5 µL of SYBR Green I master mix (Roche), 1 µl (5 µM) of forward primer, 1 µL (5 µM) of reverse primer, and 3 µl of diluted (1:10) cDNA. The qPCR reactions were carried out using an initial step for 10 min at 95 °C to activate the *Taq* polymerase, followed by 42 cycles consisting of 10 s at 95 °C (denaturation), 10 s at 60 °C (annealing), and elongation at 72 °C for 6 s. After every elongation, the fluorescence was measured and used to determine the Cq. Based on cDNA dilution curves, qPCR efficiencies (E) were determined. Combined with Cq values, E were used to calculate the relative expression [51,52] of each sample:

$$\text{Relative expression} = E_{\text{target}}{}^{\Delta Cq(\text{calibrator} - \text{sample})} * E_{\text{reference}}{}^{\Delta Cq(\text{sample} - \text{calibrator})}$$

Specificity of the qPCR assay was confirmed by agarose gel electrophoresis and amplicon sequencing at assay set-up, and by melting curve analysis based on slowly heating the reaction mixture from 65 °C to 98 °C directly following each individual PCR reaction.

2.6. Statistical Analysis and Data Presentation

Statistical analysis was performed using the JMP Pro14 software (SAS Institute Inc, Cary, NC, USA). As gene expression and cell viability occur at the level of the cell, and each culture well consisted of their own unique mixture of cells from multiple animals, individual wells were treated statistically as samples. Technical replicates from individual wells were averaged into one observation. Technical replicates had a variation of 0.26% (±0.69), while average variance between wells per treatment was 17.1% (±9.63). Fold changes of exposed samples relative to their respective solvent control mean were calculated and used in the subsequent analysis for both gene expression and viability data. To maintain control variance in the analyses, control samples were calculated in the same manner. All data were tested for normality and equal variances by Shapiro–Wilk W test and Levene's test, respectively. If the criteria for parametric testing was met, a one-way ANOVA was performed, followed by Dunnett's method comparing individual groups to the solvent control if these were found to have originated from different populations. However, most data did not meet the assumptions of parametric testing, even after transformation, so non-parametric testing was used. To assess population differences, the Wilcoxon rank-sum test was used to compare solvent control with control blank, whereas the Kruskal–Wallis test was used to compare treatment groups. In the latter case, if the null hypothesis was rejected, statistical differences between treatment groups and the solvent control were assessed by the Steel method. The significance level for rejection of the null-hypothesis was set to 0.05. Scatter plots were made using Prism 8 (GraphPad Software, San Diego, CA, USA). In each graph, the mean ± standard error of the mean (SEM) is indicated. Outliers of more than 2-fold difference to the group mean were excluded from the dataset, while all outliers were included if the statistical test was non-parametric.

3. Results

3.1. Solvent Control vs Control Blank

For every culture prepared, control wells with and without 0.2% EtOH (solvent control and control blank, respectively) added to the media were included. The AB and CFDA-AM viability assays performed on these cells revealed that the solvent had statistically significant negative effects on both metabolic activity and membrane integrity (Supplementary data, Figure S1). No influence on gene expression was detected from the solvent (Supplementary data, Figure S2). Note that data from exposed cells in the following sections are compared to the effects seen in solvent control cells.

3.2. BPA Exposure

BPA at all experimental doses (10^{-9} M to 10^{-3} M) affected cell metabolic activity (Figure 1A), while all except 10^{-9} M affected membrane integrity (Figure 1B). A hormesis effect was detected in both assays, with low doses stimulating and high doses inhibiting cell viability. At 10^{-3} M, no membrane integrity was detected. In the AB assay, lower BPA concentrations increased data variance compared to higher concentrations. At 10^{-4} and 10^{-3} M, the cellular outgrowths visible at lower concentrations and control had disappeared completely (see example in Supplementary data, Figure S3).

Gene expression levels of gonadotropin subunits *FSHb* and *LHb* (Figure 2A,B, respectively), and of GnRH receptor *GnRHr1b* (Figure 3A), were unaffected by BPA exposure at all doses tested (10^{-9} M to 10^{-5} M). In contrast, doses from 10^{-7} M to 10^{-5} M increased *GnRHr2a* transcription levels (Figure 3B).

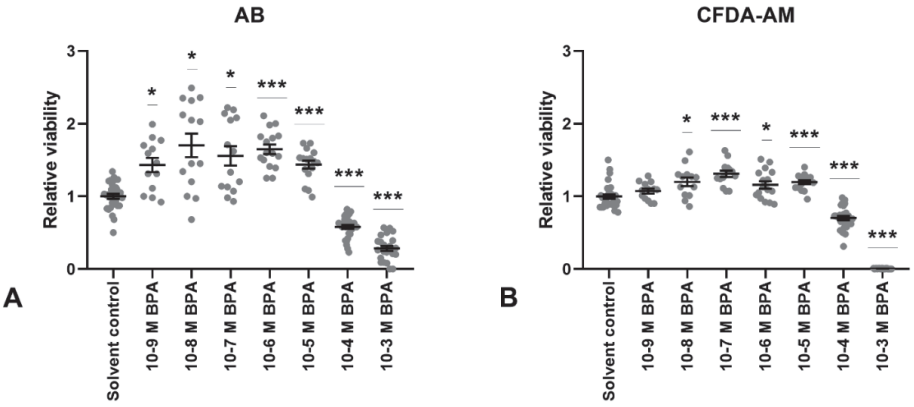

Figure 1. Viability in terms of metabolic activity (AlamarBlue (AB) assay) (**A**) and membrane integrity (5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) assay) (**B**) in Atlantic cod pituitary cells after 7 days of primary culture w/wo Bisphenol A (BPA) added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 13–30). Statistical significance (* $p < 0.05$, *** $p < 000.1$) was assessed using the Kruskal–Wallis test followed by the Steel method.

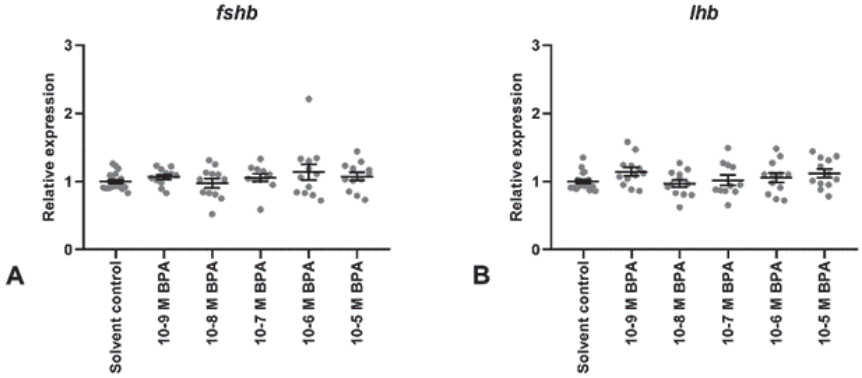

Figure 2. Gene expression of gonadotropin subunits (*FSHb*; (**A**), *LHb*; (**B**)) in Atlantic cod pituitary cells after 7 days of primary culture w/wo BPA added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 11–18). No statistical differences between groups were detected (tested by Kruskal–Wallis; (**A**), or one-way ANOVA; (**B**)).

3.3. TBBPA Exposure

All experimental doses of TBBPA (10^{-9} M to 10^{-4} M) significantly affected cell viability, both in terms of metabolic activity and membrane integrity (Figure 4A,B, respectively). As with BPA, a hormesis effect was detected for both parameters. TBBPA cytotoxicity appeared ten times more potent than that of BPA on these cells, as negative effects were detected from 10^{-5} M TBBPA. At 10^{-4} M, no membrane integrity was detected. At 10^{-5} and 10^{-4} M, no cellular outgrowths were visible through the microscope.

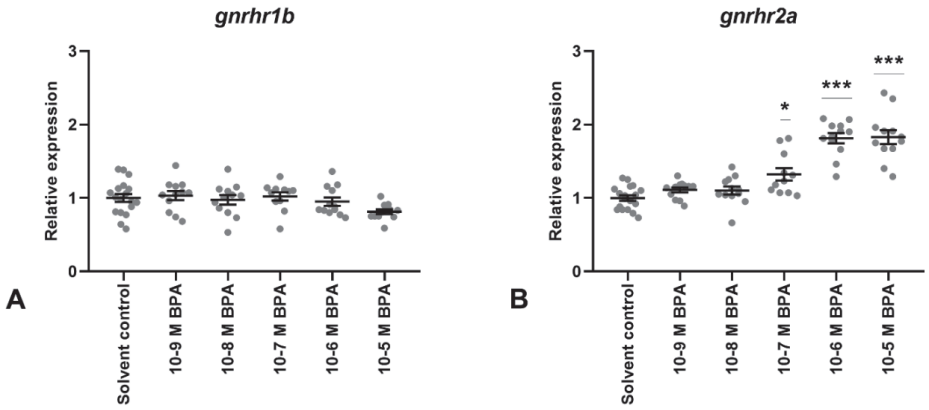

Figure 3. Gene expression of two gonadotropin-releasing hormone receptors (*GnRHr1b*; (**A**), *GnRHr2a*; (**B**)) in Atlantic cod pituitary cells after 7 days of primary culture w/wo BPA added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 11–18). Statistical significance (* $p < 0.05$, *** $p < 000.1$) was assessed using a one-way ANOVA followed by Dunnett's method.

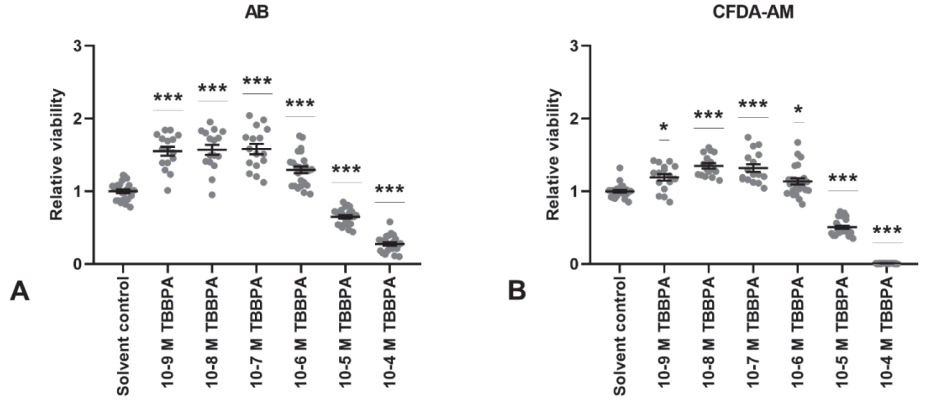

Figure 4. Viability in terms of metabolic activity (AB assay; (**A**)) and membrane integrity (CFDA-AM assay; (**B**)) in Atlantic cod pituitary cells after 7 days of primary culture w/wo tetrabromobisphenol A (TBBPA) added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 16–24). Statistical significance (* $p < 0.05$, *** $p < 000.1$) was assessed using the Kruskal–Wallis test followed by the Steel method.

Small, but statistically significant, increases in *FSHb* expression levels (Figure 5A) were observed in samples treated with 10^{-9} M to 10^{-7} M TBBPA. Similarly, a non-significant tendency was seen after 10^{-6} M exposure ($p = 0.11$). Levels of *LHb* mRNA (Figure 5B) increased significantly after 10^{-9}, 10^{-8}, and 10^{-6} M treatment, while a small, non-significant, increase was observed in 10^{-7} M ($p = 0.17$) samples. No changes were detected after 10^{-5} M TBBPA exposure.

Gene expression levels of both GnRH receptors (*GnRHr1b* and *grnrh2a*; Figure 6A,B, respectively) was unaffected by TBBPA concentrations of 10^{-9} M to 10^{-6} M, but decreased significantly after 10^{-5} M TBBPA treatment. In terms of gene expression, TBBPA exposed samples displayed larger in-group variation than BPA exposed samples.

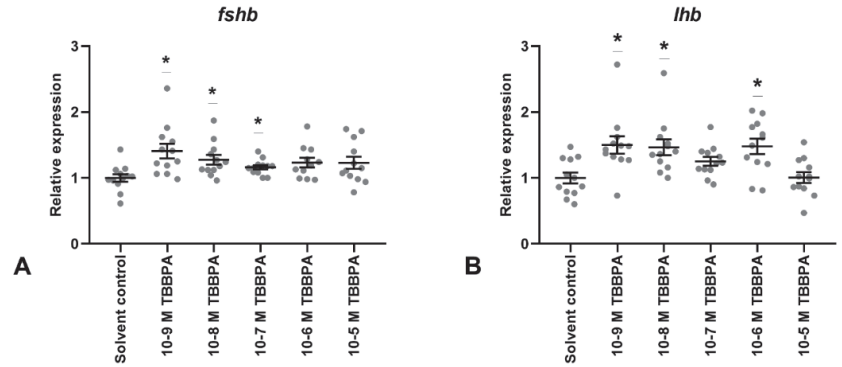

Figure 5. Gene expression of gonadotropin subunits (*FSHb*; (**A**), *LHb*; (**B**)) in Atlantic cod pituitary cells after 7 days of primary culture w/wo TBBPA added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 11–12). Statistical significance (* $p < 0.05$) was assessed using the Kruskal–Wallis test followed by the Steel method.

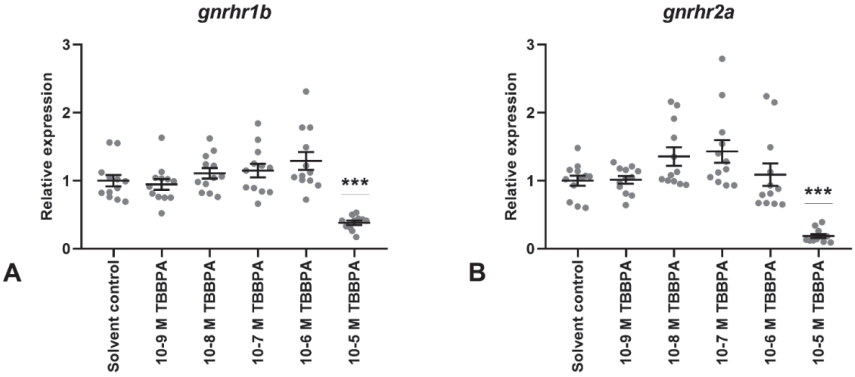

Figure 6. Gene expression of two gonadotropin-releasing hormone receptors (*GnRHr1b*; (**A**), *GnRHr2a*; (**B**)) in Atlantic cod pituitary cells after 7 days of primary culture w/wo TBBPA added to the culture media for the last 72 h. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 11–12). Statistical significance (*** $p < 000.1$) was assessed using the Kruskal–Wallis test followed by the Steel method.

4. Discussion

All wildlife is potentially susceptible to the impacts of anthropogenic activity. The substantial use and subsequent environmental release of synthetic compounds are examples of such activity and a source of increasing concern. This study demonstrates that single, acute exposure to either BPA or TBBPA at environmentally relevant concentrations can affect cell viability and reproduction-related gene expression in Atlantic cod pituitary cells, potentially affecting the reproductive capacities of this species.

4.1. Methodological Considerations

To obtain physiologically relevant results using in vitro systems for toxicological testing, it is crucial that cells are kept under physiologically relevant conditions [45,53]. Nevertheless, it is common practice to use mammalian protocols on fish tissue cultures, with only the incubation temperature adjusted to accommodate differences between mammalian and fish physiology. The present study

followed protocols optimized for Atlantic cod physiology, regarding not only temperature, but also cell density, pCO_2, and pH and osmolality of the working solutions and incubation media [45]. These optimized conditions allow cultured pituitary cells to maintain stable membrane potentials, fire action potentials, and to exhibit steady GnRH responses for at least two weeks. Moreover, cell viability is significantly improved compared to traditional cell culture conditions [45].

To be able to expose the pituitary cells to high concentrations of BPA and TBBPA, the bisphenols were initially dissolved in EtOH. The working concentration of 0.2% did not affect gene expression of any gene analyzed, but did negatively affect cell viability compared to those incubated in EtOH free media (control blank). Consequently, all exposed cells were compared to that of the solvent control. Most studies use either EtOH or dimethyl sulfoxide (DMSO) as the solvent for BPA and TBBPA, both of which are able to induce cytotoxicity [54,55]. Despite this, effects from the solvent itself are rarely reported, making comparison between studies difficult in this regard. For future studies, we would recommend lower EtOH working concentrations, as negative effects were detected here. The water solubility of BPA and TBBPA is 120–300 mg/L and 4.16 mg/L at 25 °C, respectively, with decreasing solubility at lower temperatures [5,15]. The cell medium used for the cod cultures was prepared and incubated at 12 °C. The highest doses administered to the cells thus exceed the water solubility threshold for these compounds and are unlikely to be environmentally relevant for Atlantic cod. They may still, however, be mechanistically informative. It should also be noted that high concentrations, similar to the higher doses administered in this study, have been detected in landfill leachates (17.2 mg/L for BPA) [9] and freshwater sediment (9.8 mg/kg DW for TBBPA) [10], and could potentially reach other wildlife. In marine waters, levels are usually below 1 µg/L (4.39 nM) for BPA and below 1.8 µg/L (3.3 nM) for TBBPA [7,12,16], which corresponds roughly to the two lowest doses, 10^{-9} and 10^{-8} M (0.23 and 2.28 µg/L for BPA, and 0.54 and 5.44 µg/L for TBBPA, respectively), used in this study. In Atlantic cod liver, detected levels of BPA and TBBPA have been 107.2 and 9 ng/g lipid weight (LW), respectively [56].

Cells were exposed once (at 4 day post plating) and incubated for 72 h prior to harvest and analysis. While the half-life of BPA and TBBPA in fish is rather short, <1 day [5,15], in a pituitary cell culture, the metabolism is expected to be slower than in the intact body, as the main site of metabolism is in the liver. The final concentrations of the compounds or possible metabolites formed during the incubation were not measured. Metabolites may have differential properties than the parent compound, and we cannot rule out the possibility that any observed effect described here is a result of such.

4.2. BPA

BPA, often referred to as a xenoestrogen because of its ability to act as an estrogen agonist, binds to estrogen receptors (ERS) and promotes endogenous 17β-estradiol (E2) effects in vertebrates [9]. Not surprisingly, the vast majority of reproductive studies on BPA in fish has been on ovaries or hepatic vitellogenin production, which are important sites of estrogenic activity. The studies reveal that in many teleosts, including Atlantic cod, BPA is able to induce vitellogenin production and act as an estrogen [35,37,57,58]. In addition to the gonads and liver, estrogen acts on the higher levels of the reproductive axis. Correspondingly, in the brain of developing zebrafish (*Danio rerio*) larvae, BPA has been shown to activate estrogenic markers, such as enhanced transcription of brain aromatase (*cyp19a1b*), through Er-dependent transcription [19,21]. In a recent study using Atlantic cod pituitary cultures, 72 h of 25 ng/mL E2 exposure had no effect on gene expression levels (*FSHb*, *LHb*, *GnRHr1b*, *GnRHr2a*) in cells from sexually maturing donors [3]. In the current study, BPA had no effect on *FSHb*, *LHb*, or *GnRHr1b* at any concentration tested but was able to stimulate *GnRHr2a* expression at 10^{-7} to 10^{-5} M (22.8–2282.9 µg/L), the lowest dose being similar to the E2 dose previously used by von Krogh et al. [3]. This indicates that BPA is able to induce *GnRHr2a* expression through some Er-independent pathway. While E2 did not induce gene expression in maturing Atlantic cod pituitary cells, it stimulated the expression of both *FSHb* and *GnRHr2a* in cells from sexually mature fish [3].

Keeping this is mind, it is possible that BPA exposure at different stages of the reproductive cycle could, like E2, induce differential effects.

Another physiological aspect of E2 is the ability to stimulate cell proliferation, including at the pituitary level [59]. In sexually maturing Atlantic cod pituitary cultures, 25 ng/mL, but not 2.5 ng/mL, E2 stimulated cell viability [3]. Here, however, BPA was able to stimulate viability at lower concentrations than did E2, increasing metabolic activity from 10^{-9} M and membrane integrity from 10^{-8} M. Similar proliferative potency has been seen in the offspring of female mice, with increased pituitary proliferation and gonadotrope numbers when exposed prenatally to just 0.5 µg/kg/day of BPA [60]. In concurrence with concentrations considered safe by authorities (Predicted no effect concentration (PNEC) for marine waters; 0.15 µg/L) [6], no adverse effects were detected after BPA exposure at this level. However, metabolic activity was stimulated by concentrations close to the safe limit (i.e., 10^{-9} M/0.228 µg/L), indicating that cellular mechanisms may be affected even at this level. Both through visual inspection and cell viability testing, doses of $\geq 10^{-4}$ M were found to induce cytotoxicity, and at 10^{-3} M, no membrane integrity was detected. Similar results have been measured in a zebrafish hepatocyte cell line (ZFL), with 24h LC_{50} and 96h LC_{50} for BPA of 367.1 and 357.6 µM, respectively [61]. In chicken embryonic hepatocytes, the 36h LC_{50} for BPA was somewhat lower, calculated at 61.7 µM [62].

In the present study, BPA exposure did not affect transcript levels of *FSHb*, *LHb*, or *GnRHr1b* at any dose but increased *GnRHr2a* expression at $\geq 10^{-7}$ M. To our knowledge, there are no other studies looking at this in vitro, making direct comparison difficult. There are, however, some studies in vivo. The first teleost study looking at the effect of BPA exposure on gonadotropins was conducted by Rhee et al. [22], using the mangrove killifish (*Kryptolebias marmoratus*) as the model. Waterborne BPA exposure increased transcript levels of the gonadotropin β-subunits in both juvenile (300 µg/L for 24 h) and adult fish (600 µg/L for 96 h). Similar results have been reported from zebrafish. Qiu et al. [23] investigated BPA exposure during development (from 2 to 120 hpf) in zebrafish larva and found increased mRNA levels of *FSHb* after 1000 µg/L and *LHb* after 10, 100, and 1000 µg/L treatments. In juvenile zebrafish, exposure to 10^{-5} M BPA for 20 days increased *LHb*, but decreased *FSHb* pituitary expression [24]. In contrast, the recent work of Wang et al. [25], showed that female goldfish (*Carassius auratus*) exposed to 1, 50, and 500 µg/L BPA for 30 days had reduced levels of *FSHb* and *LHb* transcripts, whereas no effect was detected in males. The discrepancies between the studies described above and also our own results could be due to species-specific differences, for instance in BPA Er affinity, uptake, or metabolism. Furthermore, experimental design (e.g., in vivo vs in vitro), age of the experimental animals, and exposure time could all affect the outcome. Conflicting results have also been reported on the effects of BPA in mammalian studies. Results are conflicting, also in mammalian reports. For instance, rats treated postnatally from day 21–35 with 2.4. µg/kg/day had decreased serum LH and pituitary *LHb* levels [63], while adult male rats exposed to 5 and 25 mg/kg/d for 40 days, had reduced serum FSH and LH, but increased *FSHb* and *LHb* transcript levels [64]. Moreover, female mice treated with BPA at 0.5 µg/kg/day had increased gonadotropin mRNA levels, whereas mice treated with 50 µg/kg/day had decreased levels [60].

The GnRH receptors mediate hypothalamic GnRH responses on the pituitary cells, and in Atlantic cod, *GnRHr2a* is thought to be the main modulator of gonadotropin regulation [65]. In Atlantic cod, expression of *GnRHr1b* and *GnRHr2a* are susceptible to glucocorticoid regulation, and the latter also to sex steroid regulation in Atlantic cod [3,47], making them both likely targets for endocrine disruption. Presently, *GnRHr1b* expression was unaffected by BPA, while *GnRHr2a* transcription increased following exposure to 10^{-7}, 10^{-6}, and 10^{-5} M BPA. Acute exposure to ≥0.1 µM BPA could thereby, assuming translation into protein, increase *GnRHr2a* receptor levels, enhancing GnRH sensitivity and subsequent LH production. Also in this regard, there are no in vitro studies for comparison. However, in the killifish (*K. marmoratus*), in vivo exposure to 600 µg/L BPA for 96 h increased brain/pituitary transcript levels of *GnRHr*, an orthologue of Atlantic cod *GnRHr2a*, in secondary males, similarly to our findings [66]. In contrast, no effect was seen in hermaphrodite killifish. Li et al. [67] found no effect on brain *GnRHr*

(also a *GnRHr2a* orthologue) expression from 1, 10, 50, 125, and 250 mg/kg BPA exposure in tongue sole (*Cynoglossus semilaevis*) females, whereas a decrease was seen in males exposed to 250 mg/kg BPA. While the present study did not detect any effects from BPA on *GnRHr1b* levels, the orthologue *GnRHr1a,* was significantly upregulated in brains of adult female rare minnows (*Gobiocypris rarus*) following 15 µg/L BPA exposure for 35 days [17]. No effect was detected in males. The above mentioned studies describe brain levels, or in one case a mixture between brain and pituitary levels, of *GnRHr*, and we have not managed to find a teleost study looking solely at pituitary *GnRHr* levels. In mammals, however, pituitary *GnRHr*, the paralogue to teleost *GnRHr*, showed increased transcript levels after 40 days of 5 and 25 mg BPA/kg/d in adult male Wistar rats [64]. In female mice pituitaries, *GnRHr* transcripts increased after treatment with 0.5 µg/kg/day BPA, but decreased after 50 µg/kg/day treatment [60]. Although little information is available, it appears as if there are both species and gender differences regarding the effect of BPA on *GnRHr* gene expression. In the present study, due to a limited number of available fish and lack of external dimorphic traits in Atlantic cod, the pituitary cultures were prepared as a mixture between male and female donors. For future mechanistic studies, it seems to be worth pursuing cultures separated by sex.

4.3. TBBPA

The effects of TBBPA on fish reproduction is far less studied than that of BPA. Though studies have indicated that TBBPA can affect teleost reproductive success [28,30,31], there are no previous studies, known to us, looking at the higher levels of the BPG-axis in this animal group. Here, we demonstrate that TBBPA can stimulate pituitary cell viability and gene expression of gonadotropin β-subunits at acute, environmentally relevant concentrations.

In the present study, 10^{-9} to 10^{-6} M TBBPA exposure stimulated both metabolic activity and membrane integrity in cod pituitary cells. At some doses, the cell viability levels of TBBPA exposed cells exceeded the level of not only the solvent control, but also the control blank samples, most likely reflecting cell proliferation. Similar findings, albeit at higher concentrations, were previously demonstrated in a rat pituitary tumor cell line, GH3, where 10^{-6} to 10^{-4} M TBBPA induced cell growth and increased growth hormone (GH) production [41]. While E2 may be a stimulator of proliferation, as mentioned above, the estrogenic properties of TBBPA is still debated. Nevertheless, in the human breast adenocarcinoma cell line, MCF-7, estrogenic proliferation was seen following 24 h TBBPA exposure up to 500 µM [34]. This is a much higher concentration than that which induced cytotoxicity in cod pituitary cells, ≥10^{-5} M (10 µM), indicating differential sensitivity between cell types. However, our findings are in concert with other teleost studies. For instance, in ZFL cells, the 24 h LC_{50} and 96 h LC_{50} was 4.0 and 4.2 µM, respectively [61]. The zebrafish standard embryo assay demonstrated that TBBPA ≥ 0.75 mg/L (1.38 µM) caused lethality or malformation [68]. Similar results are reported for zebrafish larvae, where the 96 h LC_{50} for TBBPA was 5.27 mg/L (9.7 µM) [37]. Many mammalian studies also report similar findings. In GH3 cells, measuring the reduction of blue resazurin dye to red fluorescent resorufin, the same cytochrome as used in the AlamarBlue assay, cytotoxicity was detected at doses >1 µM [33]. Moreover, in primary cultures of rat cerebellar granule cells, trypan blue measurements, indicating loss of membrane integrity in stained cells, demonstrated a 24 h LC_{50} value of 7 µM TBBPA [69]. The same study demonstrated that all membrane integrity was lost at 20 µM TBBPA, in concert with the present results.

The cytotoxicity of EtOH was apparently counteracted by both bisphenols tested in the present study. However, the EtOH reduced cell viability was not reflected in reduced gene expression compared to control blank cells. Similarly, the cytotoxicity induced by 10^{-5} M TBBPA exposure did not affect *FSHb* and *LHb* expression, which both remained at the level of the solvent control. The lowered cell viability did, however, correlate with reduced gene expression of the *GnRHr* receptors that were otherwise unaffected by TBBPA treatment. It is not clear to us what might cause this discrepancy and further studies are needed for elucidation.

As mentioned above, studies on the potential effects of TBBPA at the higher levels of the BPG axis are currently very limited. To our knowledge, there are only two vertebrate studies, only one of which concerns gonadotropin levels. Further, to our knowledge, no study has investigated the effect of TBBPA exposure on GnRH receptor expression or possible direct effects at the pituitary level. van der Ven et al. [44] found no obvious changes in pituitary, nor gonadotrope, histology after TBBPA exposure in a one-generation study using Wistar rats. However, male pituitary weight dose-dependently increased. The authors could not conclude the mechanisms behind this, but suggested that it might be caused by feedback stimulation by the observed decreased levels of circulating thyroxine (T4). In a recent study by Zhang et al. [43], adult male frogs (*Rana nigromaculata*) were exposed to 0.001–1 mg/L TBBPA for 14 days. Doses 0.001–0.1 mg/L decreased serum LH, while 0.1 and 1 mg/L decreased serum FSH. This is in the opposite direction of our findings, where TBBPA stimulated gene expression of both gonadotropins, even at environmentally relevant concentrations. On the other hand, TBBPA treatment also led to increased serum testosterone and E2 levels in the male frogs. It is therefore possible that the reduced FSH and LH levels were a result of negative feedback from the sex steroids at the brain and pituitary level, rather than direct effects from TBBPA. It is also possible that the steroid action masked potential direct effects from TBBPA at the pituitary level. While direct effects on gonadotropin expression are evident from the present findings, it remains to be seen if this could lead to physiological alterations in vivo for Atlantic cod.

The mechanisms at which TBBPA affects gene expression and other reproductive endpoints remains to be assessed. However, TBBPA has the potential to act as a thyroid hormone agonist [41], and disruption of the thyroid axis can lead to sexual dysfunction [70]. Furthermore, thyroid hormones are thought to be involved in the seasonal regulation of reproduction [71]. It would therefore be interesting to investigate possible TBBPA effects throughout the reproductive cycle. Not only for the connection to thyroid hormones and because available information on the reproductive effects of TBBPA is limited, but also because the sensitivity of potential endocrine targets might differ through the different stages of sexual maturity [3,47].

5. Conclusions

The present study demonstrates that single, acute, and environmentally relevant doses of BPA or TBBPA are able to directly affect both cell viability and gene expression in Atlantic cod dispersed pituitary cells in vitro. Cell viability was stimulated by low dose exposure to both phenols, while cytotoxicity was evident only at high, non-environmentally relevant doses. Bromination seemingly increased cytotoxicity, as TBBPA exposure decreased pituitary cell viability at 10-fold lower doses than BPA. BPA stimulated gene expression of the GnRH receptor 2a, the main gonadotropin modulator in Atlantic cod, while TBBPA stimulated gene expression of both gonadotropins. The differential effects seen from BPA and TBBPA exposure at the transcriptional level indicate that they act on different receptors or activate different pathways.

Supplementary Materials: The following are available online at http://www.mdpi.com/2410-3888/4/3/48/s1, Figure S1: Viability assays, controls, Figure S2: Gene expression, controls, Figure S3: Morphological changes, control cells vs high BPA exposure cells.

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Conflicts of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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In vitro effects of BPA and TBBPA on cell viability and reproduction-related gene expression in pituitaries from sexually maturing Atlantic cod (*Gadus morhua* L.)

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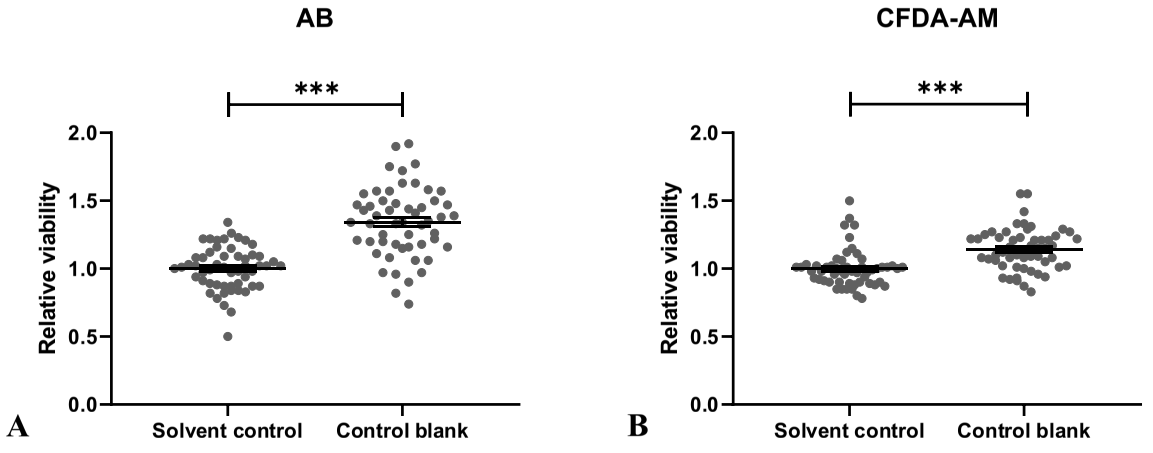
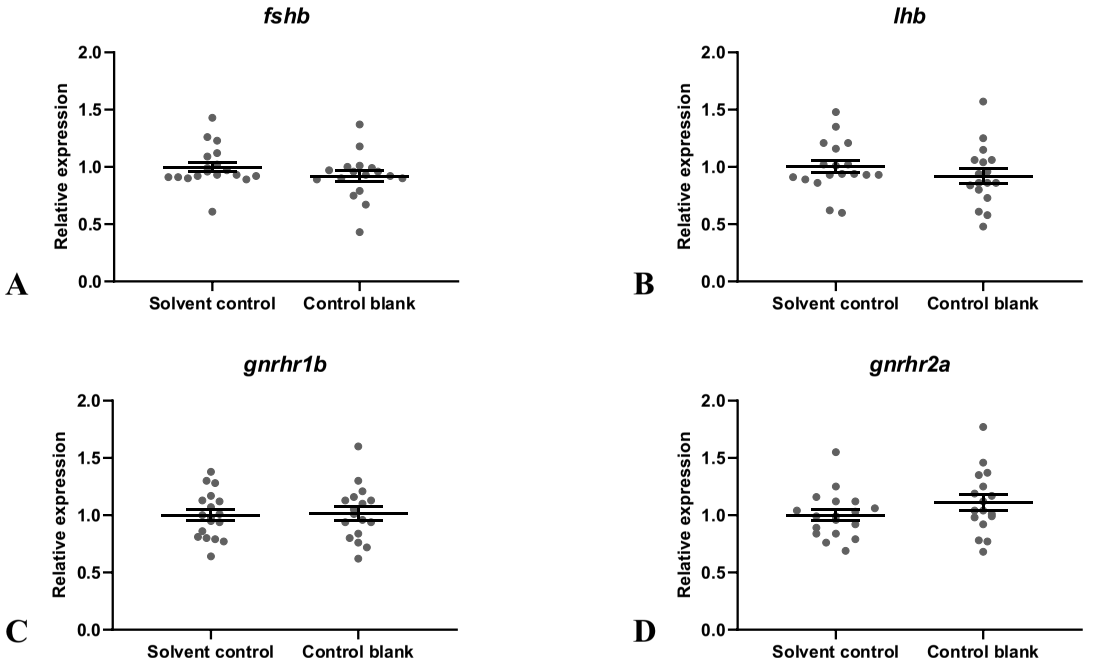
Supporting data, figures S1, S2 and S3

Viability assays, controls

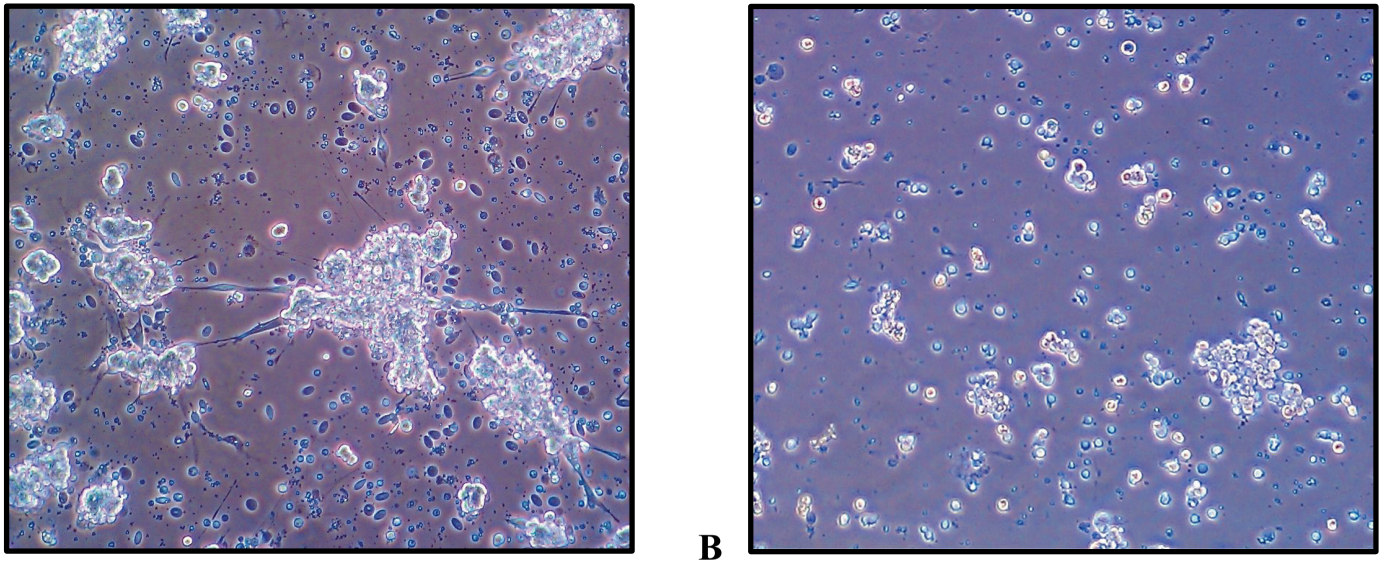


Supplementary Figure 1 Viability in terms of metabolic activity (AB assay; A) and membrane integrity (CFDA-AM assay; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2 % EtOH (solvent control/control blank) added to the culture media for the last 72 hours. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 54). Statistical significance (***; p<000.1) was assessed using the Wilcoxon rank-sum test.



Supplementary Figure 2 Gene expression of gonadotropin subunits (*fshb*; A, *lhb*; B) and Gnrh receptors (*gnrhr1b*; C, *gnrhr2a*; D) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2 % EtOH (solvent control/control blank) added to the culture media for the last 72 hours. Each data point represents the sample fold change relative to mean solvent control. For each column, the group mean ± SEM is indicated (n = 17-18). No statistical differences between groups were detected (by Student's t-test).

Morphological changes, control cells vs high BPA exposure cells



Supplementary Figure 3 Atlantic cod pituitary cells after seven days in culture.
A; Control cells, and B; Cells exposed to high dose (10^{-4} M) BPA for 72h. In the BPA exposed cells, cell numbers are significantly reduced and the cellular outgrowths visible in control cells are absent.

Lista Errata

Side	Line	Original text	Corrected text
22	28	Gc	GC
44	Fig. 1.8	**B**) schematic sagittal view of the brain	**B**) Schematic sagittal view of the brain
58	1	Gc	GC
58	13	stage sexual maturity	stage of sexual maturity
58	15	stress have been shown	stress has been shown
65	Fig. 2.2	Im; immature, Mg; maturing, Me; mature, Sp; spent	**Im**; immature, **Mg**; maturing, **Me**; mature, **Sp**; spent
66	14	grown a physiologically ambient environment	grown in a physiologically ambient environment
72	8	pool pituitaries from the different sexes,	pool pituitaries from the different sexes separately,
81	12	paper I	**paper I**
91	7	*grnrh1b* and *grnrhr2a*	*gnrhr1b* and *gnrhr2a*
114	2	The expression of *grnhr2a* was more prominent than that of *grnhr1b*	The expression of *gnrhr2a* was more prominent than that of *gnrhr1b*

It has been calculated that if no accident prevented the hatching of the eggs and each egg reached maturity, it would take only three years to fill the sea so that you could walk across the Atlantic dryshod on the backs of cod

- Alexandre Dumas, *Le Grande Dictionnaire de cuisine*, 1873
(from M. Kurlansky, «Cod. A biography of the fish that changed the world», 1997)

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